THE LEUKOCYTE APOPTOSIS ASSAY: A CLINICAL PREDICTOR OF RADIOSENSITIVITY ?

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The Leukocyte Apoptosis Assay: A clinical predictor of radiosensitivity?

by

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DECLARATION

I, **Wendy Lynn Solomon**, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Wendy Lynn Solomon **Date**

ABSTRACT

The main objective of radiotherapy is to completely eradicate tumours while preserving the integrity of the normal surrounding tissue. The severity of normal tissue reactions varies considerably amongst individuals. Following the same treatment regime for individuals with identical tumours in terms of location, pathology and size might result in adverse tissue reactions for some while others may experience no adverse reactions. Based on this principle some individuals who are more radiosensitive than others are receiving beyond normal tissue tolerance doses while others, who are more radioresistant, receive sub-tolerance doses. These differences in response to radiation are a concern for both the patient and the oncologist. For these reasons it is essential to predict individual responses to radiotherapy in order to optimise the treatment regime for each patient.

The development of predictive assays for normal tissue reactions are in the early stages and not routinely used in laboratories. Qualities of a predictive assay should include the capacity to distinguish between small changes in individual radiosensitivity. These assays should also be easy and quick to perform so that it can be employed during the initial treatment planning phase for therapy. Also, the assay should be inexpensive enough to be used on a daily basis in the clinic.

The Leukocyte Apoptosis Assay (LAA) is able to predict intrinsic radiosensitivity of individuals based on the apoptotic response of T-lymphocytes. Apoptosis, or programmed cell death, is a form of cytotoxicity that can be observed after radiation exposure and can be evaluated using flow cytometry. This investigation involves the application of the assay in a South African context where it had not been used before.

The principle aim of this study was to assess the feasibility of using the Leukocyte Apoptosis Assay to investigate individual variability and sensitivity to radiation within a South African population. Certain variables such as age, gender and race which could have implications on the apoptotic response of individuals have also been taken into consideration. Briefly, heparinised blood was collected and exposed *in vitro* to 0Gy (control), 2Gy and 8Gy gamma radiation. After 48 hours, lymphocytes were collected

and prepared for flow cytometric analysis using FITC-conjugated CD4 and CD8 monoclonal antibodies. Apoptosis was assessed by gradual internucleosomal DNA degradation using PI staining. Percentage radiation-induced apoptosis was determined for each sample. Radiation-induced apoptotic responses were studied in 300 healthy donors (mean age: 41.6yrs, range 19-78yrs) from the Western Province Blood Transfusion Services (WPBTS) as part of a standard curve study. Although interdonor variation was observed, intra donor variation was low. Results also showed a clear dose response curve. Significant gender, race and age differences were observed in this study. Using z-score analysis, the data showed that approximately 2% of donors had a low apoptotic response in both CD4 and CD8 lymphocytes.

South Africa is one of the leading countries in treating radioresistant and inoperable tumours using neutron beam therapy. Severe late reactions from neutron beam therapy are a particular concern. For this reason heparinised blood from 18 donors were exposed to both X-rays and neutrons and analysed using the LAA methodology. It was found that the LAA assay was able to distinguish cellular radiation damage induced by X-ray and neutron treatment modalities. When comparing a neutron dose half that of X-rays, the ratio of biological effect was approximately 1. The LAA endpoint data is therefore consistent with previously published RBE data and is able to reflect the difference in ionisation density of the different treatment modalities.

The difference in methodology between the LAA and the more routinely used, Annexin V-FITC Apoptotic assay, was also investigated. Heparinised blood from 3 donors was irradiated with X-rays and neutrons and labelled with Annexin V-FITC and PI for the Annexin V assay. The same 3 donors were previously analysed using the LAA assay. The Annexin V data obtained did not show a clear dose response curve. There were also not the expected differences noted between the neutron and X-ray data. The Annexin V assay did not work for this study and the investigation was not continued. After doing both the Annexin V and the LAA assay, the LAA assay is the preferred method to measure intrinsic radiosensitivity.

The identification of radiosensitive individuals using test radiation doses to blood samples also allow one to manage the detriment of exposure to radiation workers. For

this reason blood from 10 donors were analysed using the LAA as well as the micronucleus assay. No significant relationship was found between the apoptotic response of an individual and their micronuclei frequency. Further investigation into this would however be most informative for radiation protection practices.

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I dedicate this thesis to my husband, Noel,
my daughter, Emma
and my parents, Mathew and Irene.

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ABBREVIATIONS

α	alpha
β	beta
γ	gamma
µg	microgram
µl	microlitre
Bq	bequerel
CBMN	cytokinesis-blocked micronucleus assay
Cyt-B	cytochalasin-B
DNA	deoxyribonucleic acid
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
Gy	Gray
IGRT	Image Guided Radiotherapy
IMRT	Intensely Modulated Radiotherapy
keV	kilo electron volt
LAA	leukocyte apoptosis assay
LET	linear energy transfer
LINAC	linear accelerator
meV	milli-electron volt
Ml	millilitres
mM	millimolar
MN	micronucleus
mu	monitor unit

NDI	nuclear division index
PBS	phosphate buffered saline
PE	phycoerythrin
PI	propidium iodide
PMT	photomultiplier tube
PS	phosphadtidylserine
RBE	relative biological effectiveness
RIA	radiation-induced apoptosis
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RTOG/EORTC	Radiation Therapy Oncology Group/ European Organisation For Research And Treatment Of Cancer
SSC	side scatter

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Radiation

Radioactivity is the transformation of the nucleus of radioactive atoms together with the emission of radiation in the form of particles (alpha and beta particles) or photons (gamma rays). The unit for radioactivity is the Becquerel (Bq) which corresponds to one radioactive disintegration per second (Hall, 2000).

Ionising radiation is the transfer of energy in the form of electromagnetic waves or particles as it passes through matter. This energy can displace electrons from atoms or molecules, thereby producing ions (Hall, 2000). The presence of these charged or ionised atoms in living tissue has the potential to disrupt normal biological processes.

1.1.1 Electromagnetic radiation

X- and gamma-rays are 2 forms of electromagnetic radiation routinely involved in the study of biological damage in various systems. As these are both photons, they do not differ in their nature or properties, but differ in the way in which they are produced. It is however generally accepted that gamma-rays are more energetic than X-rays. X-rays are produced extranuclearly in an electric device that accelerates electrons to high energy and can stop abruptly in a target, such as tungsten or gold. Gamma rays are produced intranuclearly by the spontaneous decay of radioactive material such as ^{60}Cobalt. A gamma ray can penetrate deeply into human tissue and has been widely used for cancer radiotherapy (Hall, 2000).

1.1.2 Particulate radiation

Alpha rays, beta rays, protons and neutrons are examples of particulate radiation. Alpha rays are produced by the spontaneous decay of certain radioactive atoms such as radium, plutonium, uranium and radon. Due to its large mass and positive charge, the alpha ray can only pass a short distance. It is only harmful once it has entered the body, however it can be stopped by a filter, as thin as a sheet of paper. (Hall, 2000)

Beta rays are produced by the spontaneous decay of certain radioactive materials such as tritium, ^{14}C, ^{32}P and ^{90}Sr. It has a single electric charge, either positive (positron) or negative (negatron). Beta particles are harmful if they enter the body; however they are easily stopped by a thin sheet of metal (Hall, 2000).

Neutrons are released following nuclear fission of heavy metals such as uranium or plutonium. Neutrons are also produced using particle accelerators by bombarding a beryllium target with protons or deuterons. Neutrons do not carry any electric charge and therefore hardly damage cells. However, when neutrons impinge on the nucleus of hydrogen, it causes ionisation in the body, leading to various types of damage (Hall, 2000). Neutrons also interact with the nuclei of carbon, nitrogen and oxygen. This produces charged particles that are generally large in mass and in charge (Tubiana et al, 1990).

1.1.3 Absorbed dose

The basis of generating a dose absorbed in tissue is the energy transfer processes (ionisation events) that occur when radiation pass through matter. Dose is defined as $D = \Delta\varepsilon/\Delta m$ where $\Delta\varepsilon$ is the mean energy transferred by the radiation to a mass Δm of the medium (Report 33 of the International Commission on Radiation Units and Measurements (ICRU)) (Tubiana et al, 1990). The SI unit of radiation dose absorbed in tissue is the gray (Gy). One gray equals one joule per kilogram. Although cellular damage increases as a function of the dose absorbed, the quantity of the macroscopic dose (joules/kilogram) is not sufficient to fully predict the level of biological damage. For example, a unit dose of alpha particles and neutrons produce much more biological damage than a unit dose of x-rays or gamma rays (Price et al, 1990). Since neutrons have also been used in this study and cellular damage is compared to that of X-rays, the microscopic dose distributions for the different treatment modalities need to be considered.

1.1.4 Linear Energy Transfer (LET)

Linear Energy Transfer is the rate at which energy is deposited per unit distance of the path travelled by a particle. That is, $L = dE/dl$ (Hall, 2000). LET is measured in kiloelectron volts per micrometer (keV/μm) and is a function of the mass, charge and energy of the radiation (Travis, 2000). LET is proportional to mass and charge and

inversely proportional to the energy of the radiation. X-rays and gamma-rays are examples of low-LET radiation since it produces sparse ionisations separated by relative long distances. High-LET radiation includes neutrons and alpha particles which produce many ionisations in very short distances (Travis, 2000). All radiation types induce microscopic dose distributions that can vary over many orders of magnitude when expressed as keV/μm. (Tubiana et al, 1990). Therefore it is not prudent to assign a mean LET value to a particular type of radiation but this is often done. Examples are ^{60}Cobalt gamma-rays at 0.2 keV/μm and 250keV X-rays at 2.0 keV/μm (Hall, 2000). A large overlap is evident when the lineal energy transfer of neutrons and ^{60}Cobalt gamma rays are compared. The lineal energy produced by neutrons that impinges on a proton when interacting with a hydrogen atom is for a large part low-LET in nature. The largest part of a unit dose of neutrons is the result of such knock-on protons. Smaller fractions of a unit dose of neutrons are made up by alpha particles and heavy recoil fragments. These particles are the result of neutrons interacting with the nuclei of carbon, nitrogen and oxygen atoms. These particles induce very high-LET ionisation densities. Although the very high-LET portion of a neutron dose is relatively small, it is this part that is responsible for most of the biological damage (Tubiana et al, 1990).

1.1.5 Radiobiological Effectiveness (RBE)

Equal doses of different types of radiation do not produce equal levels of biological effect. RBE is defined as the ratio of a dose of test radiation to the dose of a reference radiation that produces the same level of biological response (Travis, 2000). The reference radiation in the past has often been 250 keV X-ray or ^{60}cobalt gamma rays (Hall, 2000). With high energy linear accelerators, now commonly available, 6 MeV X-rays is used.

RBE is expressed in the following formula:

$$\text{RBE} = \frac{\text{Dose in Gy from reference radiation}}{\begin{array}{l}\text{Dose in Gy from test radiation delivered}\\ \text{under the same conditions that produces}\\ \text{the same level of biological effect.}\end{array}}$$

(Hall, 2006)

RBE values are always calculated at a level of isoeffect when comparing the biological effectiveness of radiations of different ionisation densities (Travis, 2000). For a particular charged particle, the lower the LET, the lower the biological effect (Dainiak, 2002). RBE is not a constant. It is dependant on various biological and physical parameters. Most fundamental to this is the dose. The lower the dose of the test radiation, the higher the RBE that can be calculated. RBE also changes with the type of cells or tissue studied as well as the particular endpoint that is being followed.

1. 2 Effects of Radiation

1.2.1 Interaction of Radiation on cells

When ionising radiation interacts with cells it produces ionisations and excitations that could directly or indirectly affect cellular DNA. Direct action occurs when radiation deposits its energy directly in a macromolecule (that is DNA) that results in DNA strand breaks. Both direct and indirect actions occur for all types of ionising radiation. The likelihood of a direct action following exposure to high-LET radiation like neutrons is higher compared to direct action from exposure to low-LET X-rays. Indirect action is when ionisations are absorbed in water molecules that result in unstable unpaired chemical species called free radicals. Free radicals have the ability to initiate chemical reactions and therefore indirectly cause damage to cells (Travis, 2000).

1.2.2 Radiation effects on the DNA molecule

DNA is a relatively large molecule with a double helix structure. It consists of two strands held together by hydrogen bonds between the bases (Hall, 2000). Radiation can lead to different types of damage to the DNA molecule. The loss or change of a base on the DNA chain can lead to a mutation as the sequence of bases are responsible for storing and transmitting genetic information. Breaks in just one strand of DNA molecules are efficiently repaired after irradiation with little or no long term consequences to the cell. Breaks in both chains of the DNA are more difficult for the cell to repair accurately. If repair does not take place after irradiation, the DNA chain can separate and lead to cell death. Radiation can also produce crosslinks between two regions of the same DNA strand, between two complementary DNA strands or between different DNA molecules. These lesions could be detrimental if not repaired properly (Travis, 2000).

1.2.3 Radiation effects on the chromosome

When cells are radiated, chromosomal breaks are produced, resulting in "sticky" ends which could rejoin with other sticky ends. Breaks may rejoin in their original configuration with no effect on the cell or they may fail to rejoin resulting in aberrations which are deleted at the next mitosis. Broken ends could also reassort and join to form distorted chromosomes (Hall, 2000).

Chromosome aberrations occur when a cell is irradiated before the chromosomal material has doubled. The radiation-induced break occurs in a single strand of chromatin which is replicated leading to a chromosomal aberration visible in the next mitosis. When the cell is irradiated after the DNA material has been doubled, the radiation-induced break occurs in the two chromatin strands of the chromosome. The aberrations produced are called chromatid aberrations (Hall, 2000).

Chromosomal aberrations have long been linked with carcinogenesis and in certain cells e.g. peripheral lymphocytes; they act as biomarkers of radiation exposure (Bonassi et al, 2000).

1.2.4 Haemopoietic system

The haemopoietic system is highly sensitive to ionising radiation. Radiation damage to stem cells of the bone marrow affects the circulating blood cells and platelets, as well as the lymphoid tissue found in the spleen, liver, lymph nodes and thymus (Hall, 2000). Ionising radiation causes irreversible gene mutations and chromosomal aberrations in stem cells which could increase the risk of leukaemia (Wright, 2002).

Circulating red blood cells, platelets and granulocytes are radioresistant whereas lymphocytes are more sensitive to radiation. After radiation, a drop in lymphocytes can give an indication of radiation exposure and dose levels (Travis, 2000).

1.2.5 Repair of radiation damage

Studies have shown that after cells have been exposed to ionizing radiation, some DNA damage is repaired. The cellular repair involves the restoration of the normal nucleotide sequence of DNA after damage (Bohr *et al*, 1989; Giver et al, 2001).

There are two types of repair namely sublethal damage (SLD) repair and potentially lethal damage (PLD) repair. Sub-lethal damage repair is demonstrated when cells are irradiated with two or more doses separated by time. Potentially lethal damage repair occurs when post-irradiation conditions are altered in order to delay cells entry into mitosis. Cell damage is usually expressed at cell division and by delaying this, repair of DNA damage is allowed. Both sub-lethal damage and potentially lethal damage repair is not as prevalent after high-LET radiation (for example neutrons, carbon ions and alpha particles) (Travis, 2000).

1.3 Radiotherapy

Approximately 60% of cancer patients are treated with radiation therapy and it has been regarded as the second treatment of choice after surgery (Torres-Roca & Stevens, 2008). The success of radiotherapy involves destroying malignant tumours while minimizing damage to the surrounding normal tissue (Symonds, 2001). As radiation doses are increased, more cells are destroyed until a sufficiently high dose has completely eradicated the tumour (West & Hendry, 1992). This same dose of radiation however will also increase the amount of damage to the normal surrounding tissue. For this reason, the amount of radiation used to treat tumours should be limited by the tolerance of the surrounding normal tissue and not that of the tumour itself (Papiez et al, 2002). If the patient's level of tolerance could be identified prior to treatment, it could lead to an administration of high doses for patients with higher tolerance. Therefore tissue prediction assays can lead to an improvement in tumour control rates for patients receiving higher doses (West & Hendry, 1992).

1.3.1 Types of radiotherapy

Different treatment modalities are selected depending on the type and location of the tumour. This investigation focussed on X-ray and neutron therapy.

1.3.1.1 X-ray therapy

Since the discovery of X-rays by Roentgen in 1895, many developments have been made regarding the use of X-rays in medical applications. Since the 1950's, conventional X-ray radiotherapy has grown worldwide and today most hospitals have a

radiotherapy service (Scharf & Chomicki, 1996). Traditional radiotherapy requires that conventional X-rays be delivered into the body in doses sufficient to assure that enough damage has occurred to all the cancer cells. If given in sufficient doses, X-ray therapy can control many cancers but the risk is that healthy tissue may receive a similar dose and can be damaged. This usually results in a less than desired dose to reduce damage to healthy tissue and avoid unwanted side effects. These limitations in X-ray therapy have led to the use of the heavy charged-particles, protons in radiotherapy. The advantage of proton treatment over conventional X-ray treatment is that the energy distribution of protons can be directed and deposited in tissue volumes designated by the physician in a three dimensional pattern directed from each beam used (Habrand et al, 1995). This provides greater control and management of the cancer while it reduces damage to healthy tissue and vital organs. Heavy particle therapy, on the other hand, is more expensive than conventional X-ray therapy. High dose, precision X-rays can these days be delivered through new technology which includes image guided (IGRT) and intensity-modulated radiotherapy (IMRT) (Torres-Roca & Stevens, 2008). For the purpose of this study though, conventional X-ray therapy measures were used.

1.3.1.2 Neutron therapy

Fast neutrons are more effective in treating large, slow-growing tumours which are resistant to conventional X-ray radiation (Jones & Wambersie, 2007). Examples of these tumours include salivary gland tumours, prostate cancer and soft tissue sarcomas.

The reasons for using neutron therapy can be explained by the (i) oxygen effect and the (ii) cell cycle effect. In larger tumours, the blood supply is reduced and cells a distance from a capillary are deprived of oxygen. Cells lacking oxygen are much more resistant to low-LET X-rays than to high-LET neutrons. The oxygen enhancement ratio for X-rays is a factor of 2.5-3. For neutrons this value is reduced to 1.6-1.7 (Fowler, 1981). Hypoxic cells are therefore less protected from ionising radiation when treated with neutrons. The cells of slow growing tumours spend less time in the dividing phase of the cell cycle where they would be more sensitive to radiation. Cells that spend more time in the DNA synthesizing phase are more resistant to conventional X-ray radiation. Fast growing cells that have a high percentage of cells in S-phase, have been found to be more resistant to X-rays but not to neutrons (Slabbert et al, 2000).

Wambersie et al (1994) asserts that 10-15% of patients treated with ionizing radiation benefit from neutron therapy. These patients however first need to be identified due to the more severe side effects of neutron therapy in comparison to conventional treatment. Wambersie et al (1994) also reports that in most studies higher percentages of late complications have been found for patients treated with neutrons. Factors such as poor technical conditions could be the cause for these late complications. Treatment modalities should therefore be evaluated to give the best tumour control without severe side effects.

A continuing neutron therapy program has been conducted at iThemba LABS for the past 20 years. This was done, not withstanding the effect that Neutron Therapy facilities worldwide have closed down. Lessons learnt from neutron therapy studies are very applicable to modern particle therapy that is been established worldwide. For example, carbon ion therapy has ionisation densities similar to that of neutrons. This is the reason why many researchers from outside South Africa use the neutron beam at iThemba LABS in Faure..

1.3.2 Side Effects of Radiation

Radiation effects on normal tissue are usually divided into early and late reactions (Williams *et al*, 2003). Early or acute reactions include any treatment-related morbidity that occurs within 90 days after commencement of radiotherapy. Depending on the size and area being treated, characteristic symptoms include mucositis, dermatitis, vomiting, diarrhoea, epilation (hair loss) and depletion of the cellular compartments of the bone marrow. Late reactions, which are usually severe and irreversible, occur several months or even years after radiotherapy and may jeopardize the success of therapy (William *et al*, 2003). Typical examples include lung or subcutaneous fibrosis, chronic myelopathy, telangietasia, bone necrosis and radiation nephropathy.

Studies with patients on radiotherapy have indicated a correlation between the radiosensitivity of normal tissue fibroblasts cultured *in vitro* and late reactions in the same patients from whom the cells were taken (Hall, 1997). Side effects need to be considered when assessing the benefits of the treatment modality to be used. The Radiation Therapy Oncology Group/European Organisation for Research and Treatment

of Cancer (RTOG/EORTC) classification system was designed to document late side effects in more detail and classify symptoms and treatment using a six-point scale ranging from nil (0) to fatal (6) (Hoeller *et al*, 2003). It is important to have an assay that could predict late effects as it is more critical to prevent late effects than early effects.

1.3.3 Fractionation of doses

Dividing the treatment dose into smaller fractions reduces the damage to normal tissue due to the repair of sub-lethal damage between doses. Fractionation however increases the damage to tumour cells because of re-oxygenation and redistribution of tumour cells in the cell cycle. Re-oxygenation and redistribution after the first dose of radiation causes tumour cells to move from a resistant phase of the cell cycle into a more sensitive phase within a few hours (Hall, 1997). Fractionated irradiation could therefore shorten the total time of treatment as well as reduce the risk of tumour repopulation and long term toxicity (Brizel *et al*, 1998). During fractionated radiotherapy, the total dose, size of the fractions, number of the fractions and the time over which the treatment is spread are determined by both the tumour and the tolerance of the surrounding normal tissue (Travis & Tucker, 1987).

Dose fraction schemes are employed in radiotherapy to exploit the differences in reparable radiation damage between early-responding (for example skin and mucosal) and late-responding tissue (for example spinal cord) (Hall, 2000). The relative importance of fractionated radiotherapy for different types of tissue is reflected in their respective α/β (alpha/beta) ratios. α/β ratios for a particular tissue type reflects the reparability of the tissue (Steel, 2002). Late responding tissues have smaller α/β ratios and as such a higher capacity to repair sub-lethal radiation damage (Withers et al, 1982). By contrast tumours and early responding tissue have higher α/β ratios and are less protected by fractionated treatment protocols.

Typically 30 fractions at 2Gy per fraction are used in X-ray therapy. Fractionation schemes are less important for neutron therapy. Cells and tissue are more sensitive to high-LET radiation. This results in higher levels of alpha-type damage and α/β ratios for all tissue types are generally very high values (10-20Gy). As a result less reparable damage is induced by neutrons in fractionated schemes. Neutron therapy is only given

in 12 fractions using 1.7Gy per fraction. Fractionated neutron therapy is less likely to protect late-responding tissue because complications in neutron therapy can easily be induced (Joiner and Johns, 1987).

1.4 Apoptosis

Apoptosis was first described by Kerr and colleagues (1972) as a particular set of changes associated with cell death. Apoptosis is derived from the Greek words *apo* for "apart" and *ptosis* for "fallen" which had been used to describe the autumnal dropping or shedding of leaves.

Apoptosis or "programmed cell death" is a normal physiological process aiding in the process of embryogenesis, normal tissue turnover, immune development and defence and the prevention of carcinogenesis (Fleischer, 1997). This process of cell death can be induced by a number of internal and external stimuli. Internal stimuli include exposure to radiation and toxins as well as metabolic and cell cycle disturbances whereas external stimuli include the removal of growth factors and hormones and reactions with cell surface receptors like FAS protein (Wang, 2005).

Radiation-induced apoptosis in certain cells, especially peripheral blood lymphocytes, is thought to result from initial DNA damage. When faced with DNA damage, lymphocytes could either repair the DNA damage or undergo apoptosis in order to reduce the carcinogenic risk of radiation exposure (Cregan et al, 1999; Petrovic et al, 2005). Cells from the haematopoetic- and lymphoid systems are highly sensitive to radiation and cells from epithelial origin are less sensitive. After irradiation, lymphocytes do not enter mitosis but die rapidly by interphase death due to apoptosis. This apoptotic response explains the rapid loss of lymphocytes after radiation treatment (Steel, 2002).

Radiation-induced apoptosis can initiate signalling in different parts of the cell including the nucleus, cytosolic elements and plasma membrane. Studies have shown that the tumour-supressor gene, p53, plays an important role in the cell's response to DNA damage (Verheij and Bartelink, 2000). Activation of p53 after radiation damage causes cell cycle arrest at G1, allowing the DNA damage to be repaired before replication and mitosis occurs. If repair fails, p53 triggers the apoptotic deletion of the cells with DNA

damage (Kerr et al, 1994; Verheij and Bartelink, 2000; Wang, 2005). Any inhibition or mutation of p53 and the other proteins involved in the induction of apoptosis (ATM, Bcl-2 and bax) can result in compromised apoptosis (Crompton et al, 2001; Mallick, 2005).

Morphological changes during apoptosis starts with cellular shrinkage due to cytoplasmic condensation. This is followed by chromatin condensation and fragmentation of the nucleus. Eventually the cells separate into a number of small fragments or apoptotic bodies which are then phagocytosed by neighbouring cells (Bras et al 2004). Apoptosis should not be confused with another form of cell death, necrosis. Necrosis is a series of changes occurring in response to irreversible cell injury. It involves enzymatic degradation of the cellular membrane causing the cellular contents to leak out of the cell and evoke an inflammatory response (Kumar et al, 2007). During apoptosis the cell membrane remains intact but is altered in such way that the cell and its fragments attract phagocytes resulting in the cell being cleared without evoking an inflammatory response (Bras et al, 2004).

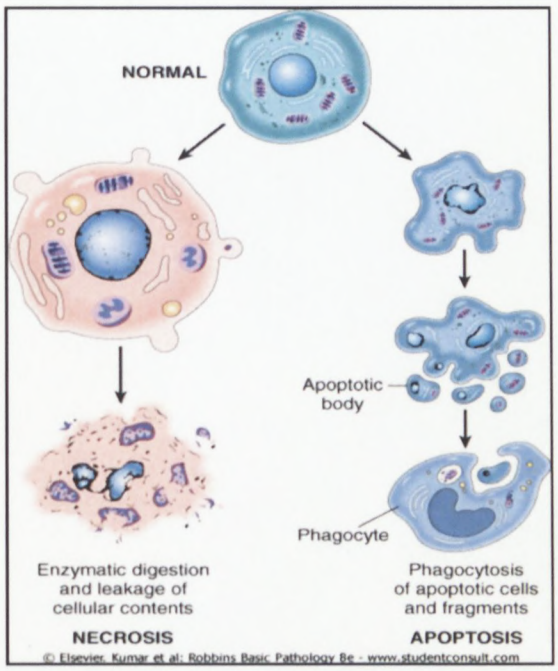


Figure 1.1: Cellular features of necrosis (left) and apoptosis (right) (Kumar et al, 2007).

Molecularly, apoptosis is initiated by two major pathways which converge on caspase activation, namely the **receptor**-mediated pathway and the **mitochondria**-mediated pathway. The cascade of caspases (cystein-dependent, aspartate-specific proteases) are responsible for cleaving the intracellular proteins in both the cytoplasm and nucleus, thereby digesting the cell from within (Fleisher, 1997). The TNF (tumour necrosis factor) group of death receptors in the receptor-mediated apoptosis pathway activate upstream (initiator) caspase-8 while cytochrome c, released from mitochondria in the mitochondria-mediated apoptosis pathway activates upstream caspase-9 (Rubel et al, 2006). The actions of the upstream caspases both activate a major downstream (effector) caspase, caspase 3 (Schimmer et al, 2001). A third minor pathway of caspase activation has also been identified, where Granzyme B, synthesized by cytotoxic T-cells, directly activates caspase-3 (Schimmer et al, 2001).

Besides the caspase group of proteases, another proteolytic system is involved in apoptosis. The ubiquitin-mediated proteolytic system is involved in the degradation of short-lived proteins within eukaryotic cells. Many of these cellular proteins fulfil normal functions within the cell and therefore the ubiquitin system plays an important role in regulating key proteins by selective degradation (Calatayud et al, 2005). This system has been associated with the control of apoptosis induced by ionizing radiation in human lymphocytes (Masdehors et al, 2000).

1.4.1 Measuring Apoptosis

Apoptosis could play a role in measuring intrinsic radiosensitivity. Studies have shown that pre-radiotherapy apoptotic levels in normal tissue correlate with apoptotic levels seen post-irradiation (Levine et al, 1995). If the response of tissue to irradiation can be predicted, it could lead to a decrease in the early and late morbidity seen after radiotherapy as well as improve overall local control and survival.

Several methods have been developed to study the different aspects of the apoptotic cascade. Due to its speed and reliability, flow cytometry has become a popular method for assessing apoptosis in peripheral blood lymphocytes (Crompton & Ozsahin, 1997).

1.4.1.1 The Leukocyte Apoptosis Assay

Crompton and Ozsahin (1997) developed the leukocyte apoptosis assay (LAA) to predict intrinsic radiosensitivity of normal tissue based on the radiation-induced apoptotic response of CD4 and CD8 T-lymphocytes. The LAA measures apoptosis with the use of flow cytometric analysis. Fluorescein isothiocyanate (FITC) conjugated CD4 and CD8 antibodies are used to identify cell type and propidium iodide (PI) is used to quantify cellular DNA content. Apoptotic lymphocytes are therefore identified as those cells staining positive for their cell type specific antibodies and displaying reduced DNA content (Menz *et al*, 1997).

1.4.1.2 The Annexin V-FITC Apoptotic Assay

The Annexin V assay is an easy flow cytometric assay that distinguishes between early apoptosis, late apoptosis and necrosis when cells are labelled with both Annexin V and PI (Wilkins et al, 2002b). An early event of apoptosis is the translocation of the phospholipids phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer (Louagie *et al*, 1998a). Annexin V is a Ca^{2+} dependant, phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS (Raynal et al, 1997). Annexin V can be conjugated to a fluorochrome such as fluorescein isothiocyanate (FITC) and used in the flow cytometric analysis of apoptosis.

1.5 Apoptosis vs. induction of micronuclei in T-lymphocytes

1.5.1 The Cytokinesis-block micronuclei assay.

The cytokinesis-block micronucleus (CBMN) technique in human lymphocytes has been adopted by laboratories world-wide as a sensitive and reliable method for assessing chromosomal damage (Fenech, 2000). Micronuclei formation in isolated lymphocytes was studied by using the CBMN assay. Cells were isolated, stimulated with a mitogen, irradiated and incubated for 44 hours. Cytokinesis was then blocked by means of Cytocalasin B. After a total of 72 hours incubation, cells were prepared for fluorescent microscopic analysis.

CHAPTER TWO
LARGE EQUIPMENT USED IN THE STUDY

2.1 Linear Accelerator (LINAC)

A 6 MV linear accelerator at iThemba LABS, Faure, South Africa was used to perform X-ray irradiation. A dose-rate of 2Gy/min was delivered at a source surface distance (SSD) of 100cm, a field size of 30x30 cm^2 and a gantry angle of 0°. Blood samples were placed side-by-side in the centre of the radiation beam on a 7.4cm thick backscatter Perspex block. A sheet of 30x30 cm^2 by 20mm thick polyethylene was used as build-up material. The dose at the position of the samples was verified by using a Farmer ionisation chamber, type 1490 and a Farmer electrometer, model 822. The calibration was done using the TRS 398 protocol. Diodes were set up on the backscatter and exposed to 100 monitor units (mu) for 1 minute as part of the consistency checks. The output factor readings were approximately 0.977 Gy/100mu. The consistency check was to ascertain whether or not the LINAC was functioning in a normal manner.

2.2 Neutron beam

Neutron irradiation was performed using the cyclotron at iThemba LABS, the only nuclear particle therapy facility in the Southern Hemisphere. It is also the only facility in the world where both high energy neutrons and protons are used for patient treatment. The neutron beam at iThemba LABS is produced by bombarding a beryllium target with high energy protons. A dose rate of 0.4Gy/min was delivered by 66 MeV protons bombarding a 19.6mm thick beryllium target. A p(66)/Be(40) reaction is used to produce the neutron therapy beam. This neutron production method produces a range of neutron energies with a mean energy of 29MeV (Jones, 2001).

The neutron therapy facility includes an isocentric gantry capable of ± 185° rotation and has a dose rate of about 0.4Gy/min. The dose rate is influenced by the current measured from the target and this can vary slightly during irradiations. For safety reasons, this is always limited between 25-30 micro amperes. A rotating collimator (360°) with a continuously variable rectangular aperture provides field sizes ranging from 5.5 x 5.5 cm^2 to 29 x 29cm^2 at a source surface distance of 150cm. A manually-

controlled moving floor allows full rotation of the gantry (Jones et al, 1988 and 1994). A field size of 29x29cm^2 and SSD of 150cm was used at a gantry angle of 0°. Blood samples were placed side-by-side in the centre of the beam on a 15cm thick backscatter Perspex block with 20mm thick polyethelene used as build-up material. The dose at the position of the tubes was verified by means of a Far West ionisation chamber (Far West Technology) and current digitiser (Berkeley Nucleomics, Corporation, USA). Daily dosimetry checks verify the output factor of 0.845 monitor units per gray.

2.3 Flow cytometer

Samples were analysed on a FACScan flow cytometer (Beckton Dickinson) equipped with a 488nm Argon laser as the light source. The principle of flow cytometry is as follows. Scattered light from cells and particles are converted to electrical pulses by optical detectors. Collimated (parallel light waveforms) light is collected by confocal lenses focused at the intersection point of cells and the light source. Light is then sent to different detectors by using optical filters. The electrical pulses originating from the light detected by the photomultiplier tube (PMT) is processed by a series of linear and log amplifiers. Logarithmic amplification, as was the case with this study, is often used to measure fluorescence in cells. This type of amplification expands the scale for weak signals and compresses the scale for 'strong' or specific fluorescent signals. The different signals are processed by an Analog to Digital converter (ADC) which allows for events to be plotted graphically.

Morphological changes of dying cells can be detected by analysing light scattered as cells move through the laser beam. The intensity of light scattered at a forward direction (forward scatter) correlates with cell size while the intensity of light scattered at a 90° angle to the laser beam (side scatter) correlates with granularity (Darzynkiewicz, 1997). As cells flow through the laser beam, the bound fluorochromes are excited, giving green fluorescence from Fluoroscein Isothiocynate (FITC) at 530nm and red fluorescence from Propidium iodide (PI) at 620nm.

Three-colour Calibrite beads (Beckton Dickinson) were run as controls on a daily basis before analysing samples. This was done to remove any residual spectral overlap. PMT voltage and spectral compensation was initially set using single-stained beads with FITC

alone, Phycoerythrin (PE) alone and PI alone. Settings on the flow cytometer were further optimized by using a combination of the different fluorochrome stained beads to compensate for their interactions.

Analysis of the data was performed with CELLQuest Pro software (Beckton Dickinson Immunocytometry Systems).

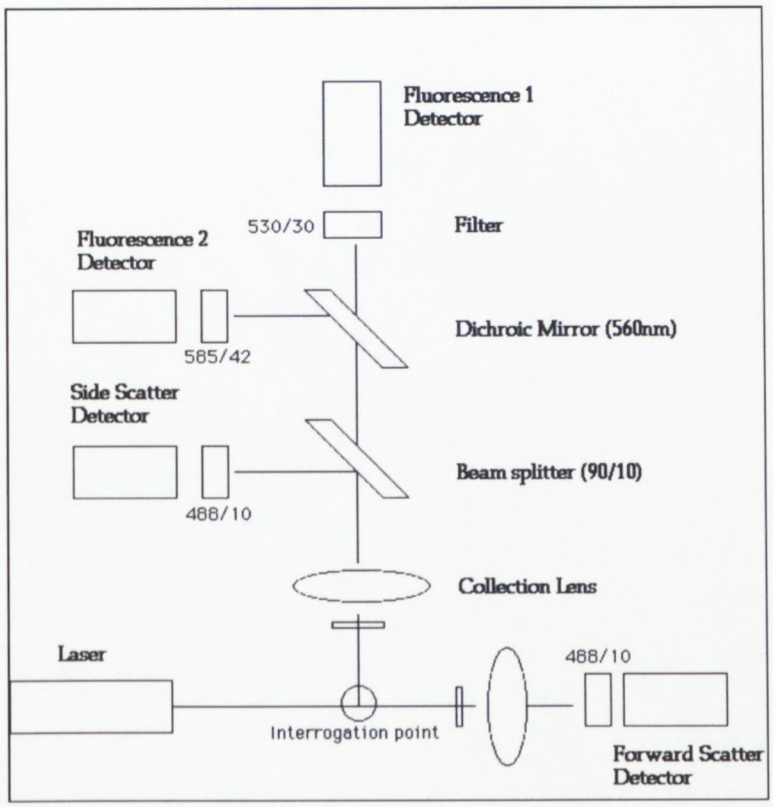


Figure 2.1: A simplified layout of a typical analytical flow cytometer (Ormerod, 1994).

CHAPTER THREE
THE LEUKOCYTE APOPTOSIS ASSAY (LAA)

3.1 Introduction

Crompton and Ozsahin (1997) developed the leukocyte apoptosis assay (LAA) to identify individuals who were genetically predisposed to radiation injury. The assay is based on the radiation-induced apoptotic response of a number of white blood cells. Radiation-induced cytotoxicity is expressed in the form of apoptosis within hours of radiation damage. This form of radiation induced apoptosis is prominent in lymphoid tissue. Darzynkiewicz and co-workers (1992) have previously found that the evaluation of lymphocyte apoptosis can be measured quickly and reliably by using flow cytometry. By using flow cytometry blood analysis, 10 000 cells or more can be rapidly assessed as well as a number of different cell types thereby enhancing the specificity of the assay (Crompton et al, 1999).

The various leukocyte cell types display differences in radiosensitivity. Monocytes have been found to be the most radioresistant with B-lymphocytes, NK lymphocytes and T-lymphocytes being more radiosensitive (Crompton & Ozsahin, 1997). Subsets of T-lymphocytes have also been found to have different responses to radiation with CD8 T-lymphocytes being more radiosensitive than CD4 T-lymphocytes (Wilkins et al, 2002a).

CD4 - and CD8 T-lymphocytes were chosen for the LAA because they show better flow cytometrical separation compared to other lymphocyte subsets. Lymphocyte assays for predicting intrinsic radiosensitivity have been found to be rapid, reproducible and less time-consuming than using non-blood cells like skin fibroblast (Ozsahin et al, 2005). At a fixed dose (8Gy) and time after radiation exposure (48 hours), Ozsahin and co-workers (2005) found that lymphocyte apoptosis reflects the rate at which a physiological response to radiation damage is initialised. Individuals who express high levels of T-lymphocyte apoptosis initialise physiological responses more rapidly whereas individuals with a low T-lymphocyte response initialise physiological responses more slowly.

Cellular response to DNA double-strand breaks is a major factor influencing radiosensitivity in humans (Shiloh, 1997). Individuals displaying high levels of sensitivity

have an abnormality in their ability to recognize or repair DNA double-strand breaks induced by ionising radiation. These individuals may have a predisposition to cancer. This can be seen in patients suffering from the genetic disorder, ataxia telangiectasia (AT) (Shi et al, 2001). Crompton et al (1999) found that patients with ataxia telangiectasia displayed less radiation-induced apoptosis than expected when compared to age-matched controls. When the body is unable to respond physiologically to radiation damage, it can lead to enhanced toxicity as well as late toxicity (Crompton et al, 2001). Approximately 5-10% of patients receiving radiotherapy show signs of late toxicity (RTOG/EORTC scale) many months after therapy has been completed (Bentzen & Overgaard, 1994). As these patients have not been previously identifiable, radiotherapy doses have been conservative. Should the LAA be able to identify radiosensitive patients, the oncologist would be able to stratify each patient and plan an effective radiotherapy regime. This could provide an indication of the expected scale of patient-specific late toxicities. Patients who display less sensitivity (radioresistant individuals) could receive a higher dose whereas those presenting as sensitive (radiosensitive individuals) would have the advantage of receiving alternative treatment options. Therefore using the LAA which measures physiological response (in this case apoptosis) at a cellular level can be a good predictor of the response to radiation therapy.

Fractionated radiotherapy exposures tend to use 2Gy high dose rate fractions. Ozsahin and co-workers (1997) therefore developed the LAA technique which uses high dose rate exposures but could also yield information at low doses. The technique could be used for doses ranging from 0.5-8Gy. After 8Gy irradiation, the levels of radiation-induced apoptosis rose to a maximum between 48 and 72hrs after irradiation (Crompton & Ozsahin, 1997). Studies by Radojcic & Crompton (2001) also found 8Gy to be the dose at which apoptosis reaches a plateau maximum.

The LAA uses propidium iodide (PI), a dye most often used to detect dead cells by flow cytometry, as it carries two positive charges and is prevented from entering intact cells (King, 2000). It is only during late apoptosis that the plasma membrane of cells becomes permeable to propidium iodide (Carbonari et al, 1995). The LAA is based on the fact that reduced DNA fluorescence of the apoptotic lymphocytes result in a population distinct from debris and non-apoptotic cells. The reduction in DNA fluorescence is caused by

apoptotic-associated changes of the cell that limits the uptake of the propidium iodide stain (Crompton et al, 2001).

3.1.1 Aims of the LAA study:

A series of experiments have been performed to implement and validate the LAA assay. This includes:

(i) Pilot study: The purpose of the pilot study was to standardize the LAA assay in South Africa and to determine the reliability of the assay. This was necessary as it is the first time that the LAA assay has been set up in South Africa. Heparinised blood from 4 volunteer colleagues was collected. Later heparinised blood from 9 volunteer colleagues was collected.

(ii) Preliminary Standard curve study: This is to look at the distribution of radiation-induced apoptosis of 100 donors within a healthy Western Cape population.

(iii) Final Standard curve study: This study population was further increased to 300 healthy donors within a Western Cape population.

(iv) Age, gender and race study: The effects of age, gender and race on the radiosensitivity of the 300 healthy donors was analysed

(v) LAA readings for cells treated with X-rays and neutrons: This is to look at the differences in radiation induced apoptosis when using both neutron and x-ray radiation. Heparinised blood from 18 donors was collected.

(vi) LAA readings following fractionated doses of X-rays: This is to look at what effect fractionation of doses has on radiation- induced apoptosis. Heparinised blood from 4 volunteers was used for this study.

3.2 Materials and Methods

3.2.1 Sample Collection

Nurses collected blood in lithium heparin tubes from healthy volunteers on the day of irradiation. Each donor received a participant information sheet (Appendix A) informing them about the study and its objectives. Donors willing to participate had to sign a consent form (Appendix B) before 5ml heparinised blood was collected from each of them.

3.2.2 Sample preparation for irradiation procedure

The whole blood from each donor collected was subdivided into 6 smaller samples. For this, 6 round-bottomed culture tubes were prepared as follows: Each tube contained 0.5ml blood diluted in 4.5ml growth medium (RPMI 1640). The growth medium was supplemented with 20% foetal calf serum, 2g/l $NaHCO_3$ and antibiotics (1% Penstrep).

3.2.3 Irradiation set-up

The LINAC set-up for X-ray irradiation and the neutron beam set-up for the neutron irradiation are described in Chapter 2.

3.2.4 Irradiation procedure

Samples were irradiated with 0Gy (control), 2Gy and 8Gy X-rays for both the pilot and the standard curve study. 0Gy (control), 1Gy and 4Gy neutrons were applied for the neutron study. For the fractionation of doses study, tubes were irradiated with 8Gy as well as a fractionated dose of 4Gy + 4Gy X-rays separated by 3 hours.

3.2.5 Cell Culture Procedure

Immediately after exposing the samples to radiation, the culture tubes were placed in a Forma Series II water-jacketed CO_2 incubator (Model 3110, Labotec (Pty) Ltd) at 37°C in 5% CO_2. Total culture time was 48hrs.

3.2.6 Labelling and Staining of lymphocytes

During the incubation, the blood cells separated from the serum. After incubation, the serum was removed from the whole blood sample using a plastic pipette. To the remaining cell fraction with a volume of approximately 1ml, 20µl of anti-CD4 and anti-

CD8 Fluoroscein Isothiocynate (FITC)-conjugated monoclonal antibodies (Beckton Dickinson) was added. The mixture was then incubated for 20 minutes at 37°C. The erythrocytes were lysed using 4ml FACS Lysing solution (Beckton Dickinson). This treatment also permeabilised the leukocyte membranes. The samples were washed by adding 4ml phosphate buffered saline (PBS) (Sigma-Aldrich) and then centrifuged at 2250rpm for 10 minutes. The leukocyte pellet was then resuspended in 400µl of FACS Flow (Beckton Dickinson). DNA of the permeabilised leukocytes was stained with 5µl Propidium Iodide (PI) (Beckton Dickinson) not more than 30 minutes before performing flow cytometric analysis.

3.2.7 Flow Cytometric Analysis

Two-colour flow cytometric analyses were performed on the FACScan (Beckton Dickinson) at iThemba LABS. The flow cytometer set-up is described in Chapter 2. Lymphocytes were identified and gated in region 1 (R1) (Figure 3.1a) on a 2-dimensional (2D) scatter plot. This gate eliminates a significant proportion of erythrocytes, debris and unwanted leukocytes during data acquisition. The intensity of colours for each cell (green fluorescence from FITC at 530nm and red fluorescence from PI at 620nm) was measured as a total of 10 000 events was collected within the lymphocyte region. A second 2D scatter plot of conjugated-antibody fluorescence (FL1-height) vs. cellular DNA content (FL2-height) is used to identify either CD4 or CD8 positive T- lymphocytes in region 2 (R2) (Figure 3.1b). A third 2D scatter plot of cellular DNA content (FL2-height) vs. cell size (FSC-height) was then used to determine the proportion of apoptotic cells in region 3 (R3) (Figure 3.1c-d) by identifying the population of cells with reduced DNA content and slightly reduced cell size. Percentage radiation-induced apoptosis was calculated by subtracting background apoptosis at 0Gy from apoptosis measured at 2Gy and 8Gy. Data analysis was performed using CellQuest Pro software (Becton Dickinson Immunocytometry Systems)

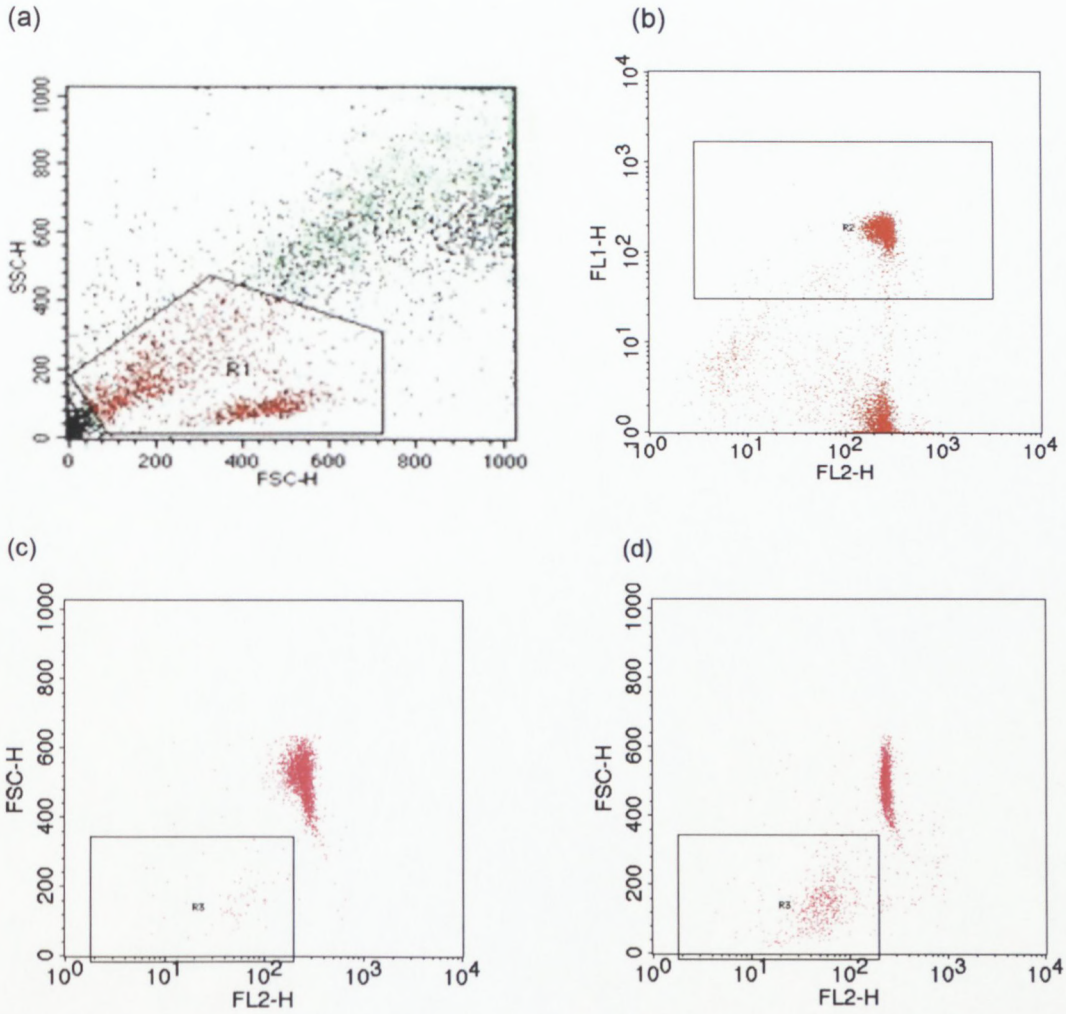


Figure 3.1a-d: LAA scatter plots showing the (a) selection of lymphocytes; (b) selection of CD 4 lymphocytes (FITC +); (c) apoptotic lymphocytes at 2 Gy and (d) apoptotic lymphocytes at 8 Gy.

3.2.8 Statistical Analysis

Statistical analysis was done by using Microsoft Excel, GraphPad Prism 4 and the Statistical Programme for Social Sciences (SPSS 16).

3.3 Results and Discussion

3.3.1 Pilot Study

3.3.1.1 Interdonor and Intradonor studies for Donors 1-4

The LAA analysis for a pilot group of 4 volunteers (Donors 1-4) is listed below. Firstly the 4 samples were exposed to 0Gy (control) and 8Gy X-ray radiation and the apoptotic response of CD4 and CD8 T-lymphocytes were measured using the LAA. In each one of these donors the radiation-induced apoptosis for CD4 and CD8 T-lymphocytes after 8Gy X-rays are tabulated below (Table 3.1).

Table 3.1a-d: Percentage radiation-induced apoptosis of Donors1-4's CD4 and CD8 T-lymphocytes after background level (0Gy) subtraction.

(a) Radiation-induced apoptosis for Donor 1: White male aged 30 years.				
Day	**0Gy CD4**	**0Gy CD8**	**8Gy CD4**	**8Gy CD8**
1	1.8	2.34	11.75	15.45
2	1.47	2.68	10.85	14.13
3	1.19	1.87	6.82	16.81
4	1.06	1.97	7.15	16.25
Mean	**1.38**	**2.22**	**9.14**	**15.66**

(b) Radiation-induced apoptosis for Donor 2: Black male aged 25 years.				
Day	**0Gy CD4**	**0Gy CD8**	**8Gy CD4**	**8Gy CD8**
1	2.62	2.83	24.65	48.89
2	2.41	2.84	26.14	47.56
3	3.08	3.13	18.58	32.85
4	2.81	3.04	14.16	29.9
Mean	**2.73**	**2.96**	**20.88**	**39.8**

(c) Radiation-induced apoptosis for Donor 3: White male aged 50 years.				
Day	**0Gy CD4**	**0Gy CD8**	**8Gy CD4**	**8Gy CD8**
1	2.51	2.41	8.71	20.54
2	2.5	2.47	8.49	21.59
3	1.96	2.36	9.12	21.73
4	2.09	2.25	8.46	20.04
Mean	**2.27**	**2.37**	**8.70**	**20.96**

(d) Radiation-induced apoptosis for Donor 4: Coloured female aged 27 years.				
Day	**0Gy CD4**	**0Gy CD8**	**8Gy CD4**	**8Gy CD8**
1	3.89	5.35	17.42	24.55
2	3.63	5.26	16.42	23.79
3	3.42	4.8	13.96	26.99
4	2.62	4.93	18.62	26.07
Mean	**3.39**	**5.09**	**16.61**	**25.35**

The age of the 4 donors ranged from 25 to 50 years with a mean age of 33 years. There were 3 males and 1 female donor. Table 3.1a-d shows the results of apoptosis observed in control samples (0Gy) and that induced by a dose of 8Gy X-rays. Data for CD4 and CD8 T-lymphocytes after background (0Gy) apoptotic level were subtracted are listed. Radiation-induced apoptosis for CD4 and CD8 T-lymphocytes were repeatedly analysed on 4 different days.

The mean background readings for CD4 T-lymphocytes are marginally lower than that of CD8 T-lymphocytes for all donors. With the exception of readings taken on the first two days for Donor 3, all CD4 background apoptotic readings are slightly less than CD8 lymphocytes. Radiation-induced apoptosis at 8Gy for CD4 lymphocytes ranged from 8.7% (Donor 3) to 20.9% (Donor 2) and for CD8 lymphocytes it ranged from 15.7% (Donor 1) to 39.8% (Donor 2). Donor 2 consistently showed high apoptotic responses on all 4 days measured.

The ranking order of apoptotic response differs for CD4 and CD8 lymphocytes. CD8 lymphocytes are more sensitive and more consistent in the ranking order for the 4 days. For example, Donor 1 has the lowest apoptotic response on each day followed by Donor 3, Donor 4 and lastly Donor 2 always has the highest apoptotic response. CD4 lymphocytes on the other hand do not have a consistent ranking order on each of the 4 days. Slight differences are noted for all 4 donors in the CD4 lymphocytes after 8Gy irradiation with X-rays. When the 4 readings for each donor are averaged, the CD4 lymphocytes for Donor 3 have the lowest apoptotic response followed by Donor 1, Donor 4 and lastly Donor 2. It is concluded that when one averages the values, CD4 lymphocytes do not follow the ranking order of CD8 lymphocytes.

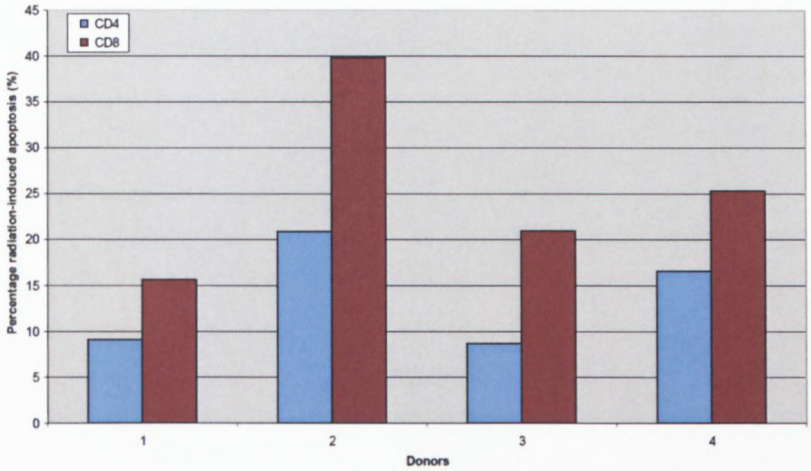


Figure 3.2: Interdonor variation of radiation-induced apoptosis following a dose of 8Gy of X-rays observed at 48 hours in CD4 and CD8 T-lymphocytes.

Interdonor variation is shown in Figure 3.2. Although interdonor variation was observed, apoptotic responses were consistent for the different cell types. For example, Donor 1 showed a low apoptotic response for both CD4 and CD8 lymphocytes when compared to the other 3 donors. Donor 2 consistently showed a high apoptotic response.

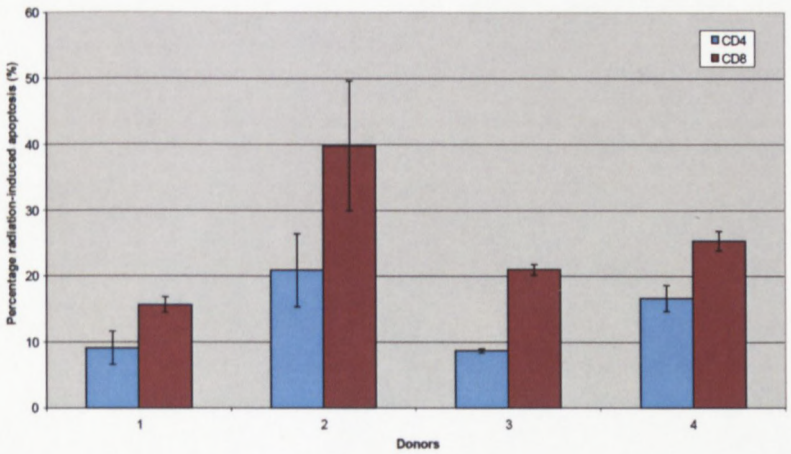


Figure 3.3: Intradonor variation of 4 donors at 8Gy for CD4 and CD8 T- lymphocytes. Error bars represent the standard deviation.

Intradonor variations are shown in Figure 3.3. These are relatively low with a mean variation of 2.6 % (range 0.3-5.6%) for CD4 lymphocytes. The mean variation for CD8 lymphocytes was 3.31% (range 0.8-9.1%).

Repeated analysis of the 4 donors on different days showed that variance between individuals was found to be higher than variance within individuals as can be seen in Figure 3.2 and Figure 3.3 where interdonor variation exceeded intradonor variation. These results are supported by Schmitz and co-workers (2003) who reported similar findings.

The low intradonor variation amongst the 4 donors (Figure 3.3) is comparable to those found by Ozsahin et al (1997) who conducted a study to determine intradonor variation from one donor over a period of 3 months. The mean variation was low at 1.41% for CD4 lymphocytes and 2.78% for CD8 lymphocytes after 8Gy X-ray irradiation.

3.3.1.2 Dose Response Curves for Donors 1-4

Figure 3.4 a-d shows the dose response curves for radiation induced apoptosis. Values for CD4 and CD8 lymphocytes

(a)

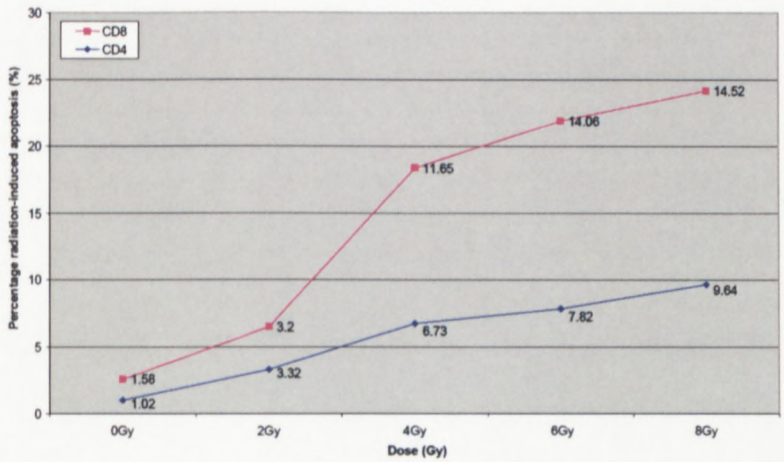
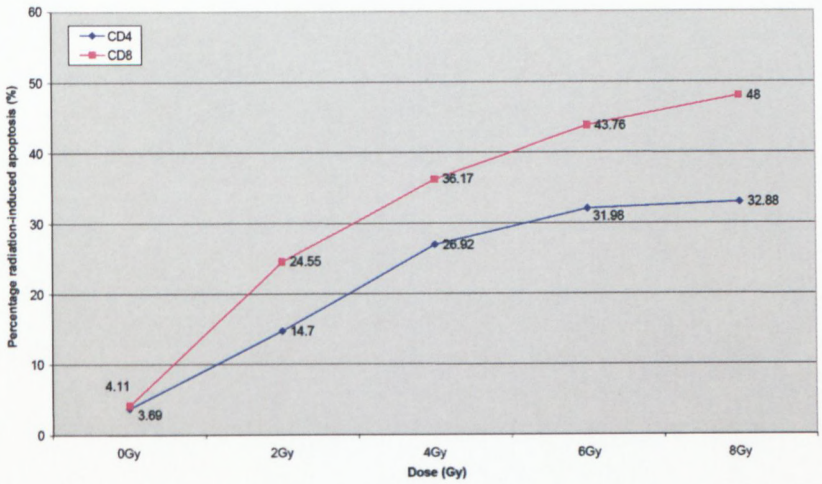


Figure 3.4a: Dose response curves after exposure to 0Gy (control), 2Gy, 4Gy, 6Gy and 8Gy X-rays for Donor 1.

(b)



(c)

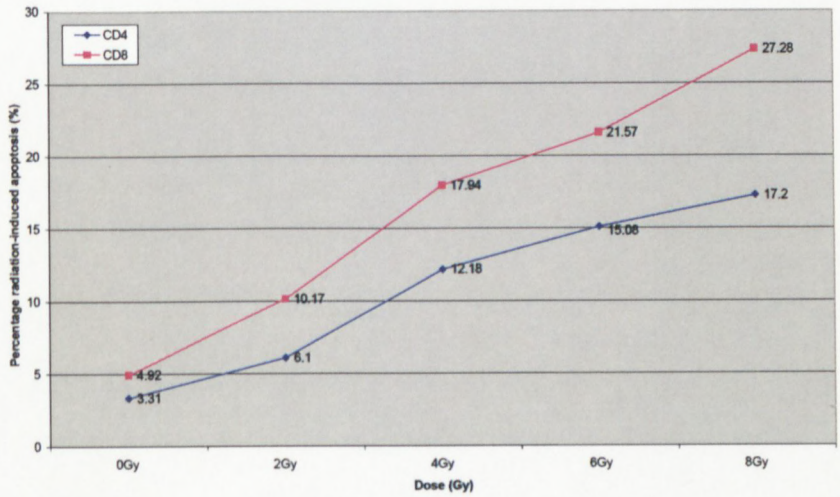


Figure 3.4b-c: Dose response curves after exposure to 0Gy (control), 2Gy, 4Gy, 6Gy and 8Gy X-rays for (b) Donor 2 and (c) Donor 3.

(d)

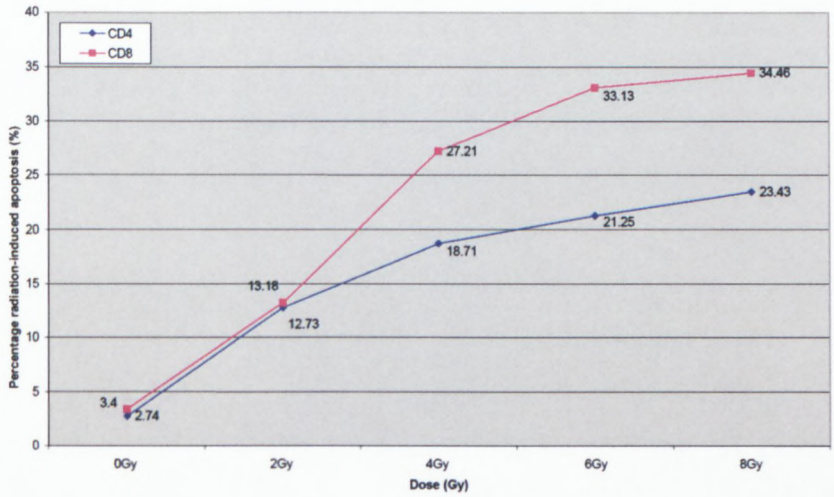


Figure 3.4d: Dose response curves after exposure to 0Gy (control), 2Gy, 4Gy, 6Gy and 8Gy X-rays for Donor 4.

The LAA readings show a clear dose response curve (Fig 3.4 a-d) for each of the 4 donors at 0Gy (control), 2Gy, 4Gy, 6Gy and 8Gy for both CD4 and CD8 lymphocytes. The apoptotic response increased with increasing dose for each of the 4 donors. Irrespective of the dose, radiation-induced apoptosis for CD8 lymphocytes is consistently higher than that noted with CD4 lymphocytes. Apoptotic responses after 2Gy is significantly higher than at 0Gy (control). For 3 out of 4 donors, a plateau in the rate of apoptotic induction at 4Gy is observed.

In most cases in radiobiology when looking at residual radiation damage, a linear quadratic is observed. When looking at these dose response curves (Figure 3.4a-d) a linear quadratic can be seen up to 4Gy but at doses higher than 4Gy, a plateau occurs. This could simply mean that doses of 6Gy and 8Gy are too high to observe a linear quadratic curve as most radiobiology studies use smaller doses.

3.3.1.3 Interdonor variation studies for Donors 1-9

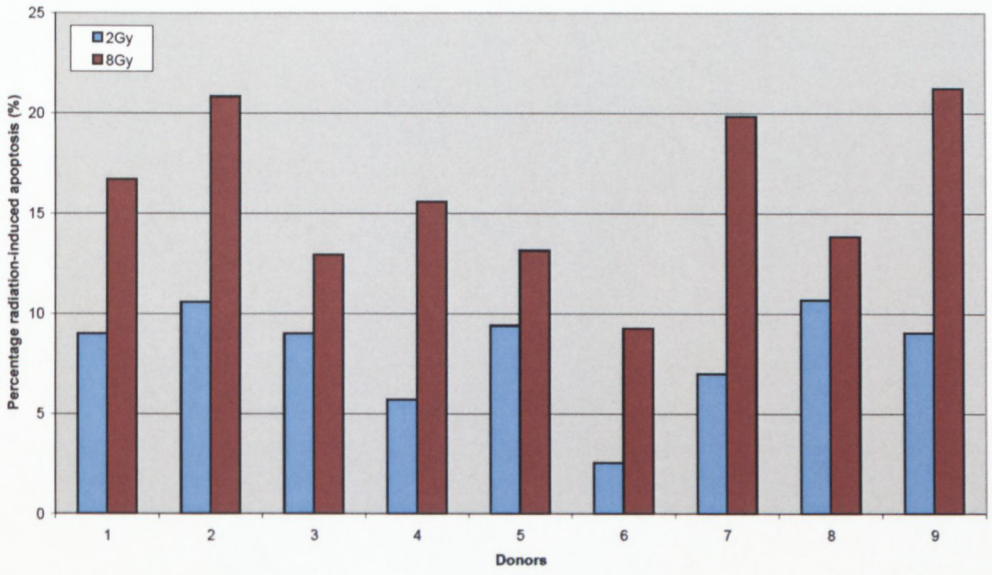
Heparinised blood was drawn from 9 volunteer colleagues and analysed to further assess the interdonor variation. The 9 samples were exposed to 0Gy (control), 2Gy and 8Gy of X-ray radiation and the apoptotic response of CD4 and CD8 lymphocytes were measured. A dose of 2Gy was included in the analysis as this is the dose commonly used in fractionated radiotherapy.

Table 3.2: Percentage radiation-induced apoptosis of 9 donor's CD4 and CD8 T-lymphocytes after background level (0Gy) subtraction.

Percentage radiation-induced apoptosis for Donors 1-9						
	CD4			CD8		
Donors	**0Gy control**	**2Gy**	**8Gy**	**0Gy control**	**2Gy**	**8Gy**
1	7.49	9	16.72	7.98	12.62	30.66
2	8.21	10.6	20.83	13.27	14.76	24.38
3	3.76	9.02	12.96	8.63	16.45	20.45
4	5.6	5.72	15.6	5.82	10.39	34.57
5	7.31	9.42	13.16	12.39	16.8	21.2
6	1.7	2.56	9.28	2.21	2.68	14.47
7	3.69	7.01	19.85	4.81	11	38.77
8	0.73	10.7	13.85	0.59	12.11	24.25
9	3.45	9.04	21.24	4.34	11.46	23.97
mean	**4.66**	**8.12**	**15.94**	**6.67**	**12.03**	**25.86**
Standard deviation	**2.64**	**2.62**	**4.08**	**4.3**	**4.21**	**7.54**

Table 3.2 shows the results of apoptosis observed in control samples (0Gy) and induced by a dose of 2Gy and 8Gy X-rays. A large variation in background counts is evident ranging from 0.7 to 8.2% for CD4 lymphocytes and 0.6 to 13.3% for CD8. Larger variations in background readings are thus evident when the population size increases. The relative increase in apoptotic response between 2Gy and 8Gy X-rays is the same for CD4 and CD8 lymphocytes. For CD4 lymphocytes, the mean ratio of the apoptotic response at 2Gy and 8Gy is 1.96 whereas for CD8 lymphocytes, it is 2.1. Results of the study showed CD8 lymphocytes to be more sensitive to radiation-induced apoptosis than CD4 lymphocytes (Table 3.2), at 2Gy and 8Gy for all 9 donors. The data is supported by Wilkins and co-workers (2002) and Crompton and Ozsahin (1997) who also observed clear differences in radiosensitivities between CD4 and CD8 lymphocytes.

(a)



(b)

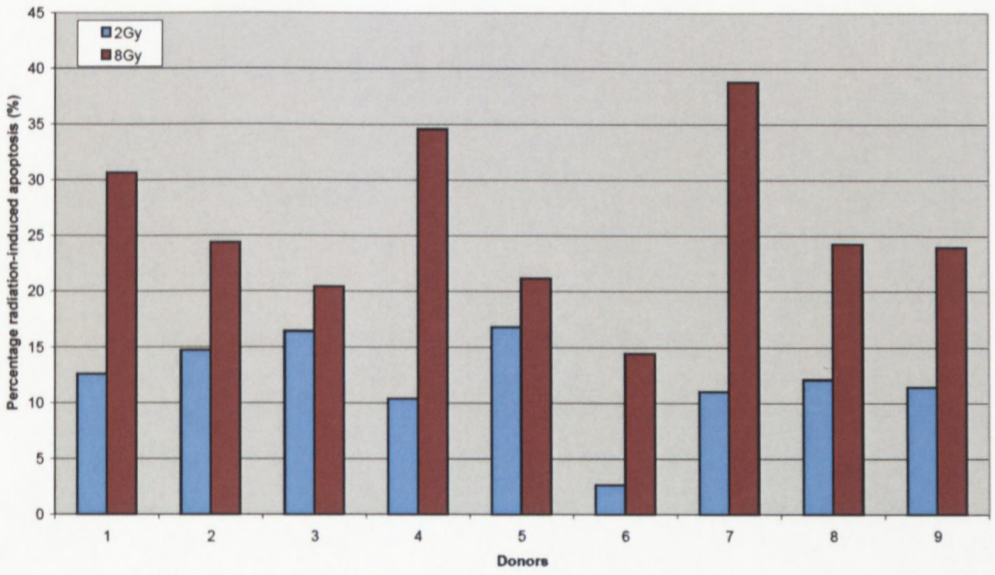


Figure 3.5a-b: Interdonor variation of radiation-induced apoptosis at 2Gy and 8Gy in (a) CD4 T-lymphocytes and (b) CD8 T-lymphocytes.

The results for each of the 9 volunteer donors are presented graphically in Figure 3.5a-b. The mean for CD4 0Gy (control) was 4.7% (range 0.7-8.2%) and for CD8 0Gy (control) it was 6.7% (range 0.6-13.3%). The mean radiation-induced apoptosis at 2Gy for CD4 was 8.1% (range2.6-10.7%) and for CD8 it was 12% (range 2.7-16.8%). The mean radiation-induced apoptosis at 8Gy for CD4 was 15.9% (range9.3-20.8%) and for CD8 it was 25.9% (range 14.5-38.8%). Interdonor variation was observed amongst the 9 donors. Donor 6 showed a low apoptotic response for both CD4 and CD8 lymphocytes at 2Gy and at 8Gy. The highest apoptotic responder is not consistent for both CD4 and CD8 lymphocytes. Results showed an increase in percentage apoptosis as the dose of X-rays increased.

3.3.1.4 Conclusion

The study yielded sufficient data to base radiosensitivity measurements on CD4 and CD8 lymphocytes and from the results obtained, it is observed that this pilot study could therefore be applied to a standard curve study in South Africa.

3.3.2 Preliminary standard curve study.

A standard curve study was conducted in order to establish a baseline with which to compare radiation-induced apoptosis in lymphocytes from patients displaying enhanced toxicity to radiotherapy. Variation in radiation-induced apoptosis amongst different donors can not be adequately described by the data from 9 donors in the pilot study. In order to perform z-score analysis to finally identify radiosensitive and radioresistant individuals, heparinised blood from 100 donors was analysed.

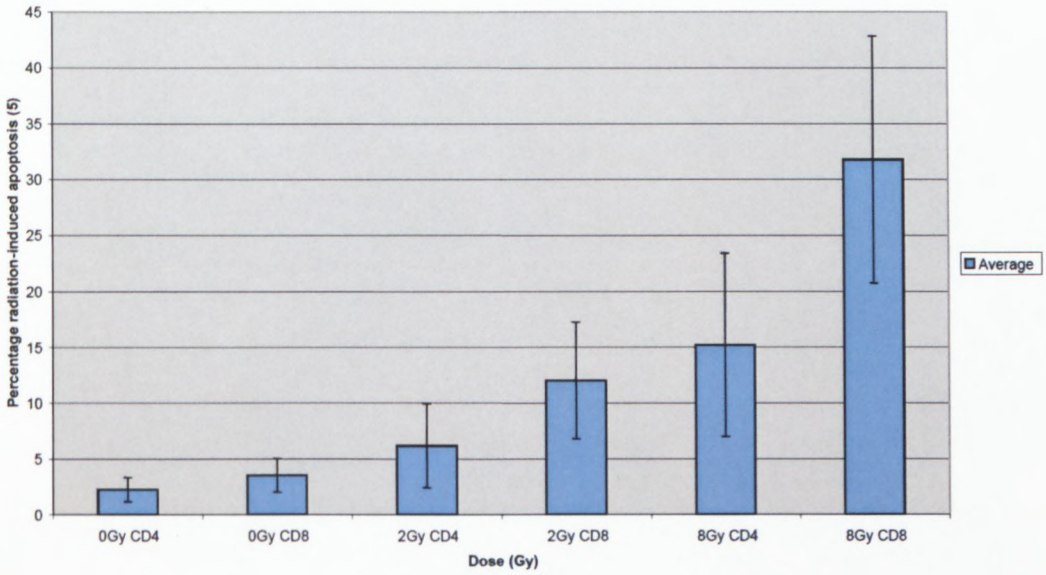


Figure 3.6: The average percent apoptosis induced by 2Gy and 8Gy X-rays in CD4 and CD8 T-lymphocytes for 100 healthy donors. The background (0Gy) level of apoptosis was subtracted. Error bars display standard deviation.

Figure 3.6 shows the average percent of radiation-induced apoptosis for the 100 healthy donors (mean: 41.6yrs, range 19-78yrs) in CD4 and CD8 T-lymphocytes after 2Gy and 8Gy X-rays. A clear dose response curve is observed as the percentage radiation-induced apoptosis increases with every dose of radiation. The percent apoptosis following a dose of 2Gy minus the background (2-0Gy) for CD4 lymphocytes ranges from 1.21-21.19% with a mean of 6.20%. Radiation-induced apoptosis for CD8 lymphocytes ranges from 1.01-30.55% with a mean of 12.03%.

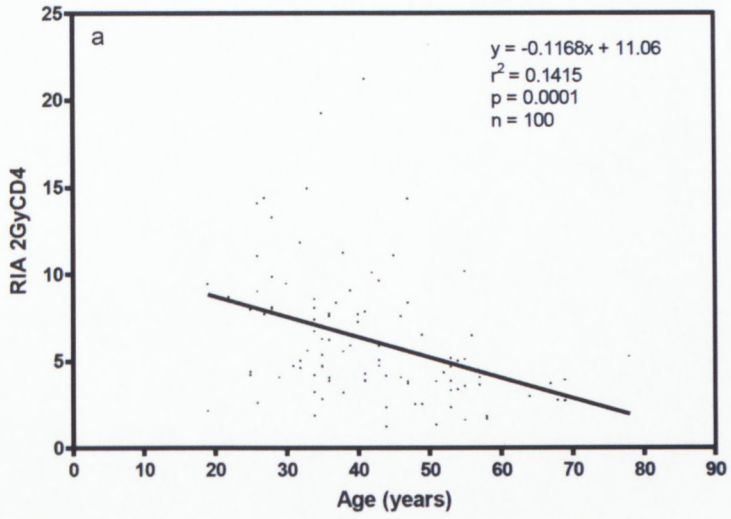
The percent apoptosis following a dose of 8Gy minus the background (8-0Gy) for CD4 lymphocytes ranges from 2.64-46.68% with a mean of 15.22% and for CD8 lymphocytes it rages from 11.2-63.43% with a mean of 31.79%. At each dose point, there is a higher rate of apoptosis observed with CD8 lymphocytes when compared to that of the CD4 lymphocytes.

Radiation-induced apoptosis data obtained from the 100 donors can be used to perform z-score analysis. The procedure below describes the way in which the analysis was performed. The method is the reference method used by Crompton et al (1999).

3.3.2.2 Z-score analysis to include influence of age:

- Radiation-induced apoptosis (RIA) was plotted against age for the 100 donors (Figure 3.7a-d).
- A different regression analysis was performed for each cell type (CD4 and CD8) and radiation dose (2Gy and 8Gy).
- The parameters from each of the 4 regression lines were used to determine the expected RIA for that age for each lymphocyte cell subset at each dose:
  RIA = (slope x age) + y-intercept
- The calculation above allows one to estimate the RIA as a function of age.
- The expected contribution of age, as determined by the regression line formula, was subtracted from the original value of RIA observed for each individual. This results in a RIA-age value.
- To show that the above data corrected for age is meaningful, a scatter plot of RIA-age vs. age should yield a trendline that hugs the x-axis (Figure 3.8a-d).
- The RIA-age data can now be used to determine z-scores (number of standard deviations from the mean) using the following equation:
  z = (RIA-age – average RIA-age for 100 donors) /standard deviation of the sample population.
- The 2 CD4 z-scores for 2Gy and 8Gy can be averaged. The 2 CD8 z-scores for 2Gy and 8Gy can also be averaged. One can also keep the 2Gy and 8Gy data separately.
- The final 2 z-score values for CD4 and CD8 lymphocytes are plotted against each other as a scatter plot. (Figure 3.9).

The graphs obtained from the outline above are listed below.



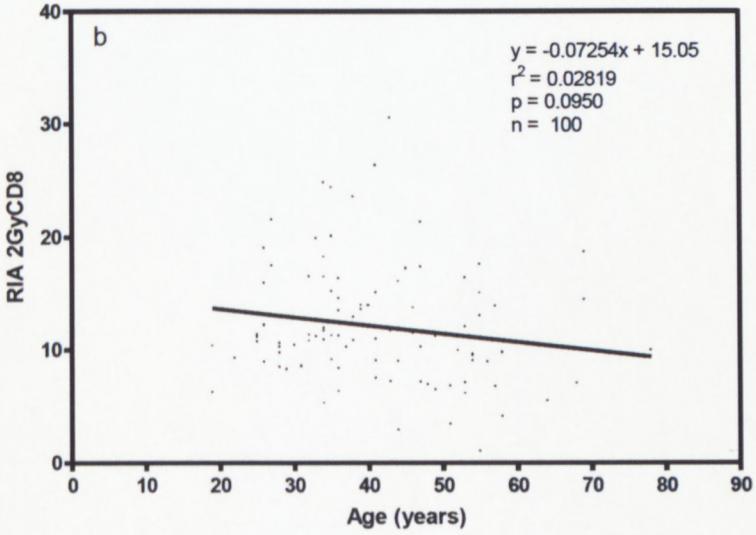


Figure 3.7a-b: Radiation-induced apoptosis (RIA) vs. age for (a) 2Gy CD4 and (b) 2Gy CD8 for 100 donors.

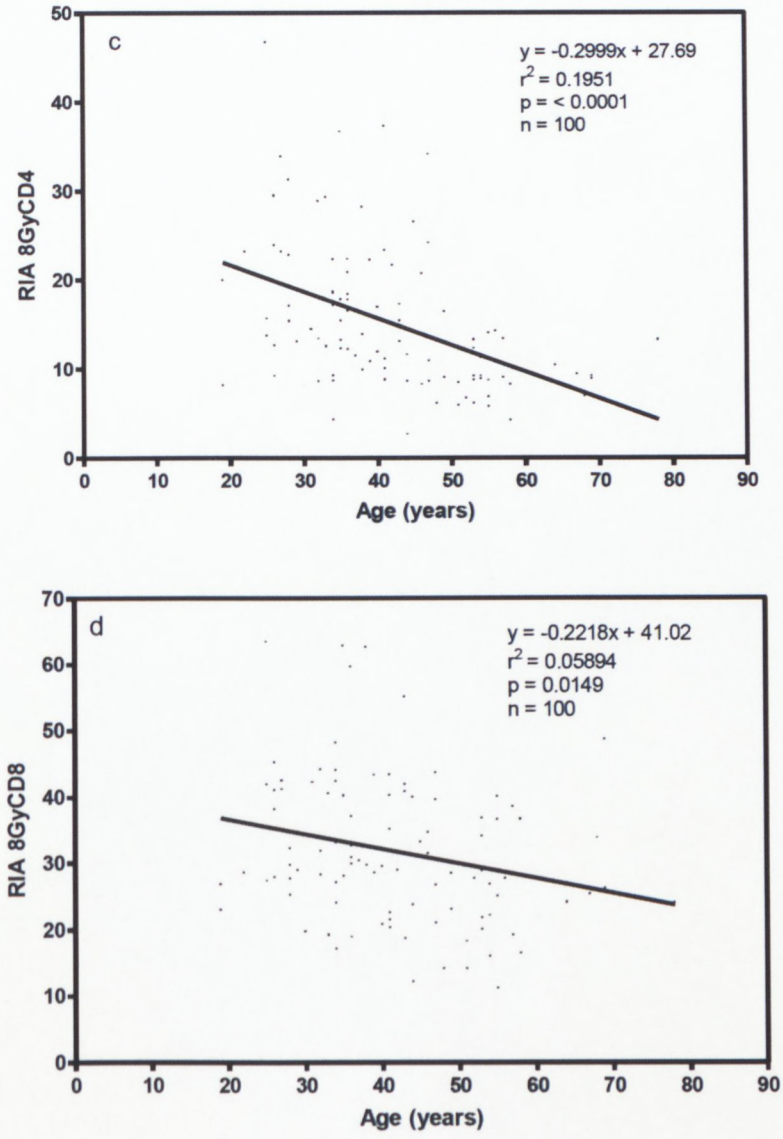


Figure 3.7c-d: Radiation-induced apoptosis (RIA) vs. age for (c) 8Gy CD4 and (d) 8Gy CD8 for 100 donors.

Radiation-induced apoptosis for the 100 donors was plotted against age for each cell type and each dose point. At 2Gy, CD4 lymphocytes (Figure 3.7a) show a negative slope when the radiation-induced apoptosis was plotted against age. A significant correlation exists between RIA and age with a r^2 value of 0.14 (p= 0.01). The regression line formula obtained that could be used to determine the expected RIA for a given age is RIA = (-0.1168 x age) + 11.06.

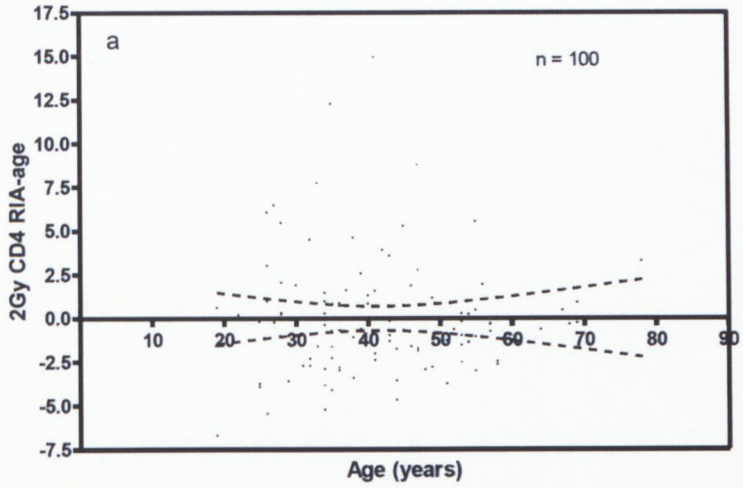
At 2Gy, CD8 lymphocytes (Figure 3.7b) also show a negative slope when RIA was plotted against age (graph1b). The relationship between RIA and age is insignificant with a r^2 value of 0.03 ($p = 0.09$). Even so, the regression line formula used to correct the data was RIA = (-0.0725 x age) + 15.05.

At 8Gy, CD4 lymphocytes (Figure 3.7c) also show a negative slope when RIA was plotted against age (graph1c). A significant correlation exists between the variables with a r^2 value of 0.19 ($p = <0.0001$). The regression line formula obtained to determine the RIA at a given age is RIA = (-0.2999 x age) + 27.69.

At 8Gy, CD8 lymphocytes (Figure 3.7d) also show a negative slope when RIA was plotted against age (graph1d). A significant correlation exists with a r^2 value of 0.06 ($p = 0.015$). The regression line formula obtained to determine the RIA at a given age is RIA = (-0.2218 x age) + 41.02.

It is evident from the above that the influence is most pronounced for CD4 lymphocytes receiving a dose of 8Gy.

The regression line formula, RIA = *(slope x age) + y-intercept,* was applied to all the RIA data for the 100 donors. The expected RIA values (y) obtained is then subtracted from the actual RIA data points observed to obtain a value for radiation-induced apoptosis corrected for age (RIA –age). These RIA-age values are then plotted as a function of age for all the 100 donors.



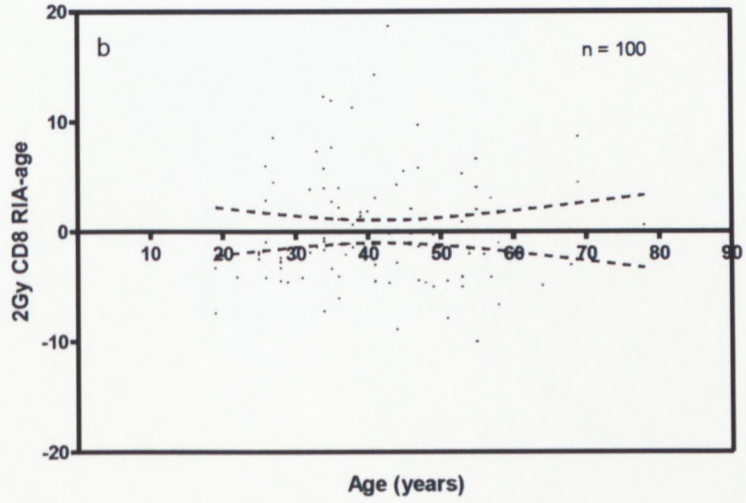


Figure 3.8a-b: Radiation-induced apoptosis with age contribution (RIA-age) vs. age for (a) 2Gy CD4 and (b) 2Gy CD8. The dotted lines indicate the 95% confident intervals of the fitted parameters.

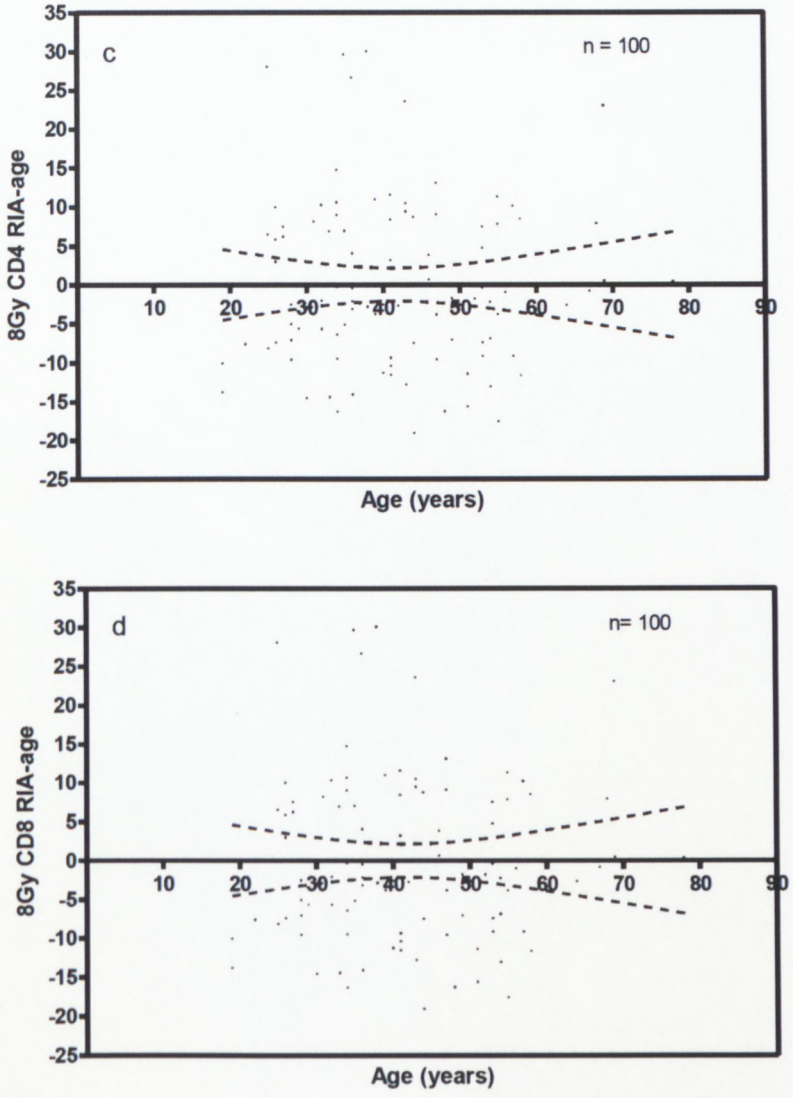


Figure 3.8c-d: Radiation-induced apoptosis with age contribution (RIA-age) vs. age for (c) 8Gy CD4 and (d) 8Gy CD8. The dotted lines indicate the 95% confident intervals of the fitted parameters.

In all cases, the scatter plots of RIA-age vs. age yield a trendline that hugs the x-axis (Figure 3.8 a-d).

The RIA-age data can now be used to determine z-scores with the following equation:

z = (RIA-age – average RIA-age for 100 donors) /standard deviation of the sample population.

This equation is applied to all the RIA-age data points for the 100 donors. The z-score values for CD4 and CD8 lymphocytes for 2Gy and 8Gy were then averaged. The final 2 z-score values for CD4 and CD8 are plotted against each other as a scatter plot (graph 3).

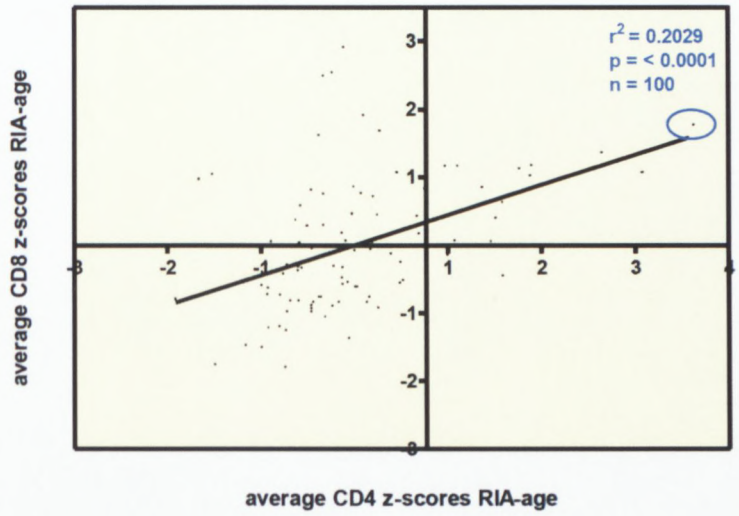


Figure 3.9: The average z-score dose correlation for 100 healthy donors from CD4 and CD8 T-lymphocyte data after a combination of 2Gy and 8Gy X-rays. Regression analysis is displayed as a solid line. The blue circle indicates radioresistant donors.

Figure 3.9 shows the average z-score analysis for CD4 and CD8 after the z-score data were pooled and averaged. This analysis show that z-score values are significantly correlated with a r^2 value of 0.20 ($p = <0.0001$). The interpretation of this scatter plot (Figure 3.9) is as follows. Z-score values can be looked up on a standard distribution table. When doing this, a z-score value of 1.645 represents the cut off point for the 95% confident interval. Any z-score value above 1.645 indicates individuals who fall within the 5% most resistant category. That is, individuals with high levels of RIA that is unlikely to develop late effects. The z-score values below -1.645 indicate the individuals who fall within the 5% most sensitive category. That is, individuals with less RIA who are more likely to develop late effects.

In Figure 3.9, there was 1 donor who displayed z-score values beyond 1.645 and none who displayed values below -1.645. The donor information and z-score values for both CD4 and CD8 cells are depicted in the Table 3.3.

Table 3.3: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of 1 donor who showed variation beyond ± 1.645 after 2Gy and 8Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
60	41	Coloured	Male	3.63	1.77

About 5% of individuals tested can be expected to have z-score values that indicate radiosensitivity (Ozsahin et al, 2005). As not a single individual was indicated to be radiosensitive in the above analysis, z-score values were again calculated separately for the 2Gy and 8Gy data sets.

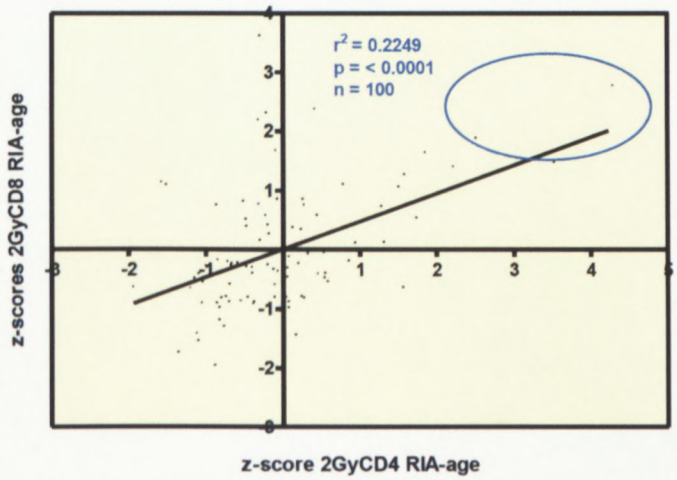


Figure 3.10: Average z-score dose correlation for 100 healthy donors from CD4 and CD8 T-lymphocyte data after 2Gy of X-rays. Regression analysis is displayed as a solid line. The blue circle indicates radioresistant donors.

Figure 3.10 shows data from both cell types, CD4 and CD8, after 2Gy irradiation that has been combined for z-score analysis. The results are significantly correlated with a r^2 value of 0.22 (p= <0.0001). Two individuals displayed variation above 1.645 and the donor information and z-score values are depicted in Table 3.4. No radiosensitive individuals could be identified in this analysis.

Table 3.4: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of 2 donors who showed variation beyond ± 1.645 after 2Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
60	41	Coloured	Male	4.30	2.77
63	47	White	Male	2.52	1.88

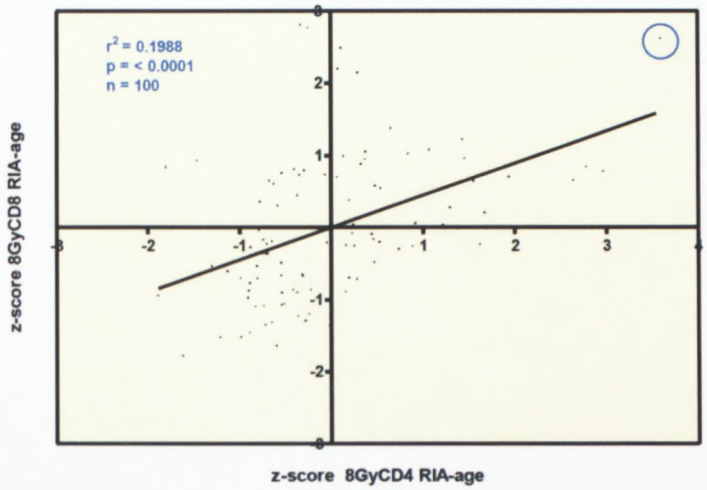


Figure 3.11: Average z-score dose correlation for 100 healthy donors from CD4 and CD8 T-lymphocyte data after 8Gy of X-rays. Regression analysis is displayed as a solid line. The blue circle indicates radioresistant donors.

Figure 3.11 shows data from both cell types, CD4 and CD8, after 8Gy irradiation that has been combined for z-score analysis. The results are significantly correlated with a r^2 value of 0.19 (p = <0.0001). One individual displayed variation above 1.645 and the donor information and z-score values are depicted in Table 3.5. No radiosensitive individuals could be identified in this analysis.

Table 3.5: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of 1 donor who showed variation beyond ± 1.645 after 8Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
15	25	Coloured	Male	3.60	2.61

After doing z-score analysis on 100 donors, it was found that there was not any subpopulation of sensitive individuals, that is individuals displaying z-score values below -1.645 for both CD4 and CD8. The only outliers were in the upper right region of the z-score analysis plot (above 1.645 for both CD4 and CD8) indicating that there were a number of individuals who displayed high levels of radiation-induced apoptosis after exposure to 2Gy and 8Gy X-rays.

Research ethics requirements state that sample sizes should increase till the required data is seen. The study was therefore increased to include a total of 300 donors in order to see whether or not population existed who displayed low levels of radiation-induced apoptosis.

3.3.3 Final Standard curve study

The standard curve study was extended to include a total of 300 healthy donors. A high number of donors were also needed to investigate the effects of age, gender and race on radiation-induced apoptosis.

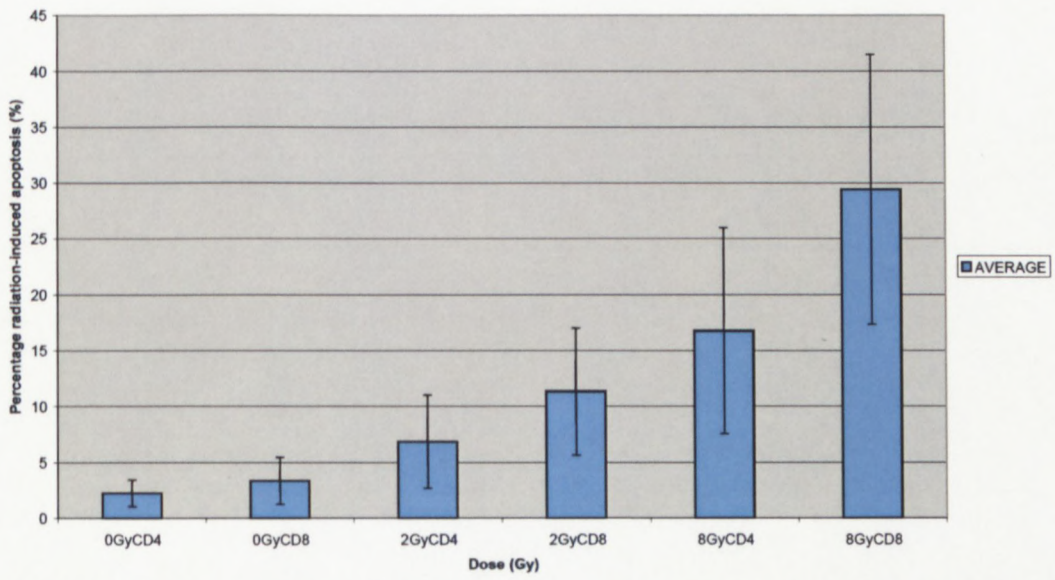


Figure 3.12: The average percent apoptosis induced by 2Gy and 8Gy X-rays in CD4 and CD8 T-lymphocytes for 300 healthy donors. The background (0Gy) level of apoptosis was subtracted. Error bars display standard deviation.

Figure 3.12 shows the average percent of radiation-induced apoptosis for the 300 healthy donors (mean: 39.2yrs, range 17-78yrs) in CD4 and CD8 T-lymphocytes after 2Gy and 8Gy X-rays. A clear dose response curve is observed as the percentage radiation-induced apoptosis increases with every dose of radiation. The percentage apoptosis at 2-0Gy for CD4 ranges from 1.0-23.65 with a mean of 6.86 and for CD8 it ranges from 1.01-30.55 with a mean of 11.34. The percentage apoptosis at 8-0Gy for CD4 ranges from 2.3-46.68 with a mean of 16.77 and for CD8 it rages from 5.61-67.58 with a mean of 29.43. Once again at each dose point, there is a higher rate of apoptosis observed with CD8 lymphocytes when compared to that of the CD4 lymphocytes.

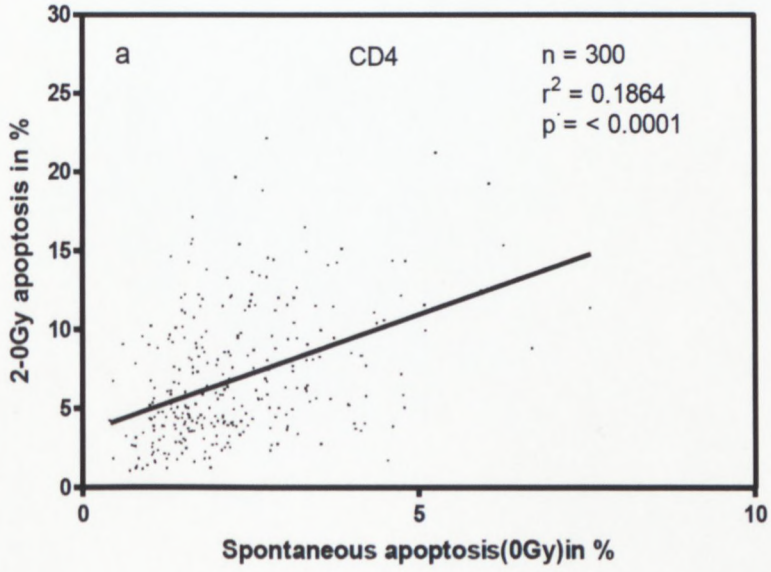
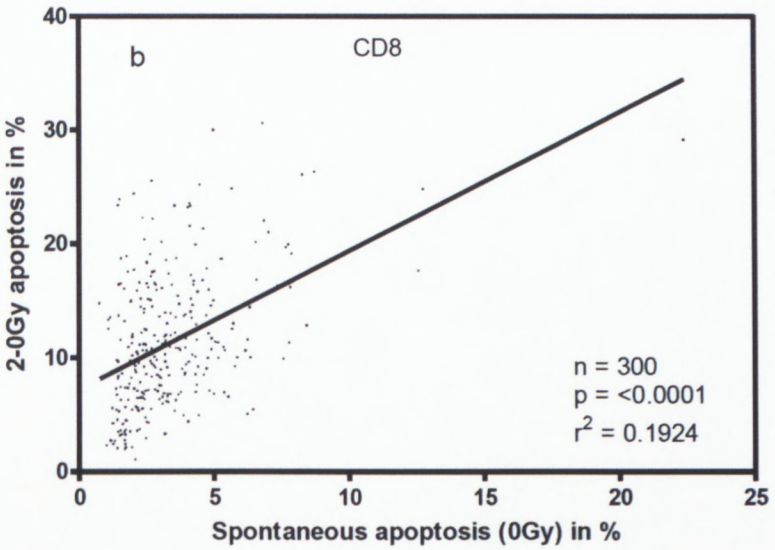


Figure 3.13a: An interdose comparison between spontaneous apoptosis (0Gy) and the percentage radiation- apoptosis in 300 donors. CD4 T-lymphocyte correlation between spontaneous and 2Gy radiation-induced apoptosis. The background (0Gy) level of apoptosis was subtracted. The solid line represents the linear regression.



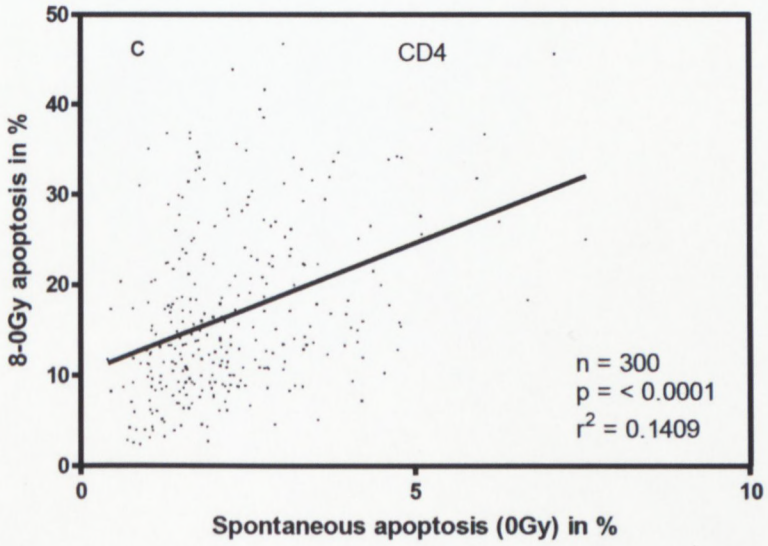


Figure 3.13b-c: An interdose comparison between spontaneous apoptosis (0Gy) and the percentage radiation- apoptosis in 300 donors. (b) CD8 T-lymphocyte correlation between spontaneous and 2Gy radiation-induced apoptosis and (c) CD4 T-lymphocyte correlation between spontaneous and 8Gy radiation-induced apoptosis. The background (0Gy) level of apoptosis was subtracted. The solid line represents the linear regression.

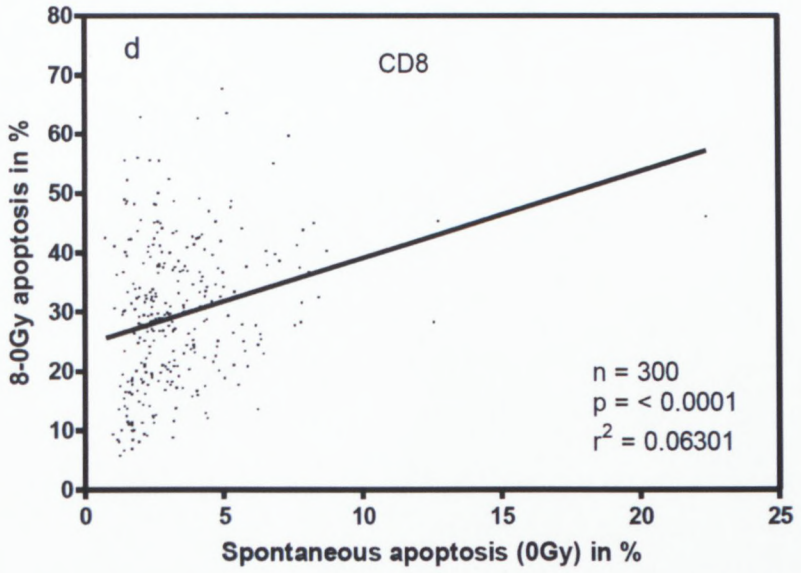


Figure 3.13d: An interdose comparison between spontaneous apoptosis (0Gy) and the percentage radiation- apoptosis in 300 donors. CD8 T-lymphocyte correlation between spontaneous and 8Gy radiation-induced apoptosis. The background (0Gy) level of apoptosis was subtracted. The solid line represents the linear regression.

Figure 3.13 a-b shows the correlation between spontaneous apoptosis (0Gy) and radiation-induced apoptosis at 2Gy for both CD4 (Figure 3.13a) and CD8 (Figure 3.13b) lymphocytes. No strong correlation exists with low r^2 values of 0.1864 for CD4 lymphocytes and 0.1924 for CD8 lymphocytes. Figure 3.13 c-d shows the correlation between spontaneous apoptosis (0Gy) and radiation-induced apoptosis at 8Gy for both CD4 (Figure 3.13c) and CD8 (Figure 3.13d) lymphocytes. No strong correlation exists with low r^2 values of 0.1409 for CD4 lymphocytes and 0.06301 for CD8 lymphocytes.

Although significant, the correlation between spontaneous (0Gy) and radiation-induced apoptosis at 2Gy and 8Gy was observed to be weak with r^2 values less than 0.2. (Figure 3.13a-d). This was also found by Ozsahin et al (1997) and could indicate that factors which influence spontaneous levels of apoptosis should not influence radiation induced apoptosis.

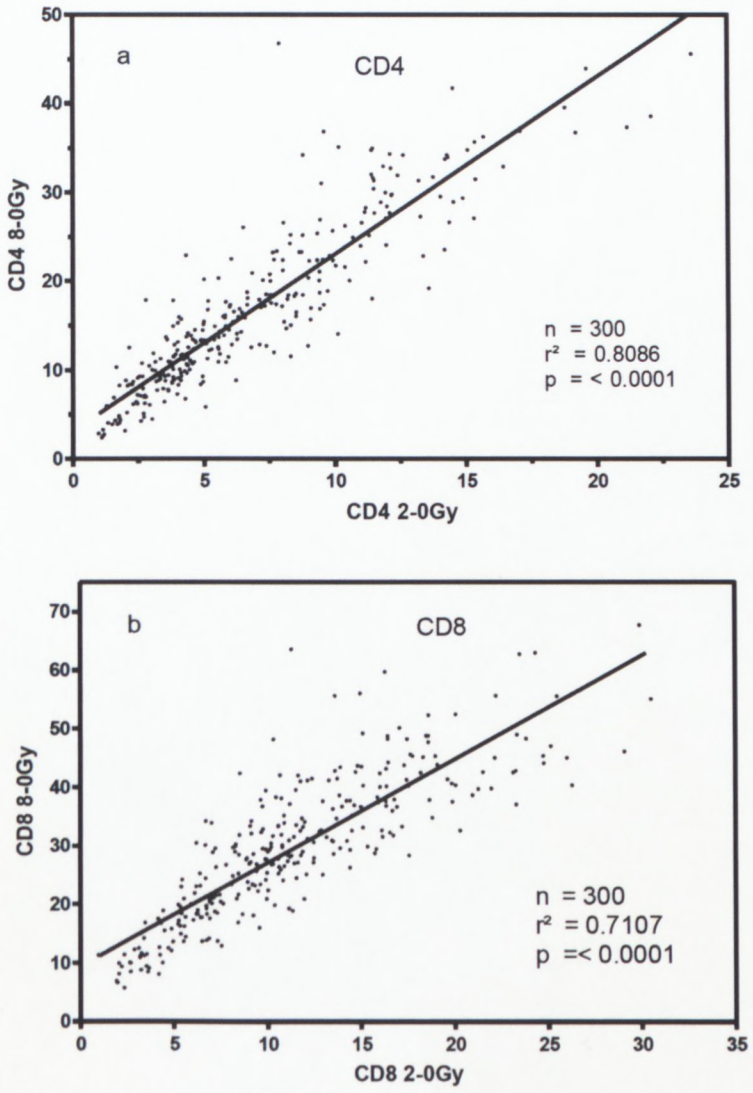


Figure 3.14a-b: An interdose comparison of the percentage apoptosis induced by 2Gy and 8Gy X-rays in (a) CD4 and (b) CD8 T-lymphocytes of 300 healthy donors. The background (0Gy) level of apoptosis was subtracted. The solid line represents the linear regression.

Interdose comparisons of the percentage apoptosis induced by 2Gy and 8Gy X-ray radiation in CD4 and CD8 lymphocytes were examined using linear regression analysis (Figure 3.14 a-b). A significant correlation between doses for CD4 (r^2 value = 0.8086, $p<0.0001$) and CD8 (r^2 value = 0.7107, $p<0.0001$) was seen. Interdose comparisons of the radiation-induced apoptosis at 2Gy and 8Gy X-rays in CD4 and CD8 lymphocytes conducted by Crompton and co-workers (1999) using 105 healthy donors compared well

to this study. Their study resulted in r^2 values of 0.81 for CD4 compared to our r^2 value of 0.80 and for CD8 their r^2 value was 0.55 compared to ar^2 value of 0.71 in this study.

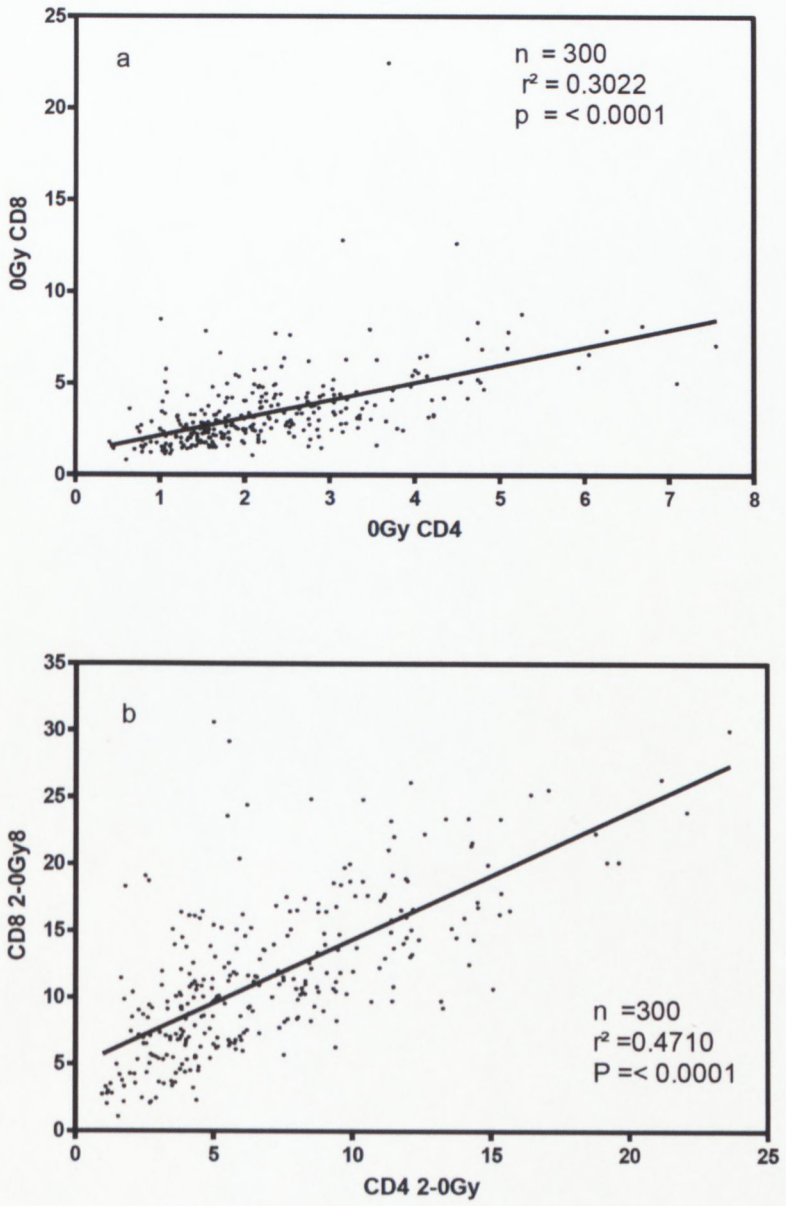


Figure 3.15a-b: A comparison of the percentage apoptosis induced in CD4 and CD8 T-lymphocytes of 300 healthy donors. (a) CD4 and CD8 correlation for background (0Gy) apoptosis and (b) CD4 and CD8 correlation after 2-0Gy X-rays. Regression curves are displayed as solid lines.

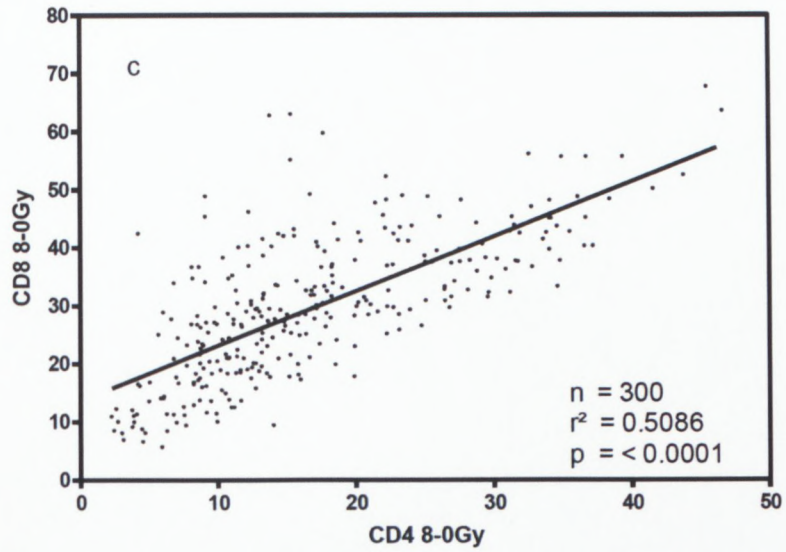


Figure 3.15c: A comparison of the percentage apoptosis induced in CD4 and CD8 T-lymphocytes of 300 healthy donors. CD4 and CD8 correlation after 8-0Gy. Regression curves are displayed as solid lines.

A range of background apoptosis was observed for the cohort of 300 healthy donors (Figure 3.15 a). The CD4 values ranged from 0.41-7.56 with a mean of 2.28. The CD8 readings ranged from 0.76-22.41 with a mean of 3.37. Apoptosis was observed without any radiation exposure and this could be due to physiological reasons. The y-intercept is higher than zero for the CD8 at 1.176. The coefficient of variation is 61.9% for CD8 compared to 52.3% for CD4. This shows that there is greater variation in the CD8 values than in the CD4 values for the 300 donors. Background apoptosis is significantly correlated with a r^2 value of 0.3022 ($p<0.0001$).

A significant correlation exists between the apoptotic response of CD4 and CD8 lymphocytes at 2Gy (Figure 3.15 b) with a r^2 value of 0.4710 ($p<0.0001$). The CD4 values range from 1.0-23.65 with a mean of 6.86. The CD8 values range from 1.01-30.55 with a mean of 11.34. A significant correlation exists between the apoptotic response of CD4 and CD8 lymphocytes at 8Gy (Figure 3.15 c) with a r^2 value of 0.5086 ($p<0.0001$). The CD4 values range from 2.30-46.68 with a mean of 16.77. The CD8 values range from 5.61-67.58 with a mean of 29.43. There is no significant difference

between CD4 and CD8 interdose comparisons. This indicates that the predicting of radiosensitivity by analysing radiation-induced apoptosis in either CD4 or CD8 lymphocytes is basically identical.

The significant correlation between the CD4 and CD8 values for both 2Gy and 8Gy compares well with Ozsahin et al (1997). This leads one to believe that the apoptotic values for individuals at the different doses (2Gy and 8Gy) could be averaged to obtain the overall apoptotic response of an individual. When looking at the z-score analysis done on the first 100 donors, there were some contradictions involved. For example, more outliers were noted when data for the 2Gy and 8Gy were analysed separately. This procedure was once again repeated for the 300 donors.

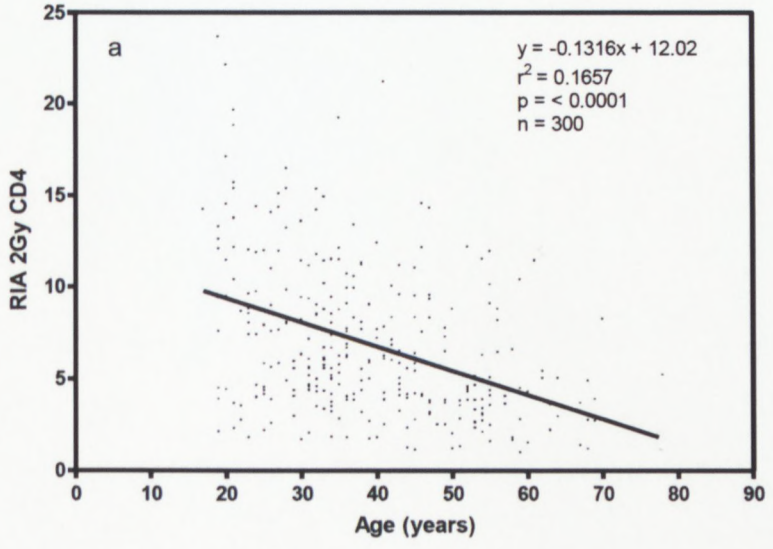
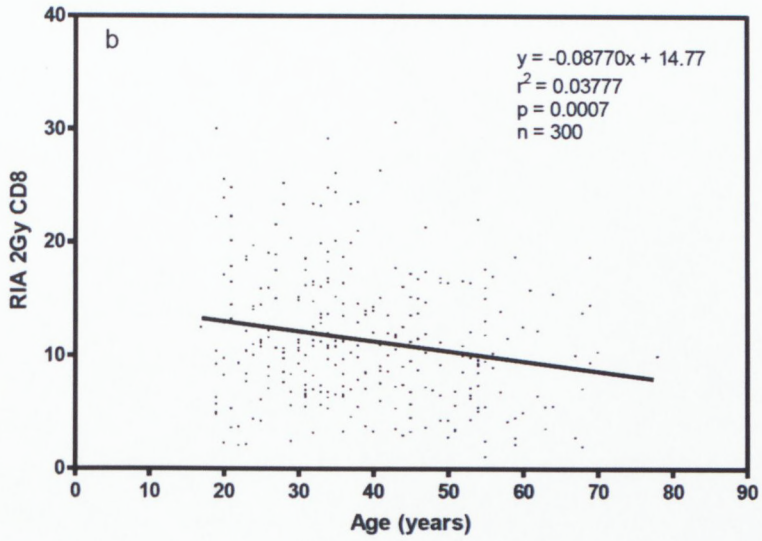


Figure 3.16a: Radiation-induced apoptosis (RIA) vs. age for 2Gy CD4 for 300 donors.



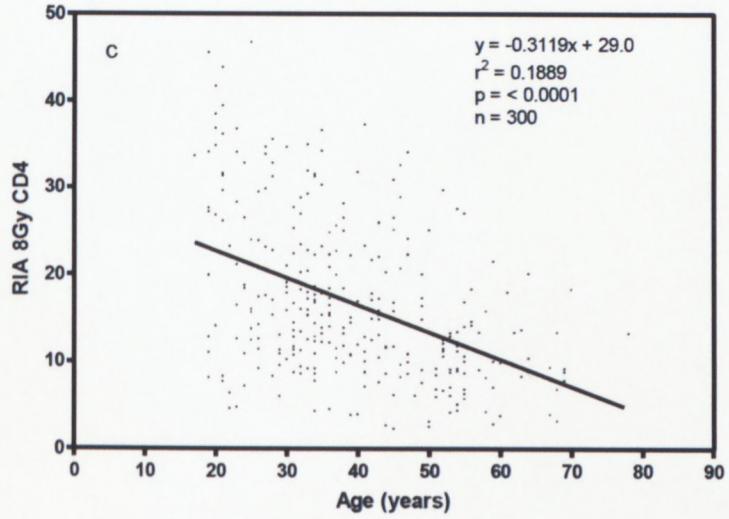


Figure 3.16b-c: Radiation-induced apoptosis (RIA) vs. age for (b) 2Gy CD8 and (c) 8Gy CD4 for 300 donors.

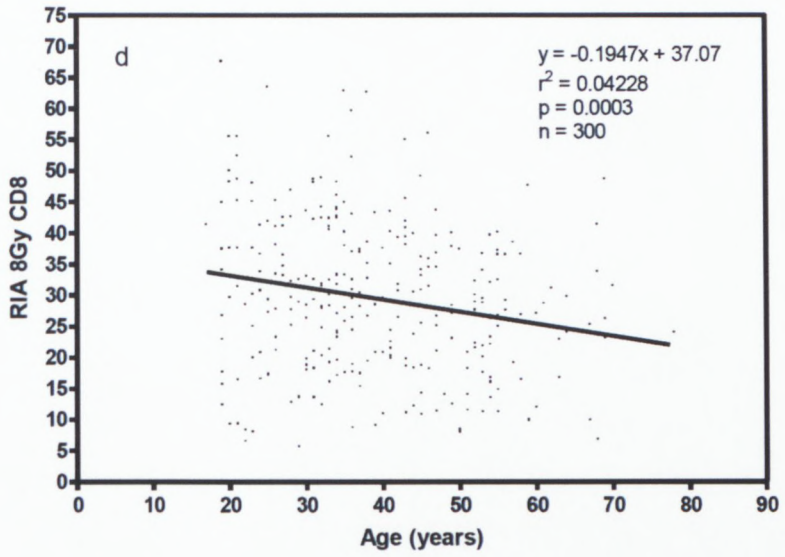


Figure 3.16d: Radiation-induced apoptosis (RIA) vs. age for 8Gy CD8 for 300 donors.

Radiation-induced apoptosis for the 300 donors was plotted against age for each cell type and each dose point. At 2Gy, CD4 lymphocytes (Figure 3.16a) show a negative slope when the radiation-induced apoptosis was plotted against age (graph 1a). A significant correlation exists between RIA and age with a r^2 value of 0.17 ($p<0.0001$). The regression line formula obtained that could be used to determine the expected RIA for a given age is RIA = (-0.1316 x age) + 12.02.

At 2Gy, CD8 lymphocytes (Figure 3.16b) also show a negative slope when RIA was plotted against age (graph1b). The relationship between RIA and age is significant with a r^2 value of 0.03 ($p = 0.0007$). The regression line formula used to correct the data was RIA = (-0.0877 x age) + 14.77.

At 8Gy, CD4 lymphocytes (Figure 3.16c) also show a negative slope when RIA was plotted against age (graph1c). A significant correlation exists between the variables with a r^2 value of 0.19 ($p = <0.0001$). The regression line formula obtained to determine the RIA at a given age is RIA = (-0.3119 x age) + 29.

At 8Gy, CD8 lymphocytes (Figure 3.16d) also show a negative slope when RIA was plotted against age (graph1d). A significant correlation exists with a r^2 value of 0.04 ($p = 0.0003$). The regression line formula obtained to determine the RIA at a given age is RIA = (-0.1947 x age) + 37.07.

It is evident from the above that the influence is once again most pronounced for CD4 lymphocytes receiving a dose of 8Gy.

The regression line formula, RIA = *(slope x age) + y-intercept*, was applied to all the RIA data for the 300 donors. The expected RIA values (y) obtained is then subtracted from the actual RIA data points observed to obtain a value for radiation-induced apoptosis corrected for age (RIA –age). These RIA-age values are then plotted as a function of age for all the 300 donors.

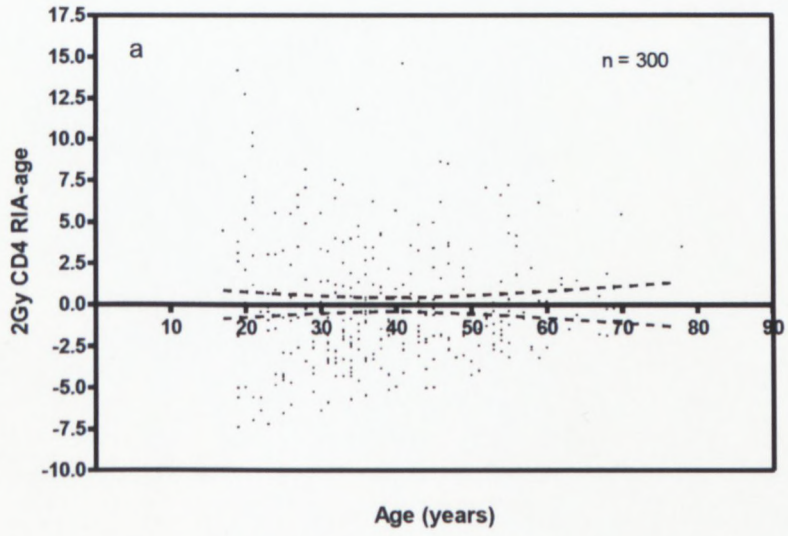
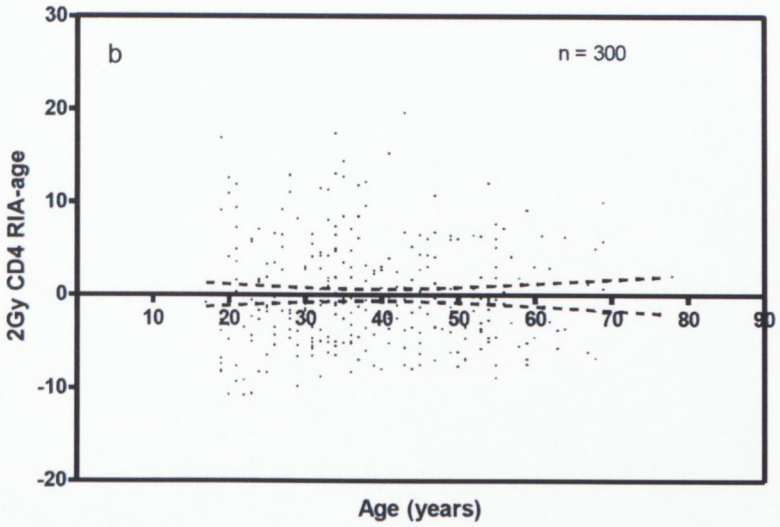


Figure 3.17a: Radiation-induced apoptosis with age contribution (RIA-age) vs. age for 2Gy CD4 for 300 donors. The dotted lines indicate the 95% confident intervals of the fitted parameters.



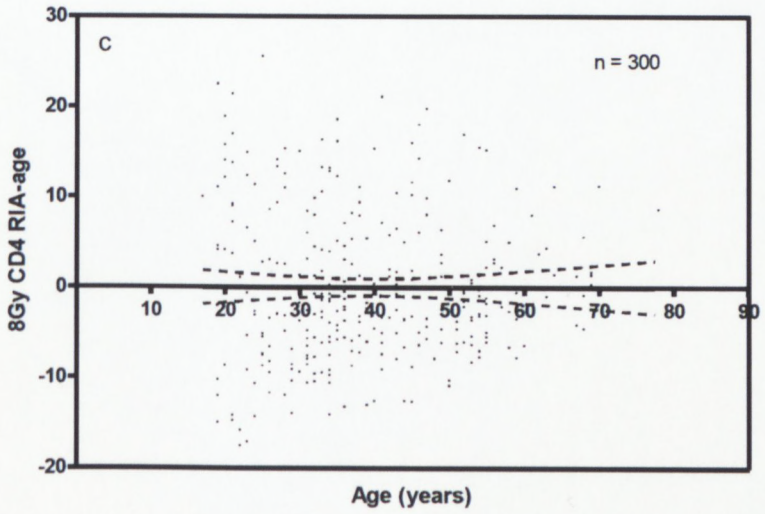


Figure 3.17b-c: Radiation-induced apoptosis with age contribution (RIA-age) vs. age for (b) 2Gy CD8 and (c) 8Gy CD4 for 300 donors. The dotted lines indicate the 95% confident intervals of the fitted parameters.

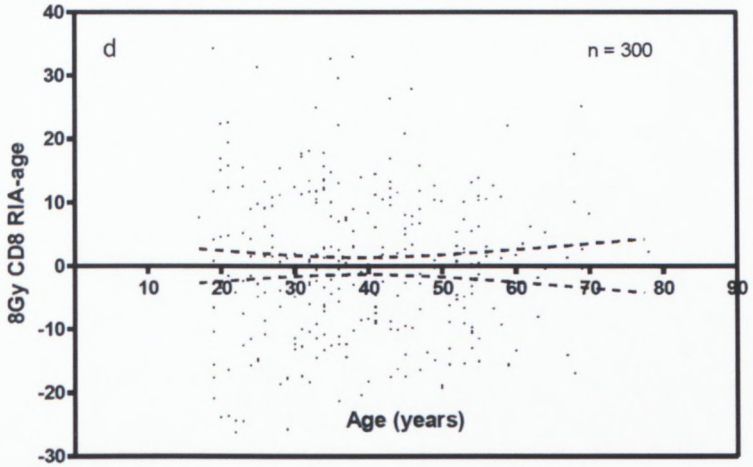


Figure 3.17d: Radiation-induced apoptosis with age contribution (RIA-age) vs. age for 8Gy CD8 for 300 donors. The dotted lines indicate the 95% confident intervals of the fitted parameters.

In all cases, the scatter plots of RIA-age vs. age yield a trendline that hugs the x-axis (Figure 3.17a-d).

The RIA-age data can now be used to determine z-scores with the following equation:

z = (RIA-age – average RIA-age for 300 donors) /standard deviation of the sample population.

This equation is applied to all the RIA-age data points for the 300 donors. The z-score values for CD4 and CD8 lymphocytes for 2Gy and 8Gy were then averaged. The final two z-score values for CD4 and CD8 are plotted against each other as a scatter plot (Figure 3.18).

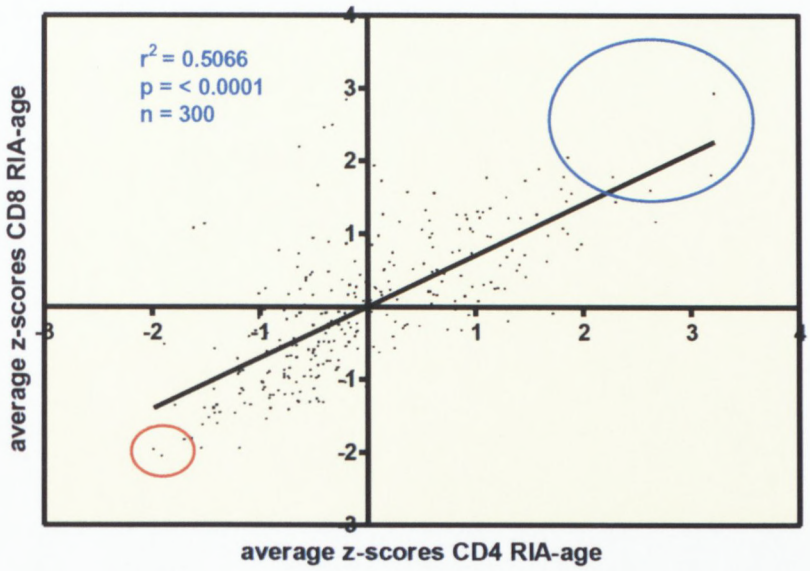


Figure 3.18: Average z-score dose correlation for 300 healthy donors from CD4 and CD8 T-lymphocyte data after both 2Gy and 8Gy X-rays. Regression analysis is displayed as a solid line. The blue circle indicates radioresistant donors and the red circle indicates radiosensitive donors.

Figure 3.18 shows the average z-score analysis for CD4 and CD8 after the z-score data were pooled and averaged. This analysis show that z-score values are significantly correlated with a r^2 value of 0.51 ($p = <0.0001$).

There were 8 donors who displayed abnormal z-score values beyond 1.645 and -1.645 in Figure 3.18. The donor information and z-score values for both CD4 and CD8 cells are depicted in Table 3.6.

Table 3.6: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of 8 donors who showed variation beyond ± 1.645 after 2Gy and 8Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
				Radioresistant	
60	41	Coloured	Male	3.193	1.802
189	19	Black	Male	3.221	2.925
251	21	Coloured	Male	2.286	1.768
264	20	Black	Female	1.864	2.038
272	28	Black	Male	1.829	1.775
				Radiosensitive	
155	22	White	Female	-1.695	-1.840
175	22	White	Female	-1.904	-2.060
176	23	White	Female	-1.983	-1.973

Five donors displayed z-score values above 1.645 for both CD4 and CD8. Three radiosensitive donors were identified who displayed z-score values below -1.645 for both CD4 and CD8.

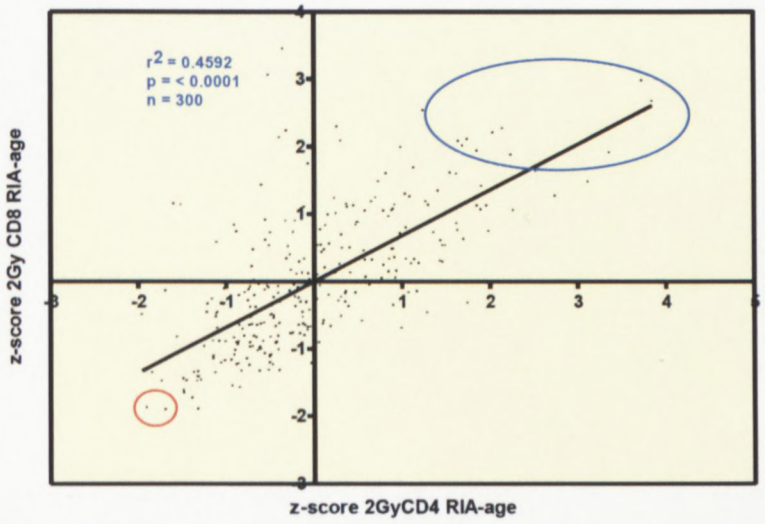


Figure 3.19: Average z-score dose correlation for 300 healthy donors from CD4 and CD8 T-lymphocyte data after 2Gy of X-rays. Regression analysis is displayed as a solid line. The blue circle indicates radioresistant donors and the red circle indicates radiosensitive donors.

Figure 3.19 shows data from both cell types after 2Gy irradiation that has been combined for z-score analysis. The results are significantly correlated with a r^2 value of 0.46 ($p =<0.0001$). Twelve individuals displayed extreme variation and the donor information and z-score values are depicted in Table 3.7.

Table 3.7: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of 12 donors who showed variation beyond ± 1.645 after 2Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
				Radioresistant	
60	41	Coloured	Male	3.847	2.667
63	47	White	Male	2.238	1.876
189	19	Black	Male	3.732	2.970
243	20	White	Male	3.360	1.908
264	20	Black	Female	2.036	2.201
267	54	White	Male	1.747	2.108
272	28	Black	Male	2.145	2.265
273	37	Black	Male	1.647	2.079
295	28	White	Male	1.857	1.937
300	32	Coloured	Male	1.690	2.007
				Radiosensitive	
175	22	White	Female	-1.689	-1.909
176	23	White	Female	-1.905	-1.878

Ten donors displayed z-score values above 1.645 and 2 radiosensitive donors were identified with z-score values below -1.645.

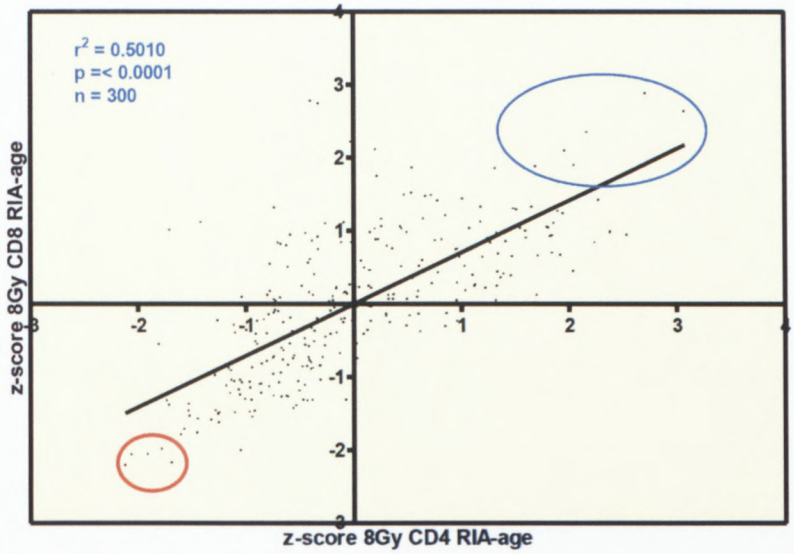


Figure 3.20: Average z-score dose correlation for 300 healthy donors from CD4 and CD8 T-lymphocyte data after 8Gy of X-rays. Regression analysis is displayed as a solid line. The blue circle indicates radioresistant donors and the red circle indicates radiosensitive donors.

Figure 3.20 shows data from both cell types after 8Gy irradiation that has been combined for z-score analysis. The results are significantly correlated with a r^2 value of 0.50 ($p = <0.0001$). Eleven individuals displayed extreme variation and the donor information and z-score values are depicted in Table 3.8.

Table 3.8: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of 11 donors who showed variation beyond ± 1.645 after 8Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
				Radioresistant	
15	25	Coloured	Male	3.076	2.629
189	19	Black	Male	2.711	2.880
195	46	Coloured	Male	2.697	2.343
210	33	Coloured	Male	1.965	2.0912
251	21	Coloured	Male	2.049	1.897
264	20	Black	Female	1.692	1.876
				Radiosensitive	
155	22	White	Female	-1.907	-2.0591
164	29	Coloured	Female	-1.687	-2.173
174	21	White	Female	-1.777	-1.992
175	22	White	Female	-2.119	-2.211
176	23	White	Female	-2.060	-2.068

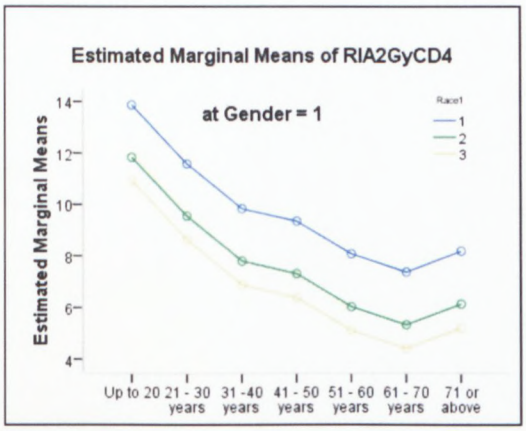
Six individuals displayed z-score values above 1.645 while 5 radiosensitive donors were identified with z-scores values below -1.645 for both CD4 and CD8. Z-score data obtained from this study showed that approximately 2% of the 300 donors had a low apoptotic response in both CD4 and CD8 lymphocytes (Figure 3.20).

After separating the z-score for 2Gy and 8Gy, the number of radiosensitive individuals has increased from 3 individuals to 5 individuals. These healthy individuals displaying reduced CD4 and CD8 z-scores indicate 2% of the total donor population with an enhanced radiosensitivity to radiation exposure.

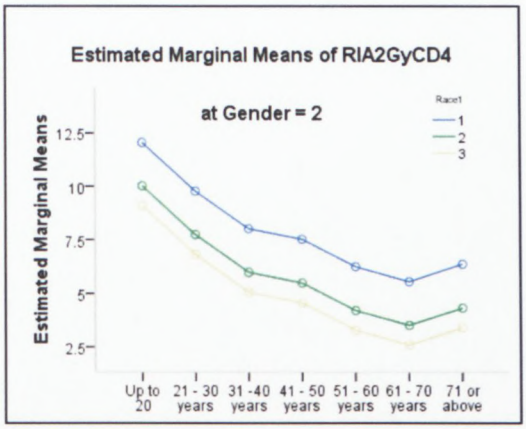
3.3.4 Age, Gender and Race study.

3.3.4.1 The effect of age, gender and race on Radiation-induced apoptosis (RIA)

Univariate Analysis of Variance was done on RIA data obtained from the 300 donors by using the SPSS 16 statistical programme. Variables included age, gender and race. The effect of these variables on the RIA of CD4 and CD8 lymphocytes at each dose point, 2Gy and 8Gy was analysed. The donor ages were grouped into 10 year intervals. The mean RIA for each 10 years of age was plotted against age. The last age group, 71 or above, only had 1 donor and therefore the results obtained for this group is not an accurate indication of the average RIA and the effects of age, gender and race. The other age groups have sufficient number of donors to enable analysis.



(a)



(b)

RIA 2Gy CD4		p-value
Age		<0.001
Gender 1	Male	<0.001
Gender 2	Female	
Race 1	Black	0.003
Race 2	Coloured	
Race 3	White	

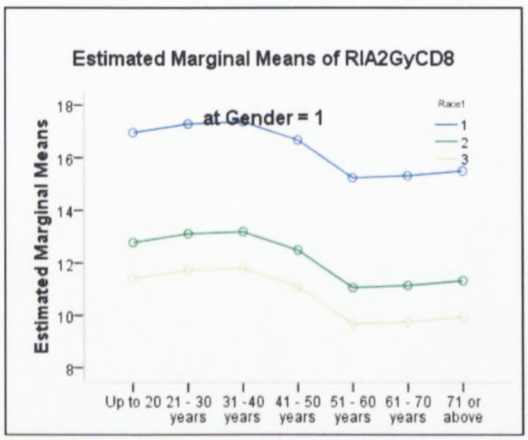
Figure 3.21a-b: The mean RIA for every 10 years plotted against age (grouped into 10 year intervals) for CD4 lymphocytes after 2Gy X-ray exposure. The effect of gender on RIA is divided into (a) males and (b) females. The different race groups are indicated by the solid lines labelled 1 (Black donors), 2 (Coloured donors) and 3 (White donors). The p-values are tabulated below the graphs.

RIA for CD4 lymphocytes after 2Gy X-ray exposure is significantly related to age (p =<0.001), gender (p = <0.001) and race (p = 0.003). After 2Gy exposure, CD4 lymphocytes show a steady decrease in RIA as age increases for all 3 the race groups. When looking at the graphs the change in RIA decreases sharply within the younger ages and then plateaus out towards the older age groups. This pattern of RIA is the same for all 3 the race groups as well as for both males and females.

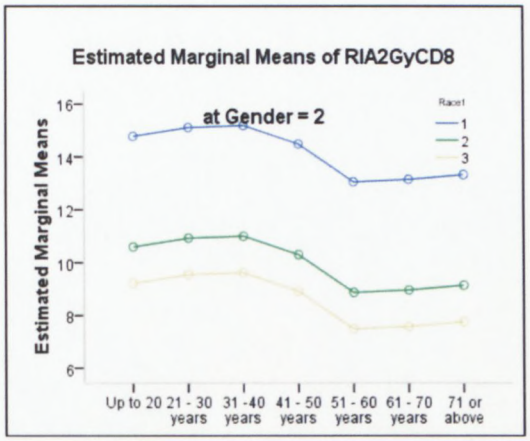
When looking at the difference between the RIA for males and females, males tend to have a slightly higher apoptotic effect after 2Gy X-ray exposure. This was noted for all 3 of the race groups. The RIA for males start at a maximum of 14% for the group up to 20 years of age and decreases to a minimum of 5% for the 61-70 year age group whereas for females it started at 12.5% at group up to 20 years and decreased to a minimum of

approximately 3 % for the 61-70 year group. There is an approximate parallel shift to lower RIA values for females when compared to males.

The effect of race on CD4 lymphocytes after 2Gy is consistent with the Black donors (1) always having the highest RIA followed by Coloured donors (2) and lastly the White donors (3) show the lowest RIA.



(a)



(b)

RIA 2Gy CD8		p-value
Age		0.460
Gender 1	Male	0.001
Gender 2	Female	
Race 1	Black	<0.001
Race 2	Coloured	
Race 3	White	

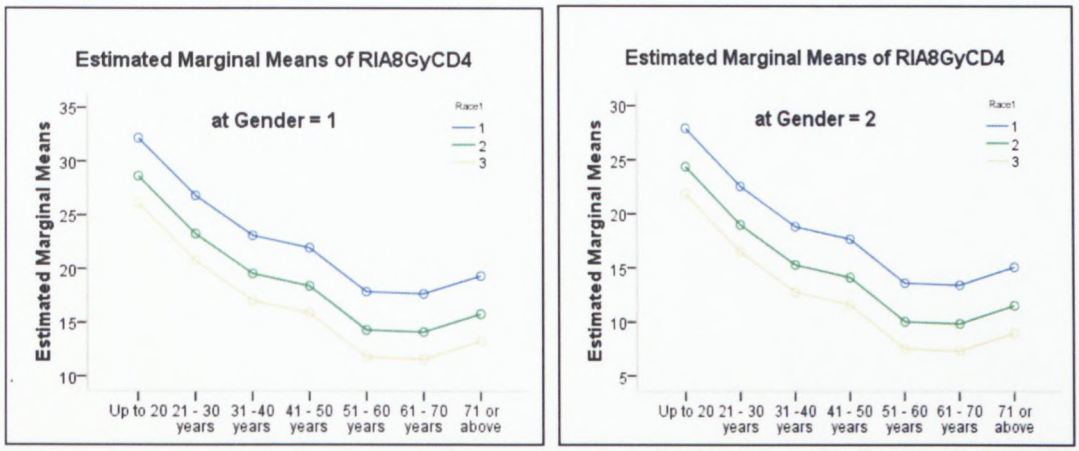
Figure 3.22a-b: The mean RIA for every 10 years plotted against age (grouped into 10 year intervals) for CD8 lymphocytes after 2Gy X-ray exposure. The effect of gender on RIA is divided into (a) males and (b) females. The different race groups are indicated by the solid lines labelled 1 (Black donors), 2 (Coloured donors) and 3 (White donors). The p-values are tabulated below the graphs.

RIA for CD8 lymphocytes after 2Gy X-ray exposure is significantly related to gender ($p = 0.001$) and race ($p = <0.001$) but not age ($p = 0.460$).

After 2Gy exposure, RIA for CD8 lymphocytes peak between 20 and 40 years of age, decreases till 51-60 years and then plateaus out after 60 years of age. This pattern of RIA is apparent for all 3 the race groups as well as both males and females. This indicates CD8 lymphocytes do not have as stable a relationship with age as CD4 lymphocytes, that is, there is not a significant decrease in RIA with an increase in age after 2Gy X-rays.

The effect of gender on RIA for CD8 lymphocytes is similar to that of CD4 lymphocytes. Males tend to have a slightly higher apoptotic effect than females after 2Gy X-ray exposure (Figure 3.22a-b). The maximum RIA at younger ages and minimum RIA at older ages for males are both higher than those of females for all the race groups.

The effect of race on CD8 lymphocytes after 2Gy is consistent with the Black donors (1) always having the highest RIA followed by Coloured donors (2) and lastly the White donors (3) show the lowest RIA. It can be seen that the RIA difference between the Black donors and the White and Coloured donors is much higher for CD8 lymphocytes than CD4 lymphocytes after 2Gy exposure.



(a) (b)

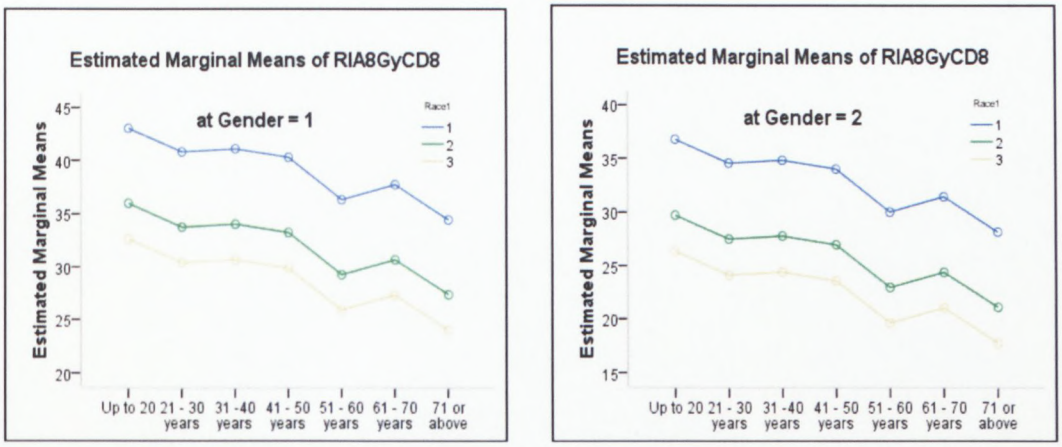
RIA 8Gy CD4		p-value
Age		<0.001
Gender 1	Male	<0.001
Gender 2	Female	
Race 1	Black	0.003
Race 2	Coloured	
Race 3	White	

Figure 3.23a-b: The mean RIA for every 10 years plotted against age (grouped into 10 year intervals) for CD4 lymphocytes after 8Gy X-ray exposure. The effect of gender on RIA is divided into (a) males and (b) females. The different race groups are indicated by the solid lines labelled 1 (Black donors), 2 (Coloured donors) and 3 (White donors). The p-values are tabulated below the graphs.

RIA for CD4 lymphocytes after 8Gy X-ray exposure is significantly related to age (p =<0.001 , gender (p = <0.001) and race (p = 0.003). After 8Gy exposure, CD4 lymphocytes also show a steady decrease in RIA as age increases for all 3 the race groups. Similarly to 2 Gy exposure graphs, the RIA decreases sharply within the younger ages and then plateaus out towards the older age groups. This pattern of RIA after 8Gy X-rays is the same for all 3 the race groups as well as for both males and females.

Once again the effect of gender on RIA is consistent, with males showing a slightly higher apoptotic effect than females after 8Gy X-ray exposure (Figure 3.23a-b). The maximum RIA at younger ages and minimum RIA at older ages for males are both higher than those of females for all the race groups.

The effect of race on CD4 lymphocytes after 8Gy is consistent with the Black donors (1) always having the highest RIA followed by Coloured donors (2) and lastly the White donors (3) show the lowest RIA.



(a) (b)

RIA 8Gy CD8		p-value
Age		0.294
Gender 1	Male	<0.001
Gender 2	Female	
Race 1	Black	0.001
Race 2	Coloured	
Race 3	White	

Figure 3.24a-b: The mean RIA for every 10 years plotted against age (grouped into 10 year intervals) for CD8 lymphocytes after 8Gy X-ray exposure. The effect of gender on RIA is divided into (a) males and (b) females. The different race groups are indicated by the solid lines labelled 1 (Black donors), 2 (Coloured donors) and 3 (White donors). The p-values are tabulated below the graphs.

RIA for CD8 lymphocytes after 8Gy X-ray exposure is significantly related to gender ($p = <0.001$) and race ($p = 0.001$) but not age ($p = 0.294$).

When looking at the graphs the maximum RIA occurs at the younger age groups and then decreases until 60 years of age with a slight peak between 60 and 70 years of age. This pattern of RIA is the same for all 3 the race groups as well as for both males and females. Once again the CD8 lymphocytes do not have as stable a relationship with age

as CD4 lymphocytes, i.e. there is not a significant decrease in RIA with an increase in age after 8Gy X-rays.

The effect of gender on RIA for CD8 lymphocytes is similar to that of CD4 lymphocytes. Males tend to have a slightly higher apoptotic effect than females after 8Gy X-ray exposure (Figure 3.24a-b). The maximum RIA at younger ages and minimum RIA at older ages for males are both higher than those of females for all the race groups.

The effect of race on CD8 lymphocytes after 2Gy is consistent with the Black donors (1) always having the highest RIA followed by Coloured donors (2) and lastly the White donors (3) show the lowest RIA. It can be seen that the RIA difference between the Black donors and the White and Coloured donors is much higher for CD8 lymphocytes than CD4 lymphocytes after 2Gy exposure.

After looking at the combined effects of age, gender and race on RIA of CD4 and CD8 lymphocytes, individual age, gender and race studies were done.

3.3.4.2 The effect of gender on Radiation-induced apoptosis

The influence of gender on radiosensitivity was analysed by doing a Mann-Whitney analysis using Graph Pad Prism 4. There were 193 males and 107 females in the standard curve population of 300 donors.

Table 3.9: Influence of gender on radiosensitivity.

Cell type	Dose (Gy)	Mean (Male) n = 193	Standard deviation (male)	Mean (Female) n = 107	Standard deviation (Female)	p value
CD4	2-0	7.31	4.4	6.03	3.55	0.02
	8-0	17.86	9.46	14.8	8.44	0.003
CD8	2-0	12.02	5.81	10.06	5.55	0.008
	8-0	31.45	12.14	25.67	11.25	<0.0001

Table 3.9 shows the differences between the average male and average female radiosensitivity. This is a direct comparison between male and female RIA without binning the ages. On average males display a higher RIA mean than females for both

CD4 and CD8 lymphocytes after 2Gy and 8Gy X-ray exposure. Large variations in RIA are noted and are possibly the result of not correcting radiation response as a function of age. Not withstanding this significant difference between male and female radiosensitivity was observed for CD4 lymphocytes after both 2Gy ($p = 0.002$) and 8Gy X-ray exposure ($p = 0.003$). Similarly a significant difference was found between male and female radiosensitivity for CD8 lymphocytes after both 2Gy ($p = 0.008$) and 8Gy X-ray exposure ($p = <0.0001$). Ozsahin and co-workers (1997) did not find significant differences between male and female radiosensitivity.

The above would indicate that severe reactions to radiotherapy would likely occur more in women than in men. This applies to the combined analysis of all South Africans in this study.

3.3.4.3 The effect of age on Radiation-induced apoptosis

The influence of age on RIA was analysed. A histogram of the average RIA for each 10 years of age was plotted against age using the GraphPad Prism 4 software.

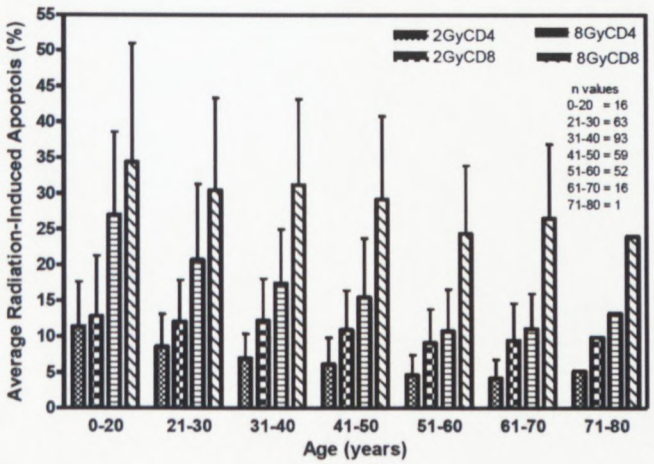


Figure 3.25: Histogram of the average radiation-induced apoptosis in CD4 and CD8 lymphocytes after 2Gy and 8Gy X-rays. Seven age groups are compared: 0-20 years, 21-30 years, 31-40 years, 41-50 years, 51-60 years, 61-70 years and 71-80 years. The background (0Gy) levels of apoptosis were subtracted. Error bars display standard deviation.

On average, reduced apoptosis was observed with increasing age in both cell types. The last age group, 71 or above, only had 1 donor and therefore the results obtained for this

group is not an accurate indication of the average RIA and the effects of age. There was no fixed decrease percentage RIA with every 10 years of age as was found with Crompton et al (2001). In their study only CD4 lymphocytes had a significant relationship with RIA and showed a 6.5% decrease in CD4 RIA after 8Gy of X-rays. The percentage change between each age group for the South African study is tabulated below (Table 3.10).

Table 3.10: The percentage change in mean radiation-induced apoptosis for 300 donors.

Cell & Dose	Age group (years)	Mean RIA	Percentage Change (%)
2Gy CD4	Up to 20	11.40	-
	21-30	8.62	24
	31-40	7.00	19
	41-50	6.17	12
	51-60	4.67	24
	61-70	4.19	10
	71 & above	5.22	-
2Gy CD8	Up to 20	12.82	-
	21-30	12.13	5.3
	31-40	12.26	-1.1
	41-50	10.96	10.6
	51-60	9.18	16.2
	61-70	9.52	-3.7
	71 & above	9.94	-
8Gy CD4	Up to 20	27.03	-
	21-30	20.75	23
	31-40	17.45	16
	41-50	15.51	11
	51-60	10.81	30
	61-70	11.10	-2
	71 & above	13.26	-
8Gy CD8	Up to 20	34.43	-
	21-30	30.49	11
	31-40	31.24	25
	41-50	29.20	6.5
	51-60	24.39	16
	61-70	26.54	-8.8
	71 & above	24.01	-

Table 3.10 shows the average RIA for each age group as well as the percentage change of RIA as age increases. CD4 lymphocytes once again show a more stable relationship with RIA. The average percentage RIA for CD4 lymphocytes decrease with the increase of age. The percentage change between the different age groups starts high after both 2Gy and 8Gy exposure and then gradually decreases until 50 years of age. Between 50 and 60 years of age there is an increase in the percentage change which indicates a larger drop in apoptotic response.

CD8 lymphocytes do not show a steady decrease in RIA with the increase of age. RIA decrease until 30 years of age, then increases slightly till 40 years before decreasing again. The percentage change between the different age groups fluctuates greatly after both 2Gy and 8Gy exposure. It is apparent that the CD8 lymphocytes, the more sensitive subpopulation to radiation, do not change with age as clearly as the CD4 subpopulation. CD8 lymphocytes, being more sensitive, does not necessary make these cells a better indicator in variation of sensitivity.

The reduced apoptotic response with the increase of age is an important factor to consider before commencing radiotherapy. It is evident that as people age, their lymphocytes are less able to mount an apoptotic response to ionising radiation damage. As most cancer patients tend to be elderly, the risk of developing late effects should be assessed in order to plan the most effective treatment regime.

3.3.4.4 The effect of race on Radiation-induced apoptosis:

The effect of race on RIA without binning the RIA into different age groups was analysed by using t-test analysis (SPSS 16). A comparison was made between Black (n = 22), Coloured (n = 120) and White (n = 158) groups within the study population of 300 donors.

Table 3.11a-d: The relationship between mean RIA and the different race groups for (a) 2Gy CD4, (b) 2Gy CD8, (c) 8Gy CD4 and (d) 8Gy CD8.

(a) RIA 2Gy CD4		
Race groups	**Mean RIA**	**p-value**
White	5.77	< 0.001
Black	10.91	
White	5.77	< 0.001
Coloured	7.54	
Black	10.91	0.01
Coloured	7.54	

(b) RIA 2Gy CD8		
Race groups	**Mean RIA**	**p-value**
White	10.03	< 0.001
Black	16.33	
White	10.03	0.002
Coloured	12.10	
Black	16.33	0.001
Coloured	12.10	

(c) RIA 8Gy CD4		
Race groups	**Mean RIA**	**p-value**
White	14.08	< 0.001
Black	25.33	
White	14.08	< 0.001
Coloured	18.75	
Black	25.33	0.01
Coloured	18.75	

(d) RIA 8Gy CD8		
Race groups	**Mean RIA**	**p-value**
White	26.54	< 0.001
Black	38.93	
White	26.54	0.001
Coloured	31.39	
Black	38.93	0.008
Coloured	31.39	

Table 3.11a-d shows the relationship between the 3 race groups for both CD4 and CD8 lymphocytes after 2Gy and 8Gy X-ray exposure. After 2Gy exposure, a significant difference in RIA for CD4 lymphocytes (Table 3.11a) was found between the White and Black donors ($p = < 0.001$); the White and Coloured donors ($p = < 0.001$) and the Black and Coloured donors ($p = 0.01$). A significant difference in RIA for CD8 lymphocytes (Table 3.11b) was also found between the three race groups: White and Black donors ($p = < 0.001$); White and Coloured donors ($p = 0.002$) and Black and Coloured donors ($p = 0.001$).

After 8Gy exposure, significant differences in RIA for CD4 lymphocytes (Table 3.11c) were found between White and Black donors ($p = < 0.001$); White and Coloured donors ($p = < 0.001$) and Black and Coloured donors ($p = 0.01$). Significant differences in RIA for CD8 lymphocytes (Table 3.11d) were also found between White and Black donors (p

= < 0.001); White and Coloured donors (p =0.001) and Black and Coloured donors (p = 0.008).

The Black donors always displayed the highest apoptotic values followed by the Coloured donors and lastly the White donors. The above data shows that the effect of race on radiosensitivity of individuals should be considered when treating patients within a South African population. Race studies have not previously been done with the LAA as studies by Crompton and co-workers used a European gene pool (Crompton et al 2001). It is clear from the analysis above that White individuals are mostly at risk for developing late effects in normal tissue following radiation therapy. For this reason the LAA assay should be set up for the different race groups to avoid influences of race in z-score analysis.

3.3.4.5 Z-score analysis for the different race groups

Z-score analysis was then performed on the RIA data of the 3 different race groups because of the differences noted above. The contribution of age for the specific race groups was taken into consideration and z-scores calculated are corrected for this variable. The cohort of Black donors for this study is only 22. Numerous attempts have been made throughout the study to increase the number of Black donors but to no avail.

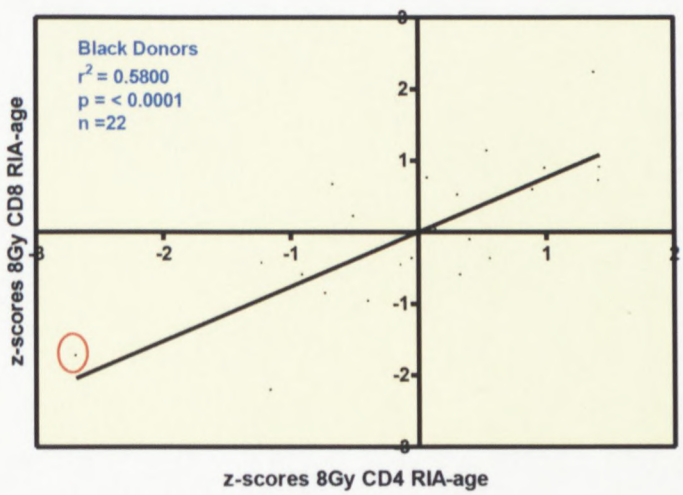


Figure 3.26: Average z-score dose correlation for 22 Black donors from CD4 and CD8 T-lymphocyte data after 8Gy of X-rays. Regression analysis is displayed as a solid line. The red circle indicates radiosensitive donors.

Figure 3.26 shows the z-score data for the 22 Black donors from both cell types after 8Gy irradiation. The results are significantly correlated with a r^2 value of 0.58 ($p = <0.0001$). No donors displayed z-score values above 1.645. Only one donor displayed z-score values below -1.645. The donor information and z-score values are tabulated below (Table 3.12).

Table 3.12: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of 1 Black donor who showed variation beyond - 1.645 after 8Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
				Radiosensitive	
19	19	Black	Female	-2.69	-1.73

The 1 radiosensitive donor within the Black group of donors represents 5% of the total Black population for this study. This percentage is more than the 2% radiosensitive individuals identified in the z-score analysis for the 300 study population where the individuals were not divided into race groups. The 5 % of radiosensitive Black individuals is more comparable to 5-10% sensitive individuals identified by Crompton (1998) and Ozsahin et al (2005). The Crompton studies were also limited to 1 race group. The radiosensitive individual within this Black population is a female donor which is consistent with previous observations.

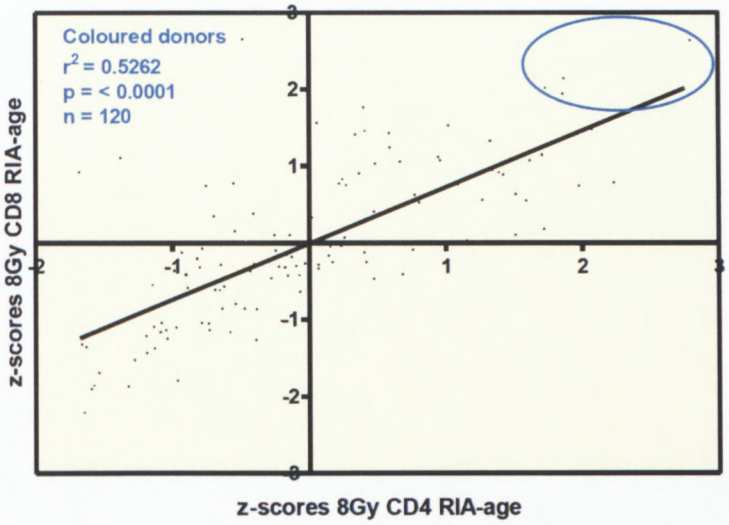


Figure 3.27: Average z-score dose correlation for 120 Coloured donors from CD4 and CD8 T-lymphocyte data after 8Gy of X-rays. Regression analysis is displayed as a solid line. Radioresistant individuals are identified by the blue circle.

Figure 3.27 shows the z-score data for the 120 Coloured donors from both cell types after 8Gy irradiation. The results are significantly correlated with a r^2 value of 0.53 (p = <0.0001). Four donors displayed z-score values above 1.645. There were no radiosensitive donors in the coloured population.

Table 3.13: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of 4 Coloured donors who showed variation beyond 1.645 after 8Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
				Radioresistant	
15	25	Coloured	Male	2.80	2.63
195	46	Coloured	Male	1.87	2.14
210	33	Coloured	Male	1.74	2.01
251	21	Coloured	Male	1.86	1.93

When comparing the z-score analysis of the coloured individuals to the z-score analysis of 300 study population, there are some differences. In the 300 study population, 1 Coloured female was identified as being radiosensitive. In the Coloured z-score analysis, no radiosensitive individuals were identified. It is unclear why this individual is not

showing up again in the more differential analysis. Possibly it is the influence of using different curve fitting parameters when correcting for age.

On the other hand 4 of the Coloured male donors identified as radioresistant donors in the 300 study population were also identified within the Coloured z-score analysis.

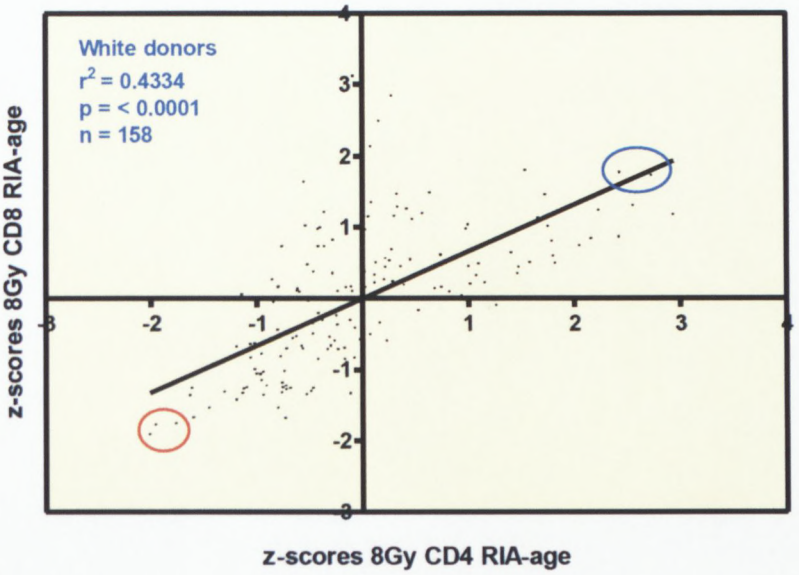


Figure 3.28: Average z-score dose correlation for 158 White donors from CD4 and CD8 T-lymphocyte data after 8Gy of X-rays. Regression analysis is displayed as a solid line. The blue circle indicates radioresistant donors and the red circle indicates radiosensitive donors.

Figure 3.28 shows the z-score data for the 158 White donors from both cell types after 8Gy irradiation. The results are significantly correlated with a r^2 value of 0.43 ($p = <0.0001$). Two donors displayed z-score values above 1.645. There were 3 donors who displayed z-score values below -1.645 (Table 3.14).

Table 3.14: Donor information and z-score values for both CD4 and CD8 T-lymphocytes of the White donors who showed variation beyond ±1.645 after 8Gy X-ray radiation.

Donor	Age	Race	Gender	CD4 z-score	CD8 z-score
				Radioresistant	
243	20	White	Male	2.73	1.72
250	21	White	Male	2.43	1.76
				Radiosensitive	
155	22	White	Female	-1.76	-1.75
175	22	White	Female	-2.01	-1.92
176	23	White	Female	-1.96	-1.78

Three White females were identified as being radiosensitive compared to the 4 White females identified within the z-score analysis for the 300 study population. These 3 individuals indicate that 2% of individuals within the White population are radiosensitive. This is the same as observed when doing z-score analysis for the 300 study population. This close comparison is likely the result of having 158 White donors within the 300 study population. The above results indicate that when doing z-score analysis of a particular race group, a lesser number of donors could be analysed to identify radiosensitive individuals. However, in the 300 study population, no radioresistant White donors were identified compared to the 2 radioresistant White males identified in the differential z-score analysis. This is perhaps more realistic of what is happening within the White donor population.

When looking at the radiosensitive individuals, the majority are White females. This correlates with the data obtained from the univariate analysis previously done where the combined effects of age, gender and race on RIA was analysed. The z-score analysis for the individual race groups also compare well with the z-score analysis done for the study population of 300 donors. The majority of radiosensitive donors found then were also White females.

Radiosensitivity and radioresistance can be identified by using the LAA within individuals and within cells CD4 and CD8 lymphocytes. The question then arises if the leukocyte apoptosis assay can also detect an increase in radiosensitivity when using radiation with high ionisation density. For this reason a number of readings were conducted comparing X-rays and neutrons. Also neutron damage is particularly important in SA as inoperable

breast cancer (Murray et al, 2004) is treated with neutrons. With neutrons there is less repair of radiation damage due to the nature of neutrons. To identify patients with possible late effects is important in neutron therapy.

3.3.5 Neutron study

When conducting this study, the assumption was made that the RBE for X-rays and neutrons was 2. This was based on previous studies done by Slabbert et al (2000) using the same neutron beam at iThemba LABS, Faure. As a result the neutron doses applied to the blood samples were half that of the X-ray doses. Vral et al (1998) has found that high-LET or densely ionizing radiation such as neutrons is more effective than low-LET radiation in causing cytogenetic damage (for example micronuclei, chromosomal aberrations). Vral et al (1998) also stated that the differences in RBE found between low-LET and high-LET are caused by the degree of initial DNA damage and the extent to which they are repaired or misrepaired.

(a)

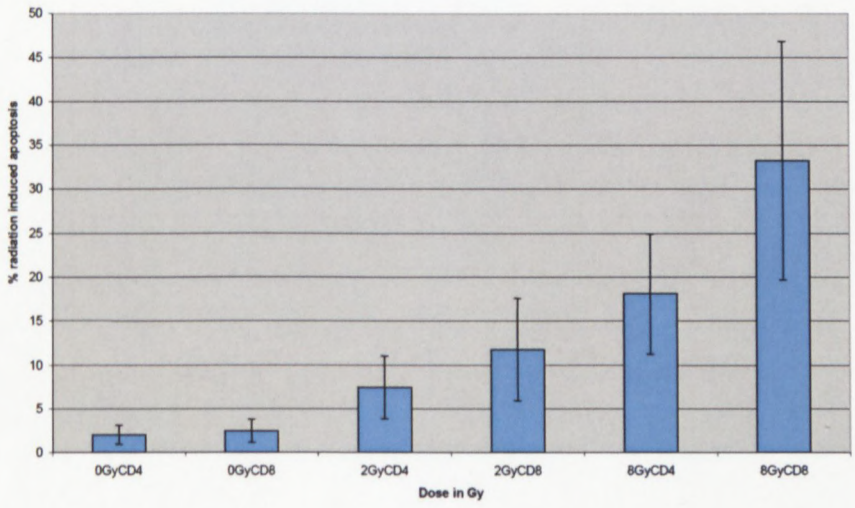


Figure 3.29a: The average percent apoptosis induced by 2Gy and 8Gy X-rays in CD4 and CD8 T-lymphocytes for 18 healthy donors. The background (0Gy) level of apoptosis was subtracted. Error bars display standard deviation.

(b)

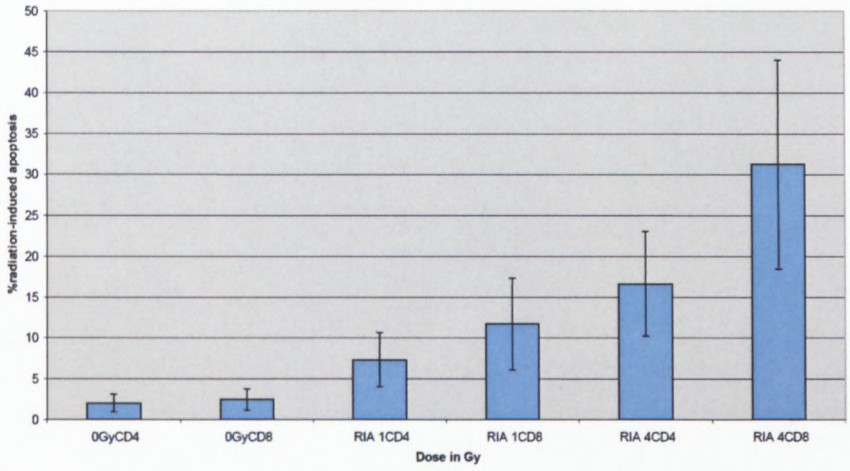


Figure 3.29b: The average percent apoptosis induced by 1Gy and 4Gy Neutrons in CD4 and CD8 T-lymphocytes for 18 healthy donors. The background (0Gy) level of apoptosis was subtracted. Error bars display standard deviation.

Figure 3.29a shows the average percent of radiation-induced apoptosis for the 18 healthy donors (mean: 42.6yrs, range 25-70 years) in CD4 and CD8 T-lymphocytes after 2Gy and 8Gy X-rays. A clear dose response curve is observed as the percentage radiation-induced apoptosis increases with every dose of radiation. The percentage apoptosis following a dose of 2Gy minus the background (2-0Gy) for CD4 lymphocytes ranges from 2.17-12.73% with a mean of 7.47%. Radiation-induced apoptosis for CD8 lymphocytes ranges from 3.2-24.55% with a mean of 11.75%.

The percentage apoptosis following a dose of 8Gy minus the background (8-0Gy) for CD4 lymphocytes ranges from 8.28-32.88% with a mean of 18.08% and for CD8 lymphocytes it rages from 14.52-55.94% with a mean of 33.24%. At each dose point there is a higher rate of apoptosis observed with CD8 lymphocytes when compared to that of the CD4 lymphocytes.

Figure 3.29b shows the average percentage of radiation-induced apoptosis for the 18 healthy donors in CD4 and CD8 T-lymphocytes after 1Gy and 4Gy neutrons. A clear dose curve was also observed as the percentage radiation-induced apoptosis increased with every dose of radiation. The percentage apoptosis following a dose of 1Gy minus

the background (1-0Gy) for CD4 lymphocytes ranges from 2.87-15.55% with a mean of 7.34%. Radiation-induced apoptosis for CD8 lymphocytes ranges from 3.69-17.14% with a mean of 11.73%.

The percentage apoptosis following a dose of 4Gy minus the background (4-0Gy) for CD4 lymphocytes ranges from 7.6-27.94% with a mean of 16.66% and for CD8 lymphocytes it rages from 13.48-52.5% with a mean of 31.26%. At each dose point there is once again a higher rate of apoptosis observed with CD8 lymphocytes when compared to that of the CD4 lymphocytes.

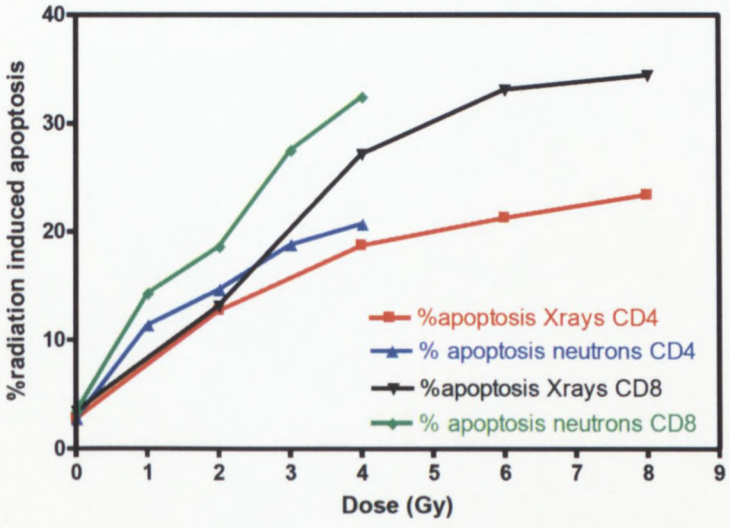


Figure 3.30: Dose response curve for one donor after 0Gy, 2Gy, 4Gy, 6Gy, 8Gy X-rays and 0Gy, 1Gy, 2Gy, 3Gy, 4Gy Neutrons for both CD4 and CD8 T-lymphocytes.

Figure 3.30 shows the dose response curve for 1 donor after exposure to 0Gy (background), 2Gy, 4Gy, 6Gy and 8Gy X-rays as well as 0Gy (background), 1Gy, 2Gy, 3Gy and 4Gy neutrons. The results were compared for both CD4 and CD8 lymphocytes. When looking at the dose response curve for 1 donor (Figure 3.30), it is apparent that there is not a simple linear quadratic model. The lymphocyte apoptotic response increases with increasing doses after both the X-ray and neutron exposure. Similar results were obtained by Slabbert et al (1996) where they found that cells that were resistant to photons (x-rays) were also resistant to neutrons.

Table 3.15: The average ratio of x-rays: neutrons for CD4 and CD8 lymphocytes after 2Gy and 8Gy X-rays as well as 1Gy and 4Gy neutrons for 18 donors.

Low dose radiation					
2Gy X-rays CD4	**1Gy Neutrons CD4**	**Ratio of biological effect**	**2Gy X-rays CD8**	**1Gy Neutrons CD8**	**Ratio of biological effect**
7.47	7.34	**1.02**	11.75	11.73	**1.01**
High dose radiation					
8Gy X-rays CD4	**4Gy Neutrons CD4**	**Ratio of biological effect**	**8Gy X-rays CD8**	**4Gy Neutrons CD4**	**Ratio of biological effect**
18.08	16.66	**1.1**	33.24	31.26	**1.08**

The assumed RBE of 2 used in the study was correct, as the ratio between the apoptotic response after X-ray and neutron exposure was approximately 1. This applies to both low doses (2Gy X-rays vs. 1Gy neutrons) and high doses (8Gy X-rays vs. 4Gy neutrons).This indicates that approximately equal levels of biological damage occurred when the neutron doses were halved. The CD4 and CD8 lymphocytes, although different in sensitivities, displayed similar biological effect ratios. Unlike residual radiation damage endpoints, for example cell survival and micronuclei assays, the RBE for apoptosis do not vary with radioresistance.

At higher doses the damage of 8Gy X-rays is higher than the assumed equivalent of 4Gy neutrons. This calculates to a lower RBE which is consistent with data from other endpoints. This, not withstanding the fact that the dose response curves are not linear quadratic in nature.

Radiotherapy fractionated doses are approximately 1.3Gy neutrons compared the standard 2Gy X-rays per fraction (iThemba LABS). Based on the data above, it is fair to assume that patients undergoing 1.3Gy neutron fractionated treatments will equally benefit from the LAA .The LAA endpoint data is therefore consistent with previously published RBE data (Slabbert et al, 2000). It's able to reflect the difference in ionization density of the different treatment modalities.

3.3.6 Fractionation of doses

Heparinised blood from 4 donors was exposed to a single dose of 8Gy X-rays as well as fractionated dose of 4Gy + 4Gy separated by 3hours.

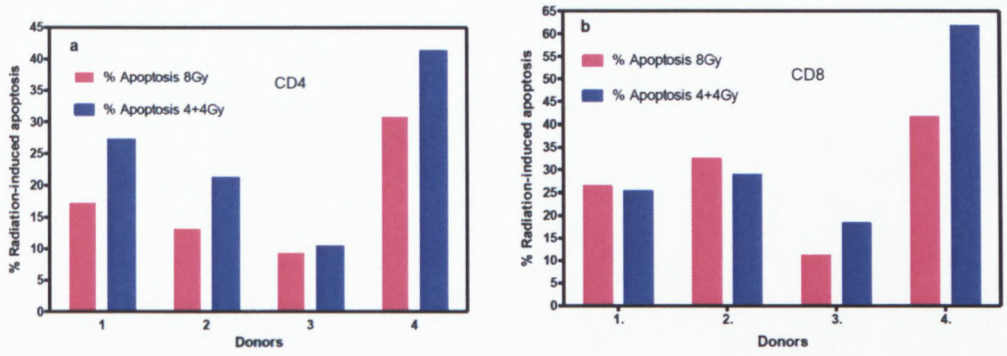


Figure 3.31a-b: The lymphocyte apoptotic response of 4 donors after 8Gy X-rays followed by a fractionated dose of 4Gy + 4Gy X-rays for (a) CD4 lymphocytes and (b) CD8 lymphocytes.

Figure 3.31 compares the apoptotic response of CD4 and CD8 lymphocytes after 8Gy X-rays and a fractionated dose of 4Gy + 4Gy. Less apoptosis is expected after the fractionated dose due to reparable damage. The results obtained with the LAA however, contradicts this theory of classical residual damage usually found with other endpoints e.g. micronuclei assays (Vral et al, 2004) where the micronuclei frequency decrease when a dose is given in two fractions separated by 3 hours. Also, cell survival studies as an example of a stochastic effect (Hall, 2000), increase with fractionation. The total dose needed to obtain a particular level of skin reaction (a deterministic effect) increase vastly when the treatment dose is delivered with more fractions (Fowler et al, 1963).

3.3.6.1 Conclusion

On average there is more apoptosis displayed after the 4Gy + 4Gy fractionated X-ray exposure. The LAA shows an increase in apoptosis with fractionation of doses and therefore does not reflect anything about reparable damage.

3.4 Conclusions of LAA studies

Cells respond to ionising radiation by activating a number of programmes including cell cycle arrest and cell death (Crompton, 1998). The unrepaired radiation-induced damage could either lead to mutations and cellular transformations or the cell could undergo apoptosis. Apoptosis plays a significant role in maintaining the body's healthy condition during radiotherapy (Gonser et al, 2004). To date there are no routine tests used within the clinical setting to predict the normal tissue response i.e. apoptosis, to radiotherapy (Fernet and Hall, 2008).

The LAA study started with a pilot study to standardize the assay within a South African population. The assay proved to be rapid and reproducible as analysis was repeated for 4 core donors on different days. The ranking order for the donors was more consistent with CD8 lymphocytes than the CD4 lymphocytes. Although differences were noted between CD4 and CD8 lymphocytes, the assay consistently identified the donor who displayed more radiation-induced apoptosis as well as the donor who displayed less radiation-induced apoptosis. Interdonor variation was found to be higher than intradonor variation and studies by Ozsahin et al (1997) and Schmitz et al (2003) were comparable to this study. Clear dose response curves were observed for the 4 core donors indicating that radiation-induced apoptosis increased with increasing doses.

The donor number was increased to include a total of 9 donors to further assess the assay. Once again interdonor variation was noted. CD8 lymphocytes also always displayed more apoptosis than CD4 lymphocytes. The pilot study yielded enough data to prove the reliability of the LAA assay and therefore a standard curve study was conducted.

Heparinised blood from 100 donors was analysed in order to establish a baseline with which to compare radiation-induced apoptosis in lymphocytes. The aim was to identify individuals displaying enhanced toxicity to radiotherapy. Z-score analysis was performed. By using z-scores, an individual's lymphocyte apoptotic response can be expressed as a number of standard deviations away from the expected value for someone of that age. To prevent experimental error, results from two different cell types can be compared during z-score analysis. This is better than using the absolute

apoptotic response values in percentage as this could vary from laboratory to laboratory and result in incorrect radiosensitivity predictions. Z-scores are less susceptible to prediction errors and can be used by the clinician with more certainty.

Previous studies have indicated that 5% of individuals tested can be expected to have z-score values that indicate radiosensitivity (Ozsahin et al, 2005). After averaging z-score data for 2Gy and 8Gy test radiation, not a single radiosensitive individual could be identified. For this reason the z-score analysis was repeated and z-scores were calculated separately following the response to 2Gy and 8Gy X-rays. Again no radiosensitive individuals were identified, however a number of radioresistant donors were observed. This could be due to the fact that the study population was too small. The standard curve study was extended to include a total of 300 healthy donors in order to see whether or not a population existed who displayed low levels of radiation-induced apoptosis.

When mean z-score analysis was once again done for CD4 and CD8 lymphocytes after exposure to 2Gy and 8Gy X-ray exposure, 3 radiosensitive individuals were identified. This represented 1% of the total population. As this was much lower than expected, z-score analysis were separated for the 2Gy and 8Gy data sets. Doing this, the number of radiosensitive individuals that can be identified changed as follows. After 2Gy exposure, only 2 radiosensitive individuals were identified whereas after 8Gy analysis, a total of 5 radiosensitive individuals were identified. These 5 individuals represent a radiosensitive donor population of 2%. Although 2Gy is only a quarter of the 8Gy dose, the z-score analysis follows a similar trend to that for 8Gy treatments. The 2 radiosensitive individuals identified with a 2Gy X-ray dose are included in the 5 radiosensitive individuals identified after a 8Gy exposure. This indicates that the LAA assay could be used to identify radiosensitive individuals even when administering a dose of 2Gy that is standard in fractionated radiotherapy.

Irrespective of how many individuals were being analysed, it was evident that large variation in radiosensitivity existed between individuals.

Race, gender and age studies conducted with the LAA proved that these factors had to be considered before treating patients within a South African population. Firstly an

analysis of variance was done on the RIA data obtained from the 300 donors. The donor ages were binned into 10 year intervals. On average higher levels of RIA was observed at younger ages and then decreased as the donors get older. This was noted for both cell types after 2Gy and 8Gy X-rays. LAA age studies by Radojcic and Crompton (2001) are comparable to the South African age studies.

CD8 lymphocytes did not have as stable a relationship with age as CD4 lymphocytes. There was no significant decrease in RIA for CD8 lymphocytes as was noted with CD4 lymphocytes.

The effect of race on both CD4 and CD8 lymphocytes were consistent with the Black donors always having the highest RIA, followed by Coloured donors and lastly the White donors showing the lowest RIA.

Males on average showed higher apoptotic responses than females for both CD4 and CD8 lymphocytes.

Separate studies were then conducted for age, gender and race without binning the age into 10 year intervals. Significant differences between male and female radiosensitivity were again observed for both CD4 and CD8 lymphocytes. Females consistently showed a lower apoptotic response than males. This would indicate that more severe reactions to radiotherapy would likely occur in women than in men.

Race studies showed significant differences in the apoptotic responses of the 3 race groups. Once again Black donors always displayed the highest apoptotic values, followed by the Coloured donors and lastly the White donors. This data indicates that White individuals are mostly at risk for developing late effects in normal tissue following radiation therapy. For this reason z-score analysis was set up for the different race groups to see whether or not race influenced the z-score analysis. The radiosensitive individuals identified in the White z-score analysis were females. No radiosensitive individuals were identified in the Coloured z-score analysis although 4 radioresistant individuals were identified. Within the Black z-score analysis only 1 female donor was identified as being radiosensitive. This correlated well with the combined age, gender and race analysis of variance conducted. When looking at the original z-score analysis

of the 300 study population the majority of radiosensitive individuals identified were also White females.

From the above it is evident that within a South African population, the individuals most likely to develop late effects to radiation therapy are White females.

To identify patients with possible late effects to neutron therapy is important in South Africa as some are treated with this modality for inoperable tumours, especially in breast cancer. It was found that the LAA was able to distinguish cellular radiation damage induced by X-ray and neutron treatment modalities. When comparing a neutron dose half that of X-rays, the ratio of biological effect was approximately 1. It is reasonable to assume a RBE value of 2 for high energy neutrons used in therapy. The RBE for neutron treatment marginally increased at lower doses. More investigation is needed to finally decide if the radiation quality factor for apoptosis induced by neutrons increases systematically as the dose gets smaller. The biological effect ratio was similar for the CD4 and CD8 lymphocytes, even though these cells display different radiosensitivities.

Fractionation of dose studies was conducted by using the LAA assay. The results obtained with the LAA are at variance with biological endpoints that reflect residual radiation damage. For example micronuclei assays, cell survival assays and skin reactions. On average more apoptosis was displayed after a 4Gy + 4Gy fractionated treatment protocol with X-rays compared to that noted for a single 8Gy exposure. The LAA showed an increase in apoptosis with fractionation of doses and therefore does not reflect anything about reparable damage.

CHAPTER FOUR
ANNEXIN V-FITC APOPTOTIC ASSAY

4.1 Introduction

The LAA assay is a newly developed assay not routinely used by all investigators of apoptosis whereas Annexin V is a simple and easy flow cytometry method for the detection of apoptosis and is widely used in laboratories (Schnarr et al, 2007; Hertveldt et al, 1997). Previously only microscopic identification of the morphological changes occurring during apoptosis was used. These methods were found to be cumbersome and time consuming, hence the development of an easy flow cytometric assay (Koopman et al, 1994).

The loss of plasma membrane integrity is an early event of apoptosis resulting in the exposure of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer. Surface exposed PS can be detected by Annexin V, a Ca^{2+} dependant phospholipid-binding protein (Van Engeland et al, 1998) (Figure 4.1).

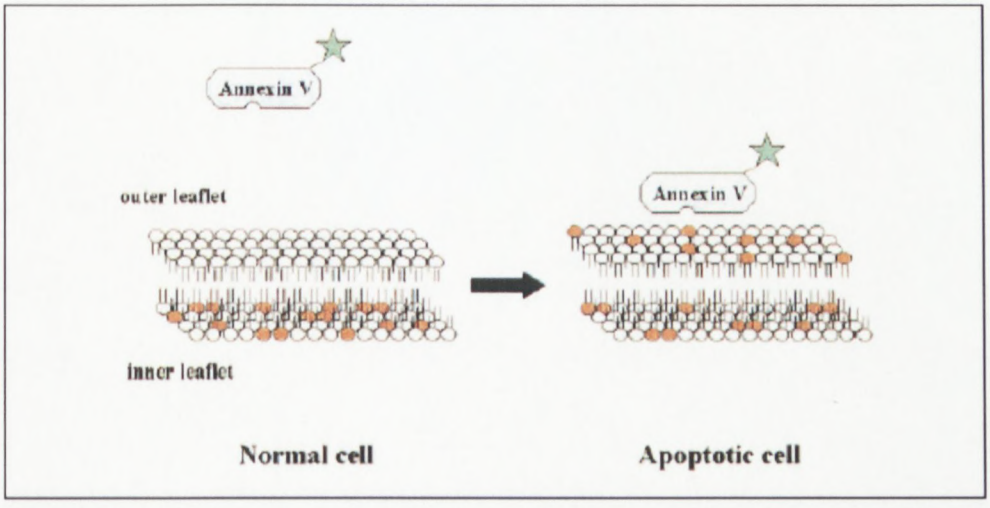


Figure 4.1: A schematic representation of the loss of membrane lipid integrity during apoptosis. Vital cells maintain a strictly asymmetric lipid bilayer composition, with PS (red circles) facing the cytosol. During apoptosis the PS molecules become exposed at the outer membrane leaflet. Annexin V can bind with high affinity to the exposed PS in the presence of Ca^{2+} (Van Engeland et al, 1998)

Annexin V staining precedes the loss of membrane integrity associated with later stages of apoptosis and is therefore used as a specific marker for apoptosis in the early phase where the cell membrane is still intact (Koopman et al, 1994). Annexin V can be conjugated to the fluorochrome, fluorescein isothiocyanate (FITC), and used in flow cytometric analysis of apoptosis. The Annexin V-FITC apoptotic assay also uses propidium iodide (PI), a fluorescent dye that stains DNA, to assess plasma membrane integrity (Hasper et al, 2000). PI acts as an exclusion dye for cell viability and does not enter cells which have maintained their plasma membrane integrity. Examples include viable cells and cells in the early stages of apoptosis. Dead cell or cells in the later stages of apoptosis have lost their plasma membrane integrity and would therefore be permeable to PI (Wilkins *et al*, 2002b). Annexin V in combination with PI can therefore distinguish between early apoptotic cells (Annexin V positive, PI negative), late apoptotic cells or necrotic cells (Annexin V positive, PI positive) (Rasola & Geuna, 2001).

The Annexin V assay may be more advantageous than the LAA assay in a number of ways. There are a number of similarities and differences between the two assays. Firstly the Annexin V assay measures more phases of apoptosis simultaneously. It can distinguish between early apoptosis, late apoptosis as well as necrotic cells whereas the LAA assay focuses mainly on late apoptosis. The Annexin V assay also uses two fluorescent signals (PI and FITC) to identify apoptotic response whereas the LAA assay only uses the one fluorescent signal (PI) to identify apoptotic bodies.

Based on previous studies (Wilkins et al, 2002b; Mirzayans et al, 2007), the Annexin V assay can measure apoptotic responses in lymphocytes after a 48 hour post-irradiation incubation period. This is comparable to the methodology used for the LAA assay. Both methods are also easy flow cytometric assays for detecting apoptosis.

The aim of this part of the study was to see if the Annexin V assay could be a better measure of apoptosis used to predict intrinsic radiosensitivity of individuals to radiotherapy.

4.2 Materials and Methods

4.2.1 Lymphocyte isolation

Lithium-heparin blood from 3 donors was used for this study. Peripheral lymphocytes were isolated by mixing the blood samples with 2ml of phosphate buffered saline (PBS) and carefully layering the blood/PBS mixture onto 3ml of Histopaque-1077 (Sigma Aldrich) in round-bottom culture tubes. The mixture was then centrifuged at 1000rpm for 30 minutes resulting in a white cloudy layer of lymphocytes between the serum/PBS and the red blood cells. This cloudy layer was collected and washed with PBS and diluted in 5ml RPMI 1640, supplemented with 15% heat-inactivated foetal calf serum (FCS) (Delta Bioproducts).

4.2.2 Irradiation

Samples were irradiated with 0Gy (control), 2Gy and 8Gy X-rays and with 0Gy (control), 1Gy and 4Gy neutrons using the LINAC and neutron beam set-up described in Chapter 2.

4.2.3 Cell Culture Procedure

Immediately after exposing the samples to radiation, the culture tubes were placed in a Forma Series II water-jacketed CO_2 incubator (Model 3110, Labotec (Pty) Ltd) at 37°C in 5% CO_2. One set of samples were cultured for 24 hours and a second set of samples were cultured for a total of 48 hours.

4.2.4 Staining Procedure

The lymphocyte/RPMI mixture was centrifuged at 1000rpm for 10 minutes, the supernatant discarded and the cells washed twice in cold PBS. The cells were then resuspended in 250µl of 1x Annexin V Binding Buffer (Beckton Dickinson). One hundred microlitres of this suspension was transferred to 5ml culture tube and 5µl Annexin V-FITC (Beckton Dickinson) and 5µl PI (Beckton Dickinson) staining solutions were added. The cells were gently mixed and incubated for 15 minutes at room temperature in the dark. Four hundred microlitres of Annexin V 1x binding buffer was added to each tube. The samples were analyzed by flow cytometry within one hour.

4.2.5 Flow cytometric analysis

Set-up of protocol: Two-colour flow cytometric analyses were performed on the FACScan (Beckton Dickinson) at iThemba LABS. A minimum of 10, 000 cells were analysed per sample. Lymphocytes were identified and gated in region 1 (R1) (Figure 4.2a) on a 2D scatter plot of cell size (Forward scatter) vs. cell granularity (Side scatter). Histograms were set up with cell count vs. Annexin V-FITC (Figure 4.2b) and cell count vs. PI (Figure 4.2c). The M1 region in each histogram indicates the Annexin V-FITC and PI positively stained cells. A second 2D scatter plot of PI vs. Annexin V-FITC with 3 gated regions was used to distinguish between the different stages of apoptosis (Figure 4.2d). The lower left ungated population of cells represent normal, undamaged cells while the lower right gated region (R4) represents cells undergoing early apoptosis. The upper right gated region (R3) represents cells undergoing late apoptosis and the upper left gated region (R2) represents the necrotic cells. Percentage radiation-induced apoptosis was calculated by subtracting background apoptosis at 0Gy from apoptosis measured at 2Gy and 8Gy.

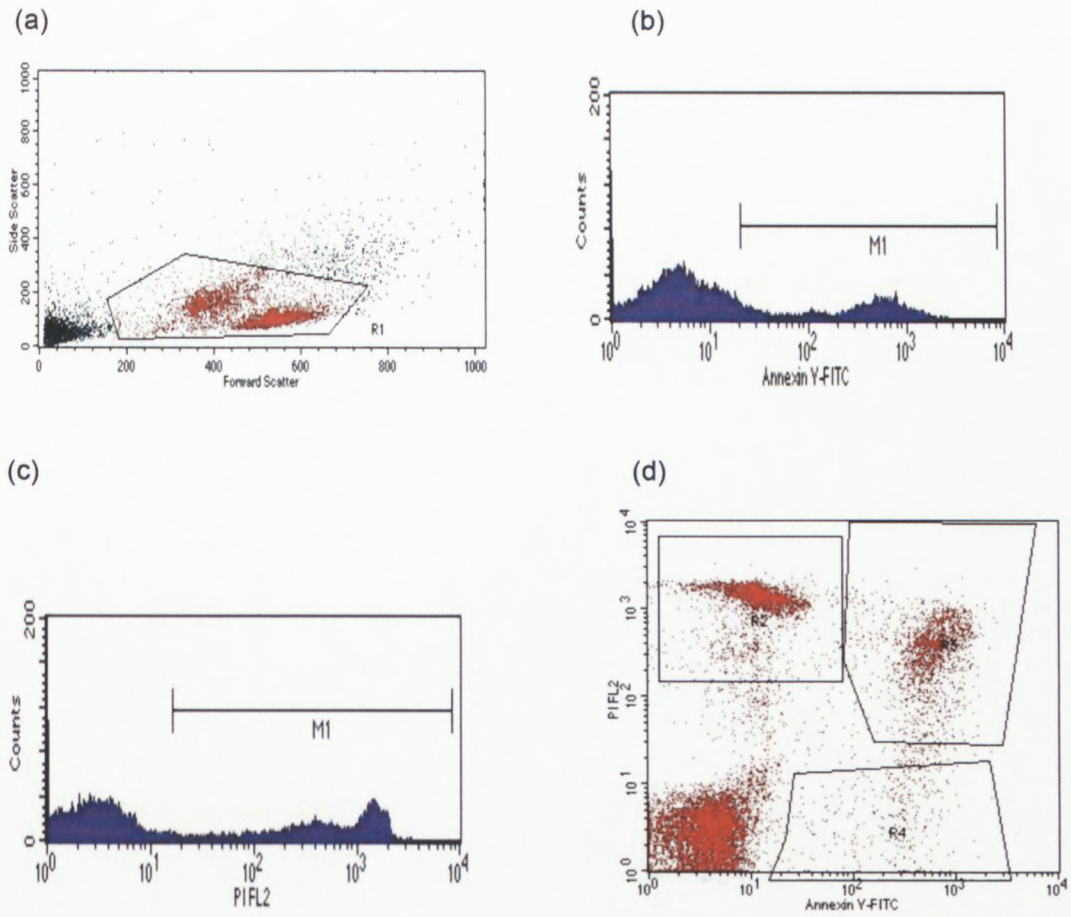


Figure 4.2a-d: Annexin V-FITC plots showing (a) the selection of lymphocytes (R1); (b) the histogram with Annexin V-FITC positive cells (M1); (c) the histogram with PI positive cells (M1) and (d) the scatter plot identifying early apoptotic cells (R4), late apoptotic cells (R3) and necrotic cells (R2).

Controls: Controls were used to set up compensation and gates by using the following samples: (i) Unstained lymphocytes; (ii) lymphocytes stained with only Annexin V-FITC and (iii) lymphocytes stained with only PI. Cells were fixed in cold methanol for 30 minutes to induce apoptosis. The control samples were then analysed in the following order. First the unstained lymphocytes (i) were analysed, followed by the cells stained only with Annexin V-FITC (ii), then the cells stained with only PI (iii). Lastly a sample of lymphocytes stained with both Annexin V-FITC and PI were analysed to see if the gates were at the correct settings.

4.3 Results and Discussion

Dose response curves were set up for each of the 3 donors to demonstrate the effect of the X-ray doses (0Gy (background), 2Gy and 8Gy) and neutron doses (0Gy (background), 1Gy and 4Gy) on apoptosis. The 3 donors were previously used in the LAA study and are known to have varied response from sensitive to resistant to radiation. As previously done in the LAA assay, the neutron doses were half that of the X-ray doses.

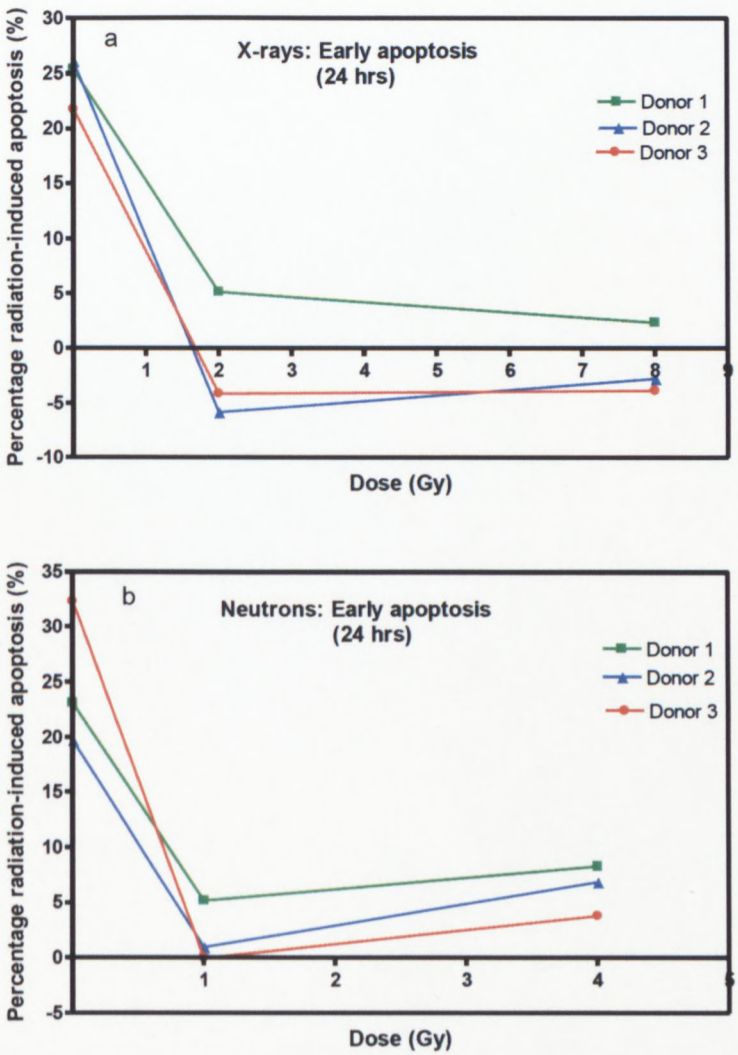


Figure 4.3a-b: Dose response curves for 3 donors after exposure to (a) 0Gy (background), 2Gy and 8Gy X-rays as well as (b) 0Gy (background), 1Gy and 4Gy neutrons. The graphs demonstrate the early apoptotic response after 24 hour incubation post-irradiation.

The dose response curves in Figure 4.3a-b show high background (0Gy) readings for all 3 donors. The radiation-induced apoptotic response then decreases for all 3 donors after 2Gy X-rays and 1Gy neutrons. One would expect there to be more apoptosis after the initial doses were applied as more radiation damage has occurred. Clear dose response curves have been found in studies done by Wilkins and co-workers (2002b) where there is an increase in apoptosis with the increase of X-rays doses. This however could not be noted in the current study.

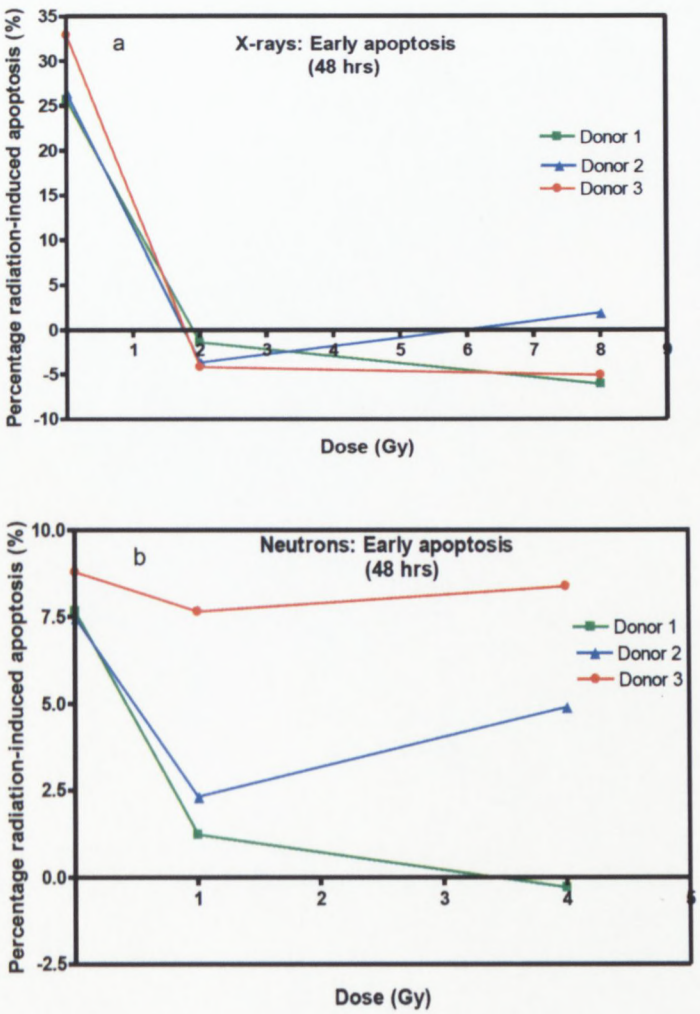


Figure 4.4a-b: Dose response curves for 3 donors after exposure to (a) 0Gy (background), 2Gy and 8Gy X-rays as well as (b) 0Gy (background), 1Gy and 4Gy neutrons. The graphs demonstrate the early apoptotic response after 48 hour incubation post-irradiation.

Similarly the dose response curves in Figure 4.4a-b show high background (0Gy) readings for all 3 donors after the 48 hour incubation. The radiation-induced apoptotic response then decreases for all 3 donors after 2Gy X-rays and 1Gy neutrons. Once again one would expect there to be more apoptosis after the initial doses were applied as more radiation damage has occurred but this is not the case after 48 hours post - irradiation. The high background readings could be explained by the pre-treatment of lymphocytes with an isolation procedure. This may have induced cells stressors and resulted in high levels of spontaneous apoptosis.

In the LAA study an approximate RBE of 2 was observed. When comparing apoptosis induced by neutrons and X-rays, values obtained do not follow the expected based on ionisation densities. The ratio between X-rays and neutrons for the Annexin V study is tabulated below (Table 4.1).

Table 4.1: The average ratio of x-rays: neutrons for lymphocytes after 2Gy and 8Gy X-rays as well as 1Gy and 4Gy neutrons for 3 donors.

Early apoptosis after 24 hour incubation					
2Gy X-rays	**1Gy Neutrons**	**Ratio of biological effect**	**8Gy X-rays**	**4Gy Neutrons**	**Ratio of biological effect**
-1.6	2.0	**-0.8**	-1.45	6.31	**-1.02**

Early apoptosis after 48 hour incubation					
2Gy X-rays	**1Gy Neutrons**	**Ratio of biological effect**	**8Gy X-rays**	**4Gy Neutrons**	**Ratio of biological effect**
-3.11	3.73	**-1.86**	-3.11	4.32	**-2.58**

The assumed RBE of 2 used in the study was not confirmed as the ratio between the apoptotic response after X-ray and neutron exposure was not as close to 1 as with the LAA study. Based on the values obtained for the 3 donors in this study, the Annexin V assay is not able to reflect the difference in ionization density of the different treatment modalities as well as the LAA assay.

4.4 Conclusion

The Annexin V data obtained did not show a clear dose response curve. There were also not the expected differences noted between the neutron and X-ray data. The

Annexin V assay did not work for this study. This could have been caused by improper set-up procedures but the investigation was not continued. After doing both the Annexin V and the LAA assay, the LAA assay is the preferred method to measure intrinsic radiosensitivity.

CHAPTER FIVE
APOPTOSIS VS. INDUCTION OF MICRONUCLEI IN T-LYMPHOCYTES

5.1 Introduction

Following radiation accidents, it's important to rapidly estimate the dose absorbed in persons exposed. Currently the most thoroughly developed biological indicator available is quantification of chromosomal aberrations in peripheral blood lymphocytes from exposed individuals (Vral et al, 1997). The micronuclei assay is a fast and easy method for determining residual radiation damage and is used by a number of investigators (Le Roux et al, 1998; Vral et al, 2004).

This method monitors chromosomal loss as well as chromosomal breakage and is also a method for testing *in vitro* genotoxicity (Kirsch-Volder et al, 2003; Fenech, 2005). The Cytokinesis Block Micronuclei assay (CBMN) distinguishes between cells that have not divided in culture (mononucleates) and cells that have divided once (binucleates) or twice (trinucleates or tetranucleates). Binucleated cells are capable of expressing accentric chromosomal fragments as micronuclei (IAEA, 2001). Micronuclei arise from acentric chromosome fragments which are not included in the daughter nuclei of a dividing cell as they are detached from the spindle. The chromosome fragment is enveloped in a nuclear membrane and is smaller than the main nuclei (Fenech, 1998). Micronuclei may also arise from whole chromosomes (Fenech, 2000).

Micronuclei are scored only in binucleated cells following phytohaemagglutinin (PHA) stimulation. PHA is a mitogen used to stimulate cell division. The binucleated cells are identified after they have been blocked from performing cytokinesis by the addition of Cytochalasin-B (Cyt-B). Cyt-B is an inhibitor of actin polymerisation which prevents the formation of the microfilament ring that constricts the cytoplasm between the daughter nuclei during cytokinesis. The use of Cyt-B enables reliable comparisons of chromosome damage between cell populations that may differ in their cell division kinetics (IAEA, 2001).

The aim of this study was to see if radiosensitivity variations between different donors are reflected by both endpoints. Of specific interest was to establish if a test dose of radiation in a donor that results in a high level of micronuclei frequency, a measure of inherent radiosensitivity, is related to the apoptotic response of the same donor.

5.2 Materials and Methods

5.2.1 Cell preparation

Lithium-heparin blood from 10 donors was used for this study. Peripheral lymphocytes were isolated by mixing the blood samples with 2ml of phosphate buffered saline (PBS) and carefully layering the blood/PBS mixture onto 3ml of Histopaque-1077 (Sigma Aldrich) in round-bottom culture tubes. The mixture was then centrifuged at 1000rpm for 30 minutes resulting in a white cloudy layer of lymphocytes between the serum/PBS and the red blood cells. This cloudy layer was collected and washed with PBS and diluted in 5ml RPMI 1640, supplemented with 15% heat-inactivated foetal calf serum (FCS) (Delta Bioproducts).

5.2.2 Irradiation

X-ray irradiation was performed using the Linear accelerator at iThemba LABS. Samples were irradiated with 0Gy (control) and 2Gy using the LINAC set-up described in Chapter 2.

5.2.3 Incubation and cell harvest

Immediately after irradiation, lymphocytes were stimulated to divide by adding phytohaemagglutinin (PHA) (Gibco) at a concentration of 3µl/ml and incubated at 37°C with loose lids in a humidified atmosphere containing 5% CO_2. After 44hrs, 150µl (3µg/ml) of Cytochalasin B (Cyt-B) (Sigma Aldrich) was added to each tube to block cytokinesis. Cultures were re-incubated for 28hrs resulting in a total of 72hrs incubation time. Tubes were then centrifuged for 5min at 1000rpm. The supernatant was removed and the cell pellet was mixed. Five millilitres of 75mM KCL were added drop-wise while tubes were vortexed at low speed. Tubes were centrifuged for 8 minutes at 800rpm, the supernatant discarded and the pellet resuspended in the remaining fluid. Five millilitres of freshly prepared 0.9%Nacl/Methanol/Acetic acid (ratio of 5:4:1) was added in the same manner as the KCL. Tubes were placed in the fridge at 4°C overnight and then

centrifuged at 800rpm for 5minutes. Five millilitres of Methanol/Acetic acid (ratio of 4:1) was added in the same manner as the KCL and the tubes were refrigerated at 4°C until slides were prepared for analysis.

5.2.4 Slide preparation

Tubes were centrifuged at 800rpm for 5 minutes, the supernatant discarded and the cells resuspended in the remaining fluid. Forty microlitres of the suspension was placed onto a slide and left on a flat surface at room temperature to dry for at least 24 hrs. Slides were stained with acridine orange at a concentration of 40µg/ml for 2 minutes, washed in distilled water and Gurr buffer pH 6.8 for 1 minute. The slide was covered with a cover slip and sealed with cutex to prevent drying. Slides were analysed immediately using a fluorescence microscope.

5.3 Microscopic Analysis

5.3.1 Nuclear division index

The nuclear division index (NDI) is an indication of the efficiency of the PHA to stimulate lymphocytes in G_0 to undergo mitotic division.

The formula for calculating the NDI is:

$$NDI = \frac{N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4}{\text{Total cells counted}}$$

where N1-4 indicates scorable cells containing 1 to 4 nuclei.
Two hundred viable cells were analysed and reported as containing 1, 2, 3 or 4 nuclei per cell.

5.3.2 Scoring of micronuclei

The number of micronuclei in 500 binucleated cells was scored.

5.3.3 Scoring criteria

The scoring criteria used in this study were according to the criteria adopted by the Human MicronNucleus (HUMN) project (Fenech et al., 2003). The 2 nuclei in a binucleated cell were situated within the same cytoplasmic boundary. The 2 nuclei were approximately equal in size, staining pattern and staining intensity. The diameter of the micronuclei was not greater than one third of the main nucleus and had the same staining intensity as the main nucleus. The micronuclei could touch but not overlap the main nuclei. Apoptotic and necrotic cells, identified by chromatin condensation and cytoplasmic vacuoles respectively, were excluded from analysis.

5.4 The LAA methodology

The same LAA methodology described in Chapter 3 was used. The blood sample used for the micronuclei assay was also used for the LAA.

5.5 Results and Discussion

Micronuclei counts and LAA analysis was done on blood from 10 donors (27-50 years, mean age 38 years). Histograms of the apoptotic and micronuclei response were performed (GraphPad Prism 4).

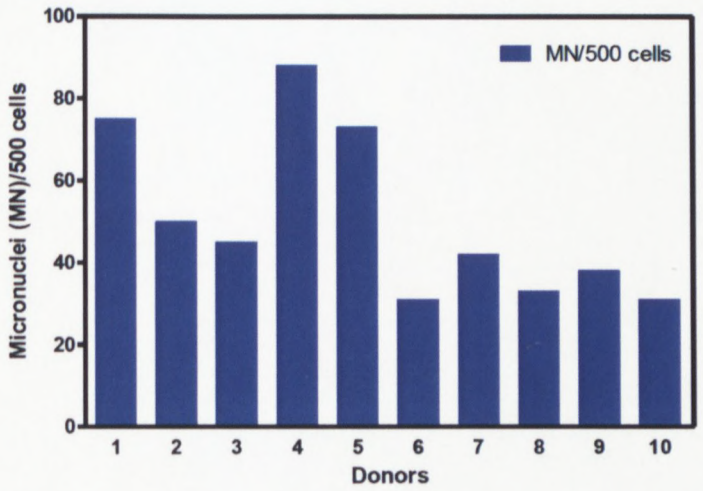


Figure 5.1: Histogram of the micronuclei count for 10 donors after 2Gy X-ray exposure. The micronuclei count is indicated by micronuclei per 500 binucleated cells.

A large variation in micronuclei yield is observed for the different donors (Figure 5.1). The micronuclei count ranges from 31 to 75 micronuclei per 500 binucleated cells. This, notwithstanding the fact that the same physical dose (2Gy) of X-rays was given in each instance.

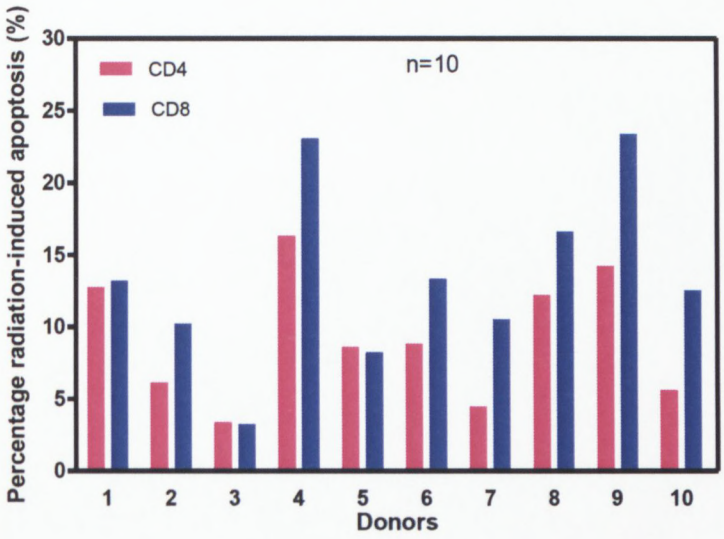


Figure 5.2: Histogram of the radiation-induced apoptotic response of 10 donors for both CD4 and CD8 lymphocytes after 2Gy X-ray exposure.

The apoptotic response for both CD4 and CD8 lymphocytes after 2Gy X-ray exposure varied between the 10 donors (Figure 5.2). The radiation-induced apoptosis for CD4 lymphocytes ranged from 3.3 to 16.3% (mean: 9.2%). Radiation-induced apoptosis for CD8 lymphocytes ranged from 3.2 to 23.4% (mean: 13.4%).

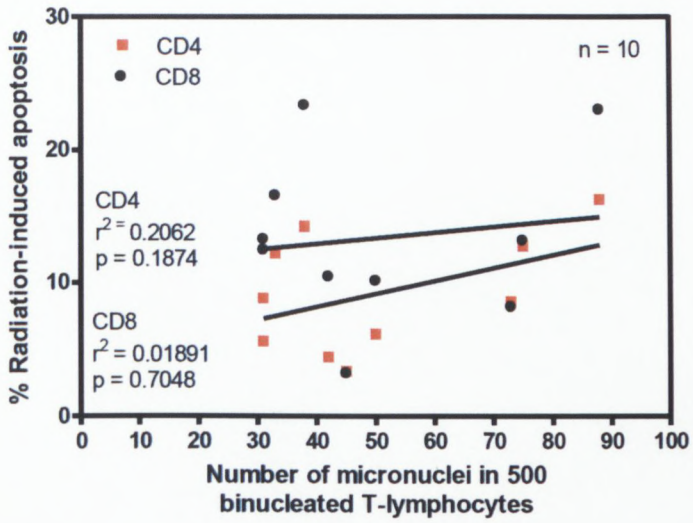


Figure 5.3: A comparison between the radiation-induced apoptotic response and the micronuclei count of 10 donors after 2Gy X-ray exposure.

From the above Figure 5.3 it is clear that both endpoints (radiation-induced apoptosis and micronuclei count) vary considerable between different donors. The main objective of this investigation was to establish if there is any relationship between the apoptotic response and micronuclei frequency of an individual. For both CD4 and CD8 lymphocytes there are on average a small increase in the ability of individuals to process the initial radiation damage when they are more sensitive to ionizing radiation as defined by micronuclei frequency. This gradual increase is however not statistically significant. Even so, the findings observed clear up two matters about radiosensitivity as defined by the respective endpoints. Firstly, residual radiation damage as quantified by micronuclei frequency is not related to the apoptotic response of cells subjected to radiation damage. Louagie and co-workers (1998b) reported similar findings in their studies conducted. Secondly, if we define inherent radiosensitivity as micronuclei frequency following a test dose, radiation workers may benefit marginally from their greater apoptotic response.

5.6 Conclusion

Although there is no significant relationship between the apoptotic response of an individual and their micronuclei frequency, the study would be most informative for radiation workers. It would be most valuable to follow micronuclei frequencies over many

years in subjects accidentally exposed to high doses of ionising radiation and who have different apoptotic responses. This is to see if the rate at which micronuclei frequencies decline after an initial dose, is related to the apoptotic ability of an individual.

CHAPTER SIX
CONCLUSION AND DIRECTIONS FOR FUTURE STUDIES

The long term goal of identifying factors that influence radiosensitivity is to be able to individualize therapy and therefore limit adverse reactions. Crompton et al (1999) reported altered apoptotic responses when comparing the reactions of hypersensitive irradiated patients with increased toxicity to healthy donors and cancer patients displaying normal toxicity. This suggested that the LAA might be a useful predictor of individuals likely to display late adverse effects to radiotherapy.

The aim of this study was to assess the feasibility of using the Leukocyte Apoptosis Assay to investigate individual variability and sensitivity to radiation within a South African population.

From the studies conducted, it is evident that the LAA assay could be implemented in oncology clinics within South Africa. The pilot study showed the assay to be reliable and reproducible, as repeat analysis of the same donors produced similar results. The assay could also identify the variation in radiosensitivity amongst individuals by distinguishing between radiosensitive and radioresistant donors. The results obtained from the pilot study were similar to findings of Crompton and Ozsahin (1997) and further studies were therefore conducted within the Western Cape population.

The study population was increased to 300 healthy donors within the Western Cape region. Using z-score analysis, the data showed that approximately 2% of the 300 donors had a low apoptotic response in both CD4 and CD8 lymphocytes. Although this percentage is much lower than results obtained by Crompton et al (2001), it shows that the LAA is able to identify individuals with increased toxicity to radiation, within a South African gene pool. This standard curve study furthermore proved that factors such as age, gender and race play an important role in an individual's response to radiotherapy. The study concluded that within a Western Cape population, the individuals most likely to develop late effects to radiation therapy are White females. However, the study could

be further expanded to include more Black donors, as the number of these donors was quite low compared to the other race groups.

The data obtained in the standard curve study indicates that the LAA is ready to be used for retrospective and prospective patient studies for individuals undergoing radiotherapy.

Neutron therapy is important for a large number of patients in South Africa suffering from inoperable cancers as well as slow growing tumours. It was found that the LAA was able to distinguish cellular radiation damage induced by X-ray and neutron treatment modalities. This study was unique as nowhere else has intrinsic radiosensitivity been assessed by leukocyte apoptosis. The study needs to be expanded to include neutron therapy patients in a retrospective study.

The CBMN assay has long been considered as the 'golden standard' for assessing radiosensitivity. The aim of this study was to see if radiosensitivity variations between different donors are reflected by endpoints of both the CBMN and LAA. South Africa has a large number of people working in the applied radiation industry. Many radiation workers absorbed radiation doses too close to the 20 mSv allowed each year. Also, accidental over-exposures to ionising radiation are far too frequent in South Africa. These include people both in the health profession as well as industries such as mining and industrial radiography. Workers that are more sensitive to radiation exposure could be allocated jobs where the risk of high dose exposure is lower. Although there was no significant relationship between apoptotic response of an individual and their micronuclei frequency found, the study would be most informative for radiation workers. The LAA assay as a predictor of radiosensitivity could be important in implementing radiation protection practices that are tailored specifically for individuals.

Fractionation of dose studies was conducted using the LAA. On average more apoptosis was displayed after the 4Gy + 4Gy fractionated X-ray exposure when compared to the single 8Gy X-ray exposure. The LAA showed an increase in apoptosis with fractionation of doses and therefore does not reflect anything about reparable damage.

Part of the study was to see if the Annexin V assay could be a better measure of apoptosis used to predict intrinsic radiosensitivity of individuals to radiotherapy. Although the Annexin V assay is widely used in laboratories, it did not work in this study. High levels of background apoptotis were observed and as a result the dose response curves were indistinct. Apoptotic levels were expected to increase as the amount of radiation damage increased but this was not the case after irradiation. Instead high background levels were followed by low and sometimes negative levels of radiation-induced apoptosis. The lymphocytes were pre-treated with an isolation procedure which could have contributed to the high levels of spontaneous apoptosis. The study also showed that the Annexin V assay was not able to reflect the difference in ionization density of the different treatment modalities as well as the LAA assay. When comparing apoptosis induced by neutrons and X-rays, the Annexin V values obtained did not result in the expected RBE value of 2. After conducting the study, it was concluded that the LAA assay was the preferred method to measure intrinsic radiosensitivity.

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APPENDIX A:

Participant Information Sheet

Prediction of intrinsic radiosensitivity by the evaluation of radiation induced toxicity of T CD4 and CD8 Lymphocytes Study of a group of 300 normal healthy donors to establish a standard curve

Dear Sir /Madam,

You are hereby asked to participate in a study to determine an individual's response to radiotherapy. Your results will form part of a database of a standard curve from normal healthy donors. Radiotherapy can induce early or late toxicity dependant on the illness and the anatomical area receiving radiation. The majority of side effects which can occur are generally temporary and can be resolved after treatment has ended. However, around 5 to 10 % of patients receiving radiotherapy will have serious late complications. The patients who develop these complications are those who have a hypersensitivity to radiation compared to "normosensitive"patients. At this stage there has been no way to predict the sensitivity to radiation and the potential long-term side effects and why they differ from patient to patient.

The Study

A test, the Leukocyte Apoptosis Assay (LAA), has been developed in Europe and the USA that allows the laboratory to estimate the intrinsic radiosensitivity of an individual by using lymphocytes (white blood cells) and exposing them in a test tube to radiation. The test will measure the toxic response of the lymphocytes to radiation. This has not yet been applied in South Africa. Your participation in this study and that of other participants will allow us to establish a standard curve of radiosensitivity in a healthy population in South Africa and furthermore to clinically evaluate this test. If at the end of this study this test proves valuable in measuring the radiosensitivity of the individual patient, we will be in a better position to predict and adapt the dose of radiation for future cancer

patients according to the results obtained. In order to conduct this study we need a 5ml blood sample taken at the time of your routine blood donation. We need to establish whether patients from different ethnic groups present with differences in their radiosensitivity as the results of this study may be applied in the oncology clinic. For this purpose we would need to analyse the data against all ethnic groups in South Africa.

Voluntary participation

Participation is totally voluntary and will not affect any routine blood donation procedures.

Risks and Benefits

There are no known risks associated with the procedure of blood collection. No additional venepuncture will be performed. There will be no direct benefit to you as a participant.

Compensation

No compensation is offered to participants in this part of the study.

Confidentiality and additional information

The study has been approved by the Ethics Committee of the Cape Peninsula University of Technology, Cape Town campus. Information regarding your ethnic group is important to the success of the study. The data, however, and treatment of your results will be managed in a confidential manner and your personal details will remain anonymous. You can obtain additional information from the head of the study Dr Kathleen Meehan at anytime. Thank you for your valuable participation.

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APPENDIX B:

CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

WP Blood Transfusion Service
WP Bloedoortappingsdiens

Consent Form

Prediction of intrinsic radiosensitivity by the evaluation of radiation induced toxicity of T CD4 and CD8 Lymphocytes Study of a group of 300 normal healthy donors to establish a standard curve

I have been informed of the objectives of the proposed study. A participant information document has been provided and explained to me. I have had enough time to ask the necessary questions and to make the decision to take part in the study. I understand that there will be no direct benefit or compensation, financial or otherwise, for participation in this study. I give permission for one (1) extra 5ml heparinised blood sample to be taken at the time of my routine blood donation for inclusion in the LAA study. I reserve the right at all times to remove myself from the study without any reasons or any resulting inconvenience.

Place.....

Date.....

Signature of Donor.....

Signature of Sister.....