2014•2015
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef
Radiation-induced molecular modulations in thyroid cells cultured under iodine-deficiency

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Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.
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<tbody>
<tr>
<td>ATC</td>
<td>Anaplastic Thyroid Carcinoma</td>
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<td>ATM</td>
<td>Ataxia-Telangiectasia Mutated</td>
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<tr>
<td>BCA</td>
<td>BicinChoninic Acid assay</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-Associated Agonist of cell Death</td>
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<tr>
<td>BCR</td>
<td>Belgian Cancer Registry</td>
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<tr>
<td>BCL-2</td>
<td>B-Cell lymphoma 2</td>
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<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Breaks</td>
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<tr>
<td>EMS</td>
<td>Ethyl MethaneSulfonate</td>
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<td>ERK</td>
<td>Extracellular signal-Regulated Kinases</td>
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<td>FTC</td>
<td>Follicular Thyroid Carcinoma</td>
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<td>FIR</td>
<td>Fractionated Ionizing Radiation</td>
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<td>FRTL-5</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>Gamma-Histone 2A variant X</td>
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<td>Gy</td>
<td>Gray</td>
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<td>ID</td>
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<td>Inhibitor-κB</td>
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<td>JAK</td>
<td>Janus Kinase</td>
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<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MTC</td>
<td>Medullary Thyroid Carcinoma</td>
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<td>MAPK</td>
<td>Mitogen-Activated Protein Kinases</td>
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<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
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<tr>
<td>Na2EDTA</td>
<td>Sodium Ethylene Diamine Tetraacetic Acid</td>
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<tr>
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<td>Sodium Chloride</td>
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<tr>
<td>NaI</td>
<td>Sodium Iodide</td>
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<tr>
<td>NF-KB</td>
<td>Nuclear Factor kappa B</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>NIK</td>
<td>NF-κB-Inducing Kinase</td>
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<td>PCCL-3</td>
<td>Continuous normal rat thyroid cells</td>
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<td>PTC</td>
<td>Papillary Thyroid Carcinoma</td>
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<tr>
<td>PI3K/AKT</td>
<td>Phosphatidylinositol-3-Kinase and Protein Kinase B</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SSB</td>
<td>Single Strand Break</td>
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<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>Sv</td>
<td>Sievert</td>
</tr>
<tr>
<td>TBHP</td>
<td>Tert-Butyl HydroxyPeroxide</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TPC-1</td>
<td>Papillary Thyroid Cancer cells</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF Receptor-Associated Factor</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid Stimulating Hormone</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Acknowledgement

During the 8 months research period at SCK•CEN, I faced many challenges but I must say that I enjoyed them all to the most. However, this journey would never have proceeded without the help and support of several people to whom I am deeply grateful.

I particularly want to express my sincere gratitude to my supervisor at SCK•CEN Dr. Hanane Derradji for her guidance, encouragement and critical analysis of the data throughout the entire research process and to Prof. Sarah Baatout for her useful feedback and for her encouragement and support. I would like to thank Prof. Veerle Somers, Dr. Patrick Vandormael and Prof. Niels Hellings for following up on my thesis and for their useful and constructive feedback and encouragement.

I would also like to express my great gratitude to my family members and husband Dr. Mohamed Mysara who always gave me motivation and support, and many thanks to my daughter “Lilly” as we achieved this tough journey together.

Thanks to my

Last, but not least, I offer especial thanks to my friends at SCK•CEN and at Hasselt University for the great time and the friendly environment that encouraged me to finish this work.
Summary

Introduction: Iodine Deficiency (ID) and Ionizing Radiation (IR) are known risk factors for development of thyroid cancer, however there is little knowledge on their combined effects on thyroid cells especially when considering low doses of radiation. We hypothesize that IR and ID would have a harmful effect on thyroid cells as both of them induce ROS production in the cells, and this would eventually lead to increased apoptosis/cell death, activation of precancerous pathways such as cell survival and proliferation pathways (NF-κB, PI3/AKT and JAK/STAT).

Materials & methods: A normal thyroid cell line was subjected to low (0.1 Gy), intermediate (0.5 Gy) and high doses of radiation (3 Gy) under iodine sufficient or deficient condition. Apoptotic/dead cells were assessed by flow cytometry, ROS production was assessed using a fluorescent probe, DNA damage was detected using comet assay and protein expression were evaluated using western blot. Statistical significance of differences ($p < 0.05$) was evaluated with the ANOVA test followed by Bonferroni post-tests.

Results: When IR and ID assessed separately 24 and 48 hours post treatment, IR up to 0.5 Gy failed to induce apoptosis in the thyroid cells but a higher dose of 3Gy did. When applied together, apoptosis was only increased at the highest dose of 3Gy. Regarding, late-apoptotic/dead cells, the co-treatment induced an increase in these populations 48 hours post co-treatment in a dose dependent manner. However, for both apoptosis/dead cells, no synergetic/additive increase was observed. After co-treatment, activation of markers of apoptosis (active caspase 3), cell survival (NF-κB p65 and pIKKa), cell growth (STAT-3) and cell cycle regulator (Cyclin-D1) were only observed at the highest dose of 3Gy. Although the co-treatment increased ROS production in a dose dependent manner and induced DNA damage starting from 0.1 Gy, the expression of p53, the guardian of the genome, was elevated starting from 0.5 Gy.

Conclusions: These data suggest that thyroid cells are relatively resistant to apoptosis, and show little DNA damage as induced by the co-treatment even when the latter involves intermediate or high doses of radiation. It should be noted, that the majority of the cells remained alive after the co-treatment and this specific population may bear chromosomal aberrations of cancer related genes which may promote their malignant transformation.
Introduction

Thyroid cancer is the most common type of endocrine malignancy which originates from the follicular or non-follicular cells of the thyroid gland. Thyroid cancer that arises from follicular cells can be either differentiated or undifferentiated tumor. Differentiated thyroid tumor includes papillary thyroid carcinoma (PTC) which is the most common type of thyroid cancer that accounts for 80%, and follicular thyroid carcinoma (FTC). Undifferentiated tumor includes medullary thyroid carcinoma (MTC) or anaplastic thyroid carcinoma (ATC). The treatment of the differentiated thyroid tumor is highly successful, while undifferentiated thyroid cancer is more aggressive, can spread easily to the lymph nodes of the neck and metastasize to other organs in the body. The undifferentiated thyroid tumor can occur de novo or can derive from PTC and FTC [1, 2]. The incidence of thyroid cancer is rapidly increasing in the last three decades all over the world [3]. According to the Belgian Cancer Registry (BCR), the incidence of thyroid cancer is significantly increased in the Flemish region from 2.7 cases per 100000 in 1999 to 5.2 cases per 100000 in 2008 in females and from 1.4 cases per 100000 in 1999 to 2.0 per 100000 in 2008 in males. The increase in the thyroid cancer burden is mainly in PTC. There is little knowledge about the aetiology of thyroid cancer and there is a need to identify the risk factors for a better prevention against it.

1. Thyroid cancer risks

1.1 Radiation exposure

Radiation exposure, iodine diet, the age, the gender and the genetic background are important determinants in thyroid cancer development. High doses of radiation exposure are well established as a risk factor for thyroid cancer. This is shown by epidemiological studies performed after the Chernobyl nuclear work station accident in 1986. The risk of thyroid cancer was dramatically increased in children and adult that were exposed to radiation and particularly to the radioactive iodine isoform (I\textsuperscript{131}) [4-6]. The radiation doses that people were exposed to after Chernobyl range from 0.1 Gy to 6 Gy depending on their location at the time of the accident [7]. A number of epidemiological studies reported that thyroid cancer can occur due to radiation exposure to less than 0.1 Gy [8]. Radiation are classified into two main categories: ionizing radiations or non-ionizing radiations. Ionizing radiations have enough energy (>13.6 eV) to ionize the matter by liberating an electron from atoms or molecules, this includes alpha and beta particles, neutrons, X-rays and γ-rays. Non-ionizing radiations are relatively low energy radiations (<13.6 eV) that can’t ionize the matter and they are considered to be less dangerous (ie,
microwaves, radiowaves, infrared, UV and visible light). The basic unit to measure exposure to ionizing radiations is a Sievert (Sv), this unit measures the biological effect of absorbed radiations (referred to as an "effective dose"). Radiations can also be measured by Gray unit (Gy), that measures the absorbed dose and it is defined as energy per mass (1Gy = 1 Joule/kilogram). High doses of ionizing radiations may have serious effects on the body. They can cause skin lesions, cancer, heart disease, and in pregnant women they can harm the fetus, induce miscarriage, and cause congenital malformations or mental retardation depending on the gestational stage at the moment of irradiation. High doses of radiation have a harmful effects on the cells as they can induce DNA damage resulting in single strand break (SSB) or double strand breaks (DSB), protein/DNA cross linking, alter DNA methylation and reduce DNA repairing capacity, which lead to cell damage or mutations. In addition, they induce Reactive Oxygen Species (ROS) production, which damages the cell structure and thereby may activate precancerous pathways. The most common precancerous pathways that are induced by high radiation doses are proliferation pathway (JAK/STAT and MAPK/ERK), pro-survival pathways (PI3K/AKT and NF-KB), in addition to radiation effect on different molecules acting upstream/downstream of these pathways such as tumor necrosis factor alpha (TNF-α), p53, IL-6 and VEGF. [9-12].

Unlike high radiation doses, the effects of low doses of radiations are still unclear. The risk of low doses of radiation exposure is a public concern as we are exposed to low doses of radiation from different sources on a daily basis such as exposure to sunlight, cosmic rays or radon gas that is found in rocks and soil in many places such as in the southern area of Belgium. In addition to the background radiations, some people receive artificial radiation for diagnostic purposes such as diagnosis using x-ray and Computer Tomography Imaging (CT-scan) or for therapeutic purposes. The exposure to radiation has increased in the last decades mainly due to medical applications. For instance, the mean effective exposure to radiation in the United States in 1980 was 3 mSV per year and it has increased to 6.2 mSV per year in 2006 (figure 1). High doses of radiation have a linear dose-response curve as increasing the radiations doses will increase the harmful effects on human cells and thereby increasing the cancer risk. For low doses of radiation the situation is much less clear, however to estimate the cancer risk from intermediate to very low radiation doses a linear extrapolation in the dose-response curve appears to be the current methodology [13, 14]. Epidemiological data suggest that the lowest dose of radiation exposure that is of cancer risk in human is ≈10–50 mSv for an acute exposure and ≈50–100 mSv for a protracted exposure [14]. On the other hand, some studies showed that low doses of ionizing radiation are beneficial for human as they induce an adaptive response in the cells [9, 15]. Indeed, there are still uncertainties about the effects of low doses of radiation. In addition, there is little knowledge
available about thyroid cancer risks after low doses of radiation exposure especially when it is combined with an endocrine disturbance event like iodine deficiency.

Mean effective exposure 1980 (3.0 mSV)    Mean effective exposure 2006 (6.2 mSV)

Figure 1: Annual effective dose (mSV) of radiation for the United States population in 1980 and 2006 (UNSCEAR, 2008).

1.2 Iodine deficiency

In addition to radiation, two billion people are suffering from iodine deficiency worldwide [16]. The main source of iodine is dietary food such as seafood, meat and iodized salt. Dietary iodine is important for thyroid hormone production that regulates many processes in our body such as body temperature, heart rate, brain development and regulates lipid and carbohydrate metabolism. Consequences of iodine deficiency are endemic goiter, growth retardation and intellectual impairment. In addition, insufficient iodine intake is associated with an increase in thyroid cancer incidence [17, 18]. Indeed, iodine deficiency is a significant health problem in developed and developing countries. The most affected regions with iodine deficiency are South Asia and sub-Saharan Africa, however mild iodine deficiency remains in 50% of Europe and in the recent years the iodine status in the United states has fallen [18]. In iodine deficient areas, the incidence of thyroid cancer appears to be higher [19]. Although epidemiological data remain controversial, iodine deficiency may play a role in increasing the incidence of thyroid cancer [1, 20].

Iodine is essential for thyroid hormone synthesis. The latter mechanism is regulated by Thyroid Stimulating Hormone (TSH) released from the pituitary gland. When iodine concentration becomes insufficient, TSH level increases as a feedback mechanism to increase thyroid hormone
synthesis. Thyrocytes can also adapt to iodine deficiency by an alternative mechanism by which they regulate their own microvasculature. This mechanism occurs earlier and it is independent on TSH regulation. Iodine deficiency causes reshaping of the microvasculature of the thyroid as it induces angiogenic reaction by releasing Vascular Endothelial Growth Factor (VEGF-A) from normal thyrocytes. This occurs via ROS and hypoxia induced elements dependent pathways. There is an inverse relationship between the availability of the iodine and the expansion of the thyroid microvasculature to ensure a steady delivery of the iodine to thyroid glands, but at the same time these events may promote tumor growth if not well controlled. [21-25]

2. The combined effect of radiation exposure and iodine deficiency.

After Chernobyl accident, epidemiological studies indicate that the high incidence of thyroid cancer was found to be associated with the high radiation doses together with iodine deficiency [26-28]. In addition, according to the Belgium Cancer Registry (BCR), there are geographical differences in the incidence of thyroid cancer. For instance, the highest incidence of thyroid cancer in Belgium have been found in the Walloon region and Brussels (6.7 cases per 100 000 person per year), while the Flemish region has a lower incidence (3.3 cases per 100 000 person per year). These geographical variations are unexplained, they may be due to the different examination strategies as explained by Van den Bruel’s group [29]. However, a potential reason may be the environmental factors and the combination between them [30]. Growing epidemiological evidence indicates that radiation and iodine deficiency increase the risk for development of thyroid cancer. However, these epidemiological data are suggestive rather than persuasive due to a lack of statistical power and limited knowledge of the underlying mechanisms. Moreover, knowing the existence of thyroid microcarcinoma that range from 3-36% at autopsies [31, 32], the exposure to radiations and iodine deficiency might be risk factors to turn this very small thyroid carcinoma to bigger thyroid cancer. As both ionizing radiation and iodine deficiency induce harmful effects on thyroid cells separately and that there are little knowledge available about their combined effects, the aim of this research is to study the cellular and the molecular modulations induced by low, intermediate and high doses of radiation in combination with iodine deficiency on thyroid cells in vitro. This may result in a better understanding of the basic mechanisms of thyroid cancer after radiation exposure and iodine deficiency, and may provide preventive strategies against thyroid cancer for the general population.
3. Potential mechanisms involved in the combined effect of radiation exposure and iodine deficiency

3.1 Reactive oxygen species production
One potential mechanism by which low doses of ionizing radiation and iodine deficiency would merge their harmful effects is the increase of ROS production. ROS can be induced in a high level by environmental stress such as exposure to high doses of ionizing radiation more than 2.5 Gy. [33, 34]. Low doses of ionizing radiation exposure can induce ROS production but at lower level [35, 36]. Iodine deficiency is known to produce ROS as well [22]. The combination of radiation and iodine deficiency may act in synergy to increase ROS production reaching then deleterious levels. This overproduction of ROS may on one hand initiate cancer by its action on DNA, and on the other hand promote tumor development by its action on angiogenesis and cell proliferation. Therefore, ROS assessment will be performed in thyroid cells that are exposed to low, intermediate and high doses of radiation combined with iodine sufficient or iodine deficiency conditions to estimate the harmful effect of this co-treatment.

3.2 DNA damage response
Ionizing radiation causes atoms and molecules to become ionized or excited. These excitations and ionizations can induce DNA damage resulting in single strand break (SSB) or double strand breaks (BSB), protein/DNA cross linking, altered DNA methylation, which lead to cell damage or mutations [37-41]. Radiation can also produce ROS which cause significant damage to the DNA. When radiation is combined with iodine deficiency, this may cause more damage response to DNA as iodine deficiency induces ROS production as well. Tumor suppressor protein (p53) plays a central role in DNA damage response as it is involved in cellular and genetic stability upon DNA damage [38]. This protein is activated by ATM by phosphorylation at Ser15. Besides p53, gamma H2AX is one of the most studied markers for DNA damage especially double strand breaks. Upon DNA damage, gamma H2AX histone is phosphorylated on Ser139 and serve as a signaling platform for DNA repair molecules [39, 42, 43]. In this study, DNA damage as a result of the co-treatment will be detected using the comet assay. Furthermore, p53 and gamma H2AX expression levels in the co-treated cells will be evaluated.

3.3 Apoptosis/cell death
It is already known that ionizing radiation induces apoptosis/cell death [44, 45]. There are several mechanisms that are involved in this response. For instance, pro-apoptotic proteins activation, p53 activation, TNF-alpha activation and activation of Caspase 3 [34, 46, 47]. When different doses of radiation are combined with iodine deficiency, apoptosis/cell death rate may eventually
increase. Therefore the number of the apoptotic, late apoptotic and dead cells as a result of the co-treatment will be assessed. In addition, the expression level of active caspase 3 and pro-apoptotic protein Bad will be evaluated in the co-treated cells.

### 3.4 NF-κB pathway activation

Another potential mechanism that could be triggered by radiation exposure and iodine deficiency is the activation of the pro-survival pathway NF-κB. The transcription factor NF-κB initiates a pro-survival network and can activate a great number of genes that are involved in stress response. There are a diverse external stimuli that can activate NF-κB and its downstream genes. For instance, bacterial and fungal infection, inflammation, oxidative stress and high doses of ionizing radiation, can all activate NF-KB. There are five members of the mammalian NF-κB family: p65 (RelA), RelB,c-Rel, NF-κB p50/p105 (NF-κB1), and NF-κB p52/p100 (NF-κB2). Under no stimulating condition, NF-κB forms a complex with NF-κB inhibitors family IκB. This IκB family includes IκB-α, IκB-β, IκB-γ (NEMO), IκB-ε, p105 and p100. NF-κB/IκB complex doesn’t translocate to the nucleus and maintain NF-κB in inactive form within the cytoplasm. There are two pathways for NF-κB activation. The classical pathway which is responsible for IκB-α IκB-β and IκB-ε rapid degradation and referred to as IKK-β-dependent pathway, and the alternative pathway that leads to the processing of p100 and activation of p52/RelB and referred to as IKK-α-dependent pathway.[9, 48-51]

As highlighted by red squares in figure 2, ionizing radiation (IR) can activate NF-κB directly by inducing DNA damage via single stand breaks or double stand breaks (SSB/DSB) that leads to nuclear ATM activation and translocation to the cytoplasm. In the cytoplasm ATM activates IKK complex that leads to NF-κB activation. Ionizing radiation can also activate NF-KB through ROS production which induces DNA damage and activates NFKB via the TRAFs pathway. It was shown that ionizing radiation can induces inflammatory response that lead to production of TNF-alpha which can activate NF-κB as well. When NF-κB is activated this results in increasing cell survival, as NF-κB is responsible for the transcription of cell cycle regulator proteins such as cyclin D1 and B1, and anti-apoptotic proteins such as Bcl-2 and XIAP which are labeled by red arrow in the figure 2.

It is well known that high doses of radiations activate NF-κB pathway, but it is not clear whether low doses of radiation can activate this pathway. Low doses of radiation may activate NF-κB pathway at lower extent, however if combined with iodine deficiency that induces ROS production which may lead to NF-κB activation, may result in a synergetic/additive negative effect on thyroid cells by increasing the cell survival induced through this pathway. Therefore, in this study, the NF-κB pathway will be investigated in thyroid cells treated with low, intermediate
and high doses of radiation combined with iodine deficiency.

Figure 2: Schematic representation of the NF-κB signalling network (Ahmed, and Li, 2008). Red squares show activation of NF-κB by fractional ionizing radiations (FIR) either through direct damage to DNA via double strands breaks or single strand break (DSB/SSB), or through ROS production that activates TRAFs pathway. Blue squares show activation of PI3K/AKT and ERK pathways by ionizing radiation and their effect on NF-κB activation. Red arrows indicate different proteins that are transcribed by NF-κB.

3.5 PI3K/AKT pathway activation

Initiation and progression of thyroid cancer involves mutations that lead to activation of PI3K/AKT signaling pathway [2, 52-54]. PI3K/AKT pathway plays a role in cell proliferation and survival and plays a central role in tumorigenesis when it is aberrantly activated. It was found that ionizing radiation can activate PI3K/AKT which at the same time lead to indirect activation of NF-κB, and both have a key role in radioadaptive resistance [9, 55]. To determine whether growth signaling is modulated by radiation and enhanced by iodine deficiency, key elements of PI3k/AKT pathway will be investigated.
3.6 JAK-STAT signaling pathway

JAK-STAT signaling pathway is another potential mechanism that may be triggered by irradiation and iodine deficiency. JAK-STAT signaling pathway influences normal cell survival and growth mechanisms and may contribute to oncogenic transformation. A number of cytokines can activate JAK/STAT pathway. When cytokines bind to their receptors, they phosphorylate and activate JAK, that in turn phosphorylates and activates STAT. JAK/STAT pathway are known to be activated in papillary thyroid carcinoma specially STAT-3, the downstream effector of IL-6R, known to be activated in 60 % of papillary thyroid carcinoma. Therefore, the expression level of STAT-3 and pSTAT-3 will be monitored in the co-treated cells.[9, 56-60]

3.7 Inflammatory response

Irradiation and iodine deficiency may trigger an inflammatory response in the thyroid cells as well. Therefore, different cytokines will be monitored in thyroid cells treated with irradiation and iodine deficiency. IL-6 expression will be monitored, as it has been shown to be involved in approximately 50% of papillary thyroid carcinoma. TNF-alpha will be monitored as it is induced by irradiation and it is involved in NF-κB pathways activation. In addition, VGEF that is induced by STAT-3 activation and induced by iodine deficiency will be monitored. [9, 22, 56].

4. Hypothesis

Since iodine deficiency induces ROS production and radiation exposure induces ROS production as well, we hypothesize that iodine deficiency and radiation especially at low doses have a synergetic or additive negative effect on thyroid cells through the production of a harmful level of ROS that would eventually lead to increased apoptosis/cell death and activation of precancerous pathways such as cell survival and proliferation pathways (NF-κB, PI3/AKT and JAK/STAT).

5. Objectives and study aims

In order to test the hypothesis, normal thyroid cell line (FRTL-5) from rat origin will be subjected to radiation (low doses 0.1Gy, intermediate dose 0.5Gy and high dose 3Gy) under iodine deficient or iodine sufficient conditions. The specific objectives and the experimental aims are explained in the following work packages:

5.1 Work Package 1 (WP 1): Apoptosis detection:

The number of apoptotic cells as a result of the co-treatment with radiations and iodine deficiency will be assessed. To do that, Annexin V/Propidium Iodide (PI) assay will be performed using
flow cytometry. Annexin V/PI assay allows to differentiate between different cell populations such as the healthy, apoptotic and later apoptotic/necrotic populations.

5.2 Work Package 2 (WP 2): Protein detection:
Different proteins will be detected as described below:

(i) The expression level of pro-apoptotic proteins such as Bad and caspase 3 will be evaluated in the co-treated cells using western blot. If the co-treatment induces apoptosis/cell death, the pro-apoptotic proteins will be upregulated.

(ii) The expression level of DNA damage response markers such as p53 and gamma H2AX will be evaluated in the co-treated cells using western blot.

(iii) The expression level of proteins involved in cell survival and proliferation pathways (NF-KB, PI3K/Akt and JAK/STAT) will be evaluated in the co-treated cells using western blot. Theses pathways are activated in the cancer cells and, if they are activated in the co-treated cells, the co-treatment may increase the potential of thyroid cancer.

5.3 Work Package 3 (WP 3): ROS assessment
Detection of ROS level in the samples will be performed using the H2DCFH-DA probe which will emit a fluorescent signal once processed by ROS in the living cells.

5.4 Work Package 4 (WP 4): Cytokine detection
Detection of cytokines (IL-6, and TNF-alpha) and angiogenesis factor (VGEF) will be monitored in the co-treated cells using Luminex assay.

5.5 Work Package 5 (WP 5): DNA damage detection
The damage in the DNA as a result of the co-treatment will be detected in the samples using single cell gel electrophoresis that is known as the comet assay.

5.6 Work Package 6 (WP 6): statistical analysis
Finally, the statistical analysis will be performed for the results using 2 way ANOUVA test followed by Bonferroni post-tests.
**Materials and methods**

1. **Cell culture**

Normal rat thyroid cell line (FRTL-5) were purchased from ATCC (American Type Cell Culture) and normal rat thyroid cells (PCCL3) were obtained from a collaborator at the free University of Brussels. FRTL-5 is a tetraploid continuous normal rat thyroid cells cloned from primary cultures of rat thyroid cells. They maintained functional characteristics of normal thyroid cells such as iodide uptake, thyroglobulin synthesis and response to TSH over prolonged periods of culture [61]. PCCL3 cells have approximately the same characteristics as the FRTL-5 cells (PCCL3 cells were tested for mycoplasma and they were mycoplasma free). The cells were grown at least 7 days in Coon’s medium (5% calf serum/5 hormone mixture) (Life technologies, Ghent, Belgium) supplemented with physiological concentration of iodine (NaI at 10^{-8}M). Preparation of the medium is shown in table 1. For Annexin V/PI and Comet assays, two days before the co-treatment, the cells were transferred into a 6 well plates at a density of 2\times10^5 cells/well in three biological replicates. For western blot assay, cells were cultured in 75 cm^2 cell culture flasks in three biological replicates as a large amount of proteins was needed. For ROS assessment, cells were grown in 96 well in three biological replicates. One hour before irradiation (0.1, 0.5 and 3 Gy of X-rays), medium was refreshed for all the plates/flasks. For the control and each dose, three replicates were refreshed with medium containing NaI (Iodide) and three replicates with medium without NaI (Iodine deficiency). For Annexin V/PI and western blot assays, the cells were harvested 6, 24, 48 and 72 hours post co-treatment, while for ROS assessment and comet assay the cells were harvested 1 hour post co-treatment.

**Table 1: composition of the medium for the culture of the FRTL-5 and PCCL3 cell lines.**

<table>
<thead>
<tr>
<th>100 ml medium</th>
<th>Products</th>
<th>Final concentration</th>
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<tbody>
<tr>
<td>91.3ml</td>
<td>Coon’s medium</td>
<td>5%</td>
</tr>
<tr>
<td>5ml</td>
<td>NCS or FBS</td>
<td>5%</td>
</tr>
<tr>
<td>1ml</td>
<td>Peni-strepto</td>
<td>100U/ml</td>
</tr>
<tr>
<td>1ml</td>
<td>Fungizone</td>
<td>2.5µg/ml</td>
</tr>
<tr>
<td>1ml</td>
<td>Glutamin 200mM</td>
<td>2mM</td>
</tr>
<tr>
<td>100µl</td>
<td>Insulin 10mg/ml</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>50µl</td>
<td>Transferrin 10mg/ml</td>
<td>5µg/ml</td>
</tr>
<tr>
<td>7.2µl</td>
<td>Hydrocortisone 50µg/ml</td>
<td>3.6ng/ml</td>
</tr>
<tr>
<td>20µl</td>
<td>Glycyl-histidine-lysine 50µg/ml</td>
<td>10ng/ml</td>
</tr>
<tr>
<td>20µl</td>
<td>Somatostatin 50µg/ml</td>
<td>10ng/ml</td>
</tr>
<tr>
<td>500µl</td>
<td>TSH 1 U/ml</td>
<td>5mU/ml</td>
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</table>

Hydrocortisone, Glycyl-histidine-lysine and Somatostatin were not added to PCCL3 cells.
2. **Cell counting**

Cell counting was performed to calculate the number of cells to be seeded in each well plate and to calculate the number of cells needed. This has been done by Moxi Z automated cell counter that measures the number of cells in 1 ml. 2X10^5 cells were seeded in each well and 5x10^5 cells were used to perform the Annexin V/ PI assay and Comet assay. 5X10^3 cells were seeded in each well in 96 well-plate for ROS assessment.

3. **Apoptosis detection using Annexin V/ Propidium Iodide (PI) assay**

Annexin V/ PI assay was performed to assess the number of apoptotic cells as a result of the co-treatment with irradiations and iodine deficiency. The Annexin V/ PI assay kit was obtained from eBioscience Company (eBioscience, Vienna, Austria), it has binding buffer, Annexin V and PI dyes. 2-5 × 10^5 cells /ml were washed by PBS and then re-suspended in a binding buffer. Cells were stained with 5 μL of Annexin V-FITC to 195μL cell suspension, then incubated for 10 min at room temperature in the dark. Cells were washed with 200 μL of binding buffer, re-suspended in 190 μL binding buffer and then stained with 10 μL PI. Both living cells, early apoptotic and late apoptotic/dead cells were determined using BD AccuriC6 flow cytometer. Color compensation for the Annexin V and PI staining was performed before the analysis by using single color controls (cells treated with annexin V only, cells treated with PI only and untreated cells) and adjusting the fluorescent signals in the FL1 and FL3 channels. Cells treated with Staurosporin which induces apoptosis were used as a positive control.

4. **Protein detection using western blot assay**

Total proteins were extracted from the cell lysate using “Ready Prep” extraction buffer (Biorad, Nazareth Eke, Belgium). Protease inhibitor (Roche Life Science, Germany) and tributyl phosphate (Sigma Aldrich, Diegem, Belgium) were added to the extraction buffer before use. 100 μl of the extraction buffer were added to each sample with sonication for 30 seconds for homogenization. Protein quantification was done using BCA (bicinchoninic acid assay) method. 20μg of proteins were supplemented with Laemmli buffer (1/4 of the total volume) and beta-mercaptoethanol (1/10 of the Laemmli buffer) and were heated at 95°C for 5 minutes. Electrophoresis was performed on Sodium Dodecyl Sulfate (SDS)–polyacrylamide ready to use gels (Ge Health care life science). The gels were pre-ran with a running buffer (Contain 1% SDS) for 12 minutes at 160 volt. 5 minutes prior running the samples on the gel, they were heated at 95°C to denature the proteins, then ran along with a molecular weight marker on the gel for 30
min. The separated proteins were transferred onto a nitrocellulose membrane using the iBlot dry transfer system from Invitrogen. The membrane was blocked with a blocking buffer (contains sodium azide, Life technologies, Merelbeke, Belgium) for 1 hour at room temperature then it was washed twice/5-min with a washing buffer (0.5 M Tris, 1.5 M Nacl, 0.5 % Tween20, PH 7.4). The primary antibody (the company and the concentration used is shown in table 2) which was diluted in the blocking buffer was added to the membrane and incubated overnight at 4°C under constant agitation. The membrane was washed twice/5min then incubated with the secondary antibody (goat anti-mouse or anti-rabbit immunoglobulin G, dilution 1/4000 in washing buffer, Life technologies, Merelbeke, Belgium) for 45 min at room temperature, followed by 2 washes/5min.

For visualizing the protein-antibody complex, the Qdot streptavidin conjugate (Life technologies, Merelbeke, Belgium) was used with a dilution of 1/4000 in the blocking buffer, and incubated with the membrane for 45 min under stirring. The membrane was then exposed to UV light and the bands were visualized using Vilber Fusion imaging system. Band densitometry was performed using Bio1D analysis software and normalization against B-actin/vinculin that was detected on the same membrane of the protein of interest was performed.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Company</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFκB p52 Anticorps(K-27): sc-298</td>
<td>Santa Cruz Biotechnology, Heidelberg, Germany</td>
<td>Dilution 1/500</td>
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<tr>
<td>NFκB p65 Antibody (C-20) sc-372</td>
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<td>Santa Cruz Biotechnology, Heidelberg, Germany</td>
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<tr>
<td>p-NIK (Thr 559): sc-12957</td>
<td>Santa Cruz Biotechnology, Heidelberg, Germany</td>
<td>Dilution 1/200</td>
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<td>β-Actin (C4): sc-47778</td>
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5. ROS assessment using CM-H$_2$DCFDA probe

ROS production as a result of the co-treatment with irradiation and iodine deficiency was detected by CM-H$_2$DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) probe (Life technologies, Merelbeke, Belgium). Cells were seeded at a density of 5x $10^3$ in 96-well plate, and allowed to grow for two days. At the day of the experiment, cells were treated with medium containing NaI (Iodide) or with medium without NaI (Iodine deficiency), and the plates was X-irradiated with 0.1 Gy, 0.5Gy and 3Gy. Directly after irradiation, cells were washed twice with HEPES-buffered Hank's balanced salt solution (HEPES/HBSS buffer), loaded with 20 µM CM-H$_2$DCFDA (in HEPES/HBSS buffer, 100 µl/well), Hoechst 33258 (Life Technology, Ghent, Belgium) at a dilution of 1:1000 for normalization against the number of cells in each well, thereafter incubated in the dark at room temperature for 25 minutes. The dye was washed out by HEPES/HBSS buffer and the plates were incubated in the dark at room temperature for 30 minutes. Cells treated with 20 µM Tert-butyli hydroperoxide (TBHP) for 3 minutes were used as a positive control. CLARIOstar® microplate reader was used to analyze the fluorescent signal and to measure the mean fluorescent intensity at 485 nm excitation and 535 nm emission for CM-H$_2$DCFDA probe, and at 352 nm excitation and 461 emission for Hoechst.

6. Luminex assay for cytokine detection

Supernatants were collected from the co-treated cells that were incubated in 75 cm$^2$ cell culture flasks and 6 well/plate 6 and 24 hours post co-treatment. Supernatants were incubated in 96-well
plate with dye-injected magnetic beads conjugated with antibodies against 3 different cytokines (Millipore Co, MA, USA). Each bead has a certain ratio of infrared to red dye that helps to identify the associated cytokine (VGEF- TNF-alpha and IL-6). 25µl of detection antibody were added after washing the plate and incubated for 1 hour at room temperature. Then, 25µl of streptavidine-phycoerythrin were added to each well together with the detection antibody and incubated for 30 minutes at room temperature. The wells were washed twice and 125µl sheath fluid were added to each well and the plate was run on the Luminex 100™ machine using the HTS, FLEXMAP 3D™ software.

7. Comet assay for DNA damage detection

The Alkaline Comet assay (single cell gel electrophoresis) was performed to evaluate the DNA damage (single strand and double strand breaks) induced by the co-treatment. FRTL-5 cells were seeded at a density of 2.5x 10^5 in 6-well plates. One hour after irradiation and iodine deficiency, cells were trypsinized and suspended in PBS (1 million cells per ml). 10µl cell suspension was mixed with 0.8% low melting point agarose preheated at 37 ºC and the mix was spread on GelBond® film with a cover slip placed on the top then chilled on ice for 10 minutes. The coverslip was removed and the slides were placed in a lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 1.2g Tris, 1% sodium lauroyl sarcosinate, 1% Triton X-100, 10% DMSO and the pH was adjusted at 10 using NaOH) overnight in the dark at 4 ºC. The slides were placed then in electrophoresis buffer (300 mM NaOH and 10 mM Na2EDTA in 1000 ml MiliQ water) and denatured for 40 minutes at speed of 22 revolution/minutes. Electrophoresis was applied at 1V/cm and the volt was additionally controlled with a portable voltmeter. After neutralization with cold PBS, the slides were left to dry then stained with SYBR® Gold overnight. The stained GelBond® films were scored using a fluorescent microscope and the images were analyzed using “Metafer version 3.5.1” analysis software. Cells treated with Ethyl MethaneSulfonate (EMS) that induce DNA damage were used as a positive control.

8. Statistical analysis

Analysis of the results was performed using GraphpadPrism5 software. Results are presented as mean ± Standard Error of the Mean (SEM). Statistical differences between control and co-treated groups were determined by two-way ANOVA followed by Bonferroni post-tests. The results were considered statistically significant when p < 0.05.
Results

1. Radiation and iodine deficiency induce apoptosis/cell death in thyroid cells

AnnexinV/PI double staining assay was performed to evaluate the proportion of living, apoptotic, late apoptotic/dead cells using flow cytometry. The co-treated FRTL-5 normal rat thyroid cells grown in 6 well plates were co-stained with Annexin V/PI dyes followed by flow cytometry detection, the experiment was done in 3 biological replicates. Apoptotic cells that undergo changes in their plasma membrane (phosphatidylserine are turned at the outer side of the plasma membrane) can be labeled with the Annexin V-FITC conjugated antibody and can be seen on the flow cytometer as AnnexinV-positive PI-negative population. While late apoptotic or necrotic cells that lose the integrity of their plasma membrane will be positively stained with both PI and Annexin V to become Annexin V-positive PI-positive population. Population with aspecific membrane damage are considered dead cells and they are Annexin V-negative PI-positive (figure 3A and 3C).

Six hours after the co-treatment with irradiation and iodine deficiency, it was too early to detect the signs of apoptosis/cell death and there was no significant increase in the number of the apoptotic and late apoptotic/dead cells between the conditions compared to controls (data not shown). There was a significant increase 24 hours post co-treatment in the apoptotic cells at 3Gy iodine ($p < 0.05$) and 3 Gy iodine deficiency ($p < 0.01$) compared to control iodine and iodine deficiency respectively. For late apoptotic/dead cells, there was a significant increase after 24 hours at 0.5 Gy iodine deficiency ($p < 0.01$), 3Gy iodine ($p < 0.001$) and 3 Gy iodine deficiency ($p < 0.001$) compared to control iodine and iodine deficiency respectively (figure 3A and 3B).

48 hours post co-treatment, there was a significant increase in the apoptotic cells at 3 Gy as well for iodine ($p < 0.01$) and iodine deficiency ($p < 0.001$) compared to control iodine and iodine deficiency respectively. Moreover, there was a significant increase in the number of the late apoptotic/dead cells in a dose dependent manner for both iodine and iodine deficiency compared to control iodine and iodine deficiency respectively ($p < 0.05$) (figure 3C and 3D). The effect of the co-treatment was persistently present at 72 hours post co-treatment as there was a significant increase in the apoptotic cells at 3Gy iodine ($p < 0.001$) and 3 Gy iodine deficiency ($p < 0.001$), and a significant increase in late apoptosis/necrosis in a dose dependent manner which was significant at 0.5 Gy and 3Gy iodine and iodine deficiency ($p < 0.05$) compared to control iodine.
and iodine deficiency respectively (data not shown). Nonetheless, there was no significant difference between iodine and iodine deficiency for each dose separately for all time points.

To validate these findings, Annexin V/PI assay was performed on PCCL3 normal rat thyroid cell line with the same experimental setup. 48 hours post co-treatment with irradiation and iodine deficiency, there was a significant increase in the apoptotic cells at 3 Gy iodine ($p < 0.05$) and iodine deficiency ($p < 0.001$) compared to control iodine and iodine deficiency respectively. For the late apoptotic/dead cells, there was a significant increase in their number in a dose dependent manner that was significant at 0.5 Gy iodine and iodine deficiency ($p < 0.05$), and 3 Gy iodine and iodine deficiency ($p < 0.001$) compared to control iodine and iodine deficiency respectively. However, there was no significant difference between iodine and iodine deficiency for each dose separately (Figure 1 supplement). Nonetheless, the majority of the cells in all the conditions were still living for all time points under study. Staurosporin, induces apoptosis by activating caspase-3 pathway, was used as a positive control. 24 and 48 hours after staurosporin treatment, the cells were shifted from apoptosis to late apoptosis/dead cells (Figure 3B and 3D).

Taken together, these data indicate that the co-treatment with irradiation and iodine deficiency at early time point (6 post co-treatment) doesn’t induce a significant change in the thyroid cells concerning the number of apoptotic and late apoptotic/dead cells. However, the co-treatment induces apoptosis at 24, 48 and 72 hours post co-treatment only at 3 Gy iodine and iodine deficiency compared to controls. Furthermore, the co-treatment induces late apoptosis/cell death in a dose dependent manner especially at 0.5 and 3 Gy iodine and iodine deficiency 24, 48 and 72 hours post co-treatment compared to controls. Moreover, the majority of the cells remain living population for all time points under study. Nonetheless, there was no synergetic or additive effect between radiation and iodine deficiency as there was no significant difference between iodine and iodine deficiency among each dose separately.
24 hours post irradiation and iodine deficiency

### Control I
- 0.1 Gy I
- 0.5 Gy I
- 3 Gy I

### Control ID
- 0.1 Gy ID
- 0.5 Gy ID
- 3 Gy ID

#### 24 hours

**A**

**B**

24 hours

- Apoptotic cells (%)
- Late apoptotic/Dead cells (%)

![Graph A](image)

![Graph B](image)
C  

48 hours post irradiation and iodine deficiency

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<thead>
<tr>
<th>Dose</th>
<th>Control I</th>
<th>0.1 Gy I</th>
<th>0.5 Gy I</th>
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</table>

**Apoptotic cells (%)**

- Control
- 0.1 Gy
- 0.5 Gy
- 3 Gy
- Staurosporin

**Late apoptotic/Dead cells (%)**

- Control
- 0.1 Gy
- 0.5 Gy
- 3 Gy
- Staurosporin

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**48 hours**

<table>
<thead>
<tr>
<th>Dose</th>
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<th>0.1 Gy</th>
<th>0.5 Gy</th>
<th>3 Gy</th>
<th>Staurosporin</th>
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<tr>
<td>Late apoptotic/Dead cells (%)</td>
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<td><img src="image15" alt="Graph" /></td>
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</table>
**Figure 3: Annexin V/PI double staining.** FRTL-5 cells were double stained with Annexin V/PI to discriminate between living, apoptotic and late apoptotic/dead cells. A and C are the scatterplots while B and D are the analysis figures. A and C show the changes in the different cellular populations 24 and 48 hours post co-treatments. Living healthy population is Annexin V-negative PI-negative (lower left quadrant), Apoptotic population is Annexin V-positive PI-negative (upper left quadrant), Late apoptotic/necrotic population is Annexin V-positive PI-positive (upper right quadrant) and population with aspecific membrane damage which is considered to be necrotic or dread population is Annexin V-negative PI-positive (Lower right quadrant). The scatterplots and the figures show that the co-treatment with irradiation and iodine deficiency induces apoptosis at 3 Gy and they induce late apoptosis/cell death in a dose dependent manner especially at 0.5 and 3 Gy. However, and for each dose separately there is no significant difference between iodine and iodine deficiency. Staurosporin was used as a positive control.

I: Iodide, ID: Iodine Deficiency. * indicates significant difference ($p < 0.05$) when compared to the control groups (iodine and iodine deficiency).

2. Increased ROS production in thyroid cells treated with irradiation and iodine deficiency

To examine the ROS production as a result of the co-treatment with irradiation and iodine deficiency, a general oxidative stress indicator CM-H2DCFDA probe was used. The co-treated FRTL-5 normal thyroid cells grown in 96-well microplates were stained with CM-H2DCFDA and the experiment was performed in 3 biological replicates. The CM-H2DCFDA probe is membrane permeable and once inside the cell, the intracellular estrases cleave the ester groups of the CM-H2DCFDA probe. Thereafter, oxidation by intercellular ROS produce dichlorofluorescein (DCF) derivatives, which are highly fluorescent. The mean fluorescent intensity is proportional to the ROS level that is produced in the cells.

There was a significant induction of ROS in a dose dependent manner in both iodine and iodine deficiency treatment to reach the highest level at 3Gy iodine and iodine deficiency compared to the control iodine and iodine deficiency respectively (0.1 Gy iodine $p < 0.05$, 0.5 Gy iodine $p < 0.001$, 0.5 Gy iodine deficiency $p <0.01$, 3 Gy iodine and iodine deficiency $p <0.001$) (figure 4). Moreover, for each dose separately, the ROS level is significantly higher in the irradiated cells treated with iodine deficiency compared to the irradiated cells treated with iodine in control cells and at 3Gy condition ($p < 0.05$) (figure 4). TBHP that induces ROS production was used as a positive control and Hoechst dye was used for the normalization against the cell number.
Together these findings show that irradiation and iodine deficiency induce ROS production in a dose dependent manner compared to controls. Moreover, there was no additive or synergetic effect of the co-treatment as there was no difference between iodine and iodine deficiency for each dose separately except at high dose (3 Gy) which was also seen at the control in the co-treated cells.

![Figure 4: The co-treatment with irradiation and iodine deficiency induces ROS production in a dose dependent manner.](image)

3. **Radiation and iodine deficiency induce DNA damage**

In order to detect DNA damage that is induced by the co-treatment, the comet assay was performed on FRTL-5 co-treated cells in 3 biological replicates. The co-treated cells embedded in agarose and spread on microscope slides were lysed overnight followed by denaturation and electrophoresis. The DNA which is negatively charged at PH > 13 migrated to the positive pole and the damaged DNA formed a comet shape. The intensity of the DNA in the comet tail that can be detected with a fluorescent microscope reflects the percentage of DNA damage.

There was a significant increase in DNA damage in both iodine and iodine deficiency treatment to reach the highest level at 3Gy iodine deficiency compared to the controls (0.1 iodine $p < 0.05$, 0.5 iodine $p < 0.01$, 0.5 iodine deficiency $p < 0.05$, 3 Gy Iodine $p < 0.01$ and 3 Gy iodine deficiency $p < 0.001$) (Figure 5). However, when comparing each dose separately, there was no significant
difference between iodine and iodine deficiency (Figure 5). Cells treated with MethaneSulfonate (EMS) that induces DNA damage were used as a positive control.

These findings indicate that the co-treatment induced DNA damage that can be detected from 0.1 Gy dose and reach the highest level of damage at 3 Gy iodine deficiency condition. However, there was no combined synergetic/additive effect of the co-treatment when comparing iodine deficiency with iodine for each dose separately.

**Figure 5: Radiation and iodine deficiency induce DNA damage in the co-treated FRTL-5 cells.** A) The co-treatment induced DNA damage, however no combined synergetic/ additive effect was noticed. B) The comet shape that reflects the percentage of DNA damage. Results are expressed as comet percentage DNA in tail (mean ± SE). * Indicates significant difference ($p < 0.05$) when compared with control groups (Iodine and iodine deficiency). ID: Iodine deficiency, EMS: MethaneSulfonate

4. **Apoptosis and DNA damage markers of radiation and iodine deficiency**

As irradiation and iodine deficiency induce apoptosis/cell death and they induce DNA damage as shown previously, the molecular markers of apoptosis and DNA damage response were checked in the co-treated FRTL-5 cells using western blot assay and the experiment was performed in 3 biological replicates. The expression level of activated caspase 3, marker for apoptosis and cellular damage, was evaluated 6 hours after the co-treatment. Caspase 3 level increased in a dose
dependant manner, however it is only significant at 3 Gy iodine \((p <0.05)\) and 3 Gy iodine deficiency \((p <0.001)\). In addition, for each dose separately, there was a significant increase in caspase 3 level at 3 Gy iodine deficiency compared to 3 Gy iodine \((p <0.01)\) (Figure 6A and B). The expression level of the pro-apoptotic protein BAD was checked as well 6 hours after the co-treatment but there was no significant difference between the conditions (data not shown) and the active phosphorylated BAD was not expressed. As ROS induces DNA damage and cause double strand breaks, the expression level of gamma H2AX, marker of DNA double strand breaks, was evaluated 6 hours after the co-treatment. 6 hours was late to see the damage and there was no significant difference in gamma H2AX level between the conditions (data not shown). To further investigate the molecular response to DNA damage, p53 which plays a central role in DNA damage response was checked 6 hours after the co-treatment. The expression level of p53 and its phosphorylated form on Ser 15 increased at 0.5 Gy iodine \((p <0.01)\) and 3Gy iodine \((p <0.05)\) compared to control iodine (Figure 6A and B). In addition, there was no significant difference between iodine and iodine deficiency for each dose separately. Normalization was performed against Vinculin on the same membrane of the protein of interest to avoid pipetting errors. Papillary thyroid carcinoma cells (TPC-1) was used as a positive control.

Together these findings indicate that the co-treatment activates the molecular marker of apoptosis, active caspase 3, that is more noticed at high dose (3 Gy) and they have a synergetic/additive combined effect on caspase 3 activation at 3 Gy. In addition, the co-treatment activates the DNA damage response marker (p53 and phosphorylated p53) especially at intermediate dose (0.5Gy) and high dose (3 Gy) iodine condition. Moreover, there was no combined effect of the co-treatment on DNA damage response marker (p53 and pp53).
Figure 6: Response of different apoptosis/DNA damage marker proteins to irradiation and iodine deficiency in FRTL-5 thyroid cells. A) Western blotting of activated caspase 3, P53, pP53 (phospho S15) and housekeeping proteins B-actin and vinculin. B) Analysis of active Caspase 3 and pP53/P53 western blotting normalized against Vinculin. The expression level of active caspase 3 increased especially at 3 Gy iodine and iodine deficiency and there was a combined effect at 3 Gy. While the expression level of pP53/P53 increases at 0.5 and 3 Gy iodine and there was no combined effect of the co-treatment. * Indicates significant difference ($p<0.05$) when compared with control groups (Iodine and iodine deficiency). I: iodine, ID: iodine deficiency, TPC-1: papillary thyroid carcinoma cells.
5. Radiation and iodine deficiency activate the pro-survival NF-κB pathway

To understand the molecular mechanisms that play a role in the response to radiation and iodine deficiency, the expression level of different pro-survival NF-κB proteins were evaluated using western blot. NF-κB is a pro-survival transcription factor that regulates a great number of genes involved in cell cycle regulation and anti-apoptosis processes. NF-κB pathway is activated with high dose of radiation, however it is not clear if low dose of radiation can activate it and whether the combination between irradiation and iodine deficiency has a combined effect on activation of this pro-survival pathway.

FRTL-5 normal thyroid cells were treated with different doses of radiation and iodine deficiency, the proteins were extracted 6 hours post co-treatment and the expression level of different proteins was evaluated using western blot assay. As can be seen in figure 7, NF-κB p65 level increased in a dose dependent manner, however the increase is only significant at 3 Gy iodine and 3 Gy iodine deficiency \((p < 0.001)\) compared to control iodine and iodine deficiency respectively. Moreover, there was no significant difference between iodine and iodine deficiency for each dose separately. To further validate the results, the expression level of pIkKa/β (phosphorylated on ser 176) which is upstream of NF-κB p65 was checked. pIkKa/β increased as well in a dose dependent manner, however it is only significant at 3Gy iodine deficiency \((p < 0.01)\) compared to control iodine deficiency, and there was no significant difference between iodine and iodine deficiency for each dose separately (Figure 7 A and B). The expression level of NIK and its phosphorylated form (Thr 559) which are upstream of NF-κB were checked and there was an increase trend at 3 Gy iodine and iodine deficiency but not significant (data not shown). NF-κB p52 which is another member of the NF-κB family was checked as well, but there was no significant difference between the conditions (data not shown). Cyclin D1 is a cell cycle regulator protein and NF-κB regulates its transcription. At 3 Gy iodine, the stimuli was not enough to induce a significant increase in cyclin D1, however at 3 Gy iodine deficiency there was a significant increase in cyclin D1 expression \((p <0.05)\) (Figure 7 A and B). However, for each dose separately, there was no significant difference between iodine and iodine deficiency. As PI3K/AKT pathway plays a role in NF-κB activation and it is activated in many types of cancer including papillary thyroid carcinoma, the expression level of AKT1 and its phosphorylated form \((pAKT \text{ Thr 308})\) was evaluated 6 hours post co-treatment. There was no significant difference between the conditions compared to control iodine and iodine deficiency (data not shown). Normalization was done against vinculin or B- actin on the same membrane of the protein of
interest to avoid pipetting errors. TPC-1 papillary thyroid carcinoma cells were used as a positive control.

Together these findings indicate that high dose of irradiation and iodine deficiency activate the pro-survival pathway NF-κB especially at 3 Gy iodine deficiency. However, low and intermediate doses of irradiation (0.1 Gy and 0.5Gy) when combined with iodine deficiency don’t induce a significant effect on NF-κB activation. Nonetheless, the co-treatment doesn’t have a synergetic or additive effect on NF-κB activation.
Figure 7: Response of different NF-κB prosurvival proteins to irradiation and iodine deficiency in FRTL-5 thyroid cells. A) Western blotting of NF-κBp65, pIKKa/β, Cyclin D1 and housekeeping proteins B-actin and vinculin. B) Analysis of NF-κBp65, pIKKa/β and Cyclin D1 western blotting normalized against B-actin or vinculin. The expression level of NF-κBp65, pIKKa/β and Cyclin D1 increase especially at 3 Gy. * Indicates significant difference (p <0.05) when compared with control group (iodine and iodine deficiency). I: iodine, ID: iodine deficiency, TPC-1: papillary thyroid carcinoma cells.

6. Effect of radiation and iodine deficiency on growth, survival and differentiation pathway JAK/STAT

JAK-STAT signaling pathway influences normal cell survival, growth mechanisms and it can contribute to oncogenesis. The expression and the activation of signal transducer and activator of transcription (STAT3) and its active phosphorylated form increase in 60% of papillary thyroid carcinoma. To investigate the effect of the co-treatment with irradiation and iodine deficiency on
the precancerous JAK-STAT pathway, the expression level of STAT-3 and its phosphorylated form pSTAT-3 (Tyr 705 phosphorylated) in FRTL-5 cells was evaluated 6 hours post co-treatment. The expression level of STAT-3 and pSTAT-3 significantly increased at 3 Gy iodine deficiency ($p < 0.01$) compared to control iodine deficiency (Figure 8A and B). In addition, the expression level of pSTAT-3/STAT-3 was higher at 3 Gy iodine deficiency compared to 3 Gy iodine ($p < 0.01$) (Figure 8A and B). Normalization was performed against B-actin on the same membrane to avoid pipetting errors. TPC-1 papillary thyroid carcinoma cells were used as a positive control.

These data indicates that the co-treatment activates JAK/STAT pathway especially at 3 Gy iodine deficiency, however at low and intermediate dose there was no significant effect. Moreover, there is a synergetic/additive effect between irradiation and iodine deficiency on STAT-3 activation that can be seen at 3 Gy iodine deficiency.

Figure 8: Irradiation and iodine deficiency increase STAT-3 and pSTAT-3 expression levels in FRTL-5 thyroid cells. A) Western blotting of STAT-3, pSTAT-3 and housekeeping proteins B-actin. B) Analysis of pSTAT-3/STAT-3 western blotting normalized against B-actin. The expression level of pSTAT-3/STAT-3 increase especially at 3 Gy iodine deficiency and there was a combined effect of the co-
treatment at 3 Gy iodine deficiency. * Indicates significant difference (p <0.05) when compared with control group (iodine and iodine deficiency). I: iodine, ID: iodine deficiency, TPC-1: papillary thyroid carcinoma cells.

7. **Effect of radiation and iodine deficiency on secreted levels of TNF-alpha and VGEF**

TNF-alpha is involved in the inflammatory response and it can activate the pro-survival NF-κB pathway. Since the co-treatment may cause inflammatory response, the secreted level of TNF-alpha was assessed in the co-treated cells. In addition, we checked the secreted level of VGEF to check if the co-treatment induce angiogenesis, knowing that iodine deficiency induces the release of the angiogenic factor VGEF, possibly through the STAT-3 pathway. FRTL-5 thyroid cell were treated with different doses of radiation and iodine deficiency. Six hours and 24 hours post co-treatment, the supernatant was collected and the concentration of VGEF and TNF-alpha was assessed using the Luminex machine. The experiment was done in 3 biological replicates and 2 technical replicates. There was no significant difference in the secreted levels of TNF-alpha and VGEF 6 hours and 24 hours post co-treatment compared to control. However, for TNF-alpha, there was an increase trend at 0.5 Gy iodine and iodine deficiency compared to control iodine and iodine deficiency respectively after 6 hours and 24 hours post co-treatment. Moreover, there was no significant difference between iodine and iodine deficiency for each dose separately (data shown in supplement Figure 2). Lipopolysaccharides (LPS) that induces inflammation was used as a positive control. These findings indicate that the co-treatment has no significant effect on the secreted level of TNF-alpha and VGEF after 6 hours and 24 hours.
Discussion

Although radiation and iodine deficiency are known risk factors of thyroid cancer, there is little knowledge available about their combined effect on thyroid cells especially when considering low doses of radiation. To investigate the cellular and the molecular modulations in thyroid cells that are induced by radiation and iodine deficiency, FRTL-5 normal rat thyroid cells were treated with different doses of radiation under iodine sufficient or iodine deficient conditions, and different endpoint were investigated: ROS production, apoptosis/cell death, DNA damage and different pro-survival/proliferation proteins. Assessment of ROS production showed that the co-treatment induced ROS production in a dose dependent manner, however there was no combined effect of the co-treatment when comparing each dose separately. Evaluation of apoptosis/dead cells fractions showed that the co-treatment induced apoptosis only at 3 Gy iodine and iodine deficiency after 24, 48 and 72 hours post co-treatment. In addition, the co-treatment induced cell death in a dose dependent manner which is more significant at 0.5 and 3 Gy 24, 48 and 72 hours post treatment. However, the major population consisted of living cells for all the time points under study. Detection of DNA damage in the co-treated cells indicated that the co-treatment induced DNA damage starting from 0.1 Gy, however there was no combined synergetic/additive effect of the co-treatment and the majority of the cells remained undamaged. In addition, detection of the differences in apoptosis and DNA damage protein markers indicated that the co-treatment has a combined synergetic/additive effect on caspase 3 activation only at 3 Gy and that the co-treatment activates p53 at 0.5 Gy and 3 Gy iodine condition. Besides, evaluation of the expression level of different survival and proliferation proteins showed that the co-treatment activated the pro-survival pathway NF-κB and the proliferation pathway JAK/STAT especially at 3Gy iodine deficiency. Importantly, there was a combined synergetic/additive effect of the co-treatment on STAT-3 activation at 3Gy.

To assess ROS production as a result of the co-treatment with radiation and iodine deficiency, CM-H2DCFDA probe was used. Although the co-treatment induced ROS in a dose dependent manner even at low dose of radiation, there was no combined synergetic/additive effect observed. 2 Gy of gamma radiation was found to induce ROS production in FRTL-5 normal thyroid cell line [34]. Iodine deficiency was also found to induce ROS production in PCCL-3 normal rat thyroid cell line and in primary thyroid cells [22]. In our study, we observed that radiation and iodine deficiency separately induced ROS production which increased with increasing the radiation dose to reach the highest level at 3 Gy iodine and iodine deficiency, which is in line
with previous literature. Although ROS production at 3 Gy was significantly higher in iodine deficiency condition than in iodine condition, this observation was also observed in the control. Therefore there is no combined synergetic/negative effect between both factors on ROS production and this was not expected. This can be explained by the fact that ROS are produced in the cells immediately after the treatment and it is possible that the level declined with time. In our experiment, the combined effect was not seen and this may be due to experiment handling time (1 hours post co-treatment). These findings indicate that the co-treatment with radiation and iodine deficiency induces ROS production in a dose dependent manner but no combined synergetic/additive effect was observed. However these findings need to be further validated at earlier time point to investigate the combined effect of both factors.

In order to detect DNA damage that is induced by the co-treatment, comet assay was performed directly after the co-treatment. In addition, the expression of p53, the key molecule in DNA damage response was checked at the protein level. Radiation and iodine deficiency induced DNA damage that could be detected even at low dose of radiation, however no combined synergetic/additive effect was observed. In addition, p53 expression level was elevated at 0.5 and 3Gy iodine treatment. It has been shown that ionizing radiation induces DNA damage [41], and it was observed that there is a dose relationship between DNA damage and radiation dose that can be detect from 0.05 Gy in blood cells [40]. We observed that the co-treatment induced DNA damage that could be detect starting from 0.1 Gy iodine and reached the highest level of damage at 3 Gy Iodine deficiency, but there is no dose dependent relationship. This can be explained by the fact that DNA damage should be detected immediately after the treatment due to repair kinetics which occurs afterwards. Although we performed the experiment as soon as possible after the treatment, it took us an hour to handle the samples. In addition, at 3 Gy, there were about 20% dead cells which appeared totally damaged under the microscope. These heavily damaged cells, couldn’t be identified nor quantified by the software, therefore they were excluded manually. Measuring gamma H2AX foci under the fluorescent microscope can be a complementary approach as it was proven to be a sensitive method to detect DNA damage both in vitro and in vivo [38, 43]. Nonetheless, the percentage of DNA damage in the co-treated cells for all the conditions under study was not exceeding 11 % which is quite low considering the radiation doses used. This observation is in line with the earlier study of Yang’s research group [39] who observed that primary thyroid cells were resistant to apoptosis and DNA damage. They explained that by the fact that thyroid cells have an efficient DNA repair mechanism which is promoted by p53 in vitro and in vivo. Therefore, we checked the expression level of p53 6 hours
after the co-treatment by western blot. p53 expression level was significantly increased at 0.5 Gy and 3 Gy iodine conditions which is in line with Yang et. al’s observations. Although, p53 expression increased significantly starting from 0.5 Gy, no synergetic/additive effect was observed. This observation has to be confirmed using more replicates and at later time points (24 ad 48 hours post co-treatment). The expression level of gamma H2AX, a DNA double strand break marker, was checked at the protein level 6 hours after the co-treatment using western blotting. There was no significant change observed and this is most probably due to the fact that gamma H2AX should be detected immediately after the treatment [38, 43]. Although iodine deficiency may sensitize the cells to the DNA damage induced by radiation, there was no combined synergetic/additive effect of the co-treatment observed for DNA damage and on p53 expression, and this was not expected.

Evaluation of the apoptotic/dead cell fraction through Annexin V/PI assay, showed that the co-treated cells exhibited apoptosis only at 3 Gy iodine and iodine deficiency conditions after 24, 48 and 72 hours post co-treatment. However, the co-treatment induced late apoptosis/cell death in a dose dependent manner which was more significant at 0.5 Gy and 3 Gy iodine and iodine deficiency conditions 24, 48 and 72 hours post co-treatment. Nonetheless, there was no combined synergetic/additive effect observed and the majority of the cells remained healthy. We could confirm these results in another normal thyroid cell line (PCCL3) from rat origin. Although the co-treatment induced apoptosis/cell death at high dose, the co-treated cells showed a resistant profile to apoptosis and late apoptosis/cell death at low dose. The same observations have been made by other Terra’s groups [44] who found that UV-radiation induces apoptosis in a dose dependent manner in FRTL-5 thyroid cells that can be detected 48 hours after irradiation, however apoptosis couldn’t be detected at low doses of radiation which is in line with our data. To explore the mechanism behind the resistance to apoptosis, we investigated the expression of the active form of caspase 3, a pro-apoptotic protein. We found that caspase 3 was significantly increased at 3 Gy iodine and iodine deficiency only. The same observation was made by two research groups who reported overexpression of active caspase 3 in primary thyroid cells and in FRTL-5 cells at high radiation doses, however they didn’t observe an effect at low doses [34, 47]. Since iodine deficiency was recently found to induce apoptosis in thyroid cells [25], it was expected to find a combined synergetic/additive effect of the co-treatment on apoptosis/cell death. However no combined effect on apoptosis/cell death was observed when comparing iodine deficiency with iodine treatment for each dose separately for all time points under study. Even if there was a synergetic/additive effect on the expression of caspase 3 at 3 Gy, this wasn't enough
to trigger a synergetic/additive effect on apoptosis/cell death for the same dose. This can be related to the fact that the cellular entry into apoptosis is a balance between pro- and anti-apoptotic players and this balance determines the cellular fate. Therefore, it is worth to evaluate in the future, the expression of anti-apoptotic proteins such as BCL-2 which was found to be sensitive to ionizing radiation [45] especially in thyroid cells [38].

Induction of apoptosis by irradiation seems to be cell type dependent. Indeed, Torudd’s group [41] investigated the effect of radiation on normal human lymphocyte cells and they found that the percentage of apoptotic cells induced by 0.1-2 Gy ranged from 20% to 40% after 24, 48 and 72 hours of irradiation. In our study, we observed that the number of apoptotic/dead cells was low even at high dose of 3 Gy (apoptotic cells were ranging from 2% to 3% and late apoptotic/dead cells were ranging from 15% to 22% for 0.1, 0.5 and 3 Gy) and the biggest population remained the living cells for all the time points under study. These observations are in line with Yang et al.’s study [39] as they found that primary thyroid cells were resistant to ionizing radiation-induced apoptosis/cell death up to 5 Gy. Our data suggest that there must be molecular mechanisms turned on/off that make these thyroid cells resistant to the co-treatment. Therefore, the fact that these cells seem to be resistant to apoptosis and do not die massively after high doses of radiation implies that they must have responded to the co-treatment with a survival mechanisms and this may allow them to transmit genetic damage to the next generation. Of note, cancer may originate from a single transformed proliferating cell. Taken together, the observations on the number of apoptotic/dead cells induced by the co-treatment suggest that radiation and iodine deficiency may have a dual effect as they contribute to apoptosis/cell death at high radiation doses but they may promote resistance to apoptosis at low and intermediate radiation doses.

To understand the mechanisms of radioresistance in thyroid cells, different proteins belonging to the pro-survival NF-κB pathway were evaluated 6 hours post co-treatment using western blot. NF-κB p65 and its upstream activator pIKK-alpha were elevated in the co-treated thyroid cells especially at 3 Gy iodine deficiency. In addition, the cell cycle regulator cyclin D1 that is regulated by NF-κB was elevated at 3 Gy iodine deficiency. However, there was no combined synergetic/additive effect of the co-treatment on NF-κB activation. The transcription factor NF-κB initiates a pro-survival network and can activate a great number of genes that are involved in stress response. Ionizing radiation is known to activate the pro-survival NF-κB pathway [48]. Previous studies on different cell lines including Hela (cervical cancer), 293 (Human epithelial embryonic kidney) and keratinocytes have shown that both ionizing and ultraviolet radiations
activates the pro-survival factor NF-κB that was behind the radioadaptive resistance of these cell lines [12, 48, 62]. However, it is not clear in the literature if radiation, especially at low doses, can activate NF-κB in normal thyroid cells and whether iodine deficiency has a role in NF-κB activation. In our study, we observed that the co-treatment activates NF-κB p65, the most abundant form of NF-κB, in FRTL-5 normal thyroid cells at 3 Gy. plkk-alpha was found to be increased at 3 Gy as well but the increase was only significant at 3 Gy iodine deficiency. Moreover, Cyclin D1 exhibits the same profile like plkk-alpha and it was significantly increased at 3 Gy iodine deficiency. Although iodine deficiency induces ROS production [22] that may activate NF-κB, we couldn’t observe a significant effect of iodine deficiency on NF-κB activation as there was no significant difference between iodine deficiency and iodine treatment when comparing both treatments within each dose. Likewise, there was no effect of the co-treatment at low and intermediate doses of radiation. On the other hand, Pozdeyev’s study [63] showed NF-κB activation 72 hours post treatment in thyroid cancer cells. It is possible that irradiation and iodine deficiency have a synergistic effect on NF-κB activation that may increase the cell survival and make the cells resistant to apoptosis, therefore, this experiment needs to be repeated at 24, 48 and 72 hours post co-treatment to investigate further the effect on NF-κB activation.

PI3K/AKT pathway is another pro-survival pathway which is known to be activated by ionizing radiation especially at high doses leading to radioadaptive resistance in the cells [55]. In addition, AKT is overexpressed in thyroid carcinoma cells and considered to be molecular marker for thyroid cancer diagnosis [38, 54]. To investigate whether there is an effect of the co-treatment on PI3K/AKT, pAKT/AKT expression levels were evaluated 6 hours post co-treatment using western blot. There was no difference between the conditions since the highest level of AKT should be detected one hour post irradiation [55]. Therefore, the experiment needs to be repeated one hour post co-treatment to investigate the effect on PI3K/AKT activation.

An important observation in our study is that the co-treatment induced a synergistic/additive effect on STAT-3 expression at 3 Gy. This is of particular interest since previous studies have shown that STAT-3 up regulation is observed in many cancer cells such as breast cancer and lung cancer cells [57, 58]. In addition, Zhang’s research group observed an overexpression of STAT-3 and its phosphorylated form in human papillary thyroid carcinoma tissue that indicates the oncogenic role of STAT-3 in thyroid cancer [56]. Moreover, recent studies have observed that high doses of radiation (2 to 6 Gy) activate JAK/STAT survival pathway that may contribute to radioresistance during radiotherapy in human breast cancer and pulmonary adenocarcinoma cell lines [59, 60].
Therefore, to investigate the combined effect of radiation and iodine deficiency on thyroid cells and to investigate whether they increase the potential risk of thyroid cancer, the expression level of STAT-3 and pSTAT-3 (Tyr 705 phosphorylated) was evaluated 6 hours post co-treatment using western blot. As expected, the expression level of pSTAT-3/STAT 3 was elevated in a dose dependent manner that was significant at 3 Gy iodine deficiency. Although the effect of the co-treatment is not significant at 0.1 Gy, 0.5 Gy and at 3 Gy iodine conditions, there was an increase trend that need to be confirmed by repeating the experiment at different time points. Li and Yu research groups [59, 60] have observed the overexpression of STAT-3 at 12 hours and 24 hours post irradiation which can be applied in our experiment in the future. Although there is a little knowledge in literature studies about the role of iodine deficiency in JAK/STAT pathway activation, we observed that iodine deficiency when combined with a dose of radiation (3Gy) has a combined synergetic/additive negative effect on STAT-3 activation. This observation indicates that the stress of radiation or iodine deficiency alone is not enough to contribute to oncogenesis via STAT-3 activation, however their oncogenic potential has been observed when combined together.

We hypothesized that iodine deficiency and radiation especially at low doses have a synergetic or additive negative effect on thyroid cells through the production of a harmful level of ROS that would eventually lead to increased apoptosis/cell death and activation of precancerous pathways such as cell survival and proliferation pathways (NF-KB, PI3/AKT and JAK/STAT). These results support this hypothesis, however to further investigate the combined effect of both factors and to investigate the effect at low doses of radiation, further research is needed.

There are some limitation of this study which should be addressed in the future. First, the cell line being used. FRTL-5 is a continuous normal rat thyroid cell line and it is the most common cell line used in research studies that are focusing on thyroid hormone synthesis and production, response to TSH, iodine uptake and thyroid function in general [61, 64]. Since this research is investigating the pro-survival, proliferation and apoptosis pathway, this cell line may not be the best option. Although it would be better to use primary human cells for such a study, it is difficult to obtain such cells and they are not suitable for a large experiment set up due to their limited number of passages. Thyroid cells may be sensitive to tumorigenesis if they are exposed to radiation alone or in combination with iodine deficiency. However, a good in vitro model for investigating the response in thyroid cells to irradiation and iodine deficiency cells is not available. In addition, there are genetic defect in almost all human thyroid cell lines [39].
However, this aspect need to be addressed in the future. Another limitation of this study lies in the normalization method in western blot assay. Although it is recommended to normalize against the total protein by using a stain free gels [65], in this research, radiation induces upregulation/down regulation of different proteins, in addition high doses of radiation induce cell death and the total protein extracted in this case is from less number of cells. These factors may affect the result when we normalize the protein of interest against the total protein. Therefore, we normalized the protein of interest against housekeeping proteins (Vinculin or B-actin) after checking that radiation doesn’t have effect on them. Besides, normalization was done on the same membrane of the protein of interest to neglect pipetting errors.

The future perspectives of this study include further investigation of the pro-survival and pro/anti-apoptotic genes/proteins at different time points, investigating the effect on the gene level by performing microarray analysis to have a global view on the molecular mechanisms that are switched on/off. In addition, mTOR and MAPK/EKR signaling pathways promoting cellular growth and proliferation respectively, represent potential pathways that may be affected by the co-treatment and it is worth to investigate them in the future. Moreover, chromosomal rearrangement such as the RET/PTC which is expressed in 60% of thyroid cancer, should be checked in the co-treated cells in the future. Besides, to investigate in depth the effect of radiation, especially at low doses, repeated exposure to different doses of radiation together with multiple phases of iodine deficiency can be applied. Nonetheless, in order to confirm this research, in vivo animal models should be used.

In conclusion, these findings provide an overview on the changes occurring at the molecular level in thyroid cells following a combined treatment of radiation and iodine deficiency. The fact that thyroid cells exhibited a resistant profile to apoptosis after the co-treatment involving moderate and high doses of radiation implies that the remaining living cells may undergo malignant transformation in the future. Radiation and iodine deficiency induced ROS production, DNA damage, pro-survival and the proliferation pathways activation especially at high dose, however the effect of low doses and the synergetic/additive effect of them was not obvious in this study as the effect were investigated shortly after the co-treatment. However, it can't be excluded that the effect could be observed at later or earlier time points than the one used in this study. To be able to translate the data to the human situation, we would need to take this study further by the use of human primary epithelial thyroid cells. This research is important for radioprotection regulation, especially for those patients receiving radiation for therapeutic reasons.
References


Figure 1: AnnexinV/PI double staining. PCCL3 cells were double stained with AnnexinV/PI 48 hours post co-treatments. The co-treatment with irradiation and iodine deficiency induce apoptosis at 3 Gy I and ID, and induce late apoptosis/cell death at 0.5 Gy and 3 Gy I and ID. For each dose separately there is no significant difference between iodine and iodine deficiency. I: iodide, ID: iodine deficiency. Asterisk indicates significant difference ($p < 0.05$) when compared to control group.
**TNF-a 6hrs**

![Graph showing TNF-a levels after 6 hours exposure to different doses and conditions.](image1)

**TNF-a 24 hours**

![Graph showing TNF-a levels after 24 hours exposure to different doses and conditions.](image2)

**VGEF 6hours**

![Graph showing VGEF levels after 6 hours exposure to different doses and conditions.](image3)

- **Dose** represents the radiation exposure levels (Control, 0.1 Gy, 0.5 Gy, 3 Gy, LPS).
- **Iodine** indicates samples with iodine supplementation.
- **Iodine deficiency** indicates samples without iodine supplementation.

Statistical significance marked with *** for comparisons between groups.
**Figure 2: effect of the co-treatment on the secreted level of TNF-alpha and VGEF.** TNF-alpha and VGEF secreted levels were measured using luminx assay 6 hours and 24 hours post co-treatment. There was no significant difference between the conditions in both TNF-alpha and VGEF secreted levels 6 hours and 24 hours post co-treatment. LPS: Lipopolysaccharides.
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Richting: master in de biomedische wetenschappen-klinische moleculaire wetenschappen
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