Oral antioxidants for radioprotection during medical imaging examinations

by

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A thesis submitted in conformity with the requirements for the degree
Master of Science
Institute of Medical Science
University of Toronto

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Abstract
The oncogenic effect of ionizing radiation (IR) is clearly established and occurs in response to DNA damage. Many diagnostic imaging exams make use of IR and the oncogenic risk of IR-based imaging has been calculated. We hypothesized that the DNA damage sustained from IR exposure during medical imaging exams could be reduced by pre-medicating patients with antioxidants. First, we tested and validated a method for measuring DNA double-strand breaks (DSBs) in peripheral blood mononuclear cells (PBMCs) exposed to low doses of ionizing radiation. Afterwards, we conducted a pilot clinical study in which we administered oral antioxidants to patients undergoing bone scans, prior to radiotracer injection. We showed that oral antioxidant pre-medication reduced the number of DSBs in PBMCs induced by radiotracer injection. Our study shows proof-of-principle for this simple and inexpensive approach to radioprotection in the clinical setting.
Acknowledgments

Dr. Mikulis, thank you for your wisdom, guidance, and patience throughout my graduate studies. Your enthusiasm for science is infectious. I deeply admire your endless curiosity for all aspects of biology and medicine. I can only dream of having a sustained and distinguished academic career such as yours.

Dr. Bristow and Dr. Laposa, thank you for challenging me from the first slide of my first PAC meeting. We were privileged to have your combined expertise to guide us throughout this work. I must also extend my deep appreciation to you, Dr. Bristow, for welcoming me into your lab and allowing me to learn from your team.

Dr. Murphy, thank you for your strategic vision, ambition, and resourcefulness. Your energy has helped push this project through all of the road-bumps and above all of the hurdles.

Joe, you’ve made an immeasurable impact in what is still the fledgling part of my career. I can’t thank you enough for your academic mentorship and brotherly attention.

And finally, to my family, thank you for sharing your love, pressure, angst, and everything in between.
Contributions

Drs. David Mikulis, Joe Barfett, and Kieran Murphy conceived and initiated this research project in the University Health Network’s Joint Department of Medical Imaging. Drs. Robert Bristow and Rebecca Laposa provided scientific and methodological guidance throughout this research. Abby Skanda provided administrative support.

The STTARR program at MaRS provided laboratory space, equipment, and training for conducting our research. Deborah Scollard, Dr. Justin Grant, Salomeh Jelveh, and Dr. Patricia Lindsay must be especially acknowledged for their support with facility access, training, and dosimetry. Melanie Macasaet-Peralta from the Pathology Research Program provided laboratory services for immunofluorescence during our initial dose-response experiments. Judy Gabrys and Dr. Mihaela Ginj procured patient lists for study subject recruitment. The nuclear medicine technologists conducted blood draws for the patients.

In addition, the members of the Bristow lab provided invaluable technical knowledge and helped in the development of our full protocol, including laboratory technique, microscopy, image post-processing, and image analysis. We would like to extend our sincere gratitude to Ken, Gaetano, Jan, Daria, and Alice for their kind support.

Finally, we would like to thank the Peter Munk Cardiac Centre Foundation and the Society of Interventional Radiology Foundation for funding this work.
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<th>Description</th>
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<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>3-dimensional</td>
</tr>
<tr>
<td>53BP1</td>
<td>p53-binding protein 1</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-hydroxydeoxyguanosine</td>
</tr>
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<td>99m Tc</td>
<td>Technetium-99m</td>
</tr>
<tr>
<td>99m Tc-MDP</td>
<td>Technetium 99m Methylene diphosphonate</td>
</tr>
<tr>
<td>ALARA</td>
<td>As low as reasonably achievable</td>
</tr>
<tr>
<td>AOMF</td>
<td>Advances Optical Microscopy Facility</td>
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<tr>
<td>ATM</td>
<td>Ataxia telangiectasia</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl2-associated X protein</td>
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<td>BER</td>
<td>Base excision repair</td>
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<td>BRCA1</td>
<td>Breast cancer type 1 susceptibility protein (BRCA1)</td>
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<td>BRCA2</td>
<td>Breast cancer type 2 susceptibility protein (BRCA2)</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>cc</td>
<td>cubic centimetres</td>
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<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>CDKI</td>
<td>Cyclin-dependent kinase inhibitor</td>
</tr>
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</tr>
<tr>
<td>Chk2</td>
<td>Checkpoint kinase 2</td>
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<tr>
<td>CIHI</td>
<td>Canadian Institute of Health Information</td>
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<td>CT</td>
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<td>4',6-diamidino-2-phenylindole</td>
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<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase, catalytic subunit</td>
</tr>
<tr>
<td>DRF</td>
<td>Dose reduction factor</td>
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<td>DSB</td>
<td>double-strand break</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>eV</td>
<td>Electron Volt</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>grams</td>
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<tr>
<td>G1 phase</td>
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<tr>
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<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
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<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>hv</td>
<td>photon</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Hz</td>
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</tr>
<tr>
<td>IR</td>
<td>Ionizing Radiation</td>
</tr>
<tr>
<td>IRIF</td>
<td>Irradiation-induced foci</td>
</tr>
<tr>
<td>IRR</td>
<td>Incidence rate ratio</td>
</tr>
<tr>
<td>kVp</td>
<td>Kilovoltage peak</td>
</tr>
<tr>
<td>L</td>
<td>Liters</td>
</tr>
<tr>
<td>m</td>
<td>Metres</td>
</tr>
<tr>
<td>M</td>
<td>Moles per liter</td>
</tr>
<tr>
<td>M2M2</td>
<td>Mouse double minute 2</td>
</tr>
<tr>
<td>mA</td>
<td>milliAmperes</td>
</tr>
<tr>
<td>MBq</td>
<td>Megabecquerel</td>
</tr>
<tr>
<td>MIP</td>
<td>Maximum Intensity Projection</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MRN</td>
<td>MRE 11, RAD50, and NBS1</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetylcysteine</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NIR</td>
<td>non-irradiated</td>
</tr>
<tr>
<td>NNK</td>
<td>Nicotine-derived nitrosamine ketone</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation of Economic Co-operation and Development</td>
</tr>
<tr>
<td>OMEC</td>
<td>Oral mucosal epithelial cell</td>
</tr>
<tr>
<td>OTC</td>
<td>Over-the-counter</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP ribose polymerase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PNK/PNP</td>
<td>Polynucleotide kinase/phosphatase</td>
</tr>
<tr>
<td>PRP</td>
<td>Pathology Research Program</td>
</tr>
<tr>
<td>Pt</td>
<td>Patient</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>REB</td>
<td>Research Ethics Board</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication Protein A</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>S phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>SD; St. Dev.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
</tr>
<tr>
<td>SSB</td>
<td>single-strand break</td>
</tr>
<tr>
<td>SSBR</td>
<td>Single-strand break repair</td>
</tr>
<tr>
<td>Sv</td>
<td>Sievert</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TMDT</td>
<td>Toronto Medical Discovery Tower</td>
</tr>
<tr>
<td>UHN</td>
<td>University Health Network</td>
</tr>
<tr>
<td>× g</td>
<td>times the force of gravity</td>
</tr>
<tr>
<td>XLF</td>
<td>XRCC4-like factor</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray cross-complementing protein 1</td>
</tr>
<tr>
<td>XRCC4</td>
<td>X-ray cross-complementing protein 4</td>
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Chapter 1 – Introduction

1.1 Defining Ionizing Radiation

Ionizing radiation (IR) is defined as a beam consisting of particles or photons that have sufficient energy to eject electrons from molecules, thus temporarily turning a stable molecule into an ion. IR can consist of high energy particles or photons. For the scope of this thesis, we will primarily discuss IR in the form of high energy photons on the electromagnetic spectrum. There are differences between the way photons and heavy particles interact with biological tissues. While the latter form of IR is still of considerable interest in healthcare, it is more relevant to the fields of radiation oncology and aerospace medicine. But high energy photons are the primary form of IR used in diagnostic medical imaging.

The types of IR found on the electromagnetic spectrum are commonly referred to as x-rays and γ rays. X-rays are produced from the collision of electrons with an atomic nucleus. As the electrons lose kinetic energy from the collision, x-rays are released in accordance with conservation of energy principle. On the other hand, γ rays are created when an atomic nucleus transitions from a high energy state to a low energy state. The process is called γ decay.

Currently, there is no consensus on the physical differences between x-rays and γ rays; they simply differ by origin. In the past, x-rays were thought to categorically have longer wavelengths and lower energy than γ rays. However, since the development of linear accelerators that can produce x-rays with energy as high as 4 MeV and the discovery of radioisotopes like technetium-99m which produce γ rays with energy as low as 140 keV, there is now an overlap in terms of their properties.
Even though low levels of IR cannot be seen or felt, it is now known that we are constantly exposed to IR from our surroundings—both from Radon in the Earth and cosmic rays from outer space. Over 100 years of research has shown that IR can produce significant changes in biological tissues that can manifest as disease anywhere from days to decades after exposure. Despite these risks, ever since its serendipitous discovery in the late 19th century, the medical community has been using IR to diagnose and treat disease.
1.2 Use of Ionizing Radiation in Medical Imaging

1.2.1. Modern x-ray based medical imaging modalities

In 1895, a German physicist named Wilhelm Roentgen observed that his work with electrical discharges in vacuum tubes produced invisible emissions that were capable of penetrating solid materials. He referred to these emissions as “X-Rays.” During his experiments with the x-rays, he found that they had varying levels of penetration through different materials and he was able to capture these differences on a photographic film. On December 22nd, 1895, he used his discovery to take the world’s first radiograph—an image of his wife’s hand that showed its underlying skeletal structure, including the separations between her metacarpals and proximal phalanges. For the first time, internal anatomy, defects, and foreign objects could be observed without performing an incision. This was the birth of modern medical imaging. Roentgen’s technology has since evolved and developed into different imaging modalities based on similar x-ray production techniques.

*X-ray Planar Radiography*

Planar radiography is one of the most common imaging modalities used in clinical settings. It is often employed as a screening tool to assess for acute injuries and possible chronic diseases. The image is based on the differential absorption of x-rays by various tissues. With modern x-ray technology, the anatomical region of interest is placed in between the x-ray source and a solid-state flat panel detector. The x-rays that pass through the patient’s body are detected and converted into a digital two-dimensional projection. Since bone and calcifications attenuate x-rays more than soft tissue, these regions will appear as bright regions on the final image; soft-tissue does not absorb x-rays as much and appears dark.
The modern vacuum tube used for x-ray production consists of a negatively charged cathode as an electron source and a thin tungsten wire, heated to 2200°C, as the metal target. A positive voltage is applied to the metal target, creating a potential difference between the cathode and the anode ranging from 25 kV to 140 kV. This voltage is referred to as the accelerating potential or kilovoltage peak (kVp). A lower kVp electron beam produces photons with less energy after electron-metal collision, thereby resulting in a lower dose of radiation. Twenty-five kV is used for digital mammography whereas 140 kV is used for evaluating bone and the chest. The current of the vacuum tube can also be modulated. It typically ranges between 50 and 400 mA for planar radiography.

Digital Mammography

This variant of radiography makes slight modifications to the equipment used in traditional x-ray planar radiography in order to minimize the radiation dose to the breast while maintaining the requisite image quality for detecting small tumours and microcalcifications. The anode target is made of molybdenum, which allows for the release of photons with lower energy than tungsten. The cathode filament is flat, instead of helical, in order to produce a more focused electron beam. Also, the window of the x-ray tube is made of beryllium instead of glass in order to ensure that low energy X-rays are not filtered out. The addition of a molybdenum or aluminum filter is used to reduce the number of high energy photons (>20 keV) that the patient is exposed to, thereby reducing the radiation dose.

Digital Fluoroscopy

Fluoroscopy uses “continuous” x-ray imaging to obtain images in real-time. X-ray pulses are 5-20 ms in duration and images are acquired at a frequency of up to 30 frames per second. In
practice, this is used during interventional procedures in order to guide the placement of catheters, stents, coils, pacemakers, etc. It can also be used in conjunction with flowing contrast agents for dynamic studies of the gastrointestinal tract or cardiovascular system. Computed Tomography

Computed Tomography (CT) was invented in 1972 by Sir Godfrey Hounsfield and Allan Cormack. With this imaging modality, the patient is situated inside of a rotating circle, called the gantry, which contains an x-ray source and detectors. The gantry rotates around the patient while irradiating the patient to acquire data continuously. One-dimensional projections from all angles in a complete revolution around the patient can be reconstructed to form a two-dimensional (2D) slice. However, since one 2D axial slice of the patient’s anatomy is of limited diagnostic value, multiple sequential slices throughout an anatomical region are typically acquired. Alternatively, a helical volume can be obtained by moving the patient along the head-foot axis while the gantry rotates. Reconstruction can be done in either the x, y, or z plane to allow the radiologist to view contiguous slices of the anatomy in either the axial, coronal, or sagittal planes.

1.2.2. Modern gamma-ray based imaging modalities

In 1896, one year after the discovery of the x-ray, a French scientist named Henri Becquerel followed up on Roentgen’s work with the use of phosphorescent salts. He theorized that phosphorescent materials could absorb sunlight and subsequently generate x-rays. Surprisingly, he observed that the uranium salt was able to leave a distinct imprint on photographic plates even without any prior exposure to sunlight. Pierre and Marie Curie expanded upon Becquerel’s observation to determine that the atomic nucleus of certain elements such as Uranium, Thorium,
Radium, and Polonium naturally produced ionizing radiation; they called this phenomenon “radioactivity.”  

Although Becquerel and the Curies were jointly awarded a Nobel Prize in 1903 for their discovery of radioactivity, it took nearly 40 years to establish nuclear medicine as a medical specialty and create radionuclides that could be safely used in medical care. Technetium-99m, the most commonly used radionuclide today, was developed in 1937\(^\text{15}\) and nuclear medicine’s most public breakthrough occurred in 1946 when Iodine-131 was successfully used to treat thyroid cancer\(^\text{16}\).

Today, radioactive molecules are commonly used both diagnostically and therapeutically. We will now briefly examine some of the most common diagnostic modalities in nuclear medicine.

*Planar Scintigraphy*

Analogous to x-ray planar radiography, this is the most basic nuclear medicine imaging modality. A radioactive material is injected into a patient’s blood circulation and it distributes throughout the body. A typical radionuclide used for this type of scan is Technetium-99m, which emits gamma-rays at 140 keV. The gamma-rays are detected and the final result is a two-dimensional image which displays the distribution of radionuclide throughout the body\(^\text{9}\).

*SPECT*

Single Photon Emission Computed Tomography (SPECT) produces a series of consecutive two-dimensional images that represent the distribution of radiotracer throughout an organ or the body. Just as a planar scintigraph can be compared to an x-ray radiograph, SPECT can be compared to CT. Instead of imaging from one angle, the gamma camera captures the gamma ray
energy signal from all angles around the patient in order to reconstruct two-dimensional slices from one-dimensional projections\textsuperscript{9}.

**PET**

Positron Emission Tomography (PET) involves injecting a radiotracer that emits positrons, the antiparticles of electrons. When positrons interact with electrons, an annihilation event takes place, resulting in the emission of 511 keV gamma rays. These gamma rays are captured and three-dimensional images can be reconstructed\textsuperscript{9}. Currently, PET and SPECT machines are being combined with traditional CT scanners so that the new hybrid scanners can obtain functional information from the nuclear medicine component and morphological information from the CT component in quick succession\textsuperscript{17, 18}.

1.2.3. Medical Imaging Utilization Statistics

Four million four hundred thousand CT examinations were conducted in Canada during the most recently surveyed 12 month period, spanning 2011 to 2012\textsuperscript{19}. The data shows a 58% increase in the total number of CT scans over the preceding 9 years, which appears to be closely linked with a 57% increase in the total number of CT scanners available over that same time period\textsuperscript{20}. This trend illustrates a sharp rise in the number of CT scans conducted in Canada over the past decade.

When considering usage statistics in USA, the trend is even more striking. While the frequency of scans for Canadians is 126 for every 1000 people nationwide, USA conducts 265 scans per 1000 population\textsuperscript{21}. But high CT utilization is not specific to North America. Across 16 out of 34 countries in the Organisation of Economic Co-operation and Development (OECD) for which national data is available, the average CT scan frequency is 134 per 1000 population\textsuperscript{21}.
Medical imaging accounts for half of all radiation exposure in USA, and half of that medical imaging related radiation exposure is a result of CT scanning\textsuperscript{22}. Although x-rays are performed more frequently, with estimates higher than 300 million medical x-rays per year in USA (assuming the current usage rate per population is still similar to 2000)\textsuperscript{23}, radiation doses are low. A single intraoral radiograph only delivers an average effective dose of 0.005 mSv, which is equivalent to approximately one day of natural background radiation that the average American is exposed to; and an x-ray of the extremity only delivers 0.001 mSv, equivalent to just a few hours of background radiation\textsuperscript{24}. On the other hand, nuclear medicine examinations can deliver a much higher dose per scan—for example, a cardiac stress-rest test with thallium 201 chloride delivers 40.7 mSv\textsuperscript{25}. However, nuclear medicine scans are conducted much less frequently than CT (only 17 million per year in USA\textsuperscript{26} vs 85 million CTs\textsuperscript{21}).
1.3 Hazards of Ionizing Radiation

Although the use of IR in medical imaging has vastly improved the quality and delivery of healthcare, it is not completely harmless to the patient. In this section we will examine how IR can damage sub-cellular structures and cause long-term effects in patient.

1.3.1. Mechanism of Injury

There are three main mechanisms that photons initiate to cause damage to biological molecules. At energies below 0.1 MeV, the photoelectric process is dominant\textsuperscript{27}. A photon imparts its energy into an electron and ejects it from the atom it was bound to. The incident photon is extinguished during this collision and the electron takes on most of the energy as kinetic energy.

At medium photon energies, between 0.5 MeV and 3.5 MeV, Compton scattering dominates\textsuperscript{27}. In this process, the incoming photon’s energy is much greater than the electron-binding energy of the target atom. Therefore, rather than being extinguished, the photon scatters with some energy intact and can undergo further Compton scattering until the energy is low enough that the photoelectric process occurs. At high energies above 1.02 MeV, pair production also occurs\textsuperscript{28}. In this process, a photon impacts the nucleus of an atom and its energy is converted into a positron and an electron. These two oppositely charged particles can go on to ionize other molecules but the positron ultimately interacts with an electron, resulting in an annihilation event. The particles are extinguished and two 0.51 MeV photons are emitted, which can go on to produce further ionizations through Compton scattering and the photoelectric process as described above.

When IR enters tissues or cells, there is a possibility that one of the aforementioned events will be initiated directly through an interaction between a photon and the atom of a critical biological
structure. This type of damage would be classified as a direct action of radiation. Direct actions are not common with photon-based IR. They only represent approximately 30% of the damage that occurs to DNA from x-ray and γ-ray radiation \(^{29}\). Before the photon can come into contact with a biological structure, it is likely to first ionize a water molecule since 70% of a cell is composed of water \(^{30}\).

When a photon (denoted as \(h\nu\)) ionizes a water molecule, the following reaction occurs:

\[
H_2O + h\nu \rightarrow H_2O^+ + e^-
\]

The \(H_2O^+\) molecule produced from the primary photon interaction is an ion radical. It is a charged ion, due to the loss of an electron; and it is a radical, due to its unpaired electron. Primary ion radicals are extremely reactive and have a very short lifetime, on the order of \(10^{-10}\) seconds \(^{1}\). Hence, the \(H_2O^+\) molecule almost immediately interacts with another water molecule in the following reaction:

\[
H_2O^+ + H_2O \rightarrow H_3O^+ + OH^-
\]

This reaction produces the hydroxyl radical (OH\(^-\)). The hydroxyl radical is not charged but it has an unpaired electron, which makes it highly reactive. It has an intracellular lifetime of approximately \(10^{-9}\) seconds \(^{1}\), which is one order of magnitude longer than the primary ion radical. In this time-span, the hydroxyl radical can diffuse across short distances. If a hydroxyl radical is formed through this mechanism within approximately 1 nm of a biological structure, it can ionize, radicalize, or break bonds within that target.

There is another set of chemical reactions that can result in indirect actions on biological structures, but without the primary ionization event occurring within 1 nm of the target. It
involves the creation of peroxides, which are stable precursors to hydroxyl radicals and are diffusible across longer distances within the cell.

The first step of the reaction is initiated the same way:

$$H_2O + h\nu \rightarrow H_2O^+ + e^-$$

However, the ejected electron reduces a nearby water molecule in the following step:

$$H_2O + e^- \rightarrow H_2O^-$$

Then, two hydroxyl radicals are formed independently in the following reactions:

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH \cdot$$

$$H_2O^- + H_2O \rightarrow H_3O^- + OH \cdot$$

Finally, the two hydroxyl radicals combine to form hydrogen peroxide:

$$2OH \cdot \rightarrow H_2O_2$$

Hydrogen peroxide can be a powerful oxidizing agent. But when it reacts with metal ions, it can become even more dangerous to biological structures by forming a hydroxyl radical through the Fenton reaction, which is shown below:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH \cdot + OH^-$$

Now that we understand how an incident photon from an x-ray or γ-ray source interacts with biological tissues to ionize structures or create hazardous free radicals, we can begin to explore why this process leads to deleterious effects. For this examination, the DNA molecule is the most important target to consider.
1.3.2. DNA Damage

Studies have shown that DNA is the primary target for radiation-induced cell death. An experiment with a small polonium-needle that produced short-range alpha particles showed that high doses of radiation could be delivered to the plasma membrane and cytoplasm while maintaining viability of the cell, but even a few alpha particles delivered to the nucleus caused cell death. In addition, an experiment comparing nucleus, cytoplasm, cell membrane, and whole cell irradiation using β-, x-, and γ-rays showed that cell death only correlated with dose to the nucleus.

Evidently, DNA is the most important biological molecule in the cell for sustaining life. In order to understand how free radical attack leads to the different types of DNA damage, we must first understand its molecular structure.

DNA is a large molecule composed of two strands. The backbone of each strand consists of an alternating pattern of deoxyribose (sugar) and phosphate groups. Attached to each sugar molecule is one of four nitrogenous bases: adenine, thymine, cytosine, and guanine. The order of the bases defines the genetic code of the cell. Each base has a complementary base that it pairs with on the opposite strand; adenine pairs with thymine and guanine pairs with cytosine. The two strands are held together with hydrogen bonding between the complementary bases. The strands are arranged in a “double helix” shape and the diameter of the DNA molecule is approximately 2 nm. With this structure in mind, the four major categories of damage that occur to the DNA molecule are as follows: base damages, crosslinks, single-strand breaks (SSBs), double-strand breaks (DSBs).
A base damage occurs when a hydroxyl radical reacts with the electron-rich double bond of a nitrogenous base. Typically, the hydroxyl addition does not result in a strand break. However, the structure of the base is still altered\textsuperscript{33}.

The second major category of DNA damage, crosslinks, comes in two forms: DNA-DNA crosslinks and DNA-protein crosslinks. Furthermore, DNA-DNA crosslinks can be subdivided into intrastrand crosslinks and interstrand crosslinks\textsuperscript{34}. DNA relies on its precise two-strand double helix structure for normal function. A disruption of this structure due to non-complementary nucleotides forming covalent bonds with each other, or one strand forming a kink by binding to itself, can prevent DNA replication or transcription. DNA-protein crosslinks occur when a protein is added to a nucleotide\textsuperscript{35}.

SSBs arise from damage to the deoxyribose component of the DNA backbone. A hydroxyl radical can react with the hydrogen atoms of the sugar molecule. This destabilizes the structure of the backbone, results in a strand breakage, and releases a nitrogenous base.

When a ray of IR photons deposits energy into biological tissues, it is not done uniformly. Rather, the energy is localized along the tracks of the ejected electrons. Ninety-five percent of the energy deposition tracks from x-rays and γ-rays are classified as “spurs.” Each spur consists of a 4 nm diameter cluster of 3 ion pairs and contains up to 100 eV of energy. Less frequently, a deposition event will result in a “blob.” Blobs are 7 nm in diameter, consist of 12 ion pairs, and contain 100-500 eV of energy. Given the similarity in diameter between these clusters and the DNA molecule, energy deposition events that take place near DNA can often result in multiple SSBs to DNA in nearby locations. When this occurs on opposite strands, a DSB arises.
1.3.3. DNA Damage Response

Due to the importance of the DNA molecule for maintaining the life of the cell and guiding cellular operations, cells have developed a series of complex pathways for responding to injury. The DNA damage response (DDR) varies depending on the type and level of damage that is sustained. There are three broad categories of DDR pathways, listed as follows: programmed cell death, cell cycle blockade, and DNA repair \(^{36}\).

It is unlikely that minor damage to the genome leads to either of the former two pathways. That would be incompatible with long-term sustained life, since it is estimated that cells naturally undergo tens of thousands of base damages and single strand breaks per day without any exposure to ionizing radiation \(^{37}\). Instead, the cell uses repair mechanisms such as Base Excision Repair (BER), Single Strand Break Repair (SSBR), Mismatch Repair (MMR), and Nucleotide Excision Repair (NER) to repair these small injuries.

In BER, a glycosylase removes the damaged base and APE1 (apurininc/apyrimidinic endonuclease 1) removes the sugar residue. Afterwards, DNA polymerase β inserts the correct replacement base and repair is completed by DNA ligase III-XRCC1-mediated ligation (XRCC1 is X-ray Cross-complementing protein 1) \(^{38}\).

SSBR involves Poly ADP ribose polymerase (PARP) for break detection. Polynucleotide kinase/phosphatase (PNKP) then processes the ends of the break prior to base replacement and strand ligation occurs in a manner similar to BER \(^{39}\).

MMR helps fix incorrect nucleotide base pairings and NER resolves bulky adducts such as pyrimidine dimers. However, since neither of these pathways is used for repairing damage from
ionizing radiation, they will not be further explored in this thesis. Knockout experiments involving MMR- and NER-deficient cells have not shown any increase in radiosensitivity.

The DDR and ensuing pathways following DSBs are the most pertinent to our research, due to the long-term carcinogenic potential of DSBs. However, before studying the pathways, we must examine the initial cellular response to this type of DNA damage. After a DSB occurs, various proteins are recruited into a cluster at the site of the break. These proteins can be microscopically visualized as a “focus.” Each focus is the signalling platform for initiating a DDR pathway.

One of the first protein responses following a DSB involves the ataxia telangiectasia mutated (ATM) protein and the MRN protein complex—MRN consists of MRE11, RAD50, and NBS1. MRN binds to ATM and relocates it to the DSB, where ATM can phosphorylate the H2AX histone that is closest to the site of the break. H2AX is a variant of histone H2A, which is one of the five major histones found in DNA. Histones are one of the core structures of the nucleosome. When H2AX is phosphorylated, it is referred to as γH2AX. In cells that lack ATM, H2AX phosphorylation can still take place. The DSB is sensed by the Ku70-Ku80 protein complex and a kinase called DNA-PKcs (DNA-dependent protein kinase, catalytic subunit) phosphorylates H2AX.

Although H2AX phosphorylation is one of the earliest steps of the DSB DDR, as many as 700 proteins can be targets for kinase activity following DNA damage. The phosphorylation of these other proteins acts as the signaling pathway for DDR outcomes. For this reason, the ATM kinase is known to be the most important regulator in the DDR. The figure below (Figure 1), adapted from a 2008 review exploring γH2AX’s role in cancer, outlines some of the key proteins involved in this process.
Red boxes highlight two of the main proteins of interest in our research, γH2AX and 53BP1. Their roles in the DNA damage response are described in sections 1.3.3 and 1.5. This figure is adapted from Bonner’s 2008 review, “γH2AX and cancer.”

**Apoptosis**

Apoptosis is an important anti-tumorigenic mechanism. In cells that have sustained significant DNA damage, dangerous mutations may arise. It is more beneficial for the organism if these cells are eliminated, rather than repaired improperly and allowed to proliferate. In a normal unstressed cell, proteins p53 and MDM2 (mouse double minute 2) are bound to each other.
MDM2 ubiquitinites p53 and tags it for destruction by proteasomes. Thus, although p53 is constantly produced, it is also rapidly destroyed, which means it is inactive in a healthy cell\(^4^3\).

After DNA damage, ATM phosphorylates both MDM2 and p53, causing them to disassociate\(^4^4\). P53 is no longer tagged for degradation and in its phosphorylated state, it becomes a transcription factor for pro-apoptotic genes such as BAX (Bcl2-associated X protein) and PUMA (p53 upregulated modulator of apoptosis). P53 can also regulate the cell cycle checkpoint through the p21 gene\(^4^5\).

**Checkpoint Activation**

There are several checkpoints throughout the cell-cycle. These are stages at which progression into the next phase of the cell cycle can be prevented or slowed down, in order to allow the cell to repair any damage. The checkpoints are controlled by a set of proteins called cyclin-dependent kinases (CDKs). CDKs phosphorylate other proteins in order to allow normal progression through the cell cycle. As their name implies, they require an association with a specific cyclin in order to be active\(^3^6\).

For a checkpoint to be activated, the cyclin-CDK complex needs to be de-activated. This can be achieved by activating/transcribing CDK inhibitors (CDKIs) or by changing the phosphorylation status of the CDK. There is one checkpoint in Growth 1 phase (G1), one in Synthesis phase (S phase), and two in Growth 2 phase (G2 early and G2 late). The G1 checkpoint is activated by the production of CDKIs whereas the G1, S, and G2 checkpoints are activated by the phosphorylation of Chk1/2 proteins, which go on to phosphorylate the phosphatase inhibitors (CDC25A/C) and prevent them from maintaining the activated state of CDKs\(^3^6\).
DSB Repair

Ideally, cells with DSBs repair all damage and restore both DNA strands back to their original state. The repair process called “homologous recombination” (HR) attempts to do this in an error-free fashion.  

One of the first steps of HR involves resection of both sides of the DSB by MRE11 and other endonucleases to create 3’ DNA single strands on both sides. Then, replication protein A (RPA) coats the single strands to prevent them from binding to themselves or forming secondary structures. As mentioned earlier, ATM phosphorylates many proteins during DSB recognition. One of these is breast cancer type 1 susceptibility protein (BRCA1). After it is phosphorylated, it is recruited to the site of the break and it attracts breast cancer type 2 susceptibility protein (BRCA2). BRCA2 loads Rad51 on to the RPA-coated single-strands. Subsequently, Rad52 is recruited to protect against degradation and Rad54 is recruited to unwind the double-stranded DNA molecule. The two 3’ prime ends can now invade an undamaged sister chromatid, with the assistance of helicases, so that DNA polymerases can synthesize nucleotides around the break-site using an accurate template. The whole process takes approximately 6 hours to complete.

Non-homologous end joining (NHEJ) is another process by which cells can repair DSBs. This does not require a homologous DNA sequence as a template and it can be completed faster than HR. However, NHEJ is less accurate—it can lead to nucleotide deletions and insertions. Although this poses a risk for mutations to arise and persist, it allows the cell to survive; DSBs are often lethal if they are left unrepaired.

NHEJ begins with the recruitment of Ku70 and Ku80 to the site of the break. This preserves the ends of the DNA strands from degradation and also helps recruit DNA-PKcs to the site. DNA-
PKcs forms a physical bridge between both ends of the DNA and keeps them close together to allow repair to take place. In addition, it recruits Artemis to the site and phosphorylates it to stimulate nuclease activity. PNK is another protein that is recruited during NHEJ to help process the ends of DNA strands to prepare them for ligation. If there are missing nucleotides, they can be filled by polymerases such as Polλ, Polμ, and TdT. Finally, ligase IV, XRCC4, and XLF (XRCC4-like factor) work to ligate both ends of the DNA and complete repair. This process is briefly outlined in the figure below (Figure 2).

---

**Figure 2** – **Brief visual schematic of non-homologous end joining.** Figure is adapted from Figure 2.8 in Basic and Clinical Radiobiology, 4th Ed.
Although HR is a more accurate method for repairing DSBs, it requires the presence of a homologous template. Thus, it is not always feasible for the cell to rely on HR for DNA repair. For example, in G1 phase, the homologous chromosome is too far away for DNA repair proteins to detect and use. However, in S phase and G2 phase, the sister chromatid is in close proximity and provides a homologous template for DNA repair. Therefore, NHEJ is the dominant repair process during G1 and HR is the dominant process during S and G2. In cells that are slowly-dividing or non-dividing, NHEJ is the primary repair mechanism for DSBs

Depending on the number of cells that sustain DNA damage and the post-damage pathways that are activated, the organism can experience a wide variety of deleterious effects. In the next section, we will examine some of these effects.

1.3.4. Short-term effects

The short term effects of high radiation doses are considered deterministic. In other words, all subjects who are exposed to a certain threshold dose across a specific body region can be expected to experience symptoms that increase in severity with increasing dose.

*Acute Radiation Syndrome*

High doses of whole-body radiation are lethal. At extremely high doses, above 100 Gy, death occurs 24 to 48 hours after exposure due to cerebrovascular and cardiovascular breakdown. In the range between 5 and 12 Gy, death occurs nine to ten days after exposure due to destruction of the gastrointestinal mucosa. At doses between 2.5 and 5 Gy, death can occur anywhere from a few weeks to two months after exposure due to bone marrow failure. For humans, the lethal dose for 50 percent of a population in 60 days with whole body radiation, without any medical care, is approximately 4 Gy.
Acute Radiation Syndrome is not a realistic short-term risk from medical imaging. The above dose ranges are several orders of magnitude beyond what patients are typically exposed to during diagnostic and interventional imaging examinations. Even when doses approach this range in healthcare, they are seldom used in whole-body radiation. For example, during radiation oncology treatments, high-dose radiation is precisely localized to the site of the tumour and only a small number of healthy cells are exposed 48.

_Cutaneous Radiation Injury_

Prolonged radiation exposure or a high dose of radiation to a single anatomical region can lead to acute skin injuries that extend into the subcutaneous muscle and fat. Although major radiation injury is estimated to only occur at a rate between 1/10,000 and 1/100,000 during fluoroscopically guided interventional procedures, this risk can be considerably higher in obese patients since their bodies are in closer proximity to the x-ray source (dose varies with the square of distance) 49. Absorbed dose at the skin entrance site can be 10 times higher for obese patients 50.

Certain complex procedures require more time under fluoroscopy than others. The skin entrance dose threshold for experiencing acute effects from radiation is 2 Gy 51. At this threshold, patients experience transient erythema due to increased capillary permeability and proteolytic enzyme release within 48 hours. As recently as 2008, a major academic health centre in the UK reported that over one-third of their patients undergoing endovascular aneurysm repairs exceeded this threshold 52.
1.3.5. Long-term effects

Although some symptoms of high-dose radiation exposure can present as late as two months after exposure, these symptoms are still deterministic. The severity of these symptoms increases with greater doses of radiation. In addition, these symptoms are often preceded by prodromal and latent phases. Thus, they are part of the progression of the initial short-term deterministic effects.

When we refer to long-term effects of radiation exposure, we are primarily referring to stochastic effects that do not have a minimum dose threshold and do not increase in severity with dose; only the probability of an occurrence increases with dose. To clarify, a long-term stochastic symptom caused by 1 Gy of radiation will not necessarily be worse than one caused by 0.1 Gy of radiation. There is simply a higher chance that the symptom will occur when a subject is exposed to 1 Gy of radiation.

Carcinogenesis

The main long-term effect of radiation exposure is cancer. Radiation-induced cancer has been observed ever since the discovery of IR at the turn of the 20th century. Skin cancer and leukemia incidence was high in physicists, engineers, and radiologists who worked with x-rays before radiation safety standards were established. Thomas Edison’s assistant was the first American to die of radiation-induced cancer; he passed away in 1904. It is presumed that both Marie Curie and her daughter Irene Curie died of leukemia caused by their exposure to radiation during their experimental research.

Since these early anecdotal observations, the link between medical radiation exposure and the long-term development of cancer has been well-established. There was an elevated incidence of
leukemia among radiologists who began their practice before the introduction of radiation safety guidelines in 1922. In addition, thyroid and breast cancer rates were elevated in patients who received radiotherapy during infancy for an enlarged thymus between the years 1926 and 1957. These patients, referred to as the “Hempelmann cohort,” were followed up with as recently as 2010. The thyroid cancer incidence rate ratio between irradiated subjects and non-irradiated controls (their siblings) was 6.6.

Increased cancer incidence was also seen in children who received radiotherapy for tinea capitis, ringworm of the scalp, during the 1950s. Twenty thousand children were treated in Israel, which later led to increased incidence of brain, skin, and thyroid cancer as well as leukemia.

Radiation-induced carcinogenesis typically has a long latent period. Leukemia develops the most quickly, approximately 5 to 7 years after exposure. Solid tumours take anywhere between 10 and 60 years to develop.

Carcinogenesis occurs due to a mutation in a proto-oncogene, a tumor suppressor gene, or a DNA stability gene. This causes a disruption in cellular regulatory mechanisms for proliferation, self-elimination, and immortalization. Radiation-induced mutations arise from DNA damage that is mis-repaired. The most mutagenic type of DNA lesion, as previously mentioned, is a DSB. Although homologous recombination repairs DSBs in an error-free manner, non-homologous end-joining is error-prone and can result in mutations. Analysis of chromosomal translocations in acute myeloid leukemia and lymphoid leukemia shows DNA sequence homologies specific to NHEJ at the translocation breakpoint junctions, suggesting that NHEJ is responsible for ligation at the locus. This phenomenon was also observed in mixed-lineage leukemia translocation site. Researchers showed that inhibiting DNA-PKcs, and therefore NHEJ, eliminated mixed-lineage
leukemia translocations. Furthermore, sequencing of 120 acute promyelocytic leukemia patients showed homologous sequences indicated of NHEJ repair in the majority of breakpoint junctions. All of this evidence points towards NHEJ being implicated in leukemia carcinogenesis.

1.3.5.1. Risk Estimates from Atomic Bomb Survivors

Epidemiologists have attempted to relate the risk of disease—and specifically cancer—to different levels and patterns of radiation exposure. The most-studied data set consists of the 120 000 survivors of the atomic bombings in Hiroshima and Nagasaki, Japan. The subjects are an ideal source of data for determining estimates of risk from radiation exposure because they were not pre-selected for disease, occupation, sex, or age. In addition, they had a 50 year follow-up period and the Japanese family registration system ensured that mortality data was recorded for all subjects who remained in the country.

The radiation dose that each subject received was determined by their distance from the hypocenter of the bomb at the time of bombing. Dose to the colon was estimated for 86 572 subjects and the population-dose distribution is as follows in table 1 below:

<table>
<thead>
<tr>
<th>Dose (Sv)</th>
<th># of subjects</th>
<th>% of subjects (out of 86 572)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.005</td>
<td>37 458</td>
<td>43.27%</td>
</tr>
<tr>
<td>0.005-0.1</td>
<td>31 650</td>
<td>36.56%</td>
</tr>
<tr>
<td>0.1-0.2</td>
<td>5732</td>
<td>6.62%</td>
</tr>
<tr>
<td>0.2-0.5</td>
<td>6332</td>
<td>7.31%</td>
</tr>
<tr>
<td>0.5-1.0</td>
<td>3299</td>
<td>3.81%</td>
</tr>
<tr>
<td>1.0-2.0</td>
<td>1613</td>
<td>1.86%</td>
</tr>
<tr>
<td>&gt;2.0</td>
<td>488</td>
<td>0.56%</td>
</tr>
</tbody>
</table>

Table 1 – Distribution of radiation doses to atomic bomb survivors at the time of bombing. Effective dose to the colon was estimated for approximately 85 000 atomic bomb survivors and study subjects were stratified by dose range. Data is taken from the BEIR VII: Phase 2 report, which was based off of data from Preston et al.
Ninety-four percent of the subjects were exposed to doses under 500 mSv, which is the threshold for deterministic effects of radiation exposure to develop in most organs. Eighty percent were exposed to a dose range that can be compared to modern medical imaging examinations (ie, up to 100 mSv). Forty-three percent were exposed to a dose that can be compared to a modern CT scan in one anatomical region.\textsuperscript{25}

Leukemia was the first cancer that was shown to be linked with radiation exposure in the atomic bomb survivors.\textsuperscript{70} Of the 49 114 people exposed to greater than 5 mSv of radiation, 176 died of leukemia between 1950 and 1990. It is estimated that 78 of those deaths can be attributed to radiation exposure, with the risk of leukemia increasing with radiation dose. This translates to 44\% of leukemia-deaths in that group being caused by radiation.\textsuperscript{71}

There were 5502 solid cancer deaths between 1950 and 1997 in the same radiation dose group (those exposed to >0.005 Sv), but only 8\% of those deaths were attributed to radiation.\textsuperscript{69} Cancer incidence risk between 1958 and 1987 was studied, and 11\% of the solid cancer incidence was attributed to A-bomb radiation exposure.\textsuperscript{72} A dose-response trend was observed from 0 to 3 Sv and estimates based on only the low-dose portion of solid cancer mortality data showed a dose-response from 0 to 0.125 Sv.

Further extensive analysis was done on the health outcomes of the A-bomb survivors, exploring differences between sites of cancer, age of radiation exposure, and even incidence of non-neoplastic disease. However, that level of detail is not needed to understand that the people who were exposed to radiation had a higher incidence of cancer, and that this risk also applied to those who were exposed to low radiation doses.
After combining this data and the observations from basic radiobiological studies, the linear no-threshold model has become the most widely accepted model for estimating the stochastic effects of radiation exposure at low doses. Most scientific organizations agree that there is no dose at which radiation exposure can be considered completely harmless, and that the risk of disease increases linearly with dose\textsuperscript{68}.

1.3.5.2 Estimates in Medical Imaging

Due to its ubiquitous use, the sensitive patient population it is used in, and the significant effective radiation dose delivered to the patient, CT frequency has become the primary measure for studying trends in medical radiation exposure and its associated cancer risks. In addition, most of the evidence regarding the long-term effects of low-dose radiation exposure is derived from following health outcomes of the atomic bomb survivors in Japan. The short-term external radiation that they were exposed to most closely resembles a whole-body CT when compared to all of the medical imaging modalities.

Based on the linear no-threshold model and the health outcomes of atomic bomb survivors, researchers have been able to model the estimated risk for diagnostic medical imaging exams. For example, in 2007 Brenner estimated that receiving an abdominal CT at age 15 has a lifetime attributable cancer mortality risk of 0.07% and the cancer mortality risk for a head CT at the same age is 0.015%\textsuperscript{73}. Risk drops precipitously with increased age, so the risk of each individual CT scan for adults is even lower, but when tallied across all patients receiving CT scans annually, radiation exposure from medical imaging poses a significant population health issue. Based on the total number of CT exams being conducted annually and the dose of each scan,
Brenner’s 2007 calculations suggest that 1.5 to 2.0% of all cancers in USA can be attributed to CT scanning\textsuperscript{73}. This is likely an overestimation, due to the fact that he used median CT protocol settings in his model. CT protocols are often modified for pediatric patients because it is well-established that they are more radiosensitive. Nonetheless, even if 0.5 to 1% of cancers in USA can be attributed to CT scanning, it would mean that approximately 100 000 current cancers were caused by CT scanning.

1.3.5.3 Observations in Medical Imaging

Until recently, extrapolations based on A-bomb survivor data were the best estimations in terms of calculating the cancer mortality risk of CT due to the fact that CT was only developed in the 1970s and because it takes a large cohort of patients to detect differences above a baseline cancer risk of 30%. In 2012, a British retrospective cohort study examined National Health Services data to study cancer incidence and mortality in pediatric patients who received CT exams between 1985 and 2002\textsuperscript{74}. The authors found that 60 mGy of cumulative ionizing radiation to the brain from 2-3 head CTs could triple the risk of brain tumours. Also, 50 mGy of cumulative ionizing radiation to the bone marrow from 5-10 head CTs could triple the risk of leukemia. It is important to note that the baseline risk of both of these cancers is low, so when calculating the risk of one head CT in a patient under 10 years of age based on this data, this translates into approximately one extra case of leukemia and one extra brain tumour per 10 000 patients\textsuperscript{74}.

The leukemia results fall in line with the A-bomb survivor data but the brain tumour results are four-times higher than what was estimated. However, the authors of the study acknowledge this discrepancy by stating that the confidence intervals overlap, and that the patients receiving head
CTs in the retrospective cohort study could have had existing tumours that were not detected on their first CT.

The average follow-up period in this study was 10 years. While brain tumours and leukemia were chosen to be evaluated due to their shorter latency period after radiation exposure, many radiation-induced cancers could appear in this population in the future. Brenner published follow-up commentary to this cohort study and he estimates that the lifetime brain cancer risk from a pediatric head CT could be as high as 1 in 1000 and the lifetime leukemia risk as high as 1 in 7500.75

The following year, in 2013, another large retrospective cohort study was published. This study examined 680 000 Australian pediatric patients who received a CT scan at least one year before any cancer diagnosis, out of a comparable cohort of 10.9 million patients in the Australian medicare records during that time period.76 The average radiation dose was estimated to be 4.5 mSv and the mean follow-up period was 9.5 years. In this group, there was a 24% higher incidence of all types of cancer combined when compared to unexposed patients. Incidence rate ratios (IRR) were also stratified by CT site, cancer site, and number of CTs that each patient received. One of the main observations from the study is that there were approximately 60 000 cancers in the full cohort and depending on whether a lag period of 1 year, 5 years, or 10 years from radiation exposure was used to analyze the data, either 608, 402, or 209 cancers could be attributed to CT scanning.76 Therefore, 0.3% to 1% of cancers could be attributed to CT scanning in this group.

Both of these landmark studies show that epidemiological data supports the models derived from the atomic bomb survivor studies when estimating the risk from CT scanning.
1.4 Radioprotection

1.4.1. Dose exposure

The most basic strategy for protecting patients and radiation workers from the long-term risks of radiation exposure is limitation of exposure \(^77\). This means that a patient should not undergo an IR-based examination unless they present with a clinically indicated reason that requires imaging and there is also a possibility of benefit or change in treatment plan based on the information obtained from the scan \(^78\). Healthcare providers should always weigh the benefits and risks of exposing patients to radiation before ordering IR-based scans. Furthermore, the radiation dose used to conduct the scan should be the smallest exposure that produces useful images, and optimal images should be produced at the first exposure. If possible, repeat scans should be avoided. In short, doses should be kept “As Low As Reasonably Achievable” (ALARA), which is a philosophy that has been officially supported by international radiation protection agencies since the mid-1950s \(^79\). Advances in image reconstruction and post-processing have allowed for dose reduction while maintaining image quality \(^80\).

To limit occupational exposure, radiation workers should always wear dosimeters and their exposure should be regularly monitored by a radiation safety office in order to ensure that they do not exceed limits recommended by the local radiation safety board. For example, the Canadian Nuclear Safety Commission mandates that workers are not exposed to more than 50 mSv in one year, and not exposed to more than 100 mSv in a five-year period \(^81\). Furthermore, dose to radiation workers can be reduced by increasing the distance from the radiation source. For example, radioactive isotopes can be handled with forceps instead of fingers \(^4\). This can
greatly reduce radiation exposure due to the fact that dose from a point source follows the inverse-square law \(^{82}\).

1.4.2. Shielding

Electromagnetic IR has great penetrating power, unlike heavy particle IR such as alpha and beta radiation which can be stopped with paper and plastic respectively \(^{83}\). This property is what makes x-rays and \(\gamma\)-rays effective for medical imaging. However, it is important to ensure that anatomical areas that are not of interest are protected from radiation exposure. Elements with high atomic numbers and large nuclei are most effective for blocking electromagnetic IR. Personal protective equipment made out of lead is ubiquitously used by healthcare workers in clinical settings for this purpose \(^{84}\). Radiosensitive areas, such as the gonads and breasts, are especially covered in lead-lined drapes when patients are undergoing medical imaging examinations for other regions of interest \(^{4}\). If a healthcare worker does not need to be with the patient when they are undergoing the exam, such as during CT, they remain in a separate room with lead-lined windows \(^{4}\).

1.4.3. Pharmacological approaches

Dose limitation and shielding both focus on limiting the quantity and energy of photons to which patients are exposed. However, radioprotective measures can also mitigate the effects of IR after it has entered the patient’s body. This involves the administration of drugs or chemicals to improve the body’s ability to reduce the indirect damage caused by IR.

The earliest example of using pharmacological antioxidants for radioprotection was noted in 1948, when cysteine was used to protect mice from whole-body x-ray radiation \(^{85}\). In 1954, Bacq and his colleagues in Europe observed cysteamine to have a similar radioprotective effect \(^{86}\).
Animals injected with cysteamine at a concentration of 150 mg/kg had a dose reduction factor (DRF) of 1.8. They required 1.8 times higher radiation dose to produce the same level of lethality as control animals that did not receive any cysteamine. As explored in section 1.3.1, the mechanism of IR-induced DNA damage from x-rays and γ-rays occurs through an indirect mechanism that involves highly reactive free radicals. Radioprotective sulphydryl compounds such as cysteine and cysteamine have an −SH group at one end of the molecule. This functional group allows the compound to scavenge free-radicals before they can cause DNA damage. The scavenging mechanism involves a molecule donating an electron to a free radical so that the highly reactive unpaired electron in the free radical becomes paired. Once the electron is paired, the molecule will no long oxidize critical biological structures. In order for electron donation to not lead to propagation, (ie, the donating molecule becoming a free radical itself after donating an electron) the antioxidant should form a stable molecule after reducing the free radical.

Unfortunately, cysteine is also toxic; it induces nausea and vomiting at the dose required for effective radioprotection. In order to overcome this limitation, the US Army began to develop synthetic radioprotectors in 1959. WR-2721, now known as amifostine, was the most successful radioprotector that was synthesized during the military’s development program. It is the only radioprotective drug currently approved by the US Food and Drug Administration for use in radiotherapy to mitigate radiation-induced xerostomia during head and neck treatment.

1.4.4. Antioxidant nutrients

Naturally occurring dietary antioxidants as defined by the US National Academy of Science’s Institute of Medicine include carotenoids, Vitamin E, Vitamin C, and selenium. These nutrients
have been studied for their radioprotective effects against high radiation doses and their use in small animal models has been extensively reviewed\(^91,92\).

Vitamin E (\(\alpha\)-tocopherol) has been shown to have a DRF in mice ranging between 1.06\(^93\) to 1.23\(^94\), depending on the method of administration, dose administered, and dose-rate of radiation. For example, for sub-cutaneous injection of 100 IU/kg of Vitamin E 1 hour before radiation exposure, a DRF of of 1.11\(^95\) was observed when the radiation dose-rate was 0.2 Gy/min but the DRF was 1.06 when the dose-rate was increased to 1 Gy/min. Subsequent research with higher Vitamin E dosing and improved delivery showed that radioprotection could be further improved. A DRF of 1.23 was achieved at a dose-rate of 0.6 Gy/min when Vitamin E dose was increased to 400 IU/kg and injected subcutaneously 24 hours before radiation exposure using an enhanced emulsifying agent\(^94\).

Reducing the lethality of radiation is not the only radioprotective effect that has been observed with the use of dietary antioxidants. 2.5 mg/kg of orally administered beta-carotene has been shown to reduce radiation-induced chromosomal damage in mice, as measured by micronucleated polychromatic erythrocytes\(^96\). Furthermore, patients treated with beta-carotene during radiotherapy have been shown to exhibit a lower number of micronuclei in cells exfoliated from their oral cavity\(^97\).

The aforementioned studies all show proof-of-principle of the radioprotective effects of antioxidant nutrients during high radiation doses. However, they do not directly show reduction of carcinogenesis from low-doses of radiation. Over the past few years, a few important studies have been published that show that this may be a feasible approach to offer radioprotection to patients undergoing diagnostic medical imaging exams.
In 2013, Miller and colleagues at Wake Forest School of Medicine conducted a study in mice that were pre-treated with 100 mg/kg of nicotine-derived nitrosamine ketone (NNK)\(^9\). NNK is a pro-carcinogen and radiosensitizer that led to 100% tumor incidence in their animals even without radiation exposure. Radiation exposure involved weekly whole body CT scans with an 8-slice CT scanner for four weeks, leading to a total cumulative dose of 200 mGy (50 mGy/week). Mice that were fed a 0.7% (weight/weight) N-Acetylcysteine (NAC) diet leading up to and during the study period showed the same number of tumors (10 per mouse) as non-irradiated mice. In contrast, mice that did not have an antioxidant-enriched diet during the study period exhibited twice as many tumors (20 per mouse). Although both the high radiosensitivity of the animals and the high dose of antioxidants were not representative of what would be expected in a human study, this was one of the first studies showing nutraceutical antioxidant based radioprotection from diagnostic levels of radiation.

A 2012 mixed *in vivo/in vitro* study by Kuefner showed radioprotection in an experiment designed to resemble clinical imaging more closely\(^9\). Healthy volunteers ingested a nutraceutical mixture containing Vitamin C, Vitamin E, mixed carotenoids, N-acetylcysteine, alpha-lipoic acid, and L-selenomethionine. Blood was drawn from the subjects throughout the day and irradiated ex vivo at 10 mGy. In control blood samples collected before ingestion of antioxidants, radiation exposure induced 0.14 DSBs per nucleus, whereas blood collected 60 minutes after ingestion of antioxidants only exhibited 0.06 DSBs per nucleus after irradiation\(^9\). This study showed that if antioxidants are bioavailable in a tissue that is exposed to radiation, the number of radiation-induced carcinogenic lesions can be reduced. Furthermore, this effect was achieved using an antioxidant dosing regimen that was safe for human consumption.
1.5 Detecting DNA Damage

Due to the potentially multi-decade latent period between the induction of DNA damage from low-dose ionizing radiation and the development of a solid tumor\textsuperscript{100}, it is not ideal to use cancer occurrence or mortality as a metric. Furthermore, due to the high background rate of cancer in the general population\textsuperscript{101} compared to the small increase in cancer risk from a CT, as well as the multitude of factors that contribute to cancer incidence\textsuperscript{102}, it would require a very large clinical study in order to obtain the statistical power required to observe a significant reduction in cancer incidence. Hence, quantification of DNA damage has become the most commonly used strategy for conducting prospective research on the effects of low-dose ionizing radiation from medical imaging and evaluating radioprotective strategies.

*Chromosomal Aberrations*

When a DSB occurs, the two broken ends of DNA may be rejoined in their initial configuration. We examined two repair pathways for this process in section 1.3.4. However, in some occurrences, the breaks may not rejoin, resulting in distorted chromosomes that can be visualized at the next cell division.

Dicentric chromosomes are formed when breaks are formed close together in two separate chromosomes and the two broken ends join together. This interchange is replicated during the next DNA synthesis and results in a chromosome with two centromeres, as well as two acentric fragments with no centromeres\textsuperscript{103}. If two breaks occur in both arms of the same chromosome, the two broken ends can join together to form a ring\textsuperscript{103}. During DNA synthesis, the chromosome replicates to form two overlapping rings and two acentric fragments. If two chromatids of the same chromosome are damaged in G2 phase, the two “sticky ends” may join together. The
chromosome structure then becomes stretched across the cell during anaphase when centromeres are being pulled to opposite ends of the cell. This specific aberration is called an anaphase bridge.

All three of the aforementioned chromosome aberrations are lethal to the cell that they occur in. Therefore, while these specific aberrations will not lead to carcinogenesis, their incidence in a large sample of cells can imply the presence of carcinogenic DNA damage in other cells. If a sufficient number of metaphases are scored in cultured human lymphocytes, the biodosimetry technique of scoring chromosomal aberrations has the ability to detect a recent total body exposure as low as 0.1-0.2 Gy. While this might be sufficient sensitivity to triage patients during a radiation disaster, it is not an effective technique for measuring DNA damage from low-dose ionizing radiation in diagnostic medical imaging.

*Micronucleus Assay*

The micronucleus assay was developed as a simple cytogenetic technique to assess chromosome damage. It involves counting micronuclei, also known as Howell-Jolly bodies, in dividing cell populations. Micronuclei are expressed in cells that either have acentric fragments or chromosomes that are unable to move to the spindle poles during mitosis. During telophase, a nuclear envelope forms around the aberrant chromosome, similar to a normal interphase nucleus. However, this nucleus is considerably smaller—hence, the name of the assay. Due to the fact that micronuclei are only formed during nuclear division in mitosis, this method of DNA damage quantification can only be performed in dividing cells. Furthermore, in order to accurately quantify the number of micronuclei per nucleus amongst an entire population of cells, all cells must be observed during telophase. In order to facilitate this, after undergoing one nuclear
division, cells are blocked from undergoing cytokinesis with cytochalasin-B, which is an inhibitor of actin polymerization \(^{108}\).

**8-hydroxydeoxyguanosine (8-OHdG)**

8-OHdG is a product of oxidative base damage and can be mutagenic. An accumulation of 8-OHdG in DNA was shown to have predictive value for determining breast cancer risk \(^{109}\). In addition, patients with small cell lung \(^{110}\), prostate, or bladder cancer \(^{111}\) were all shown to have elevated 8-OHdG levels. Monoclonal antibodies can be bound to 8-OHdG and measured using enzyme-linked immunosorbent assay (ELISA) techniques. Also, the avidin protein binds to 8-OHdG with high specificity. With a FITC conjugate, this method can be used with a fluorescent measurement technique such as flow cytometry to quantify oxidative DNA damage. High-performance liquid chromatography coupled with electrochemical detection can be used to measured 8-OHdG levels as well \(^{112}\).

Testing for 8-OHdG provides the advantage of not needing to culture cells and arrest them at a particular phase of mitosis for analysis of DNA damage, as is needed for scoring chromosomal aberrations or micronuclei. However, a study in which rats had their mammary glands irradiated with a dose of 3.9 Gy showed an 8-OHdG increase of only 17% in the experimental group \(^{113}\). This dose is highly carcinogenic (even lethal) and several orders of magnitude above the radiation doses uses in medical imaging. Therefore, it is likely that this technique does not have the sensitivity required to evaluate DNA damage in the context of diagnostic imaging.

**Single-cell gel electrophoresis (Comet assay)**

The comet assay was developed in 1984 by Swedish scientists \(^{114}\) and was named for the shape that damaged nuclear DNA makes after lysis and electrophoresis—there is a head consisting of
intact DNA and a tail with damaged pieces of DNA. Due to the fact that each cell forms its own comet, cells can be scored independently in order to study cellular heterogeneity in response to genotoxic agents. However, the assay is not very sensitive for DSBs, which are the lesions that we are most interested in for studying carcinogenesis. The comet assay’s working range is between 50 and 10 000 DSBs per nucleus, corresponding to a lower dose threshold of approximately 1 Gy in order to detect a statistically significant difference between non-irradiated and irradiated cells.

*Irradiation Induced Foci (IRIF)*

In section 1.3.4, some of the proteins that are involved in the immediate recognition and repair of DNA during the DNA damage response were introduced. Rather than waiting for DNA damage to progress into a chromosomal aberration or micronucleus after a nuclear division, these proteins can be tagged to quantify DNA damage as early as 3 minutes after a DSB is induced in a cell\textsuperscript{117}.

The most commonly used biomarker for IRIF is γH2AX. As mentioned earlier, it is one of the first proteins phosphorylated in response to a DSB\textsuperscript{41}. Phosphorylation occurs at serine-139 and can be tagged with a monoclonal antibody. Due to the fact that phosphorylation of histone H2AX occurs across several megabases around the site of the DSB, the response is highly amplified. Therefore, γH2AX foci are readily visualized through fluorescence microscopy. In addition, antibody tagged cells can be automatically quantified for γH2AX expression through flow cytometry\textsuperscript{118}.

P53-binding protein 1 (53BP1) is one of the many proteins that localizes to sites of DSBs in order to facilitate checkpoint and repair processes\textsuperscript{119}. It is necessary for Chk2 phosphorylation
and is one of the proteins required for ATM-dependent NHEJ\textsuperscript{121}. 53BP1 has also been implicated in increasing chromatin mobility around the site of a DSB\textsuperscript{122}. A specific phosphoisoform of this protein phosphorylated at serine 1778, 53BP1[S1778], has been used similarly to $\gamma$H2AX in order to quantify the number of DSBs per nucleus after radiation exposure\textsuperscript{123}. However, $\gamma$H2AX is used as a sole DSB biomarker more commonly.

Presently, quantification of IRIF is the most sensitive method for analysis of DSBs, which makes it the most appropriate method for measuring DNA damage after exposure to low-dose ionizing radiation in medical imaging. It has been used to detect DNA damage in patient blood samples after CT\textsuperscript{124}, fluoroscopy\textsuperscript{125}, and PET\textsuperscript{126} examinations.
1.6 Objective of Study

1.6.1 Rationale

The overall goal for this research project was to determine if oral antioxidant pre-medication prior to medical imaging is a viable approach for reducing the carcinogenic risk of IR-based imaging modalities.

The first step was to establish a method for measuring DNA damage as our surrogate marker for carcinogenic risk. Since this research does not include a long-term follow up component to measure cancer incidence among subjects, our evaluation of carcinogenic risk is limited to measuring DSBs, since it is the most carcinogenic radiation-induced lesion. Although a protocol for γH2AX quantification through fluorescent three-dimensional (3D) microscopy was established by the Bristow group at the University of Toronto, their protocol was optimized for studying high radiation doses in fibroblasts. The research outlined in this thesis represents the first time at our institution that DNA damage from low-dose radiation will be quantified in primary human tissue.

After establishing a working protocol, our aim was to initiate a pilot clinical study to measure irradiation induced foci in patients who were administered oral antioxidants prior to their radiation exposure.

1.6.2 Hypotheses

(a) Peripheral blood mononuclear cells (PBMCs) isolated from irradiated whole blood exhibit a linear dose-response in terms of DSBs per nucleus at radiation doses less than 500 mGy.
(b) Patients who undergo bone scans with Technetium-99m methylene disphosphonate (99m-Tc MDP) exhibit more IRIF in their PBMCs after the injection of the radiotracer.

(c) Patients who are administered a high dose of oral antioxidants before radiotracer injection exhibit smaller increases in IRIF compared to patients who do not receive any antioxidant pre-treatment.
Chapter 2 - Methods

2.1. Dose-response experiments with protocol A

Blood Collection

With REB approval (13-7086), one healthy volunteer provided blood samples for each set of dose response experiments. Four mL of blood was acquired for each experimental condition from the antecubital vein of the subject. Blood was acquired in 2 mL tubes containing sodium citrate as an anticoagulant; two tubes were used for each experimental condition. After blood collection, the tubes were inverted gently to allow the anticoagulant to mix with the whole blood.

Irradiation

Blood tubes were placed in a custom poly(methyl methacrylate) (ie, Lucite) holder and irradiated with specific doses using the X-RAD 225Cx Small Animal Image Guided Irradiation System (Precision X-Ray, CT, USA) located in Core II of the STTARR Facility. Doses were attained for each experimental condition by adjusting the irradiation settings as indicated in the following table. Dosimetry was validated by the Radiation Medicine Program at the Princess Margaret Cancer Centre.

<table>
<thead>
<tr>
<th>Dose</th>
<th>kV</th>
<th>mA</th>
<th>Duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mGy</td>
<td>225</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>53 mGy</td>
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<td>225</td>
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<tr>
<td>159 mGy</td>
<td>225</td>
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<td>3</td>
</tr>
<tr>
<td>371 mGy</td>
<td>225</td>
<td>13</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 2 – Settings used to achieve radiation doses for 0-371 mGy on X-RAD 225 Cx. All irradiations were conducted at 225 kV and 13 mA. The durations listed above, in seconds (s), were used to achieve the desired radiation dose.
A second set of dose-response experiments was conducted using the X-RAD 320 (Precision X-Ray, CT, USA) located at Princess Margaret Cancer Centre. The following settings were used to attain the desired doses.

<table>
<thead>
<tr>
<th>Dose</th>
<th>kV</th>
<th>mA</th>
<th>Duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mGy</td>
<td>100</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9 mGy</td>
<td>100</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>18 mGy</td>
<td>100</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>27 mGy</td>
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<td>1</td>
<td>16</td>
</tr>
<tr>
<td>36 mGy</td>
<td>100</td>
<td>1</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 3 – Settings used to achieve radiation doses for 0 to 36 mGy on X-RAD 320. All irradiations were conducted at 100 kV and 1 mA. The durations listed above, in seconds (s), were used to achieve the desired radiation dose.

Irradiation was conducted in an open field manner. The Pilot® (Precision X-Ray, CT, USA) software package was used to position the platform and x-ray gantry so that the entire volume in the blood tube was irradiated on the anterior side of the tube. 30 minutes after the time of irradiation, blood tubes were placed on ice for a duration of 10 minutes. The rationale for this temperature control step was to allow the maximum number of H2AX histones to be phosphorylated into γH2AX at room temperature over 30 minutes and then prevent phosphatase activity from removing the γH2AX signal by lowering the temperature of the cells.

**PBMC Isolation**

The blood from each of the two vacutainer tubes for each experimental condition was evenly combined in a conical 15 mL tube, resulting in a single 4 mL volume for each condition. The blood was diluted 1:1 with phosphate-buffered saline (PBS), resulting in an 8 mL blood/PBS mixture. The mixture was carefully layered on top of 5 mL of Ficoll-Paque PLUS (GE Healthcare Life Sciences, Quebec) in a new 15 mL tube; Ficoll was used to aid in separating PBMCs from whole blood during centrifugation.
Each mixture was centrifuged for 30 minutes at 400 times the force of gravity (400 × g). One and a half mL of the buffy coat layer was removed and placed into new 15 mL conical tubes. It was then diluted 1:3 with PBS and centrifuged at 100 times the force of gravity (100 × g) for 10 minutes. The supernatant was aspirated and the pellet was re-suspended in 8 mL of PBS, followed by 10 more minutes of centrifugation at 100 × g.

The supernatant was removed and the pellet was re-suspended in 50 μL of PBS. Six-well plates were prepared with one coverslip in each well. 10 μL volumes of cell solution were spotted on to cover slips such that there were 3 spots from the same experimental condition per coverslip. One coverslip was prepared per condition for dose-response series A1 (0 to 371 mGy). Two coverslips were prepared per condition for dose-response series A2 (0 to 36 mGy). The cell solution was allowed to air dry for 10 minutes.

The cells were fixed by covering each coverslip with 1.5 mL of 4% paraformaldehyde (PFA)/0.2% Triton-X100/PBS. After 30 minutes of fixation, the fixation solution was aspirated and the coverslips were washed with 1.5 mL of PBS, three times, for five minutes each time. In between each wash, the existing PBS was aspirated and fresh PBS was added.

Cells were then treated with 1.5 mL of 0.5% NP40 (nonyl phenoxypolyethoxylethanol)/PBS for permeabilization of the nuclear membrane. After 20 minutes, the solution was aspirated and 2 mL of PBS was added to each well. The 6-well plates were stored at 4°C until immunofluorescence was conducted.

Due to the fact that our research group primarily operates a medical imaging dry lab, immunofluorescence was outsourced to the University Health Network’s Pathology Research Program (PRP) for the first set of dose-response experiments. Their laboratory methods are
proprietary so only a summarized version of the immunofluorescence protocol was provided to us for inclusion in this thesis.

PRP Immunofluorescence protocol

Immunofluorescence was conducted at the earliest availability of the PRP, approximately one week after cells were fixed.

Cells were washed with Tris-buffered saline (TBS), followed by 5 minutes of blocking with 10% normal goat serum. Primary staining was done overnight for 53BP1 phosphorylated at serine 1778 (53BP1[S1778]) with a polyclonal rabbit antibody (Ab# 2675, Cell Signaling Technology, MA, USA). For the first dose-response series (up to 371 mGy), a dilution of 1:100 was used. For the second dose-response series (up to 36 mGy), a dilution of 1:200 was used. This was followed by secondary fluorescent antibody staining with Alexa Fluor 555 anti-rabbit goat antibody (Life Technologies, NY, USA) for 1 hour. Subsequently, another primary staining was conducted. γH2AX was stained for 2 hours with a monoclonal mouse antibody (05-636, EMD Millipore, MA, USA) at a dilution of 1:500. Secondary immunofluorescent staining was done with Alexa Fluor 488 anti-mouse goat antibody (Life Technologies, NY, USA) for 1 hour. Finally, 4’,6-diamidino-2-phenylindole (DAPI) was applied for 10 minutes and the coverslip was mounted on to a microscope slide with VECTASHIELD HardSet Mounting Medium (VECTOR LABORATORIES, CA, USA). Slides were stored at 4°C until imaging was conducted.

Imaging

Imaging was done at the STTARR facility in the Toronto Medical Discovery Tower (TMDT) with a hardware set-up consisting of an Olympus Spinning Disk Confocal microscope (Olympus Corp., Tokyo, Japan), Cascade camera (Photometrics, AZ, USA), and PlanApo 60x/1.42 oil
objective lens (Nikon, Tokyo, Japan). Oil was placed on the objective lens before use.

MetaMorph (Molecular Devices, CA, USA) software was used to control the camera from a computer.

The following microscopy settings in table 4 and table 5 were used for the first two sets of dose-response experiments.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Filter</th>
<th>EM Gain</th>
<th>Exposure (ms)</th>
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<td>0 mGy</td>
<td>DAPI</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>FITC</td>
<td>3800/3500*</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CY3</td>
<td>3650/3400*</td>
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</tr>
<tr>
<td>53 mGy</td>
<td>DAPI</td>
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<td>25</td>
</tr>
<tr>
<td></td>
<td>FITC</td>
<td>100</td>
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</tr>
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<td></td>
<td>CY3</td>
<td>3200</td>
<td>50</td>
</tr>
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<td>25</td>
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<tr>
<td></td>
<td>FITC</td>
<td>3800</td>
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<td>CY3</td>
<td>3200</td>
<td>50</td>
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<tr>
<td>159 mGy</td>
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<td>FITC</td>
<td>3500</td>
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<td>CY3</td>
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<td>50</td>
</tr>
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<td>371 mGy</td>
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<td>25</td>
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<tr>
<td></td>
<td>FITC</td>
<td>3400</td>
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</tr>
<tr>
<td></td>
<td>CY3</td>
<td>3600</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 4 – Settings used to capture cell images for dose-response series A1, 0 to 371 mGy.

Camera settings applied on MetaMorph software for the Cascade camera in order to capture cell images for the first dose response series examining doses between 0 and 371 mGy. While settings varied slightly between experimental conditions, they were kept the same for all fields of view within each experimental condition. *This dose-response series was conducted on multiple days. One day consisted of doses 0, 53, and 106 mGy; another day consisted of doses 0, 159, and 371 mGy. Since each set of experiments had its own non-irradiated control condition, there were two sets of slides with 0 mGy of radiation. They were imaged separately with slightly different EM Gains in order to optimize image quality.
<table>
<thead>
<tr>
<th>Dose (0, 9, 18, 27, 36 mGy)</th>
<th>Filter</th>
<th>EM Gain</th>
<th>Exposure (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>1</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>FITC</td>
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</tr>
<tr>
<td>CY3</td>
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<td>50</td>
</tr>
</tbody>
</table>

Table 5 – Settings used to capture cell images for dose-response series A2, 0 to 36 mGy. Camera settings applied on MetaMorph software for the Cascade camera in order to capture cell images for the second dose response series examining doses between 0 and 36 mGy. For consistency, the same camera settings were used for all coverslips.

For every field of view, 50 consecutive slices at 0.2 μm slice thickness were obtained in the z-direction for each wavelength. The 4',6-diamidino-2-phenylindole (DAPI) excitation filter was used for nuclei, fluorescein isothiocyanate (FITC) for γH2AX tagged with Alexa Fluor 488, and cyanine3 (CY3) for 53BP1[S1778] tagged with Alexa Fluor 555. Six fields of view were captured per coverslip. For dose-response series A1 (0 to 371 mGy), one coverslip was imaged per experimental condition. For dose-response series A2 (0 to 36 mGy), two coverslips were imaged per experimental condition.

*Image Post-processing*

The 50-slice image volumes were imported into ImageJ (NIH, MD, USA) and the software was used to make maximum intensity projections (MIPs) from the 50-slice image z-stacks.

*Image Analysis*

MIPs were imported into Image-Pro Plus 6.0 (Media Cybernetics Inc., MD, USA). A pixel intensity threshold was used to segment the nuclei from the background on DAPI filtered images. Outlines were drawn around the circumference of each nucleus using an automatic software function.

The FITC and CY3 filtered images were then imported into Image-Pro Plus so that DNA DSBs could be counted. For both sets of images, a pixel intensity of 35 000 units, and an object size of
1.4 pixels was set as the threshold for counting a focus. The cell nucleus outlines were merged with the foci images and all foci outside of the nuclei were excluded from our count. A table was generated with the number of foci in each cell nucleus. The total number of foci and the total number of cells were added across each field of view from each experimental condition to create a final “foci per nucleus” value.

Statistical Analysis

Linear regressions were conducted on GraphPad Prism 6 (GraphPad Software, CA, USA) to analyze relationships between radiation dose and number of DSB foci per nucleus.
2.2 Dose Response Series with Protocol B

Blood Collection

This was done similar to section 2.1. Two blood tubes were obtained per experimental condition so that there would be two technical replicates per condition in this dose-response series.

Irradiation

The following settings were used on the X-RAD 225Cx to achieve the desired doses.

<table>
<thead>
<tr>
<th>Dose</th>
<th>kV</th>
<th>mA</th>
<th>Duration (s)</th>
</tr>
</thead>
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<td>0 mGy</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
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<td>18 mGy</td>
<td>100</td>
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<td>54 mGy</td>
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<tr>
<td>72 mGy</td>
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</tbody>
</table>

Table 6 – Settings used for achieving radiation doses 0 to 72 mGy on X-RAD 225Cx. All irradiations were conducted at 100 kV and 0.5 mA. The durations listed above, in seconds (s), represent the total irradiation times used to achieve the desired dose. Each irradiation was split into two fractions. Each half was applied to opposite sides of the blood tube so that cells would be evenly exposed.

Irradiation was conducted in an open field manner. The Pilot® software package was used to position the platform and x-ray gantry. In order to achieve uniform blood volume exposure, each irradiation was split in half so that fifty percent of the dose was administered to the anterior side of the blood tube and fifty percent was administered to the posterior side of the blood tube. For example, for the 36 mGy condition, the anterior side was first irradiated for 20 seconds. Then, the gantry was rotated 180 degrees to irradiate the posterior side for 20 seconds. Blood tubes were placed on ice 30 minutes after the time of irradiation and kept on ice for 10 minutes.
**PBMC Isolation**

The blood from each vacutainer tube was transferred into a conical 15 mL tube and diluted 1:2 with 10% fetal calf serum (FCS) in Roswell Park Memorial Institute (RPMI)-1640 media, resulting in a 6 mL blood/media mixture. The mixture was carefully layered on top of 5 mL of Ficoll in a new 15 mL tube.

Each mixture was centrifuged for 30 minutes at 400 × g. 1.5 mL of the buffy coat layer was removed and placed into new 15 mL conical tubes. It was then diluted 1:3 with 10% FCS/RPMI-1640 and centrifuged at 100 × g for 10 minutes. The supernatant was aspirated and the pellet was re-suspended in 8 mL of PBS, followed by 10 more minutes of centrifugation at 100 × g.

The supernatant was removed and the pellet was re-suspended in 50 μL of PBS. Six-well plates were prepared with one coverslip in each well. The cell solution was spotted on to coverslips in 10 μL droplets (3 droplets per cover slip) and the cells were allowed to air dry for 10 minutes. Afterwards, the cells were fixed with cold methanol (-20°C) and placed in a -20°C freezer for 20 minutes. Finally, the methanol was aspirated and the cells were fixed with -20°C acetone for 1 minute.

**Immunofluorescence Protocol**

Immunofluorescence was conducted immediately after PBMC isolation. The cells were blocked with 1% FCS/PBS on a rocker for 30 minutes, split up into three rounds of 10 minutes each. After every round, the old blocking solution was aspirated and new blocking solution would be added.
Primary antibody staining for γH2AX and 53BP1[S1778] was done concurrently at dilutions of 1:500 and 1:200 respectively in a 3% bovine serum albumin (BSA)/PBS solution. Antibody solution was spotted on to Parafilm M (Bemis, WI, USA) in 30 μL droplets. Cover slips were tapped dry on Kimwipes (Kimberly-Clark Professional, GA, USA) and carefully placed cell-side facing downwards on to the antibody solution droplets. The cover slips were stored in the dark at 4°C overnight until secondary antibody staining was performed.

The next day, the cover slips were placed cell-side facing upwards in a 6-well plate and blocking was conducted on a rocker in the dark for 30 minutes (3 rounds of 10 minutes each) with 1% FCS/PBS. Secondary antibody staining was done using a similar technique as primary antibody staining, but with Alexa-Fluor 488 and Alexa-Fluor 555 both at dilutions of 1:400 in 3% BSA/PBS. The cover slips were stored in the dark at 4°C for 1 hour. Subsequently, the cells were washed with PBS four times in the dark, for five minutes each time, on a rocker. Finally, the cover slips were mounted on to slides using VECTASHIELD Mounting Medium with DAPI.

**Imaging**

Imaging was done with the same hardware set-up as Section 2.1.

The following camera settings were used for capturing images.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Filter</th>
<th>EM Gain</th>
<th>Exposure (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 18, 36, 54, 72 mGy</td>
<td>DAPI</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(same settings used</td>
<td>FITC</td>
<td>4000</td>
<td>40</td>
</tr>
<tr>
<td>for all conditions)</td>
<td>CY3</td>
<td>4000</td>
<td>40</td>
</tr>
</tbody>
</table>

**Table 7 - Settings used to capture cell images for dose-response series B, 0 to 72 mGy.**

Camera settings applied on MetaMorph software for the Cascade camera in order to capture cell images for our third dose-response series examining doses between 0 and 72 mGy. Two coverslips were imaged per condition; the same settings were used for all coverslips and all conditions.
**Image Post-processing**

The 50-slice image volumes were imported into AutoQuantX3 (Media Cybernetics, MD, USA). Images underwent 3D de-convolution using an adaptive point spread function. Each image volume underwent 25 iterations of the de-convolution algorithm.

The de-convolved volumes were opened in ImageJ to generate maximum intensity projections. These final z-stacks were used for cell analysis and foci counting.

**Image Analysis**

This was done using the same method as section 2.1. However, a pixel intensity of 17 500 was used as a threshold to count IRIF.

**Statistical Analysis**

Linear regressions were conducted on GraphPad Prism 6 to analyze relationships between radiation dose and number of DSB foci per nucleus.
2.3 Dose Response Series with Protocol C

Blood Collection

This was done identically to sections 2.1 and 2.2 except only one 2 mL sample was collected per experimental condition, since this was simply a validation experiment using new equipment.

Irradiation, PBMC Isolation, and Immunofluorescence Protocol

This was done identically to section 2.2.

Imaging

Imaging was done at the Advanced Optical Microscopy Facility (AOMF) in the TMDT with a hardware set-up consisting of an Axio Observer microscope (Carl Zeiss, Oberkochen, Germany), CoolSNAP\textsubscript{HQ} camera (Photometrics, AZ, USA), and 63x/1.4 Oil DIC Plan-Achromat oil objective lens (Nikon, Tokyo, Japan). MetaMorph software was used to control the camera from a computer. The following camera settings (Table 8) were used.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Filter</th>
<th>Digitizer</th>
<th>Gain</th>
<th>Exposure (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 18, 36, 54, 72 mGy</td>
<td>DAPI</td>
<td>20 MHz</td>
<td>1x</td>
<td>30</td>
</tr>
<tr>
<td>(same settings used for all conditions)</td>
<td>FITC</td>
<td>20 MHz</td>
<td>4x</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 8 - Settings used to capture cell images for dose-response series C, 0 to 72 mGy. Camera settings applied on MetaMorph software for the CoolSNAP camera s for our final dose-response series examining doses between 0 and 72 mGy.

Image Post-processing and Analysis

This was done identically to section 2.2

Statistical Analysis

This was done identically to section 2.1
2.4 Pilot Clinical Study

Patient Recruitment and Group Assignment

Research Ethics Board (REB) approval was obtained (11-0553-B) and ten male patients were recruited with informed consent. Study subjects were scheduled to receive bone scans for various clinically indicated reasons. See below for patient characteristics. Patients were excluded if they received radiotherapy or chemotherapy in the past six months in order to reduce the confounding effects of these cytotoxic treatments on DSB measurements. Patients were also excluded if they consumed nutraceuticals that were being used in our experimental study on the same day as their participation.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Group</th>
<th>Age</th>
<th>Radiation dose (MBq)</th>
<th>Clinical indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>67</td>
<td>784</td>
<td>Staging for prostate cancer</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>74</td>
<td>799</td>
<td>Staging for prostate cancer</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>82</td>
<td>789</td>
<td>Staging for prostate cancer</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>70</td>
<td>814</td>
<td>Staging for prostate cancer</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>61</td>
<td>795</td>
<td>Staging for prostate cancer</td>
</tr>
<tr>
<td>6</td>
<td>Antioxidant</td>
<td>66</td>
<td>800</td>
<td>Staging for pancreatic cancer</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>72</td>
<td>829</td>
<td>Staging for prostate cancer</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>40</td>
<td>757</td>
<td>Musculoskeletal Pain</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>76</td>
<td>815</td>
<td>Staging for lung cancer</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>69</td>
<td>807</td>
<td>Staging for prostate cancer</td>
</tr>
</tbody>
</table>

Table 9 – Subject characteristics and radiation doses for pilot clinical study. Subject characteristics, including age, radiation dose from 99m Tc-MDP injection, and clinical indication for receiving the scan. The first five consecutively recruited patients were assigned to the control group and the next five were assigned to the antioxidant group.

The first five consecutively recruited patients were assigned to the control group. The next five consecutively recruited patients were assigned to the experimental group (which will be referred to as the antioxidant group from here on).
Experimental Treatment and Blood Collection

For the control group, 6 cubic centimetres (cc) of blood was drawn immediately prior to the injection of 99m-Tc MDP in one blood tube containing lithium heparin as an anticoagulant. For the antioxidant group, patients were administered 2 g of ascorbate (Jamieson Laboratories, ON, Canada), 1.2 g of N-Acetylcysteine (SISU, BC, Canada), 600 mg of alpha lipoic acid (NOW Foods, IL, USA), and 30 mg of beta-carotene (Swiss Naturals, QC, Canada) in pill form 15 minutes before radiotracer injection. Immediately prior to radiotracer injection, 6 cc of blood was drawn into a single blood tube containing lithium heparin. For both groups, another 6 cc blood sample was collected 2.5 hours after the initial radiotracer injection.

PBMC Isolation

PBMC isolation was done identically to sections 2.2 and 2.3 except for one modification. 1.0 nM of Calyculin A (Cell Signaling Technology, MA, USA) was first added to the whole blood in order to reduce phosphatase activity during the experimental protocol. This technique has been used in recent studies to preserve the phosphorylated state of γH2AX \(^{127,128}\).

Immunofluorescence Protocol

This was done identically to sections 2.2 and 2.3

Imaging

Imaging was done using the same software and equipment as section 2.2. However, the settings for capturing cell images were slightly changed. For the clinical series, the following configuration (Table 10) was used:
<table>
<thead>
<tr>
<th>Condition (same settings used for all patients)</th>
<th>Filter</th>
<th>Digitizer</th>
<th>Gain</th>
<th>Exposure (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, Experimental</td>
<td>DAPI</td>
<td>10 MHz</td>
<td>1x</td>
<td>15-25**</td>
</tr>
<tr>
<td>FITC</td>
<td>10 MHz</td>
<td>4x</td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>CY3</td>
<td>10 MHz</td>
<td>4x</td>
<td></td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 10 – Settings used to capture cell images for pilot clinical study. Camera settings applied on MetaMorph software for the CoolSNAP camera in order to capture cell images of study participants’ PBMCs. **DAPI exposure was adjusted between 15 and 25 ms to optimize image quality of cell nuclei.

Image Post-processing and Analysis

This was done identically to sections 2.2 and 2.3. However, a pixel intensity of 20 000 was used as a threshold for counting IRIF.

Blinding

Neither the patients nor the researchers were blinded for the study. Patients were aware of their group assignments. Control patients did not receive any intervention (no placebo was used). The same study personnel conducted patient recruitment, PBMC isolation, immunofluorescent preparation of cells, microscopy, and DSB foci analysis.

Statistical Analysis

A 2-way repeated measures ANOVA was used to assess the interaction between antioxidant intervention and radiation exposure. Sidak’s multiple comparisons test was used to assess the significance of differences between groups. A Student’s t-test was used to compare differences in induced foci per nucleus between groups.
Chapter 3 - Results

3.1 Dose Response Data with Protocol A

The first dose-response series was conducted with radiation doses ranging between 0 mGy and 371 mGy. At least 100 cells (ranging between 100 and 194) were analyzed per coverslip across 6 fields of view. The number of foci per nucleus and the number of cells per condition can be seen in the table below.

The experiments were done on two days, with each day having an independent non-irradiated blood sample as a control. Therefore, the non-irradiated (NIR) cell values represent an average calculation of foci per nucleus from each day—standard deviation is reported with each average. For all of the other doses, each foci/nucleus value represents foci per nucleus on just one coverslip.

<table>
<thead>
<tr>
<th>Radiation Dose (mGy)</th>
<th>γH2AX Foci/nucleus (SD)</th>
<th># of cells</th>
<th>53BP1[S1778] Foci/nucleus (SD)</th>
<th># of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIR</td>
<td>0.15 (0.03)</td>
<td>173, 100</td>
<td>1.68 (1.19)</td>
<td>173, 100</td>
</tr>
<tr>
<td>53</td>
<td>0.26</td>
<td>125</td>
<td>0.56</td>
<td>125</td>
</tr>
<tr>
<td>106</td>
<td>0.97</td>
<td>103</td>
<td>0.61</td>
<td>103</td>
</tr>
<tr>
<td>159</td>
<td>0.92</td>
<td>194</td>
<td>1.78</td>
<td>194</td>
</tr>
<tr>
<td>371</td>
<td>2.20</td>
<td>193</td>
<td>3.63</td>
<td>193</td>
</tr>
</tbody>
</table>

Table 11 - Dose-response data for γH2AX and 53BP1[S1778] from 0 to 371 mGy using protocol A. The number of γH2AX and 53BP1[S1778] foci per nucleus in our first dose-response series, from 0 to 371 mGy, is presented in this table. Two repeats were conducted for the NIR condition. The foci/nucleus value for NIR is an average of both repeats. Standard deviation is included in brackets. The # of cells analyzed from each repeat is included, separated by commas.

γH2AX foci/nucleus showed a positive linear dose-response trend from 0 mGy to 371 mGy (R² = 0.97), despite a small decrease between 106 mGy and 159 mGy. In the same cells,
53BP1[S1778] showed an increasing trend from 53 mGy to 371 mGy. However, the data for the non-irradiated controls did not follow this trend. The NIR cells (1.46 foci/nucleus) displayed much higher expression of 53BP1[S1778] compared to the cells irradiated with 53 mGy (0.56/nucleus) and 106 mGy/nucleus (0.61/nucleus). Hence, there was no linear dose-response trend overall for this biomarker ($R^2 = 0.45$). There was no significant non-zero slope ($p=0.14$).

![Dose Response A](image)

**Figure 3** – $\gamma$H2AX and 53BP1[S1778] foci/nucleus in PBMCs irradiated from 0 to 371 mGy prepared using protocol A. The number of $\gamma$H2AX and 53BP1[S1778] foci per nucleus are shown above for our first dose-response series. Standard deviation across two repeats is included as error bars in the non-irradiated (NIR) condition. Linear regressions showed a dose-response for $\gamma$H2AX ($R^2 = 0.97$) but not 53BP1[S1778] ($R^2=0.45$).

A second dose-response series was carried out at a dose range below the first series. Doses ranged from 0 mGy to 36 mGy. A different irradiation device was used at a different location for this set of experiments. Also, the concentration of 53BP1[S1778] antibody was diluted to 1:200. Apart from that change, the protocol remained the same.
<table>
<thead>
<tr>
<th>Radiation Dose (mGy)</th>
<th>γH2AX Foci/nucleus (SD)</th>
<th># of cells</th>
<th>53BP1 Foci/nucleus (SD)</th>
<th># of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIR</td>
<td>0.09 (0.05)</td>
<td>420, 348</td>
<td>0.36 (0.41)</td>
<td>420, 348</td>
</tr>
<tr>
<td>9</td>
<td>0.05</td>
<td>219</td>
<td>0.26</td>
<td>219</td>
</tr>
<tr>
<td>18</td>
<td>0.37</td>
<td>312</td>
<td>0.60</td>
<td>312</td>
</tr>
<tr>
<td>27</td>
<td>0.03</td>
<td>199</td>
<td>0.23</td>
<td>199</td>
</tr>
<tr>
<td>36</td>
<td>0.22</td>
<td>363</td>
<td>0.30</td>
<td>363</td>
</tr>
</tbody>
</table>

Table 12 - Dose-response data for γH2AX and 53BP1[S1778] from 0 to 36 mGy using protocol A. The number of γH2AX and 53BP1[S1778] foci per nucleus in our second dose-response series, from 0 to 36 mGy, is presented in this table. Two repeats were conducted for the NIR condition. The foci/nucleus value for NIR is an average of both repeats. Standard deviation is included in brackets. The # of cells analyzed from each repeat is included, separated by commas.

Two coverslips were imaged for each of the conditions. Foci/nucleus values are reported as a cumulative sum of foci divided by a cumulative sum of cells across coverslips. In other words, the number of foci and cells were counted on each coverslip, and the total number of foci across both coverslips was divided by the total number of cells. This value was treated as a single data point.

Since this dose-responses series was also conducted across two days, each day had its own non-irradiated control data-point. Therefore, the NIR values are an average of the two data-points obtained from each day. And so they are reported with standard deviation values.

A dose-response trend was not observed for γH2AX (R²=0.09) or 53BP1[S1778] (R²=0.01). There was no meaningful difference in terms of γH2AX foci/nucleus between the experimental conditions. γH2AX measurements increased drastically from 0.05 to 0.37, then back down to 0.03, and finally back up to 0.22 foci/nucleus from the first to the fourth irradiation conditions. 53BP1[S1778] measurements followed a similar pattern, with the primary difference being that
the NIR condition also displayed an unusually high number of 53BP1 foci/nucleus (0.36, SD 0.41).

Figure 4 – γH2AX and 53BP1[S1778] foci/nucleus in PBMCs irradiated from 0 to 36 mGy prepared using protocol A2. The number of γH2AX and 53BP1[S1778] foci per nucleus are shown above for our second dose-response series, examining doses from 0 to 36 mGy. Standard deviation across two repeats is included as error bars in the non-irradiated (NIR) condition. There was no dose-response trend for either DSB biomarker.
3.2 Dose Response Data with Protocol B

This dose-response series was carried out with a different experimental protocol, as described in section 2.2. Radiation doses ranged from 0 to 72 mGy. Furthermore, two replicates were conducted for each experimental condition. Cell images from this experiment are included in figures 5 and 6 on the following pages. The data is summarized in Table 13, below.

<table>
<thead>
<tr>
<th>γH2AX</th>
<th>Dose (mGy)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIR</td>
<td>18</td>
<td>36</td>
<td>54</td>
<td>72</td>
</tr>
<tr>
<td>A</td>
<td>0.15</td>
<td>0.11</td>
<td>0.16</td>
<td>0.15</td>
<td>0.63</td>
</tr>
<tr>
<td>B</td>
<td>0.16</td>
<td>0.11</td>
<td>0.09</td>
<td>0.35</td>
<td>0.64</td>
</tr>
<tr>
<td>Average</td>
<td>0.16</td>
<td>0.11</td>
<td>0.13</td>
<td>0.25</td>
<td>0.63</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.01</td>
<td>0.00</td>
<td>0.05</td>
<td>0.14</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>53BP1</th>
<th>Dose (mGy)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIR</td>
<td>18</td>
<td>36</td>
<td>54</td>
<td>72</td>
</tr>
<tr>
<td>A</td>
<td>0.18</td>
<td>0.04</td>
<td>0.32</td>
<td>0.57</td>
<td>0.77</td>
</tr>
<tr>
<td>B</td>
<td>0.24</td>
<td>0.05</td>
<td>0.13</td>
<td>0.52</td>
<td>0.64</td>
</tr>
<tr>
<td>Average</td>
<td>0.21</td>
<td>0.04</td>
<td>0.23</td>
<td>0.54</td>
<td>0.70</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.05</td>
<td>0.01</td>
<td>0.14</td>
<td>0.04</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Table 13- Dose-response data for γH2AX and 53BP1[S1778] from 0 to 72 mGy using protocol B. The number of γH2AX and 53BP1[S1778] foci per nucleus in our third dose-response series, from 0 to 72 mGy, is presented in this table. Two repeats were conducted for each condition. Data from the first repeat is labeled “A” and data from the second repeat is labeled “B.” Foci per nucleus from each repeat is included above, along with the average between both repeats and the standard deviation. The number of cells analyzed per condition in each experimental repeat ranged from 74 to 141.
Figure 5 – Non-irradiated cells from dose response experiment B. Partial microscopy field of view of non-irradiated cells. Cells were imaged at 60x magnification. The figure shows the same cells imaged with the (A) FITC channel for γH2AX, (B) CY3 channel for 53BP1[S1778] and (C) DAPI channel for cell nuclei.
Figure 6 – Cells irradiated with 72 mGy in dose response experiment B. Partial microscopy field of view of cells irradiated with 72 mGy. Cells were imaged at 60x magnification. The figure shows the same cells imaged with the (A) FITC channel for γH2AX, (B) CY3 channel for 53BP1[S1778] and (C) DAPI channel for cell nuclei. γH2AX and 53BP1[S1778] are seen co-localized in the same cells.
A linear regression was conducted to analyze the dose-response trend from 0 to 72 mGy. Both γH2AX and 53BP1[S1778] displayed significant non-zero slopes (p = 0.009 and p = 0.002 respectively). The γH2AX linear model had an R² value of 0.59 and the 53BP1[S1778] model had an R² value of 0.72. Plotted values and linear trends can be seen in the figure below (Figure 7).

![Dose Response B](image)

**Figure 7 - γH2AX and 53BP1[S1778] foci/nucleus in PBMCs irradiated from 0 to 72 mGy prepared using protocol B.** This dose-response series was conducted after significant improvements to our protocol. Standard deviation across two replicates is included as error bars in all conditions. Both γH2AX and 53BP1[S1778] showed a linear dose-response trend from 0 to 72 mGy.
3.3 Dose Response Data with Protocol C

The final dose-response series was conducted to confirm the results seen in section 3.2. We were transitioning to the use of a new microscope and we wanted to ensure that the new imaging hardware would not produce drastically different results.

Since this set of experiments was simply to replicate earlier findings, only one trial was conducted per condition.

<table>
<thead>
<tr>
<th>Dose (mGy)</th>
<th>Foci</th>
<th>Cells</th>
<th>Foci/nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIR</td>
<td>8</td>
<td>75</td>
<td>0.11</td>
</tr>
<tr>
<td>18</td>
<td>28</td>
<td>108</td>
<td>0.26</td>
</tr>
<tr>
<td>36</td>
<td>13</td>
<td>63</td>
<td>0.21</td>
</tr>
<tr>
<td>54</td>
<td>10</td>
<td>84</td>
<td>0.12</td>
</tr>
<tr>
<td>72</td>
<td>24</td>
<td>51</td>
<td>0.47</td>
</tr>
</tbody>
</table>

**Table 14 - Dose-response data for γH2AX from 0 to 72 mGy using protocol C.** The number of γH2AX foci per nucleus in our final dose-response series, from 0 to 72 mGy, is presented in this table. This dose-response series was conducted to test a new imaging hardware configuration that we planned to use for our subsequent pilot clinical study.

When analyzed with a linear regression, there was no significant dose-response trend. The slope was not significantly non-zero (p=0.26) and the $R^2$ value was 0.39. However, in line with the previous dose-response series, there was an approximately four-fold increase in γH2AX foci/nucleus in the 72 mGy condition compared to the NIR condition.
Figure 8 - γH2AX foci/nucleus in PBMCs irradiated from 0 to 72 mGy prepared using protocol C. This dose response series was simply conducted to test a new imaging hardware set-up. Hence, only one repeat was conducted, and only 50 to 100 cells were imaged per condition. There was no significant linear dose-response trend. However, there was a marked difference between the NIR and 72 mGy conditions.
3.4 Pilot Clinical Data

Our final set of results is from the pilot clinical study. Six fields of view were imaged per coverslip and three coverslips were imaged per condition. Foci per nucleus values were calculated from each coverslip and averaged across the three technical replicates to produce a mean foci per nucleus value for each condition. Figure 9 shows typical microscopy images from a control patient’s pre-radiotracer injection blood sample.
Figure 9 – PBMC nuclei from a patient’s blood sample. Partial microscopy field of view from a control patient’s pre-radiation blood sample. Cells were imaged at 63x magnification. The figure shows the same cells imaged with the (A) FITC channel for γH2AX, (B) CY3 channel for 53BP1[S1778] and (C) DAPI channel for cell nuclei. (D) A composite image of all three channels was made in ImageJ to show DSB foci in cell nuclei. A zoomed in version of (E) a cell containing a 53BP1[S1778] focus and (F) a cell containing a γH2AX focus are shown as well.
The average number of cells counted per patient (adding all 3 technical replicates together) in the pre-radiation condition was 237 (range 107 to 526, SD 119) and the average number of cells counted in the post-radiation condition was 209 (range 139 to 388, SD 75). Mean γH2AX foci per nucleus can be found in the table below.

The summarized γH2AX data from our pilot clinical study is included in this table. Foci per nucleus values were obtained from imaging three coverslips and six fields of view per coverslip, per condition. The mean foci/nucleus across the three technical replicates (coverslips) is included above. The “difference” column was calculated by subtracting pre-radiation foci/nucleus values from post-radiation values. Therefore, this column represents the induced number of foci/nucleus from radiation exposure. Data from each experimental group was averaged and standard deviations were calculated.

The mean γH2AX foci/nucleus data from each patient and the standard error of the mean (SEM) is graphed in Figure 10 below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pt #</th>
<th>Pre-radiation</th>
<th>Post-radiation</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean foci/nucleus</td>
<td>Mean foci/nucleus</td>
<td>Induced foci/nucleus</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Pt 1</td>
<td>0.23</td>
<td>0.49</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>Pt 2</td>
<td>0.10</td>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Pt 3</td>
<td>0.33</td>
<td>0.41</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Pt 4</td>
<td>0.23</td>
<td>0.88</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Pt 5</td>
<td>0.38</td>
<td>0.61</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>0.25</td>
<td>0.58</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>St Dev</td>
<td>0.10</td>
<td>0.18</td>
<td>0.21</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>Pt 6</td>
<td>0.11</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Pt 7</td>
<td>0.24</td>
<td>0.22</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>Pt 8</td>
<td>0.37</td>
<td>0.39</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Pt 9</td>
<td>0.29</td>
<td>0.27</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>Pt 10</td>
<td>0.36</td>
<td>0.35</td>
<td>-0.01</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>0.27</td>
<td>0.27</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>St Dev</td>
<td>0.10</td>
<td>0.10</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 15 - γH2AX foci/nucleus in PBMCs taken from control and antioxidant treated patients before and after radioisotope injection. The summarized γH2AX data from our pilot clinical study is included in this table. Foci per nucleus values were obtained from imaging three coverslips and six fields of view per coverslip, per condition. The mean foci/nucleus across the three technical replicates (coverslips) is included above. The “difference” column was calculated by subtracting pre-radiation foci/nucleus values from post-radiation values. Therefore, this column represents the induced number of foci/nucleus from radiation exposure. Data from each experimental group was averaged and standard deviations were calculated.
Figure 10 - \( \gamma \)H2AX foci/nucleus in PBMCs taken from patients undergoing bone scans before and after 99m Tc-MDP injection. \( \gamma \)H2AX foci in all 10 study subjects. The first five patients were assigned to the control group and the next five were assigned to the antioxidant group. \( \gamma \)H2AX foci/nucleus in PBMCs collected before radiotracer injection is shown in light green. \( \gamma \)H2AX foci/nucleus in PBMCs collected 2.5 hours after radiotracer injection are shown in dark-green. Bar graph values represent the mean of three technical replicates. Error bars represent the standard error of the mean from three technical replicates. The dotted lines connect the pre-radiation value to the post-radiation value for each patient, showing relative change in foci/nucleus. Control patients showed a marked increase in foci per nucleus whereas the antioxidant patients consistently showed minimal or no difference between pre- and post-radiation foci per nucleus values.

In the control group patients, PBMCs from blood taken before radioisotope injection showed an average of 0.25 \( \gamma \)H2AX foci/nucleus (SD 0.10), whereas PBMCs from blood taken 2.5 hours after injection showed an average of 0.58 foci/nucleus (SD 0.18). In the experimental group, pre-radiation PBMCs showed 0.27 \( \gamma \)H2AX foci/nucleus (SD 0.10), which was a similar level of DNA damage as the control group. But in contrast to the control group, 2.5 hours after radioisotope injection, PBMCs from the 5 experimental patients only showed an average of 0.27 foci/nucleus (SD 0.10).
2-way repeated measures ANOVA showed a significant interaction between treatment group and radiation exposure ($p=0.01$). Sidak’s multiple comparisons test with multiplicity adjusted $p$-values indicated a significant increase in $\gamma$H2AX foci/nucleus values from pre-radiation to post-radiation for control subjects ($p=0.003$) but not for antioxidant subjects ($p=0.9995$). Control subjects had an average increase of 0.32 $\gamma$H2AX foci/nucleus (SD 0.21) 2.5 hours after 99m-Tc MDP injection whereas antioxidant subjects only showed an average increase of 0.00 (SD 0.02) foci/nucleus. Data points grouped by experimental condition are plotted in Figure 11 below.

**Figure 11 - $\gamma$H2AX foci per nucleus in PBMCs grouped by experimental condition.** Boxplots showing $\gamma$H2AX foci/nucleus across control and antioxidants patients. Boxplot whiskers represent minimum and maximum values within each condition. The boxes show 1st quartile, median, and 3rd quartile values. Constituent data points are plotted within each box plot. 2-way repeated measures ANOVA showed a significant interaction effect between antioxidant intervention and radiation exposure. Sidak’s multiple comparisons test showed a significant increase in foci/nucleus in control subjects but not in antioxidant subjects.
53BP1[S1778] was also quantified in the same cells and the results are summarized in Table 17 below. For control subjects, PBMCs from blood taken before radiotracer injection showed an average of 0.42 γH2AX foci/nucleus (SD 0.18), whereas PBMCs from blood taken 2.5 hours after injection showed an average of 0.67 foci/nucleus (SD 0.27). In the antioxidant group, pre-radiation PBMCs showed 0.54 53BP1[S1778] foci/nucleus (SD 0.10), and post-radiation PBMCs showed 0.40 foci/nucleus (SD 0.26).

<table>
<thead>
<tr>
<th>Group</th>
<th>Pt #</th>
<th>Mean foci/nucleus</th>
<th>Mean foci/nucleus</th>
<th>Difference foci/nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pt 1</td>
<td>0.52</td>
<td>0.97</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Pt 2</td>
<td>0.20</td>
<td>0.32</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Pt 3</td>
<td>0.58</td>
<td>0.48</td>
<td>-0.10</td>
</tr>
<tr>
<td></td>
<td>Pt 4</td>
<td>0.25</td>
<td>0.71</td>
<td>0.46</td>
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<td></td>
<td>Pt 5</td>
<td>0.57</td>
<td>0.89</td>
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<tr>
<td></td>
<td>Average</td>
<td>0.42</td>
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<td>0.25</td>
</tr>
<tr>
<td></td>
<td>St Dev</td>
<td>0.18</td>
<td>0.27</td>
<td>0.24</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>Pt 6</td>
<td>0.59</td>
<td>0.19</td>
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</tr>
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<td></td>
<td>Pt 7</td>
<td>0.43</td>
<td>0.29</td>
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</tr>
<tr>
<td></td>
<td>Pt 8</td>
<td>0.66</td>
<td>0.33</td>
<td>-0.33</td>
</tr>
<tr>
<td></td>
<td>Pt 9</td>
<td>0.44</td>
<td>0.33</td>
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</tr>
<tr>
<td></td>
<td>Pt 10</td>
<td>0.57</td>
<td>0.84</td>
<td>0.27</td>
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<tr>
<td></td>
<td>Average</td>
<td>0.54</td>
<td>0.40</td>
<td>-0.14</td>
</tr>
<tr>
<td></td>
<td>St Dev</td>
<td>0.10</td>
<td>0.26</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 16- 53BP1[S1778] foci/nucleus in PBMCs taken from control and antioxidant treated patients before and after radioisotope injection. The summarized 53BP1[S1778] data from our pilot clinical study is included in this table. Foci per nucleus values were obtained from imaging three coverslips and six fields of view per coverslip, per condition. The mean foci/nucleus across the three technical replicates (coverslips) is included above. The “difference” column was calculated by subtracting pre-radiation foci/nucleus values from post-radiation values. Therefore, this column represents the induced number of foci/nucleus from radiation exposure. Data from each experimental group was averaged and standard deviations were calculated.
The mean 53BP1[S1778] foci/nucleus data from each patient and the SEM from technical replicates can be visualized in Figure 12 below.

Figure 12 – 53BP1[S1778] foci/nucleus in PBMCs taken from patients undergoing bone scans before and after 99m Tc-MDP injection. 53BP1[S1778] foci in all 10 study subjects. The first five patients were assigned to the control group and the next five were assigned to the antioxidant group. 53BP1[S1778] foci/nucleus in PBMCs collected before radiotracer injection is shown in pink. 53BP1[S1778] in PBMCs collected 2.5 hours after radiotracer injection is shown in red. Bar graph values represent the mean of three technical replicates. Error bars represent the standard error of the mean. The dotted lines connect the pre-radiation value to the post-radiation value for each patient, showing relative change in foci/nucleus. Variability between technical replicates for each condition was high with this biomarker.

2-way repeated measures ANOVA showed a significant interaction between radiation exposure and treatment group (p=0.04). However, Sidak’s multiple comparisons test with multiplicity adjusted p-values did not show a significant difference between pre- and post-radiation 53BP1[S1778] foci/nucleus values for either control subjects (p=0.11) or antioxidant subjects.
Control subjects showed an average increase of 0.25 53BP1[S1778] foci per nucleus (SD 0.24) after radiotracer injection and antioxidant subjects showed an average increase of -0.14 (SD 0.26). Data points separated by experimental group are plotted in Figure 13 below.

**Figure 13 - 53BP1[S1778] foci per nucleus in PBMCs grouped by experimental condition.**
Boxplots showing 53BP1[S1778] foci/nucleus across control and antioxidants patients. Boxplot whiskers represent minimum and maximum values within each condition. The boxes show 1st quartile, median, and 3rd quartile values. Constituent data points are plotted within each box plot. 2-way repeated measures ANOVA showed a significant interaction between antioxidant treatment and radiation exposure. However, Sidak’s multiple comparisons test did not show a significant difference between pre- and post-radiation measurements in either the control group or the antioxidant group.
Chapter 4 - Discussion

4.1. Validating our methodology

We elected to study DSBs in particular, due to the fact that radiation-induced carcinogenesis is typically initiated during the misrepair of DSBs through the NHEJ repair pathway described in sections 1.3.3 and 1.3.5. The ultimate goal of our research is to reduce the carcinogenic risk of radiation exposure in medical imaging. Elevated DSB formation is an appropriate indicator for carcinogenic risk, since it is the primary event that initiates error-prone repair. However, it is important to keep in mind that not every DSB is carcinogenic. Error-free repair from correct NHEJ or homologous recombination, cellular senescence, and apoptosis are all possible outcomes from DSB formation in a cell (all described in section 1.3.3). But our methodology is currently not capable of distinguishing between the downstream pathway of each individual radiation-induced DSB a priori. Hence, we quantified the average number of DSBs per nucleus in each experimental condition as our surrogate measure of carcinogenic risk.

Our research began with multiple dose response series in order to develop an optimal protocol for measuring DNA DSBs in PBMCs. In this section we will analyze the results in detail and address any shortcomings.

4.1.1 Dose response series A1, 0 to 371 mGy

Due to the fact that this was a new area of investigation for our research group, we did not have the expertise required to delve straight into low dose radiation in the sub 100 mGy range, which would have been most representative of the doses used in diagnostic medical imaging procedures. We began with a dose-response series consisting of a non-irradiated control and the following four doses: 53, 106, 159, and 371 mGy. The 371 mGy condition was intended to be
used as a positive control, where an increased number of foci per nucleus would certainly be observed even with a non-optimized protocol.

Our results were positive for γH2AX in this dose-response series. The NIR condition displayed 0.15 γH2AX foci/nucleus (SD 0.03), which is similar to values seen in the literature for non-irradiated lymphocytes\textsuperscript{99,126}. The positive control condition displayed a clear treatment effect, with 2.2 γH2AX foci/nucleus. These results alone showed that on a gross level, our cell preparation and immunofluorescence protocol was functional. Furthermore, there was a linear dose-response trend from 0 to 371 mGy. Although we only conducted one replicate for each of the irradiated conditions and two replicates for the non-irradiated control, we felt that the results were strong enough to move to a lower dose-range.

In this series, all cells were stained for both γH2AX and 53BP1[S1778]. Although γH2AX has become the most commonly used biomarker in this area of research and is sufficient to quantify DNA DSBs on its own, we wanted to use a secondary biomarker to validate our findings, especially because of potential assay sensitivity problems that we thought we may encounter at lower doses.

Similar to γH2AX, 53BP1[S1778] foci/nucleus in the 371 mGy positive control condition was much higher than all other conditions. However, there was no linear trend from 0 to 371 mGy. This is likely due to the data from the 0 mGy condition. Non-irradiated cells displayed an average of 1.68 foci per nucleus (SD 1.19). Even if we assume that one of the data points was an outlier (2.52 foci per nucleus) and consequently skewed the mean, the other data point still showed uncharacteristically high foci per nucleus (0.84).
Apart from the NIR data, there was a positive trend from the 53 mGy to the 371 mGy condition. The 106 mGy condition displayed more foci per nucleus than the 53 mGy condition and the 159 mGy condition displayed more foci/nucleus than the 106 mGy condition.

One explanation for these 53BP1[S1778] results is that the antibody was not diluted enough. It is possible that many of the foci we were observing represented domains where non-specific binding occurred. However, the data was only anomalous for the NIR condition. All other conditions still followed a positive dose-response trend and had foci per nucleus values within the expected range. Therefore, it is possible that there was a coincidental issue with handling both non-irradiated samples on both days of experimentation. Nonetheless, it was worth considering the use of a more dilute antibody concentration in a subsequent dose-response series.

4.1.2 Dose response series A2, 0 to 36 mGy

After completing a dose-response series from 0 to 371 mGy, we elected to study doses in a lower range in order to test the sensitivity limits of our assay. The next dose response series was conducted at doses from 0 to 36 mGy.

The results from this dose-response series were not as positive as the previous experiment. There was no linear dose-response trend from NIR to 36 mGy for γH2AX nor 53BP1[S1778].

The γH2AX foci per nucleus values for the non-irradiated control cells in this experiment were very similar to the previous experiment (0.09 foci/nucleus, SD 0.05 vs. 0.15 foci/nucleus, SD 0.03 respectively). This indicates consistency in our technique between experiments, which is a positive finding from this experiment.
As noted earlier in the results section, foci/nucleus increased from the 9 mGy condition to the 18 mGy condition, decreased for the 27 mGy condition and increased again for the 36 mGy condition. To interpret these results, it is worth noting how these experimental conditions were first grouped when irradiation and sample preparation took place. On the first day of experimentation, the 9 mGy and 27 mGy conditions were studied. One the second day of experimentation, the 18 mGy and 36 mGy conditions were studied. Therefore, it is likely that some type of systematic difference resulted in the 9 and 27 mGy conditions having similar γH2AX foci per nucleus values that were both less than the 18 and 36 mGy conditions.

Likewise, for 53BP1[S1778], foci per nucleus did not follow a positive trend in relation to radiation dose. In addition, the 9 mGy and 27 mGy conditions also followed a similar pattern of having similar foci per nucleus numbers to the NIR condition and having less foci per nucleus than the 18 and 36 mGy conditions. The 18 mGy condition displayed the most number of foci per when measuring both γH2AX and 53BP1[S1778], despite there being two conditions with higher radiation doses.

Although similar condition grouping was done in the previous dose response series that ranged from 53 mGy to 371 mGy, this pattern was not observed, likely due to the fact that the difference in radiation doses was high enough to offset any systematic differences between experiments conducted on different days.

Taken together, the results suggest that our assay sensitivity was not sufficient for observing differences in DNA damage in cells irradiated with less than 40 mGy of x-ray radiation. This experiment would have benefited from having biological or technical replicates and calculating mean values from at least three independent trials. However, there were some factors that needed
to be considered before spending additional time and money on repeating this experiment, which was not budgeted for in our operating grant.

It appears as though reducing the 53BP1[S1778] antibody concentration from 1:100 to 1:200 was beneficial in reducing the number of foci per nucleus observed in non-irradiated cells. In this experiment, the value was 0.36 (SD 0.41), compared to the previous value of 1.68 (SD 1.19). Although 0.36 foci per nucleus is still higher than expected, and a standard deviation equivalent to the mean is certainly non-ideal, this was still an improvement. Before deciding to dilute the 53BP1[S1778] further, it was more important to resolve some other issues in our experimental design.

A major factor worth considering is that the irradiations were conducted at a different location for this dose-response series. Due to long-term dysfunction with our primary irradiator that is located within our laboratory facility at MaRS, we were forced to use an irradiator located in the Princess Margaret Cancer Center building across the street. However, the initial blood draws and post-radiation PBMC isolations still took place at MaRS. Thus, the blood had to be transported a significant distance in the middle of the experiment. The transportation process may have resulted in some of the blood samples being physically agitated, thereby adding another factor that may have contributed to DNA damage.

In addition, a different model of irradiator was used. However, we do not suspect that this contributed to the fact that our results were not positive for this experiment. Dosimetry was separately calculated and validated for this irradiator, and appropriate adjustments in irradiation time were made to account for any differences. Furthermore, even if there was a difference in this irradiator compared to the one used in our previous dose-response series, it would have
affected all experimental conditions equally; the overall trend would not have been affected. Nonetheless, it would have been preferred to carry out the dose-response series on the same irradiation device for consistency and inter-experiment comparability.

From our perspective, these were some of the gross irregularities in this experiment. However, there was a large section of the protocol that we did not have intimate knowledge of, where more experimental error could have occurred.

For our first two dose-response series, the Pathology Research Program at the University Health Network (UHN) conducted the immunofluorescence section of our protocol. Due to our research group’s lack of wet lab experience, we thought it would be prudent to outsource this stage of the protocol to a third-party service with expertise in this field. Unfortunately, since they are a core service that handles orders from all departments in the hospital network, there was typically a one-week delay in between our PBMC isolation and their immunofluorescence preparation. At this point in the protocol, the cells were already fixed and permeabilized. They were being stored at 4°C in PBS. In theory, this should have preserved epitopes of interest for primary antibody labelling. However, our experiment was expected to produce only small changes in a fraction of cells between conditions. Therefore, even small changes in membrane permeability or epitope configuration over time could have altered our results. In an ideal protocol, primary antibody labelling would have taken place the same day as cell fixation.

In addition, although we provided the Pathology Research Program with our desired antibody dilutions and incubation times, we had no way of ensuring precision or general diligence in handling our samples. We considered that this could be a problem when trying to study differences as low as 9 mGy between conditions.
Due to the reasons stated above, we decided that it would be necessary to conduct all subsequent dose-response series independently from start to finish, including immuno-labelling of our PBMCs, with minimal delay between steps in the protocol.

4.1.3 Dose response series B, 0 to 72 mGy

In the next dose response series, we used doses from 0 to 72 mGy. This represented an intermediate dose range between our first and second dose-response series. The goal was to help us understand the minimum radiation dose at which we were able to detect a difference in DNA injury between irradiated and non-irradiated cells.

This was also the first dose-response experiment where we conducted immunofluorescent labelling ourselves. Additionally, we changed our PBMC isolation and cell fixation protocol to mirror the methodology used by other leading international research groups who specifically study DNA DSBs in PBMCs isolated from patients undergoing diagnostic imaging procedures. Our previous protocol was optimized by a local radiation oncology group (whose auspices we conducted this research under). However, they primarily used that protocol to study DNA injury in other cell types, such as fibroblasts, after exposure to high doses of radiation.

Finally, the last major change we made to the protocol was the addition of an image post-processing step involving 3D de-convolution of our image z-stacks. This was employed to improve the contrast between IRIF and the background in our images. This process ultimately helped us determine the most appropriate threshold for differentiating between true IRIF and other domains of high optical intensity in our images.

Two technical replicates were conducted for this dose-response series. Due to our improved technical ability in PBMC isolation, only 2 mL of whole blood was needed per condition.
Both γH2AX and 53BP1[S1778] displayed a positive linear dose-response trend from 0 to 72 mGy. However, the R² values from the linear regression in this dose-response series (γH2AX 0.59; 53BP1[S1778] 0.72) were much lower than the R² value from our first γH2AX dose-response series (0.97). This suggests that in the sub-72 mGy range, our dose-response is not as linear as it is between 0 and 371 mGy. But this is also expected since as the difference in radiation doses becomes smaller, so should the difference in the number of DSBs per nucleus. And at some point, the difference in the number of DSBs per nucleus will be too small to discriminate between conditions, or too small to observe a perfectly linear dose-response when considering variability in the data.

Since the purpose of this experiment was simply to determine the working range of our assay, we did not conduct three independent biological repeats to be able analyze dose-response data with a 1-way ANOVA and Tukey’s multiple comparisons test. Although that would have been the most accurate way to determine the sensitivity of our assay, it was not the primary goal of our research. A qualitative analysis of our dose-response graph suggests that our assay cannot distinguish between non-irradiated cells and cells irradiated with up to 36 mGy. However, there appears to be a meaningful difference in γH2AX foci per nucleus when comparing the 0 and 72 mGy conditions, and also a marked difference the non-irradiated cells and the cells irradiated with either 54 or 72 mGy when measuring 53BP1[S1778]. Taken together, this suggests that our assay’s sensitivity for detecting differences between NIR and irradiated cells has a dose threshold between 50 and 70 mGy.

It is possible that our sensitivity was limited by the relatively high variability in the small amount of data in this experiment. By analyzing more cells or conducting more replicates, it is possible that our data would have showed more linearity at doses under 50 mGy.
Although a different protocol was used for this experiment, there is some inter-experiment consistency between the data observed in this dose-response series and our first dose-response series. For example, the NIR condition in the experiment discussed in 4.1.1 displayed 0.15 $\gamma$H2AX foci per nucleus (SD 0.03), which is almost identical to the 0.16 $\gamma$H2AX foci per nucleus (SD 0.01) observed here. In that same experiment, there was a 53 mGy condition that showed 0.26 $\gamma$H2AX foci per nucleus, which is similar to the 0.25 (SD 0.14) seen in this experiment’s 54 mGy condition. This indicates that we had achieved consistency with our technique and could move forward in conducting a small clinical study in patients.

4.1.3 Dose response series C, 0 to 72 mGy

Under the advisement of the Advanced Optical Microscopy Facility (AOMF) at UHN, it was recommended that we use a different microscope with our subsequent experiments in order to improve image quality and reduce the costs of conducting our experiment. We conducted a dose response series similar to the one in section 4.1.2 to ensure that our results would not be drastically different using this new hardware set-up. Upon preliminary microscope testing with a test slide from section 4.1.2, we noted that we needed to use exposure times that were 25 times greater for the FITC and CY3 filters, and approximately 30 times greater for the DAPI filter.

Rather than simply re-image a set of old slides to create a dose-response curve, we decided to conduct a small dose-response series with new blood samples to determine whether this new microscope and camera would be sufficient for our experimental needs. We conducted one independent experiment and only labelled cells with DAPI and $\gamma$H2AX, since $\gamma$H2AX was our primary marker for DSBs. We were hoping to achieve equal sensitivity seen in section 4.1.2.
Since only one repeat was conducted, we could not compare p-values to test the significance of differences between experimental conditions. Linear regression analysis did not show a significant trend from 0 to 72 mGy in this small experiment. However, this new hardware set-up did not change our results drastically when compared to the dose-response curve produced using protocol B. 0.11 γH2AX foci per nucleus were seen in the NIR cells and 0.47 foci per nucleus were seen in the cells irradiated with 72 mGy. This is a 4.3 fold difference, which is comparable to the 3.9 fold difference seen between the NIR and 72 mGy conditions in the last dose-response series.

The data from the 18-54 mGy conditions were especially deviant from the expected linear trend. The number of foci per nucleus was higher than expected in the 18 mGy condition (0.26/nucleus) and lower than expected in the 54 mGy condition (0.12/nucleus). However, these measurements were still lower than the 72 mGy condition, which is consistent with what should be observed. Overall, the data supports our earlier observation that it is difficult to distinguish between NIR cells and cells irradiated with doses less than 50 mGy if there are not enough experimental replicates or if the number of cells being analyzed is too low. In this experiment, an average of only 76 cells was analyzed per condition (range 51 to 108, SD 22). In the previous dose-response series, an average of 100 cells (range 74 to 129, SD 26) was analyzed per condition and two technical replicates were conducted for the experiment.

Although this dose response series did not provide any new findings nor did it showcase improved sensitivity with our assay, it suggested that imaging with the new microscope and camera combination produced similar results as our previous dose-response work. Since the use of this new hardware would increase image resolution and decrease our costs, we elected proceed with this equipment for our clinical study.
4.2. Proof of principle in pilot clinical study

Notably, we made one small modification to our methodology to improve the sensitivity of our assay for the clinical study. We added a phosphatase inhibitor, Calyculin A, to the whole blood sample as the first step in our PBMC isolation protocol. Due to timeline constraints, we were unable to test this additional step in a dose-response series. However, it has been successfully used by other research groups in similar studies and we followed their exact usage \(^{127,128}\).

The IRIF biomarkers we are studying are phospho-isoforms of nuclear proteins. Though sometimes these foci can remain present for more than 24 hours after radiation exposure, the phenomenon of residual foci is typically only seen in response to high doses of radiation. At low radiation doses, these foci form at sites of DSBs but phosphatase activity during the DSB repair process results in the proteins returning back to their unphosphorylated state. After this occurs, these sites are no longer detectable by our immuno-labeling technique.

It is estimated that phosphatase activity can reduce the number of observed DSBs as quickly as 30 minutes after radiation exposure. This is problematic because our PBMC isolation protocol takes several hours to complete. Therefore in theory, by the time we fixed our cells, some of the DSB foci would have resolved through phosphatase activity. This would lead to an underestimation of the number of DSBs being induced by radiation exposure, which limits the sensitivity of our assay.

During our dose-response series, we attempted to mitigate this issue by placing our blood samples on ice for 10 minutes, 30 minutes after radiation exposure. The decrease in temperature was intended to arrest phosphatase activity. However, during some steps in the protocol, temperature could not be controlled. For example, we carried out our work in a shared lab...
facility so it was not feasible to set the centrifuge to 4°C during centrifugation, which lasted 30 minutes at the beginning of the protocol. To avoid temperature fluctuations and the crude method of working on ice to reduce phosphatase activity, we instead proceeded with a biochemical approach using Calyculin A.

4.2.1 Subject characteristics

Due to the fact that this was a small pilot study, it was important that we minimize the amount of variability amongst our subjects. In addition, we wanted our first study to involve a radiation dose on the higher end of the spectrum among routine diagnostic imaging examinations. Hence, we conducted our study in the nuclear medicine department, recruiting only male patients who were scheduled to receive bone scans with 99m-Tc MDP for clinically indicated reasons. With this imaging modality, the same quantity of radiotracer is injected for all adult male patients, which meant all of their radiation doses were similar. Subjects received an average of 799 MBq (megabecquerel) of radiation, with a standard deviation of only 2.5% of the mean (SD 19.9 MBq). In terms of effective dose, an 800 MBq bone scan is equivalent to approximately 4.5 mSv.

Nine out of the ten patients recruited for the study received bone scans to stage their cancer diagnosis. Of the nine, seven received staging exams for prostate cancer, one for lung cancer, and one for pancreatic cancer. The single patient who did not undergo a bone scan for cancer staging instead had musculoskeletal pain as his clinical indication.

Since the most common reason for males to undergo bone scans is to stage cancer, and since cancer treatments often involve agents that cause DNA damage, it was important to ensure that none of the subjecting were undergoing treatment, or had undergone treatment recently, that
could confound our measurements of DNA DSBs. One of our exclusion criteria was receiving radiotherapy or chemotherapy in the past six months. Now that we understand our study population, we can examine their results in detail.

4.2.2 γH2AX data

For our clinical series, we used six fields of view per coverslip and three coverslips per condition in order to ensure that we were capturing enough cells to obtain data that accurately reflected each condition. There was congruence between the baselines values of γH2AX foci per nucleus in the control group (0.25/nucleus, SD 0.10) and the antioxidant group (0.27/nucleus, SD 0.10). This suggests that the data from our two groups is comparable and that any difference seen in the antioxidant group is likely due to the intervention, rather than some baseline disparity between the groups.

The 2-way repeated measures ANOVA showed an interaction effect between antioxidant intervention and radiation exposure (ie, whether the cells being analyzed were from the pre-radiation or post-radiation blood draw). When examining this interaction effect further with Sidak’s multiple comparisons test, it showed that there was a significant increase in γH2AX foci/nucleus after radiation exposure in control patients, but not in antioxidant patients. The PBMCs from the post-radiation blood samples in the antioxidant group (0.27/nucleus, SD 0.10) showed γH2AX foci per nucleus values that closely mirrored the pre-radiation baseline values.

Taken together, the results suggest that 99m-Tc MDP caused additional DSBs in PBMCs of control patients undergoing bone scans. However, antioxidant pre-treatment reduced the induction of DSBs caused by radiotracer injection.
4.2.3 53BP1[S1778] data

For 53BP1[S1778], 2-way repeated measures ANOVA showed a significant interaction between radiation exposure and antioxidant treatment. However, Sidak’s multiple comparisons test showed no significant difference between pre- and post-radiation 53BP1[S1778] foci/nucleus for either control subjects or antioxidant subjects. Therefore, when using 53BP1[S1778] as a biomarker for DNA DSBs, antioxidant intervention did not confer a significant radioprotective benefit.

Additionally, the pre-radiation 53BP1[S1778] baseline values for both control subjects and antioxidant subjects were much higher than expected. The control subjects had an average baseline measure of 0.42 foci/nucleus and the antioxidant subjects had an average baseline measure of 0.54 foci/nucleus. Across all 10 subjects, the average pre-radiation 53BP1[S1778] value was 0.48 foci/nucleus (SD 0.15). Meanwhile, the healthy volunteer from our in vitro dose response experiments exhibited 0.21 foci/nucleus (SD 0.05). Although it is reasonable for patients in our clinical study to display more foci per nucleus than the healthy volunteer from our in vitro dose response experiments due to their clinical indication, a 2.3 fold difference is quite large and suggests that there could be an error with the 53BP1[S1778] measurements in our clinical study. For reference, there was only a 1.7 fold difference between study subjects and our healthy volunteer when measuring baseline γH2AX values.

While the data from our γH2AX analysis is consistent and strong, it appears as though our 53BP1[S1778] immuno-labeling technique still requires improvement. This discrepancy may be due to the fact that we added a phosphatase inhibitor to our blood samples for the clinical study.
Although this technique has been validated in other studies with \( \gamma H2AX \), it has not been tested with \( 53BP1[S1778] \).

\( \gamma H2AX \) is the phosphorylated histone at the site of a DSB whereas \( 53BP1 \) is a nuclear protein that localizes to DSBs as part of the DDR. Although it is downstream of H2AX phosphorylation, \( \gamma H2AX \) is not required for \( 53BP1 \) recruitment post-DNA damage; it is only required for \( 53BP1 \) retention. \( 53BP1 \) recruitment is primarily mediated by MDC1, RNF8, and methylated histone residues. Of these three upstream regulators, MDC1 is particularly of interest because it is also phosphorylated during the DDR and its activity is based on its state of phosphorylation. It is important to note that our phosphatase inhibitor was not specific to \( \gamma H2AX \); Calyculin A is a general PP1 and PP2A inhibitor. Therefore, since \( 53BP1 \) recruitment is mediated by the phosphoisoform of MDC1, it is possible that we altered the number of \( 53BP1[S1778] \) foci that would normally be observed by introducing a phosphatase inhibitor. Ultimately, the technical modification that we made to improve our \( \gamma H2AX \) results may have caused the abnormally high and inconsistent \( 53BP1[S1778] \) results in our clinical study.

4.2.4. Overall summary of pilot clinical data

Premedication with oral antioxidants significantly reduced formation of \( \gamma H2AX \) foci, a marker for DNA double-strand breaks, in PBMCs after injection of 99m Tc MDP for standard bone scans.

We opted for a simple combination that included N-acetyl-cysteine (NAC) and ascorbic acid, two of the best studied exogenously deliverable antioxidants, in addition to two other antioxidants, alpha-lipoic acid and beta-carotene. Since glutathione is not generally bioavailable, it was provided as its precursor NAC. The time of 2.5 hours after oral dosing was chosen for
blood sampling as it is the approximate time at which oral NAC\textsuperscript{129} and ascorbate\textsuperscript{130} both peak in terms of bloodstream concentration following oral consumption. This time point also coincides with the time of patient imaging after radiotracer injection, thus minimizing inconvenience to study subjects.

Although the study cohort is small, the results are statistically significant. The consistent mitigation of DNA double-strand breaks is encouraging validation of the approach, timing, and selection of the antioxidants that were chosen. The possibility that NAC and ascorbate peaked in bloodstream concentration at the time of the blood draw may explain the marked radioprotective effect. But in addition, alpha-lipoic acid and beta-carotene may have also provided significant radioprotection during the exposure to IR. Alpha-lipoic acid was chosen as its bloodstream concentration peaks in approximately 1 hour after oral consumption\textsuperscript{131}, and beta-carotene was chosen due to its longer pharmacokinetic profile which peaks at approximately 4 hours\textsuperscript{132}. Thus in theory, patients in our experimental group received antioxidant radioprotection for a sustained period of time, congruent to the blood pool activity profile of 99m-Tc MDP.
4.3. Future Directions

Following up on our findings from this body of work, there are both *in vitro* and clinical studies that should be conducted as a natural extension to this project in order to refine our technique for measuring DNA damage, optimize our antioxidant formulation, and ensure radioprotection across a variety of imaging modalities.

4.3.1 Methodology

Throughout these experiments, we have improved our methodology for isolating, fixing, staining, and even imaging our cells. Much of our protocol is now based off of the published work by leading groups in Germany\(^9^9\) and Switzerland\(^1^3^3\). We have even incorporated the use of a phosphatase inhibitor for improving the sensitivity of our assay. As seen in our pilot clinical data, we have achieved consistency and adequate sensitivity for bone scans with our γH2AX technique.

We were not successful in detecting significant differences in DSBs using a second biomarker. Our technique for 53BP1[S1778] still requires improvement. We attempted to do primary labeling of γH2AX and 53BP1[S1778] simultaneously with the same incubation period, but with different antibody dilutions. This technique produced a reasonable dose-response trend in our final dose-response series so we carried on with this technique in the clinical series, where it did not work as well as expected.

The literature shows that a more appropriate technique for 53BP1[S1778] may involve a higher concentration of antibody but a shorter incubation period, such as a 1:100 dilution for 1 to 2 hours\(^1^3^4\). In order to achieve this, we could modify our protocol so that only immunolabeling for γH2AX is conducted immediately after the cells are fixed and permeabilized. The subsequent day,
the cells would be washed and blocked again for 53BP1 [S1778] primary immunolabeling for 1 hour, followed by simultaneous secondary immunofluorescent labeling for both proteins. If the additional washes and coverslip manipulations deteriorate the integrity of our γH2AX labeling or coverslip preparation, we could alternatively stain separate sets of coverslips with both antibodies. Three coverslips could be stained for γH2AX and 3 coverslips could be stained for 53BP1[S1778].

Apart from improving 53BP1 immunolabeling, we believe our PBMC preparation protocol shows good results and can be maintained for the future. Our method of quantifying DSBs, adopted from the Bristow group at UHN, is also adequate and admittedly more objective than other leading laboratories in our field. We captured multiple fields of view across multiple coverslips, made maximum intensity projections, and used a consistent software-based threshold to count foci per nucleus, whereas other groups simply score foci while examining cells under the microscope.

Although scoring IRIF per nucleus using microscopy has been the most commonly used method for studying DNA damage from diagnostic imaging over the past few years, it is time consuming and has room for experimenter bias to be introduced. Flow cytometry has been used recently for the same purpose, with fluorescently labeled γH2AX. A 2014-study compared the use of microscopy and flow cytometry for quantifying DSBs in patients administered intravenous (IV) antioxidants before x-ray based examinations. There was a strong correlation between microscopy and flow cytometry results.

This suggests that a shift to a flow cytometry based approach may be appropriate. The automated nature of flow cytometry allows for more cells to be analyzed per patient or experimental
condition. This will reduce variability with results and hopefully lead to better sensitivity for low doses of radiation.

4.3.2 *In Vitro* work

Although we completed a small clinical study, there is a considerable amount of basic radiobiology work that we should conduct before moving forward with larger clinical studies. With our dose-response experiments, we were able to discriminate between NIR cells and cells irradiated with 50 mGy or more. We would like to push our sensitivity limits further. We would start off with a series comparing cells irradiated with 10, 20, 30, 40, and 50 mGy. Depending on the results, we could also explore effects in the sub-10 mGy region of the dose-response curve.

We did not conduct a dose-response series with the addition of a phosphatase inhibitor to whole blood, so that is the first modification we would make moving forward. After conducting three independent replicates and analyzing the results, we will be better informed about the sensitivity limits of our assay and about the total number of cells that should be analyzed in order to reliably find a difference between non-irradiated and irradiated cells at ultra-low doses of radiation. It is important to conduct these studies because the results will help guide us in selecting the most appropriate imaging modalities and examinations for pursuing our clinical work. For example, if we cannot achieve sensitivity with doses as low as 5 mGy in *in vitro* experiments, it would be ill-advised to study the induction of DSBs and radioprotection in patients undergoing mammography or chest x-rays.

Next, it is important that we also conduct experiments with a cell type other than PBMCs. Although PBMCs are the most convenient cells to study in our line of work, they are not the most representative cell model for studying the carcinogenic risk of diagnostic imaging.
examinations. Peripheral lymphocytes are quiescent cells\textsuperscript{135}, so even if a DSB occurs and is mis-repaired, there is an extremely low chance that the cell will divide and eventually give rise to cancerous cells. In addition, lymphocytes are highly radiosensitive in terms of their propensity for exhibiting DSBs\textsuperscript{136}. Other cells that pose a risk of developing into cancer after exposure to low-dose radiation may not show as many DSBs per nucleus as lymphocytes do, given an equivalent radiation dose. Therefore, by measuring the difference between average induced foci in control and antioxidant patients using PBMCs, we may be over-estimating the radioprotective effects of our antioxidant treatment.

Using primary oral mucosal epithelial cell (OMEC) samples from healthy human volunteers would be an appropriate first step in moving away from the PBMC model. It would be ideal to repeat the same dose-response experiments conducted in PBMCs in OMECs as well. Gonzalez and colleagues have successfully observed increased γH2AX foci after in vitro radiation of human exfoliated buccal cells\textsuperscript{137}.

Once we have completed our dose-response work with both γH2AX and 53BP1[S1778], the next stage would be to conduct \textit{in vitro} experiments with antioxidant combinations at varying doses. In our completed pilot clinical study, we administered antioxidants at the maximum daily doses recommended by Health Canada. We did not exceed these recommended doses because we wanted to ensure that patient safety would not be compromised and we also wanted to determine if radioprotection could be achieved using a simple over-the-counter (OTC) pill regimen that would not require any additional regulatory approval. However, it would be worth testing if dosing strategies beyond these limits would confer any extra radioprotective benefit.
While these *in vitro* experiments will be a good start for determining effective antioxidant combinations and estimating optimal dosing strategies, orally administered antioxidants have varying pharmacodynamic characteristics that make *in vitro* studies not perfectly translatable to the clinical setting. Therefore, future work should primarily focus on *in vivo* studies.

4.3.3 *In Vivo* studies

First, we should repeat our bone scan pilot study in a larger patient population to validate our findings. But we should conduct the study in a randomized blinded fashion, ensure we have two working biomarkers for measuring DSBs, and also collect OMECs to compare against PBMCs. Bone scans, and nuclear medicine studies in general, were an ideal imaging type for beginning our research. The fact that the entire blood pool was irradiated, that radiation exposure occurred over an extended period of time, and that we were studying DNA damage in PBMCs allowed us to observe a treatment effect even in a small study with only 10 patients. However, nuclear medicine examinations are conducted less frequently than CT and they are conducted in older patients, for whom the risk of long-term carcinogenesis from radiation exposure is lower. Therefore, the ultimate goal is to achieve radioprotection in patients undergoing CT.

CT is a more challenging modality to study because only a fraction of a patient’s blood pool is irradiated during a typical CT exam. The human body’s blood volume circulates throughout the entire body in approximately one minute, so the irradiated PBMCs will promptly be diluted with the rest of the non-irradiated PBMCs. This will make it harder to detect induced DSBs in PBMCs isolated from whole blood obtained from the antecubital vein after radiation exposure. Despite this limitation, researchers have been able to observed induced DSBs in human PBMCs after CT\textsuperscript{124}.
In our work, we observed an average of 0.31 excess γH2AX foci per nucleus in control patients injected with 99m-Tc MDP for bone scans, with a mean baseline of 0.25 foci/nucleus (SD 0.10) and mean post-radiation measure of 0.56 foci/nucleus (SD 0.17). This increase was statistically significant, even with our small sample of 5 control subjects. However, if we were to conduct a similar study in patients undergoing CT examinations, we would expect to see lower excess foci per nucleus and thus would require a study with more subjects in order to have sufficient statistical power. For example, in order to observe a statistically significant two-fold increase in foci per nucleus in this group (i.e., 0.25 excess foci per nucleus) with 95% power, we would need a control group consisting of 9 subjects. And in order to observe a roughly 50% increase in foci/nucleus, 35 patients would be required.

These sample size calculations are based on the standard deviation and paired correlation coefficient measures from our completed pilot study. However, most of our subjects were cancer patients and their average age was 67.7 years (SD 11.3). The group means, standard deviations, and paired correlation coefficients may be different for a younger cohort with different clinical indications. For example, we know from the literature and from our in vitro dose response experiments that non-irradiated PBMCs from healthy volunteers under 40 years of age typically exhibit only 0.10 to 0.15 foci per nucleus, in contrast to the 0.25 seen in our study subjects. Nonetheless, any of our future clinical studies should have more than 5 subjects per group.

In addition to using larger groups for future clinical studies, the issue of lymphocytes being amitotic and non-oncogenic means that another experimental approach should also be considered for our in vivo work. Though head CTs are on the low end of the spectrum in terms of effective dose, it may be an appropriate diagnostic exam for studying non-PBMCs, since the mouth is within the field of irradiation. If we optimize a protocol for studying DSBs in OMEC, we can
conducted a study in patients undergoing head CTs to observe whether or not there is increased DNA damage in cells obtained from a buccal swab. If we observe an increase in DSBs, we can study whether antioxidant pre-medication decreases the number of induced DSBs.

Patients undergoing a combined chest-abdo-pelvis CT are exposed to a significantly high dose of radiation (up to 20 mSv) so they are an important subset of patients to investigate antioxidant-based radioprotection in. However, if we wish to study non-PBMCs in this group, our methods will be more invasive. We will have to take multiple punch biopsies from the skin.

Before pursuing a study that involves invasive tissue procurement from patients, it would be ideal to validate radioprotection during body CT examinations in an animal model. With this approach, we can study not only the skin, but also other tissue types, including the more radiosensitive breast, stomach, lung, and colon tissues.

4.3.4 Other methods of radioprotection

We believe that administering oral OTC antioxidants, if proven to be effective, is one of the most pragmatic approaches for protecting patients from the risk of radiation exposure during diagnostic imaging exams. The approach is inexpensive, widely accessible, convenient, and poses minimal risk to the patient. However, there are some shortcomings. Typical CT exams involve radiation exposure over a short period of time, anywhere between several seconds and a few minutes. In order for oral antioxidants to be effective, they must be bioavailable and ideally at their peak concentration at the precise time of radiation exposure. In order to ensure this, patients must consume their oral antioxidants several hours before their scheduled scan time. Although the inconvenience of this can be mitigated by having patients acquire antioxidants
beforehand and consume them at home, CT scans can often be delayed, which would reduce the efficacy of a timed pre-treatment.

Intravenous antioxidant delivery has recently been tested in patients undergoing cardiac imaging and has been shown to be effective\textsuperscript{133}. It can be administered immediately before radiation exposure and be made bioavailable. Many patients are already given IV contrast agents, so this would integrate well with some CT protocols. However, for patients not receiving IV contrast, this method of radioprotection is more invasive. Furthermore, there is the risk of anaphylaxis from intravenous drug administration.

Inhalation of gas containing 1.3% hydrogen has been shown to be radioprotective as well. However, in the most recent study showing proof-of-principle, inhalation was done for 2 hours before radiation exposure and an x-ray dose of 20 Gy was used\textsuperscript{138}. If inhalation-time can be reduced and shown to be effective with low doses of radiation, this is another promising alternative to oral antioxidant pre-treatment.
Chapter 5 – Conclusion

The hazardous effects of ionizing radiation have been well documented since soon after its discovery in the early 1900s. And recently, researchers have even been able to observe an increased cancer incidence in people who received diagnostic medical imaging exams as pediatric patients. Though the risk from each individual scan is small, the frequent and rising use of medical imaging make this a public health issue, especially in more radiosensitive populations.

While considerable efforts have been made to reduce the amount of unnecessary radiation exposure that patients are exposed to—by means of advances in imaging technology and image reconstruction processes—a biological approach for improving radioprotection has not been given as much attention.

In our research, we showed proof-of-principle for an inexpensive over-the-counter approach to this problem. Patients who were administered oral antioxidants prior to radiotracer injection for bone scans showed considerably less induced DSBs in their PBMCs, compared to control patients who did not receive an antioxidant pre-treatment.

In the future, this work should be expanded to include other imaging modalities such as CT and fluoroscopy. Research should be undertaken to determine the optimal nutraceutical combination and dosing strategy for each modality. While our current methods show proof-of-principle for mitigating DNA injury, reducing the number of DSBs in PBMCs does not prove a reduction in cancer risk. Long-term follow-up should be conducted in a large patient cohort to determine whether antioxidant premedication can quantifiably reduce cancer incidence from IR exposure in medical imaging. If shown to be effective, the impact of this research would be substantial.
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