The role of Bcl-2 and Bax protein expression on individual radiosensitivity

by

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ABSTRACT

BACK GROUND:

Apoptosis is the dominant mechanism of cell death induced by radiation and is the key mechanism used to remove cells with significant DNA damage. Previous research investigated the feasibility of using the Leukocyte Apoptosis Assay (LAA) to determine individual sensitivity to radiation and it was found that an apoptotic response could be loosely linked to age, race and gender. Apoptosis is controlled by the Bcl-2 proteins and therefore the balance between Bax and Bcl-2 protein expression is important. With this background it would be relevant to know why certain individuals are more sensitive to radiation than others. The objectives of this study was to evaluate the effect of ionising radiation on apoptotic proteins, Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) expression and to explore if there is a relationship between radiation induced apoptosis (RIA) and Bcl-2 or Bax expression.

DESIGN & METHOD:

In an effort to study individual's sensitivity to radiation, lymphocytes from 42 healthy donors were exposed to various doses of radiation (0 Gy, 2 Gy, 8 Gy) from a Cobalt-60 source. After 48 hours RIA was estimated on CD4 and CD8 lymphocytes using flow cytometry and the leucocyte apoptotic assay. In addition, lymphocytes were isolated from 25 of the donors which had also been exposed to various doses of radiation (0 Gy, 2 Gy, 8 Gy) and analysed for Bcl-2 and Bax protein expression using Western Blot analysis. The experiments were performed 48 hours following exposure.

Statistical analysis was performed in order to determine if there was a difference in expression between the three doses of radiation and if a relationship existed between the amount of induced apoptosis and Bcl-2 and Bax expression.

RESULTS:

Analysis of the CD4+ lymphocytes demonstrated a significantly higher RIA after 8 Gy radiation exposure when compared to 2 Gy (Median (percentiles) respectively: 10.22% (7.12%-18.42%).and 3.00% (1.02%-6.10%) with P-value < 0.0001). The analysis of the CD8+ lymphocytes was also significantly higher after 8Gy when compared to 2Gy,

(Median (percentiles) respectively: 27.59% (16.98%-37.63%) and 8.88% (4.45%-12.87%) with P-value < 0.0001).

There was a significant difference in the Bax expression between 0Gy and 8Gy (P = 0.0361), and a near significant difference between 2Gy and 8Gy (0.0924). However no significant difference was detected between 0Gy and 2Gy (P = 1.000). (Median (percentiles) for 0, 2 and 8Gy respectively: 0.7220 (0.3882, 1.155), 0.8789 (0.5793, 1.231); 1.0051 (0.7536, 1.663).There was no significant differences in the expression of Bcl-2 between 0Gy, 2Gy and 8Gy (P = 1.000)

CONCLUSION: Both CD4 and CD8 lymphocytes demonstrated a dose dependent increase in apoptotic response. This is accompanied by an increase in Bax protein expression (a promotor of apoptosis) and no significant difference in the expression of the apoptotic inhibitor Bcl-2. Further analysis should aim at comparing expression of donors with high radiosensitivity with low sensitivity and the investigation of other proteins within the apoptotic pathway.

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LIST OF ABBREVATIONS

APAF-1	Apoptotic protease activating factor 1
APLF	aprataxin- and PNK-like factor
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related
ATRIP	ATR interacting protein
Bax	Bcl-2-associated protein X
Bcl-2	B-cell lymphoma 2
Bcl-XL	Bcl-2 like protein X
BN	binucleated
B-NHEJ	backup NHEJ
BRCA	breast cancer early onset
CA	capsid
Cdc25a	cell division cycle 25a
Cdk	cyclin dependent kinase
cDNA	complementary DNA
Chk	cell cycle checkpoint kinase
CMV	cytomegalovirus immediate-early promoter
cPPT	central polypurine tract
CT	carboxy-terminal
CTS	entral termination sequence
CV	crystal violet
Cyt C	cytochrome c
DDR	DNA damage response
DIS	dimerization signal
D-loop	displacement loop

DNA	deoxyribonucleic acid
DNAPK	DNA dependent protein kinase
DNAPKcs	DNA dependent protein kinase catalytic subunit
D-NHEJ	DNA PK dependent NHEJ
DSB	double-strand break
Fas	Fatty acid synthetase
FasL	Fas ligand
FasL	Fatty acid synthetase Ligand
FITC	Fluoroscein isothiocynate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gy	Gray
erg	unit of energy and work = 10^{-7} joules
H2AX	H2A histone family, member X
HR	homologous recombination
IR	ionizing radiation
IRIF	ionizing radiation-induced foci
kDa	kilo dalton
keV	kilo electronvolt
Ku	Ku70/80
Ku70	Ku autoantigen 70
Ku80	Ku autoantigen 80
LAA	Leukocyte Apoptosis Assay
LET	linear energy transfer
LTR	long terminal repeat
Mcl-1	Myeloid cell leukemia 1
MRN	Mre11, Rad50 and Nbs1

mRNA	messenger RNA
NHEJ	nonhomologous end-joining
p21	protein encoded by the CDK1NA gene
p53	tumour protein 53
P53	Protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
PIKK	phosphatidylinositol 3-kinase like kinase
PNK	polynucleotide kinase
Pol	polymerase
Rad	radiation sensitive
Rb	Retinoblastoma protein
RBE	relative biological ectiveness
RPA	replication protein A
RPMI	Rosewell park memorial institute
RT	reverse transcriptase
SDS-PAGE	Sulfate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TNF	Tumor necrosis factor
WPBTS	Western province Blood transfusion services
XRCC	X-ray cross-complementing

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CHAPTER 1: LITERATURE REVIEW

1.1: A brief introduction to radiation and radiotherapy

Radiation is a natural environmental phenomenon, however mankind was not directly aware of its existence until the latter part of the 19th century (West and Barnett, 2011).In 1895 Wilhelm Conrad Roentgen, a German physicist, discovered x-rays (Röntgen, 1896), however the medical use for this was only published following the observation that a hairy mole disappeared after treatment with X-rays (Hall and Giaccia, 2012).

Radiotherapy has since become a vital tool in the treatment of malignancies and is often used in combination with surgery or chemotherapy (Haubner et al, 2012; Vaiserman, 2010). It has been reported that more than 50% of cancer patients will receive radiotherapy during the course of their disease. Forty per cent are cured by this form of therapy compared to 49% by surgery and 11% by chemotherapy (Bentzen et al, 2005; Delaney et al, 2005).

Even though radiotherapy is a powerful tool and can completely eradicate tumours, its limitation is the inevitable damage to non-malignant tissue. Toxicity due to radiation is a limiting factor for treatment success and patients treated with radiotherapy often develop clinical toxicity. This limits the effectiveness of the treatment and it has been shown that 5 – 10% of patients receiving radiotherapy may develop adverse reactions (West and Barnett, 2011 and Bordón et al, 2009). Previous research has demonstrated that there is variation in radiosensitivity between individuals, and even after exposure to the same dose, patients may experience different levels of toxicity (West and Barnett, 2011; Bordón et al, 2009).

The side effects of radiotherapy are divided into acute and late effects. Acute effects may present during or just after treatment and late effects could present months to years after radiotherapy (West and Barnett, 2011). Therefore, there is a need for the development of assays to predict individual radiosensitivity which could be used to develop a treatment regime for each patient thereby reducing the side effects. These assays should be affordable, rapid and accurate in order to enable easy and fast clinical implementation (Bordón et al, 2009).

1.2: Electromagnetic radiation

X-rays and gamma rays are forms of electromagnetic radiation frequently used in radiobiology to study radiation interaction with biological material. Both gamma and X-rays result from energy produced by an unstable atom and exhibit similar properties (Khan, 1994). The terms gamma and X-rays depicts the way in which each form of electromagnetic radiation is produced. Gamma rays are generated by radioactive isotopes during spontaneous disintegration of a nucleus of radioactive isotopes. During this process energy is given off as gamma rays (Hall and Giaccia, 2006).

1.3: Production of X-rays

X-rays are generated using an electrical device that accelerates electrons to high energy level (kinetic energy) and stops them abruptly in a target with subsequent conversion of kinetic energy to X-rays (Hall and Giaccia, 2006). This process occurs in an evacuated glass tube known as an X-ray tube with two electrodes (The cathode and anode) which are both enclosed within the tube (see Figure 1.1).The cathode or negative electrode is a filament of tungsten found at one end of the tube and the anode or positive electrode which consists of tungsten target placed on a copper rod is found at the other end. When a high voltage is applied across the two electrodes, electrons

are accelerated to high velocity from the cathode towards the anode. Once electrons hit the anode target they undergo a sudden deflection and acceleration with subsequent production of X-rays (Khan, 1994).

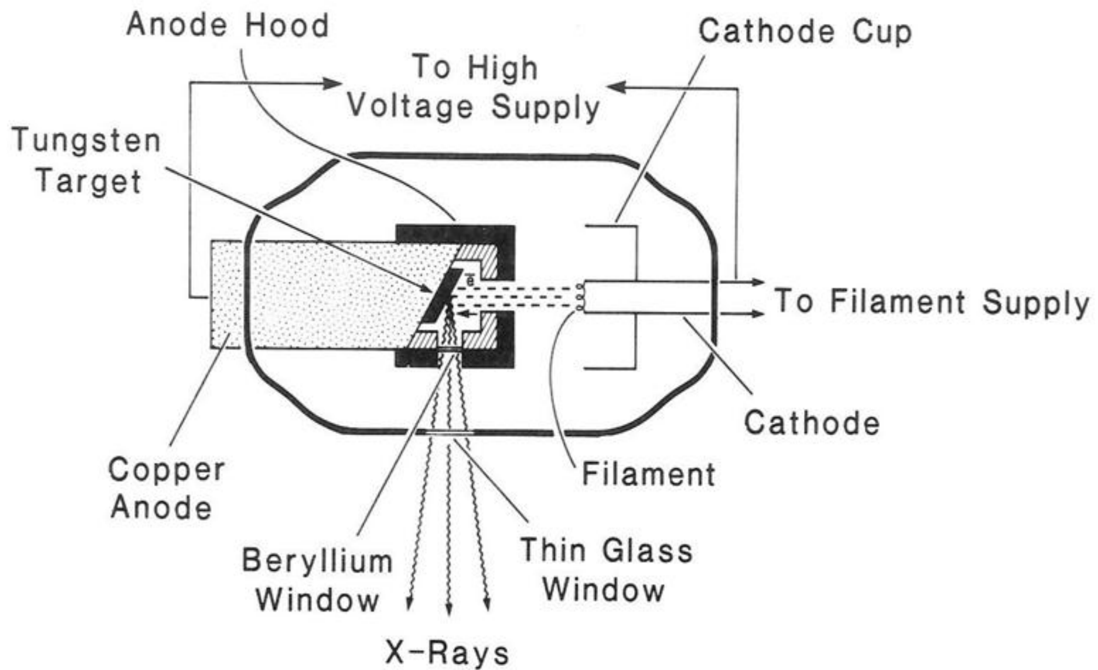

Figure 1.1: A schematic diagram of a therapy X-ray tube with hooded anode (Adapted from Khan, 1994)

1.4: Interaction of electromagnetic radiation

Electromagnetic radiation is thought to be streams of packets or bags of energy known as photons. When electromagnetic radiation passes through a material it may be transmitted without transferring any energy or alternatively its interaction with the traversed material may reduce its energy (Powsner and Powsner, 2006).There are three key processes in which photons may transfer their energy when traversing through matter: namely the Compton Effect, photoelectric effect and pair production. The Compton Effect is the most important electromagnetic radiation interaction in materials with a low atomic number (Z = 7.5) such as human tissue. The photoelectric

effect which is the main interaction in material with high atomic number and pair production is insignificant in clinical nuclear medicine as it only transpires with very high energy photons (>1.02MeV)(Stabin, 2008).

In the Compton Effect an atom of the absorbing medium is ionised due to loss of its outer shell electron. As the photon travels through the medium it interacts with the outer shell electron of the atom and some energy of the photon is transferred to the electron (Hall and Giaccia, 2012).If the photon energy transferred to the electron is higher than the electron binding energy within the atom, this results in ejection of the electron from its atom. The photon with residual energy is scattered in a different direction and the ejected electron will continue depositing its acquired energy subsequently ionising other atoms in the medium (IAEA, 2010).

The photoelectric effect occurs when a photon passing through the medium interacts with the inner shell electron with a subsequent ejection of the electron from its atom thereby ionising the atom of the absorbing medium. When the photon interacts with the medium its entire energy is transferred to the inner shell electron. Acquired energy is used to overcome the electron binding energy with ejection of the electron from the occupied shell. The vacancy in the inner shell is immediately filled by the outer shell electron and a photon disappears as its entire energy is transferred to the electron. The ejected electron or photoelectron will continue depositing its acquired energy subsequently ionising other atoms in the medium (Stabin, 2008).

1.5: Absorption of X-rays

Deposition of energy by X-rays in a medium or biological material may cause ionisation of the target molecule or atom. If the energy absorbed is enough to overcome electron binding energy of the target molecule or atom it may result in the ejection of

one or more orbital electrons of the target. This process is called ionisation (Mettler and Upton, 2008). Every ionising event generates energy of about 33 electron volts (eV) which are deposited into the absorbing medium. This energy is enough to break a strong chemical bond such as a double bond between two carbon atoms(C=C) (Hall and Giaccia, 2006; Khan, 1994).

1.5.1: Absorbed dose

In 1953 the Commission on Radiation Units and Measurements (ICRU) announced that the "absorbed dose" defined as the energy absorbed per unit mass, was to be a quantity that can be used for radiation dose quantification of any material of interest as well as biological objects. The unit "radiation absorbed dose" (rad) was initially used where rad is equal to 100 erg/g (Stabin, 2008). Later in 1972 the ICRU introduced a new unit that conformed to the International System of Units (SI) "gray" (Gy) defined as energy in Joules (J) absorbed per unit mass (kg).Therefore, 1Gy is equal to 1J/kg. Many different types of radiation dose units are used in radiobiology and some of these units are listed in table 1.1. (IAEA, 2008)

Table1.1: Summary of radiation doses and units

Dose	SI Unit	Old unit	Conversion factor
Exposure	C/kg air	Roentgen	1R = 2.58×10^{-4} C/kg air
Absorbed dose	gray (Gy)	rad	100 rad = 1 Gy
Equivalent dose	Sievert (Sv)	rem	100 rem =1 Sv

(Adapted from IAEA, 2010)

1.5.2: Linear energy transfer

Ionising radiation can be classified according to the rate at which energy is deposited as it travels through the matter or medium. This is called the linear energy transfer (LET) of radiation. It is calculated by dividing the energy deposited in kiloelectro volts (keV) by the distance travelled in micrometres (μm). X-rays and gamma rays (electromagnetic radiation) are classified as low LET because they deposit energy sparsely and are more likely to cause indirect action. Neutrons, protons and alpha particles are classified as high LET as energy deposition is dense and direct action is the dominant process of radiation (Dainiak, 2002).

Table 1.2: Typical radiation linear energy transfer values

Radiation Linear Energy Transfer, KeV/μm	
Co-60γrays	0.2
250kVpX rays	2.0
10 MeV protons	4.7
150 MeV protons	0.5
14MeVneutrons	12
2.5 MeVαparticles	166
2GeVFeions	1000

(Adapted from Hall and Giaccia, 2006)

1.6: Radiation induced cell damage

The biological effects that result from deposition of energy within the cell by ionising radiation are thought to be caused primarily by chemical modification to DNA (Jonah and Rao, 2001). When radiation interacts with biological substances it is possible that it will interact directly with targets such as DNA, resulting in ionisation of atoms of the target molecule, and initiating a chain of events which results in a biological change. Alternatively during indirect action, radiation transfers energy to ionise other atoms or molecules such as water (H_2O) which could result in the formation of free radicals (Figure 1.2). These molecules can cause critical damage to DNA and if the damage is not repaired, lead to apoptosis (Hall and Giaccia, 2012).

Apoptosis serves to prevent proliferation of genetically aberrant cells that contain significant levels of DNA damage (Stone et al, 2003). When cells are exposed to radiation, DNA strand damage is induced, which could be single or double strand breaks. Cells have to rapidly and efficiently detect these lesions and either repair them or induce apoptosis (Leobrich and Kiefer, 2006; Jackson, 2002). It is estimated that a cell exposed to a dose of 1-2Gy will suffer more than a thousand DNA base damages, about one thousand single strand breaks and approximately forty double strand breaks (Hall and Giaccia, 2012; Joiner and van der Kogel, 2009).

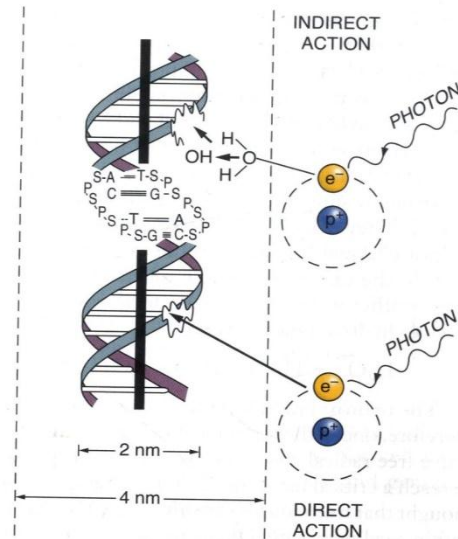

Figure 1.2: Direct and indirect action of radiation. A secondary electron resulting from the absorption of an X-ray photon interacts with DNA to produce an effect. This is known as direct action. Alternatively, in indirect action, a secondary electron interacts with a water molecule (for example) which will produce a hydroxyl radical (OH). This in turn causes damage to the DNA. (Adapted from Hall, 2012)

1.6.1: DNA strand breaks

There are many different types of cellular DNA damage that may be caused by ionising radiation which include single strand breaks (SSB) and double strand breaks (DSB) (Figure 1.3). DNA SSB shown in Figure 1.3 B are believed to have less biological effects with regards to cell killing, as these breaks are repaired almost immediately with the aid of the opposite strand being used as a template. However, if incorrectly repaired, a single strand break could result in mutations (Jonah and Rao, 2001; Mettler and Upton, 2008). DNA double strand breaks occur when breaks on both strands are opposite each other or a few bases apart (see Figure 1.3 C and D). The DNA double strand breaks are thought to be responsible for cell death due to ionising radiation (Hall and Giaccia, 2012; Joiner and van der Kogel, 2009 and Mettler and Upton, 2008).

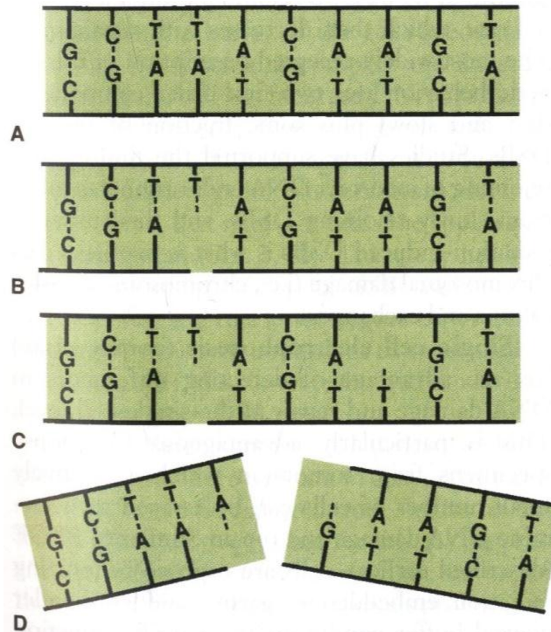

Figure 1.3: Diagram showing DNA strand breaks induced by radiation. A: Normal DNA strand. B: A single strand break. C Breaks on both stands, if a few base pairs apart may result in a double strand break or if well separated are repaired as single breaks using the opposite strand. D: Double strand break (Adapted from Hall, 2012)

1.7: The DNA Damage response

In order to prevent genomic instability and chromosomal aberrations that may be caused by radiation induced DNA damage, cells have developed mechanisms known as DNA damage responses (DDR) which determine a cell's fate after radiation exposure. DDR is a system that consists of a group of interconnected and highly coordinated pathways which regulate different effects on the cell. The system has DNA damage sensors, a group of proteins that inspect, recognise DNA damage (these include MNR, AMT, Ku, DNA-PKcs, ATRIP and ATR) and send signals to the effector pathways which then decide the cellular outcome. The major effector pathways include mechanisms which halt cell cycle progression (checkpoints), initiate DNA repair or programed cell death (apoptosis) (Joiner and van der Kogel, 2009).

1.7.1: Sensors of DNA double strand breaks

Identification of specific double strand breaks is characterised by activation and recruitment of a variety of proteins to the site of DNA damage following ionising radiation exposure. This can be demonstrated by staining with antibodies to these proteins and is referred to as ionising radiation induced foci (IRIF) (figure 1.4) (Joiner and van der Kogel, 2009). Involvement of many proteins within the DDR system in the formation of these IRIF have been demonstrated and it is believed that each of these sub nuclear regions is the basis of the mechanism in which DNA damage signalling to the DDR effector pathways occurs. Minutes after double strand breaks occur, phosphorylation of histone H2AX to γH2AX occurs. This is one of the first events known to occur in the DDR (Stucki and Jackson, 2006).γH2AX plays a central role in IRIF formation by activating and recruiting a number of proteins involved in DDR to the site of the DNA double strand break. Many other proteins such as ATM, RPA, 52BP1, MDC1, BRCA1 and the MRE11/RAD50/NBS1 complex also form IRIF and co-localise with γH2AX(Hall and Giaccia, 2012).

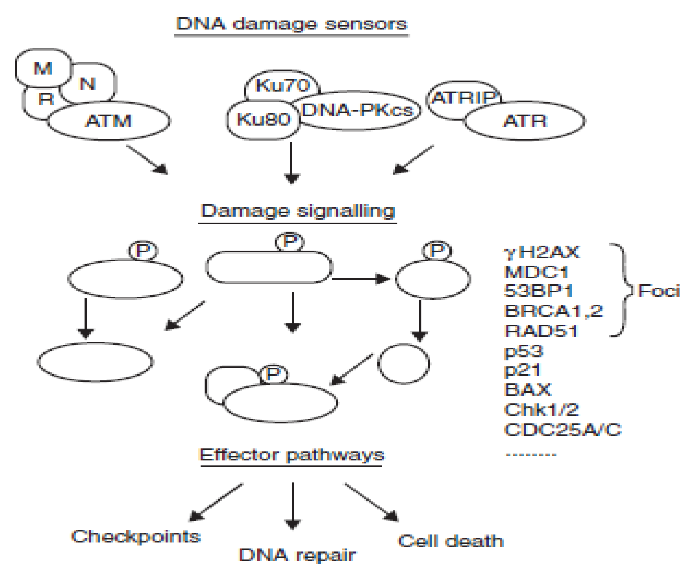

Figure 1.4: A graphic representation of DNA damage response. Double strand breaks are identified by sensors, which transmit signals to a series of downstream effector molecules. Activation of effector pathways may initiate induction of cell cycle arrest, DNA repair or cell death (Adapted from Joiner and van der Kogel, 2009)

1.7.2: DNA damage signalling

In eukaryotes, the DNA is extensively packaged into nucleosomes, which are assembled together to form a chromatin network within the nuclei. The nucleosome is an octamer that consists of two copies of the histone proteins H2A, H2B, H3 and H4 which form a 100kDa nucleosome core protein complex with 146bp of DNA wrapped around it (Takahashi and Ohnishi, 2005).The nucleosome is considered to be the fundamental subunit of the chromosome, occurring once in every 200 ± 40 bp throughout the genome. Neighbouring nucleosomes are connected by linker DNA which is complexed with linker histone H1 to make repetitive motifs (Maeshima et al, 2010). The Histone H2A has three variants H2A1-H2A2, H2AZ and H2AX.In mammalian cells H2AZ and H2AX comprise about 10% and 2-25% of H2AX respectively with H2A1-H2A2 representing the remainder (Valdiglesias et al, 2013).

Phosphorylation of H2AX to γH2AX which is induced by double strand breaks is detectable within three minutes post irradiation, and is thought to be initiated by the phosphatidyl-inositol-3-kinase-related kinases (PIKKs) family of proteins such as ATM, DNA-Pkcs and ART (Valdiglesias et al, 2013).Even though the exact sequence of events is not well defined, ATM is considered to play a central role in H2AX phosphorylation at the site of the double strand break. ATM is the product of the gene that is mutated in the autosomal recessive syndrome ataxia telangiectasia (AT) and patients suffering from this disorder are highly radiosensitive (O'Driscoll and Jeggo, 2006).

1.7.2.1 ATM-MRN interaction

DNA damage signalling is predominantly dependent on ATM activation at the site of DNA damage. The exact sequence of events leading to ATM activation following DSB are still unclear, however ATM activation via MRN complex and another new pathway that terminates with acetylation of the ATM protein have been described (Joiner and van der Kogel, 2009; Gobbini et al, 2013 and Kaidi and Jackson, 2014). ATM exists as an inactive dimer in unaltered cells and is recruited to the site of DSB by the protein complex MRN which contains three proteins MRE 11, RAD50 and NBS1.NBS1 is a product of the gene mutated in Nijmegen breakage syndrome (NBS1) (Panier and Durocher, 2013).

The MRN complex first recognises and binds to the broken ends of the DSB via RAD50 with subsequent recruitment and activation of ATM to the site of the DSB. The NBS1 C-terminus interaction with ATM elicits intermolecular autophosphorylation on Ser1981 which results in dissociation of the dimers into active monomers (Shibata and Jeggo, 2014; Gobbini et al, 2013 and Roos and Kaina 2013).Activated ATM monomers go onto phosphorylate Ser139 of H2AX forming γH2AX which consequently spreads over the region of numerous mega-bases on either side of the DSB site (Joiner and van der Kogel, 2009).

1.7.2.2 DNA-PKcs

Another kinase capable of phosphorylating H2AX and other downstream DSB response proteins is DNA-dependent protein kinase (DNA-PKcs).In a similar fashion to ATM, the DNA-PKcs is unable to detect the DSB and relies on Ku (Ku70 and Ku80) to detect the breaks (Joiner and van der Kogel, 2009).The Ku70/80 heterodimer has a high affinity for double strand DNA ends and is essential for recruitment and activation

of DNA-PKcs to the site of the DSB. Activated DNA-PKcs phosphorylate and activate other proteins such as H2AX in response to DSB (Bassing and Alt, 2004), however the exact mechanism of DNA-PKcs activation and recruitment to Ku upon ionising radiation induced DSB is not well understood (Wang and Lees-Miller, 2013). DNA-PKcs, ATM and their respective activators (Ku and MRN) play similar roles in DSB sensing and signalling. ATM-MRN is involved in all phases of the cell cycle while DNA-PKcs seem to dominate in the G1 phase (Bassing and Alt, 2004).

1.7.2.3 ATR-ATRIP

In spite of the fact that ATR (ataxia-telengiectasia and Rad3-related) protein activation is generally induced by errors in normal DNA replication it can also be activated by radiation induced DSB at all cell cycle phases (Gobbin et al, 2013). Similar to ATM and DNA-PKcs, ATR does not have the ability to detect DNA damage and relies on another protein ATRIP (ATR-interacting protein) which then recruits ATR to the site of DSB (Joiner and van der Kogel, 2009).

ATRIP bound to ATR interacts with Replication Protein A (RPA) coated single strand DNA over hangs which are generated from DSB (discussed in 1.8.2), thus recruiting and activating ATR to the site of the DSB. This activation requires an independent recruitment of a distinct protein, a clamp 9-1-1 complex (Rad9-Rad1-Hus1) by RPA and the clamp loader Rad17-RFC complex to the RPA coated single strand DNA (Shechter et al, 2004). Topoisomerase Iiβ binding protein 1 (TopBP1) forms a bridge between ATR and ATRIP via its C-terminus and interacts with the Rad9 component of the 9-1-1 complex by its BRCT-domain and thereby activating ATR (Gobbini et al, 2013; Yan and Berman, 2014). Activated ATR phosphorylates activates downstream

components of the DDR effector pathways which include cell cycle checkpoints, DNA repair and cell death (Joiner and van der Kogel, 2009).

1.7.3: Effector pathways

1.7.3.1 DNA damage cell cycle checkpoints activation

Cell cycle checkpoint activation is one of the DDR effector pathways initiated by radiation induced DNA damage which delays the cell cycle at specific points by inhibiting movement through the G1, S and G2 cell cycle phases (Kastan and Bartek, 2004 and Kang et al, 2011).A group of proteins known as Cyclin-dependent kinases (CDKs) play a vital role in the movement through all cell cycle phases by phosphorylating proteins involved in the advancement through the cell cycle (Shibata and Jeggo, 2014). As the name suggests, CDKs are only activated when they are linked to a cyclin and at each point within the cell cycle different cyclin-CDK complexes are formed. The cycle checkpoint arrest activation is accomplished by activation of cyclin-dependent kinase inhibitors (CDKIs) or by interfering with CDK phosphorylation and activity (Joiner and van der Kogel, 2009).

These checkpoint delays are thought to give the cell enough time to efficiently and timely repair DNA damage or to decide whether the damage is irreparable and therefore the cell should be eliminated (apoptosis) (Shibata and Jeggo, 2014). Checkpoint activation is the downstream process of the DDR which follows after DNA damage sensing and signalling (See **1.8.1** and **1.8.2**). The key checkpoint pathways involve the PIKK family protein kinases ATM and ATR which when activated by radiation induce DSB, elicit phosphorylation of many proteins in the DNA damage checkpoints pathways (shown in Figure1.5) (Cann and Hicks 2007). These include checkpoint kinase 1(Chk1), checkpoint kinase 2 (Chk2), p38, p53 and many others

which are essential in halting cell cycle progression, DNA repair and apoptosis (Roosand Kaina, 2006;Stiff et al., 2006 and Burrowsand Elledge, 2008).

G1/S is the DNA damage checkpoint at the boundary between the G1 and S phases which results from the activation of p53 by ATM after radiation induced DNA damage to cells in the G1 phase of the cell cycle (Kastan and Bartek, 2004). Activated p53 up-regulates a variety of genes including cyclin-dependent kinase inhibitor (p21) which prevents phosphorylation of the retinoblastoma protein (Rb) subsequently blocking cell cycle transition from G1 into S phase (figure 1.5) (Bartek& Lukas, 2003; Craig et al, 2003; Kastan and Lim, 2000).

The intra-S phase checkpoint is triggered by deactivation or dephosphorylation of CDK2 kinase, a cyclin-dependent kinase, important for progression through the S phase (Zou and Elledge, 2003). During the intra-S phase checkpoint, ATM and ATR mediated activation of Chk1 and Chk2 respectively then phosphorylates and inactivates Cdc25a and Cdc25c. Therefore by retaining CDK2 in its inactive phosphorylated form, the cell is unable to initiate progression through the S phase (Bartek and Lukas, 2003; Donzelli and Draetta, 2003). The G2/M like intra-S phase checkpoint is also mediated by the ATM-Chk1/2-Cdc25a/c pathway phosphorylating CDK1 and must therefore also be dephosphorylated to be active. This process would result in blocking transition from G2 to M phase (Cann and Hicks, 2007; Niida and Nakanishi, 2006).

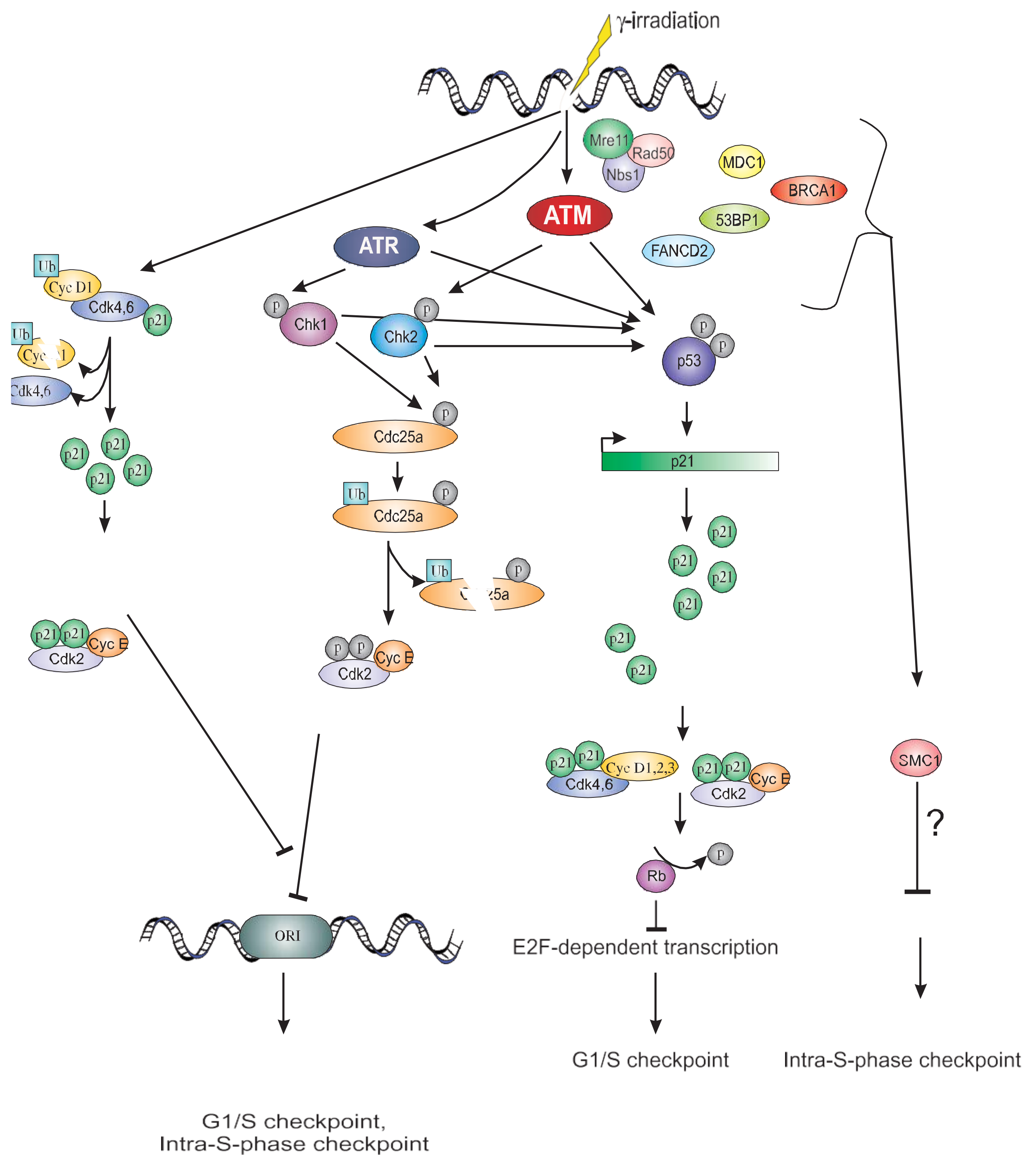

Figure 1.5: The key checkpoint pathways triggered by radiation induced DNA damage: the G1–S and intra-S-phase checkpoints are induced to prevent the DNA damage from being amplified through DNA replication. One of the principle targets of these pathways is Cdk2. Inhibition of Cdk2 can prevent E2F-dependent transcription required for S-phase entry and DNA replication. See text for detail. (Adapted from Cann and Hicks 2007)

1.8: DNA double strand break repair

Eukaryotic cells have evolved complex systems to detect and efficiently repair radiation induced DNA double strand breaks. This is achieved by delaying other cellular processes such as cell cycle progression (discussed in 1.7.3.1) giving the cell time to repair the breaks .Failure of this process may result in cell death or apoptosis (Figure 1.6) (Joiner and van der Kogel, 2009 and Roos and Kaina, 2013). There are two dominant radiation induced DNA double strand break repair pathways, namely nonhomologous end-Joining (NHEJ) and homologous recombination repair (HRR) (Nussenzweig and Nussenzweig, 2010, Hall and Giaccia, 2012).

NHEJ is an error prone DNA repair pathway that acts throughout the cell cycle. It involves resection or the addition of a few nucleotides to the broken ends prior to their ligation. In contrast HRR is an error-free repair pathway that depends on the availability of an intact homologous DNA template to repair the broken strand (Shibata and Jeggo, 2014 and Hall and Giaccia, 2012)

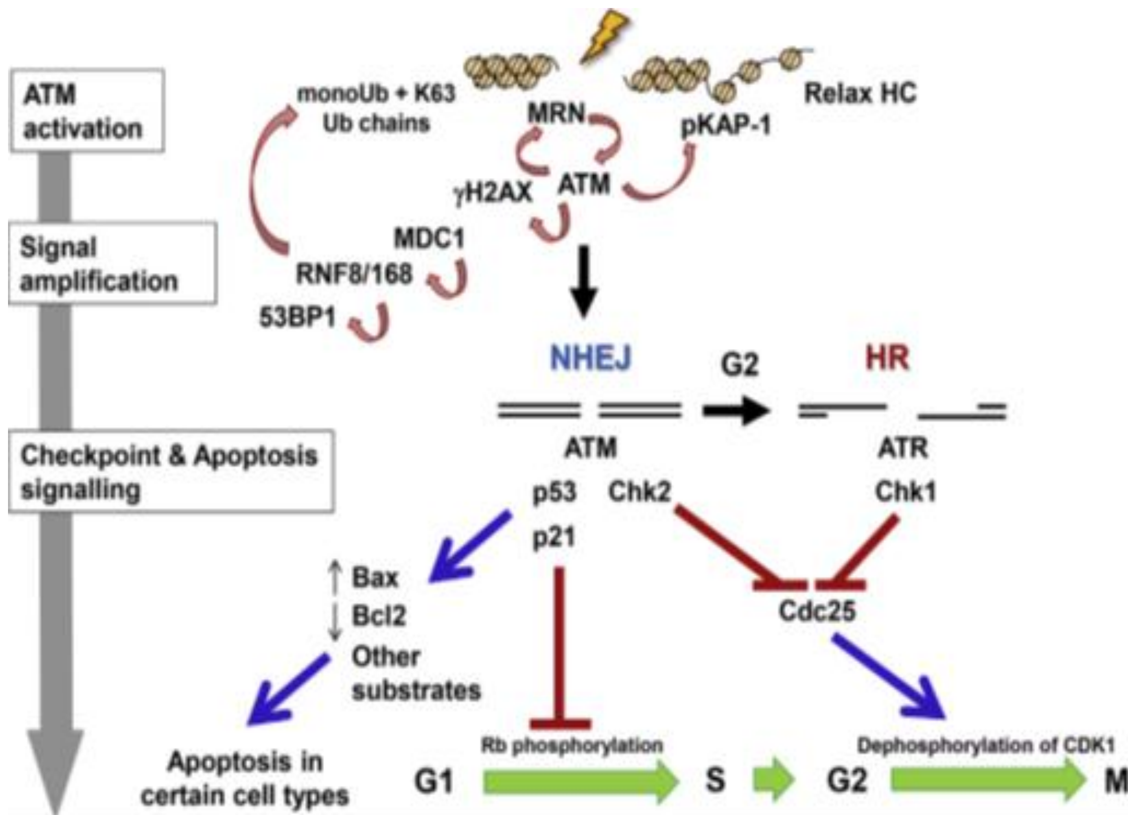

Figure 1.6: Schematic representation of DNA damage responses (DDR) effector pathways which determine a cell's fate after radiation exposure. (Adapted from Shibata and Jeggo, 2014)

1.8.1: Nonhomologous End-Joining (NHEJ)

DNA DSB activates a cascade of proteins that endorses DNA repair and inhibits cell cycle progression until the break is completely repaired. NHEJ is the major and most rapid repair mechanism in radiation induced DNA DSB that directly joins two DSB together without the use of a homologous DNA template (Lieber, 2010 and Mladenov and Iliakis, 2011). The overview of NHEJ is shown in figure 1.7: Immediately after sensing the DSB NHEJ begins by binding of Ku70/80 heterodimers that consists of a 70 kDa and 83 kDa subunits to the ends of the DSB (Hall and Giaccia, 2012; Joiner and van der Kogel, 2009; Lieber, 2010; Weterings and Chen, 2008).Binding of Ku heterodimers prevents the degradation of the DNA ends by exonucleases and also facilitates recruitment and activation of DNA-dependent protein kinase (DNA-PKcs) to the site of the DSB via its Ku80 subunit. The interaction of DNA-Ku complex with DNA-

PKcs initiates translocation of Ku heterodimers away from the DNA ends. Activated DNA-PKcs change its conformation and becomes an active holoenzyme which enables access to the DNA ends by other processing factors such as DNA polymerases (μ and λ), nucleases (Artemins) and ligase (XRCC4/LigIV/XLF) (Meek et al., 2004 ; Mladenov and Iliakis, 2011).

DNA-PKcs also occur as a complex with artemins (DNA-PKcs-Artemins). Artemins is a protein that has endonuclease activity and is recruited to the site of DSB with DNA-Pkcs. The DNA ends-Ku\DNA-PKcs complex phosphorylates artemins thus activating its endonuclease activity (Joiner and van der Kogel, 2009). Radiation induced DSB may have non-blunt ends or ends with damaged bases which are non-ligatable. In such cases DNA processing will involve resection of the DNA ends to remove damage bases by endonucleases such as artemins or filling of overhang gaps by polymerases λ and μ to make ends ligatable (Lieber, 2010; Mladenov and Iliakis, 2011, Joiner and van der Kogel, 2009; Lieber 2008). The last step in NHEJ is ligation of the DNA DSB ends which is performed by DNA Ligase IV with the aid of XRCC4 and XLF which stimulates and stabilises the activity of Ligase IV (Mladenov and Iliakis, 2011)

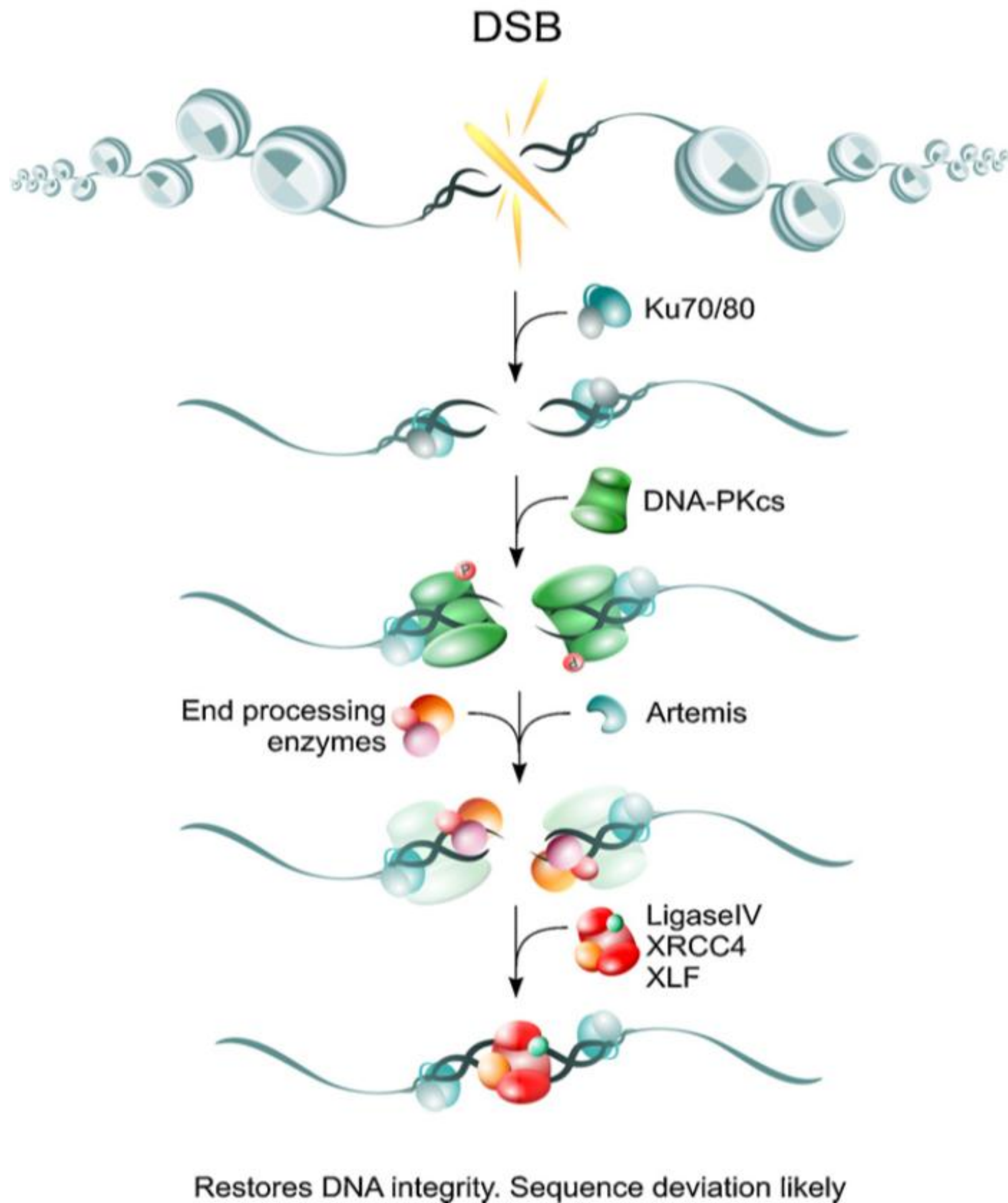

Figure 1.7: Nonhomologous End-Joining: DNA double strand breaks are sensed by the MRN complex with recruitment of ATM. Ku70/Ku80 heterodimers are then fixed to each end of DSB recruiting DNA-PKcs. Resection by Artmenis or filling by polymerases of DNA ends and blunt ends are ligated by Ligase IV, XRCC4 and XLF complex. (Adapted from Mladenov and ILiakis, 2011)

1.8.2: Homologous Recombination Repair (HRR)

In contrast to NHEJ which is active throughout the cell cycle, HRR seem to only occur during the S and G-2 phase of the cell cycle as it requires an intact homologous DNA

sequence from undamaged chromosomes or chromatids to use as a template for restoration of the lost sequence, thus repairing the DSB (Hall and Giaccia, 2012 and Mladenov and Iliakis, 2011). HRR is a slow and error free process but unlike NHEJ it is restricted to S and G-2 phases of the cell cycle. This is due to the fact that to accurately repair the damage this pathway must utilise an undamaged homologous DNA sequence acquired from sister chromatids which are only available as DNA replicates during the S phase and when they are fully synthesised in the G-2 phase of the cell cycle (Nussenzweig and Nussenzweig, 2010; Shibata and Jeggo, 2014 and Schipler and Iliakis, 2013).

The overview of HRR is shown in figure 1.8. Immediately after DSB recognition by the MRN complex and ATM phosphorylation (see 1.7.2.1 for details), BRCA 1 is also recruited to the DSB site with the initiation of HRR. The first step in HRR begins with resection of each side of the DSB blunt ends into 3'-single strand DNA overhangs by nucleases MRE11 and C-terminal binding interacting protein (CtIP). EXO1 and Bloom helicase (BLM) are also involved in this process (Shibata and Jeggo, 2014; Hall and Giaccia, 2012). The generated 3'-single strand DNA overhangs are rapidly coated with replication protein A (RPA) which is subsequently displaced by RAD 51 to form a RAD51 coated nucleoprotein filament. The BRCA 2 protein is recruited to the DSB by BRCA1. BRCA2, Rad51 paralogues (Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3) facilitates binding of RAD 51 to the RPA coated single strand (West, 2003).

BRCA2 aids the two generated Rad51 3'-single strand DNA overhang nucleoproteins invading nearby strands in search for homologous sequence in which they anneal to, forming primers for DNA synthesis and formation of the displacement loop (D-loop) (Hall and Giaccia, 2012; Joiner and van der Kogel, 2009).In the final step of HRR, the

D-loop is resolved by either of the three HRR sub-pathways which include break-induced replication (BIR), synthesis-dependent strand annealing (SDSA) and double Holliday junction (dHJ) formation (West, 2003)

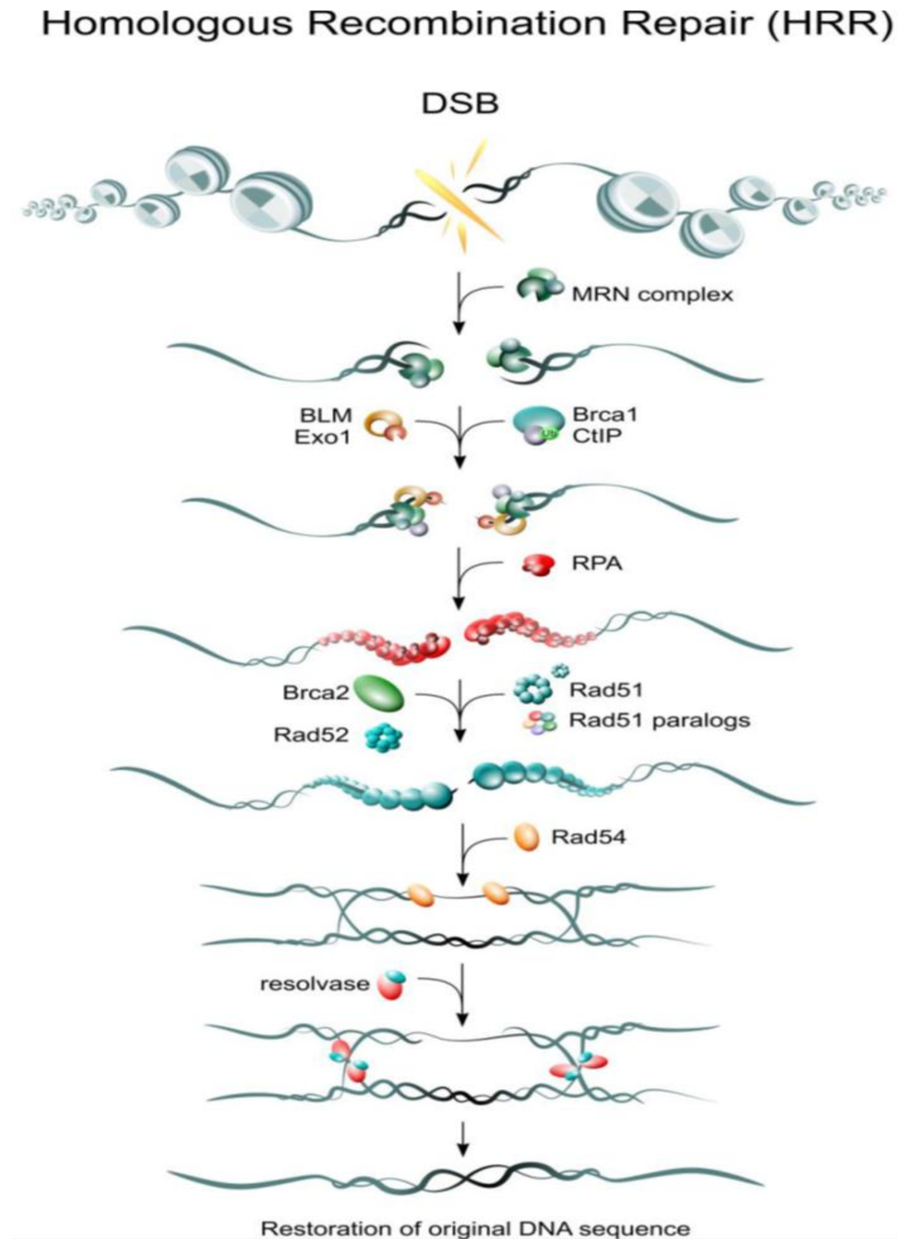

Figure 1.8: Homologous Recombination Repair: DNA double strand breaks are sensed by the MRN complex followed by resection of the DSB ends into 3'single strand DNA over hangs. RPA binds to the 3'single strand DNA over hangs, RPA is replaced by RAD51 with the aid of RAD 52 and RAD 51 paralogs. The nucleoprotein filaments invade the homologous strand forming Holliday junctions that are later resolved in to DNA duplexes. (Adapted from Mladenov and ILiakis, 2011)

1.9: Apoptosis

Apoptosis or programmed cell death was first described by Kerr and colleagues and is a physiological phenomenon that occurs spontaneously. It plays a vital role in many physiological processes including eliminating cells during normal embryonic development and is triggered by external stimuli such as radiation (Kerr et al, 1972).

Apoptosis is the dominant mechanism of cell death induced by radiation on haemopoietic cells and is the key mechanism used to remove cells with significant DNA damage, thus preventing proliferation of tumourigenic cells (Eriksson and Stigbrand, 2010; Harms-Ringdahl et al, 1996). The process of apoptosis has two major pathways, the extrinsic or death receptor pathway and intrinsic or mitochondrial pathway. Both pathways have the same endpoint involving the activation of effector caspases or cysteine-aspartate proteases (caspase-3, caspase-6 and caspase-7) (see figure 1.9). This results in nuclear degradation, cleavage of structural proteins and the formation of apoptotic bodies which are characteristic of apoptosis (Elmore, 2007).

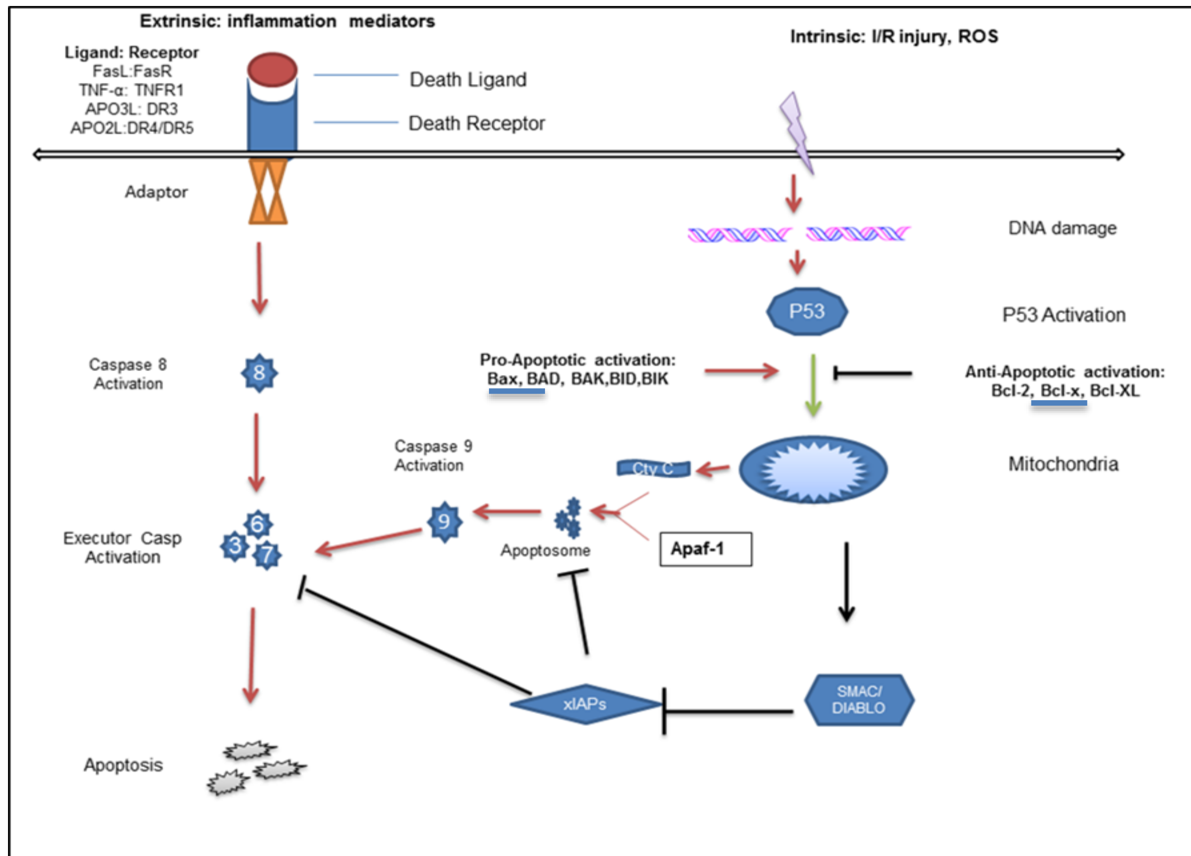

Figure 1.9: An overview of the apoptotic pathways. (Adopted from Tait and Green, 2010)

1.9.1: The extrinsic pathway of apoptosis

The extrinsic pathway is initiated by activation of transmembrane receptors such as fatty acid synthetase (Fas) receptors, which are members of the tumour necrosis factor (TNF) receptor gene family (Elmore; 2007).When a ligand binds to its corresponding death receptor (FasL/FasR, TNFα/TNF1, Apo2L /DR5), the resulting death complex initiates a downstream activation of caspase-8 and caspase-10 which cleave and activate the effector caspases (caspase-3, caspase-6 and caspase-7) (see figure 1.9) (Elmore, 2007; Eriksson and Stigbrand, 2010).The effector caspases are considered to be the executioners of apoptosis (Elmore; 2007).

1.9.2: The intrinsic pathway of apoptosis

The intrinsic pathway is initiated by a non-receptor mediated stimulus (see figure 1.9). This occurs when external stimuli such as ionising radiation act directly on DNA within

the cell which results in intracellular signals that initiate the intrinsic pathway. This pathway is mitochondria dependent and is mainly regulated by proteins of the Bcl-2 family which are divided into two groups. These consist of the pro-apoptotic protein Bax (Bcl-2-associated protein X) and BH3 only proteins and the anti-apoptotic proteins including Bcl-2 (B-cell lymphoma 2), Mcl-1(myeloid cell leukemia 1) and Bcl-XL (Bcl-2 like protein X) (Elmore, 2007; Eriksson and Stigbrand, 2010).

When DNA is damaged after exposure to radiation, p53 inhibits the anti-apoptotic *Bcl-2* gene and activates the transcription of the pro-apoptotic Bax gene (Lima et al, 2011).In some studies, Bax and BH3 only proteins have been observed to be rate limiting for radiation induced apoptosis (Erlache et al, 2005).The Bax protein results in the permeabilisation of the mitochondria outer membrane and consequently the release of Cytochrome C. Cytochrome C binds to APAF-1 to form a complex known as the apoptosome. This in turn activates caspase-9 and results in the activation of the effector caspases. The effector caspases target and cleave cell death substrates resulting in the cell exhibiting the unique phenotypic changes of apoptosis (Eriksson and Stigbrand, 2010).

1.9.3: Apoptosis regulators

The Bax and Bcl-2 proteins appear to act as the central components in apoptosis regulation with the Bcl-2/Bax ratio being an indicator of cell survival (Miyashita and Reed, 1995). In follicular lymphoma there is increased expression of the *Bcl-2* gene due to the t(14; 18) chromosome translocation. This prolongs the life span of the affected lymphocytes which proliferate uncontrollably (Peng et al, 1998). Single nucleotide polymorphism (SNP) in the promoter region of the *Bax* gene in chronic lymphocytic leukaemia (CLL) patients has been shown to affect gene expression (Saxena et al, 2002 & Oksana et al, 2005). Saxena et al.(2002) reported a guanine to

adenosine substitution at position 125 (G125A) while Moshynska et al.(2005) linked the polymorphism to reduced expression of the Bax promoter.

1.9.3.1 Bax

The Bax gene encodes a 23-kDa Bax protein which has six exons and is found in q13.3-q13.4 region of human chromosome 19 (Apte et al, 1995; Chou et al, 1996; Yildiz et al, 2013). Bax contains the Bcl-2 homology domain (BH) namely BH1, BH2 and BH3 and is therefore a member of the Bcl-2 family of proteins. This member of the pro-apoptotic Bcl-2 family has a significant role in apoptosis regulation. Bax operates by creating channels in the outer mitochondrial membrane and facilitates the release of cytochrome C and other apoptosis related factors which activate the caspases (Moshynska et al, 2005; Peng et al, 1998).

Following a death stimulus monomeric Bax proteins move to the mitochondria where they form homodimers via the BH3 domain and attach as transmembrane proteins on the outer mitochondrial membrane. This results in the formation of membrane channels by Bax homodimers with the subsequent release of cytochrome C, thus promoting apoptosis (Gross et al .1999). Heterodimerisation of Bax with anti-apoptosis proteins of the Bcl-2 family via its BH1 and BH2 domain favours apoptosis inhibition (Peng et al, 1998; Yildiz et al, 2013).The expression of the Bax gene is determined by structural changes in the gene and alterations in the promoter region which has been shown to halt apoptosis (Yildiz et al, 2013). Single nucleotide polymorphisms on the promoter region of the Bax gene have been demonstrated to reduce gene expression which results in inhibition of apoptosis (Moshynska et al, 2005; Saxena, 2002).

1.9.3.2 Bcl-2

The *Bcl-2* gene contains three exons located at chromosome band 18q21.3 and encodes for a 26-kDa Bcl-2 protein which functions as an inhibitor of apoptosis (Bachmann et al, 2007; Nückel et el, 2007). The Bcl-2 protein is the anti-apoptotic member of the Bcl-2 family and was identified due to its involvement in Human follicular B-cell lymphoma t(14; 18) (Yunis, 1983). Normally the *Bcl-2* gene is located on chromosome 18 but due to this translocation it is juxtaposed to the immunoglobulin heavy-chain locus on chromosome 14. This leads to an increase in Bcl-2 expression and prolongs the life span of the affected lymphocytes which proliferate uncontrollably (Peng et al, 1998; Young, 1993).

1.10: Flow cytometric measurement of apoptosis

Flow cytometry is the method of choice when measuring apoptosis due to its ability to analyse single cells rapidly (about 1000 to 10000 cells/s) and to produce quantitative data of different parameters such as cell membrane characteristics, DNA content, cytoplasm constituents and cell organelles (Wlodkowic et al, 2011 and Vermes et al, 2000). This method utilises measurement of forward angle light scatter (FSC) which relates to the cell size and side angle light scatter which is proportional to inner cellular structures and complexity. In the early stages of apoptosis, cells shrink due to dehydration and have low FSC signal and increased SSC (increase refraction and reflection) owing to nuclear condensation and crosslinking of cytoplasmic proteins (figure 1.10) (Wlodkowic et al, 2012). As apoptosis progresses both FSC and SSC signals are markedly reduced and the final stage of apoptosis is characterised by formation of apoptotic bodies resulting in low forward and side scatter signals (Wlodkowic et al, 2011).

It must be noted that light scatter (FSC and SSC) alone is not a definitive marker of apoptosis as similar results can be observed in necrosis, cell debris, isolated nuclei and mechanically damaged cells (Vermes et al, 2000 Wlodkowic et al, 2011 and Wlodkowic et al, 2012). However, the possibility of analysing light scatter signals in combination with a variety of more specific apoptosis cell makers is another advantage of flow cytometry.

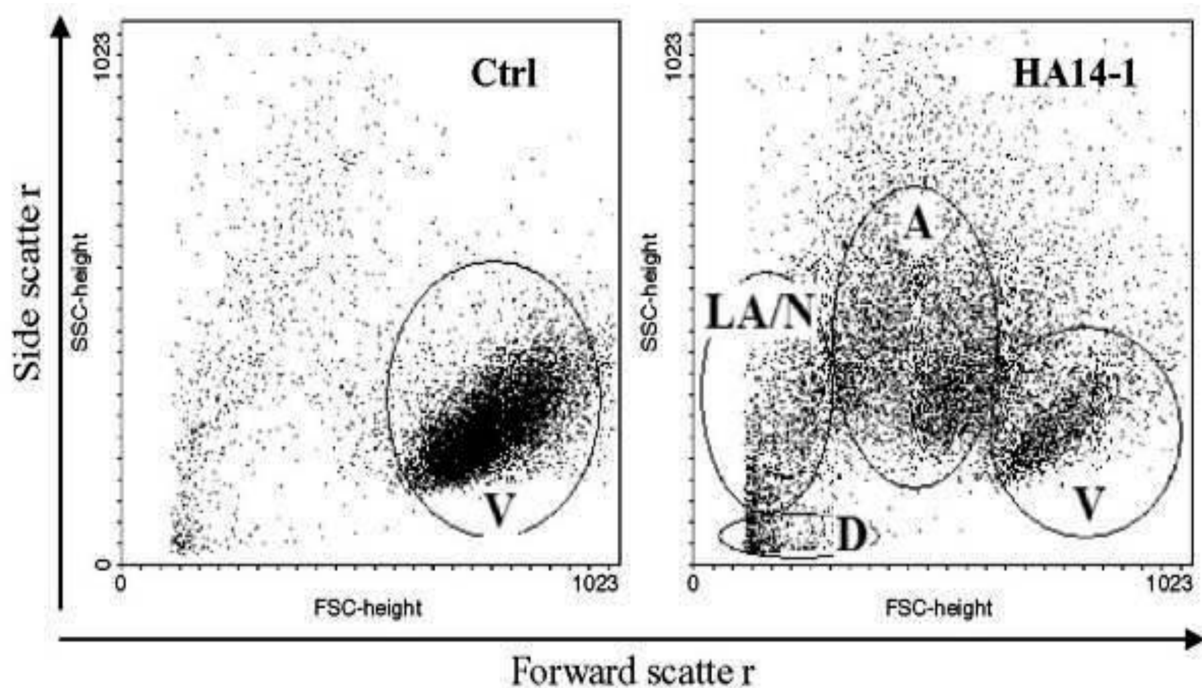

Figure 1.10: Changes in light scattering properties during apoptosis. Viable cell population (V) have similar light scattering properties as control cells. Apoptotic cells (A) have lower forward scatter while their side scatter is increased. The late apoptotic cells (LA/N) have both diminished side scatter and forward scatter properties. Apoptotic bodies and cell debris exhibit extremely low light scatter values (D) (Adopted from Wlodkowic et al, 2011).

1.10.1: Leukocyte apoptosis assay (LAA)

Permeability to certain cationic probes such as propidium iodide (PI) is one of the hallmarks observed at the final stages of apoptosis. The leukocyte apoptosis assay (LAA) was developed by Crompton and Ozsahin to predict intrinsic radiosensitivity of normal tissue based on the radiation induced apoptotic response of CD4+ and CD8+ T-lymphocytes (Ozsahin et al, 1997) .This is a flow cytometric assay that measures

apoptosis by utilising CD4 and CD8 Fluorescein isothiocyanate (FITC) conjugated antibodies to identify cell type and propidium iodide to determine cellular DNA content. Apoptotic lymphocytes are identified as cells staining for a specific antibody (CD4 or CD8) and displaying reduced DNA content (Ozsahin et al, 1997 and Ozsahin et al, 2005).

Evaluation of radiation induced apoptosis in lymphocytes has been recognised as a rapid and reliable methodology (Darzynkiewicz et al 1992, Zamai et al 1993 and Ozsahin et al, 1997). The use of lymphocytes offer significant advantages compared with other cell lines as large quantities can be obtained easily, they are easy to handle, and give rapid results as there is no need to culture. In addition, they are extremely sensitive to radiation (Henríquez-Hernández et al 2012 and Bourgier et al, 2015).

The feasibility of using the leukocyte apoptosis assay to predict the possibility of increased radiation toxicity post radiotherapy was demonstrated in a retrospective study of 12 individuals (Crompton et al, 1999). In a further study the leukocyte apoptosis assay was used to predict which patients were at risk of developing radiation toxicity. In this research 399 patients were tested prior to radiotherapy and reduced radiation induced apoptosis was observed in patients with severe toxicity (Ozsahin et al, 2005).

1.11: Western blotting (Immunoblotting)

Western blotting or immunoblotting is a technique that employs the use of monoclonal and polyclonal antibodies to detect and measure the size of proteins reacting with a specific antibody (Towbin and Gordon, 1984 and Gallagher et al, 2008). A mixture of sodium dodecyl salfate (SDS) and 2-mercaptoethanol (2-ME) or other reducing agent is used to solubilise the protein. The proteins are then electrophoretically separated

through SDS-polyacrylamide gel and transferred to a membrane (PVDF, nitrocellulose or nylon membrane) by electrophoresis using a tank (wet transfer) or semidry transfer system. Primary antibodies (monoclonal or polyclonal) specific for the target protein are then incubated with the blocked protein blot, washed and incubated with a secondary antibody (western Blotting, 2006). A variety of methods are used to detect labelled probes bound to the target protein. These include radioactive, fluorescent, colorimetric and chemiluminescence methods (Jensen, 2012 and Kurien and Scofield, 2003).

1.12: Previous studies

Since the 1980s many radiation biology studies have aimed at developing different approaches of determining radiation sensitivity to predict cancer patients' likelihood of developing radiotherapy side effects (Arlett and Harcourt, 1980; Loeffler et al, 1990; Burnet et al, 2013 and West et al, 2001). The earliest studies were aimed at establishing relationships between cellular and clinical radiosensitivity using clonogenic survival assays with skin derived fibroblasts as a model for normal tissue radiosensitivity (Henríquez-Hernández et al, 2012 and Smith et al, 1980). Most of these studies indicated that there was a correlation between normal tissue response and cellular sensitivity (Burnet et al, 1992; Burnet et al, 1994 and Geara et al, 1993). However when large similar studies were carried out similar results could not be obtained (Bentzen, 2008 and Peacock et al, 2000). Furthermore, it has been highlighted that clonogenic assays are labour-intensive and time consuming, taking about 6 to 8 weeks to produce results (Barnett et al, 2015).

This has triggered interest in exploring a number of more rapid assays that may have clinical value such as G2 lymphocyte assays (i.e. DNA damage), apoptosis

measurement and detecting chromosome damage with tests such as the micronucleus assay (Barnett et al, 2015). Currently there is no validated routine test for testing radiation sensitivity which can be applied on a clinical basis. The leukocyte apoptosis assay however has been shown to be a vital tool for predicting radiation toxicity and has demonstrated promising results in clinical practice (Ozsahin et al, 1997; Barber et al, 2000; Crompton et al, 1999; Crompton et al, 2001 and Scaife et al, 2015).

A study by Wendy Solomon assessed the feasibility of using the leukocyte apoptosis assay (LAA) to investigate individual variability and sensitivity to radiation within a South African population. The study showed that an individual's apoptotic response to radiation can be loosely linked to age, gender and race group (Solomon, 2009). In this study, donors of African descent showed an increased apoptotic response compared to donors of mixed descent and Caucasian origin. In addition, male donors had higher levels of radiation induced apoptosis when compared with female donors. Furthermore, older individuals had reduced levels of radiation induced apoptosis when compared to younger donors (Solomon, 2009). A study done by Radojcic and Crompton demonstrated similar results. In this study the youngest donor showed an increased apoptotic response compared to the oldest donor who had a decreased response (Radojcic and Crompton, 2001). This was consistent with the findings of Crompton and colleagues in which they reported 6.5% less apoptosis with each 10 years of life (Crompton et al, 1999).

Some studies have explored the use of combinations of these assays. Azria et al showed that low radiation induced apoptosis combined with genetic testing can be used to determine the possibility of developing severe late toxicity after radiotherapy

(Azria et al, 2008). Investigations of genetic disorders caused by mutations in genes involved in DNA repair pathways gave more understanding on variation in radiation sensitivity (Taylor et al, 1975). Our current understanding of individual radiosensitivity is that it's an inherited polygenic trait that is determined by a variety of genes or gene products involved in multiple cell pathways (Barnett et al, 2015).

There has been an increase in research aimed at exploring gene expression profile or signatures in response to radiotherapy using the "candidate gene approach" (Badie et al, 2008; Henríquez-Hernández et al, 2009 and Mayer et al, 2011). These studies focus on the so called "candidate genes" which are those involved in DNA damage sensing and repair (i.e. ATM, XRCC4, BRCA1\2, TP53 and Ku70/80); cell cycle control (i.e. CDKN2C, CCKND2 and CHEK2); free radical scavenging (i.e. SOD1 and CAT) and apoptosis (i.e. Bcl-2, CASP3 and Bax) (Barnett et al, 2009; Barnett et al, 2015 and West and Barnett, 2011).

In 2002 Quarmby et al published the first study investigating the relationship between radiation toxicity and gene expression. This study explored a cytokine microarray method used to identify differentially expressed gene transcripts in patients following radiotherapy. The authors concluded that similar markers could enable prediction of a patient's radiation sensitivity, thus predicting effects before treatment (Quarmby et al, 2002). Another study showed the expression pattern of 24 genes in response to X-rays that could predict radiation toxicity in nine out of fourteen patients (Rieger et al, 2004). Mayer et al explored radiation induced gene expression in lymphocytes from breast, head and neck cancer patients with radiation toxicity and compared the results to twelve matching controls. The authors reported 153 genes which were statistically significantly altered by irradiation. Sixty seven of these radiation induced genes were

identified as being likely to differentiate severe radiosensitive and normal reacting patients and more than one third were genes involved in apoptosis or cell cycle arrest (Mayer et al, 2011). It is understandable that in order to reduce toxicity and to develop individualised radiotherapy a detailed understanding of the molecular mechanisms involved in radiation induced apoptosis is required.

1.13: The present study

1.13.1: Hypothesis

The inherent ability of lymphocytes to induce apoptosis following radiation exposure differs amongst individuals of different ages, gender and race groups. It is possible that the expression of proteins involved in the activation or inhibition of apoptosis plays a role in individual radiosensitivity.

1.13.2: Aim of the study

To investigate the role of Bcl-2 and Bax protein expression on individual radiosensitivity.

1.13.3: Study objectives

- To investigate the expression of Bcl-2 and Bax at various doses of radiation (0 Gy, 2 Gy and 8 Gy).
- To investigate if Bcl-2 and Bax expression plays a role in the activation of radiation induced apoptosis in lymphocytes.
- To correlate Bcl-2 and Bax expression with the radiosensitivity of individuals.

CHAPTER 2: MATERIALS AND METHODS

2.1: Study design

This study was divided into two sections. The first part aimed to determine individual radiation sensitivity using the leukocyte apoptosis assay and quantifying the amount of radiation induced apoptosis in lymphocytes after exposure of whole blood to 2Gy and 8Gy of radiation. The second part quantified alterations in Bcl-2 and Bax protein expression within the same lymphocytes after exposure to 0Gy, 2Gy and 8Gy of radiation using Western blotting techniques.

2.2: Sample collection

Ethical clearance was obtained from the Health and Wellness Science Research Ethics Committee at CPUT, Bellville (Ref: CPUT/HW-REC 2013/H27) see Appendix A. A study participant information sheet and consent form (Appendix B) was made available to donors at the Western Province Blood Transfusion Services (WPBTS) blood donor clinics informing them about the study and its objectives. Forty two donors who were willing to participate signed a consent form before 1× 4ml heparinized and 3 × 6ml EDTA blood samples were drawn by nurses at WPBTS.

2.3: Leukocyte Apoptosis Assay (LAA)

2.3.1: Sample preparation

The LAA assay was performed according to a method described by Ozsahin and colleagues (Ozsahin et al, 1997). Briefly: Each heparinised sample was subdivided into three smaller samples containing 0.5ml of blood using six round-bottomed cell culture tubes. Blood in the cell culture tubes was diluted (1:10 dilution) in 4.5ml RPMI 1640 growth medium (Sigma-Aldrich, cat. 10771) containing 20% foetal bovine serum and 1% penicillin streptomycin. Using a Cobalt-60 source, two of the culture tubes were

exposed to 2Gy and 8Gy of radiation while the third tube was used as an untreated control (0Gy). Irradiation was at a dose rate of 0.3Gy/min and took place at iThemba LABS. After radiation exposure the culture tubes were incubated at 37°C in 5% CO_2 for 48 hours.

After 48 hours, the plasma was removed using a plastic pipette and the sample was mixed. 100µl of the sample was transferred into two new tubes. Ten microliters of anti-CD4 (BD Biosciences, Cat. 345768) or anti-CD8 (BD Biosciences Cat. 345772) Fluoroscein Isothiocynate (FITC) conjugated monoclonal antibodies were added to one of the tubes and the mixture was incubated for 20 minutes at 37˚C. Thereafter the erythrocytes were lysed by adding 4ml of 1 x FACS lysing solution (Beckton Dickinson, cat. 349202). Lysis took place by incubating the cells at room temperature in the dark for 15 minutes. This lysing solution also served to permeabilise the leukocyte membranes. Thereafter the samples were centrifuged at 300 rcf for 10 minutes. The leukocyte pellet was washed once with PBS (Lonza, cat. BE 17-517Q) and resuspended in 400µl of FACS flow (BD Biosciences, cat. 342003). The DNA of the permeabilised leukocytes was stained with 5µl Propidium Iodide (PI) (BD Biosciences, cat. 556463) and analysis was performed using a FACScan flow cytometer (Beckton Dickinson) within 30 minutes after staining with popidium Iodide.

2.3.2: Flow Cytometric Analysis

Two-colour flow cytometric analyses was performed using a FACScan (Beckton Dickinson) at iThemba LABS. Gating of the lymphocytes was performed by utilising forward and side scatter on a 2-dimentional (2D) scatter plot. A gate was placed around the lymphocytes in order to exclude red cells, debris, granulocytes and monocytes (Figure 2.1a: region 1(R1)). A total of 10000 evented were counted and the fluorescent intensity of each lymphocyte subtype (green fluorescence from FITC at 530nm and red

fluorescence from PI at 620nm) was measured. A second scatter plot measuring green fluorescence (FL1-height) vs. cellular DNA content (FL2-height) was used to identify CD4 or CD8 positive T-lymphocytes. These were included in region 2 (R2) (Figure 2.1b). A third scatter plot consisting of cellular DNA content (propidium iodide) vs. cell size (forward scatter) was used to determine the percentage of apoptotic cells. These were labelled as region 3 (R3) (Figure 2.1c) and included all cells with reduced DNA content and decreased cell size. The percentage of radiation-induced apoptosis was calculated by subtracting the percentage of background apoptosis at 0Gy from that measured at 2Gy and 8Gy. The analysis was performed using CellQuest Pro software (Becton Dickinson Immunocytometry systems).

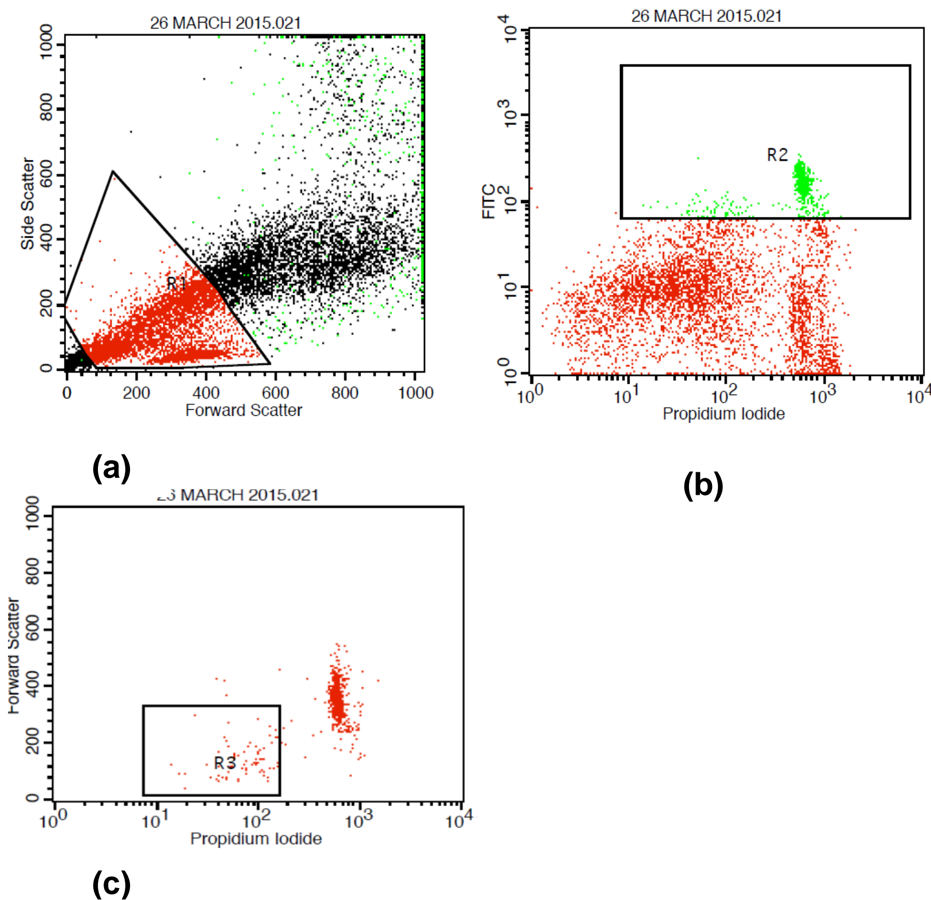

Figure 2.1: LAA scatter plots showing the (a) selection of lymphocytes (R1); (b) selection of CD4 or CD8 lymphocytes (FITC +) (R2); (c) apoptotic lymphocytes at 2 Gyor 8Gy (R3).

2.4: Lymphocyte isolation

Lymphocytes were isolated from 25 each of the three EDTA blood samples using the histopaque density gradient separation method (Sigma-Aldrich, cat.10771). One volume of blood (4ml) was diluted with an equal volume of RPMI (Sigma-Aldrich, cat. 10771) containing 20% foetal bovine serum and 1% penicillin-streptomycin to a final volume of 8 ml in a cell culture tube. Four millilitres of histopaque was poured into the bottom of a 15 ml polypropylene tube followed by 8ml of the diluted blood. Care was taken not to mix the blood with the histopaque. The 15 ml polypropylene tube was centrifuged at 2130 rpm with slow start (accelerate: 1) and slow stop (brake: 1) for 15 minutes. The mononuclear layer was carefully transferred into a new cell culture tube, washed three times by mixing with 4 ml of RPMI with 20% FBS and 1% penicillin-streptomycin and centrifuged at 1500 rpm for 10 minutes. The lymphocytes were resuspended by adding another 5 ml of RPMI with 20% FCS and 1% penicillin and streptomycin. Each of the three cell culture tubes, for Western blot analysis were then exposed to 0Gy (control), 2Gy, and 8Gy using a Cobalt source at a dose 0.3Gy/min at iThemba LABS. After radiation exposure the culture tubes were incubated at 37°C in 5% CO_2 for 48 hours.

2.5: Western Blot (immunoblotting)

2.5.1 Protein extraction

After incubation the isolated lymphocytes were transferred into a 1.5 ml tube and washed twice in ice cold 500 µl phosphate buffered saline (PBS) (Lonza, cat. BE 17-517Q). The NucleoSpin RNA/Protein extraction kit (MACHEREY-NAGEL, cat. 7404933.50) was used to extract protein according to the manufacturer’s instruction. Briefly, cells were lysed by incubation in a solution containing large amounts of chaotropic ions which inactivates all enzymes including RNases and proteases. The

lysis buffer also enhances the binding of RNA to the silica membrane and enables protein to pass through the NucleoSpin RNA/Protein Column. rDNase solution was applied to the silica membrane to remove contaminating DNA (MACHEREY-NAGEL, cat.). Salts and metabolites were removed by the washing steps which included two different buffers.

Proteins were recovered from column flow-through and a buffer (Protein Precipitator PP) was used to precipitate and denature the proteins. This was followed by a washing step that utilises a Protein Solving Buffer (PSB) with TCEP to dissolve the precipitated protein pellet. The dissolved protein pellet was then used in the Western Blot analysis

2.5.2 Total protein concentration (Bradford assay)

Bradford protein concentration determination assay, uses Coomassie brilliant blue G250 dye which under acidic conditions is predominantly in the doubly protonated red cationic form (Amax = 470 nm). When the dye interacts with proteins it is converted to a stable unprotonated form with a consequent colour change to blue (Amax = 595 nm) which is proportional to the amount of proteins present in the sample (Bradford, 1976). Protein quantification was made in comparison to assay standard values. These were prepared as a serial dilution from a known concentration of Bovine Gamma-Globulin standard (Bio-Rad, cat 500-0208). Briefly, 20 µl of serially diluted five point standards, blank and cell lysate were mixed with 1000 µl of quick start Bradford 1x dye reagent (Bio-Rad, cat 500-0205) and incubated for 5 minutes. This was followed by spectrophotometric determination of the optical density (OD) value for each sample at 595 nm. A protein standard curve was plotted. Protein concentration in the cell lysate was calculated from the standard curve using the OD values obtained.

2.5.3 SDS-poly-acrylamide gel electrophoresis (SDS-PAGE)

Appropriate volumes of lymphocyte lysates (**from 2.5.1**) were used in order to standardise the amount of protein loaded per sample to 20 µg. To determine the protein size of interest a pre-stained molecular weight marker (thermo scientific, cat. 26612) was loaded with each run and GAPDH antibody (Santa Cruze, cat. Sc-365062) was run during the experiment as a control for sample loading .The samples were separated on 12% SDS-PAGE. The samples were electrophoresed in running buffer containing 25 mM Tris, 190 mM glycine and 0.1% SDS at a constant voltage of 70 V, once the sample reached the resolving gel, the voltage was increased to 150 V for the rest of the experiment.

2.5.4 Protein Gel-Membrane Transfer

Following SDS-PAGE, the gel and polyvinylidene fluoride membrane (PVDF) (Roche, cat. 03010040001) were sandwiched between sponges and filter paper and clamped tightly together after removing air bubbles that might have been trapped between the gel and membrane. The sandwich was then submerged in ice cold transfer buffer (Tris 25 mM, Glycine 192 mM, 10% SDS, 20% methanol) and an electrical field was applied at 350 mA for 60 minutes. Successful transfer of proteins onto the PVDF membrane was checked using Ponceau S. Visual observation of short red bands was regarded as a good transfer before proceeding with the blocking step.

2.5.5 Gel staining

PageBlue™ Protein Staining Solution (fermentas, cat. R0571) was used to confirm protein transfer onto the PVDF membrane and to check if the gel ran successfully. To remove SDS and buffer salts following transfer, the gel was washed three times for five minutes with 100ml distilled water by heating in a microwave for one minute followed by gentle agitation at room temperature for four minutes. The gel was then

completely covered by PageBlue Protein Staining Solution, microwaved for thirty seconds and left to stain by gentle agitation at room temperature for twenty minutes. The stain was discarded and the stained gel was then rinsed in distilled water for five minutes and scanned with a standard colour flatbed scanner.

2.5.6 Ponceau

To optimise protein transfer from SDS-PAGE to the PVDF membrane, two PVDF membranes were used in each transfer. Both membranes were stained with Ponceau S and visual observation of short red bands on the first membrane was regarded as a good transfer. The second membrane was used to optimise transfer time and methanol concentration of transfer buffer as these may affect protein transfer. In a case where transfer time is too long or there is high methanol concentration the proteins will transfer through the first membrane into the second membrane.

The PVDF membrane was immersed in Ponceau S staining solution (Sigma, cat. P 7170) for five minutes and rinsed with distilled water to visualise the protein band with a clear background (figure 3.8).

2.5.7 Immunological protein detection

After transfer, the membrane was blocked by incubating it in Tris-buffed saline (TBS) containing 0.1% Tween-20 and 5% skim milk for 60 minutes. The blocked membrane was washed three times for five minutes with TBS-0.1% Tween-20. Thereafter it was incubated at 4°C overnight, with constant shaking, in TBS buffer (TBS plus 0.1% Tween-20, 0.5% dry milk, and 0.1% fetal bovine serum) containing the primary antibodies (1:1000) anti-Bcl-2 (mouse monoclonal, Santa cruz, cat. Sc-509); anti-Bax (mouse monoclonal, Santa cruz, cat. Sc-70405) and anti-GAPDH (mouse monoclonal, Santa cruz, cat. Sc-365062). . After being washed three times for five minutes with

TBS-0.1% Tween-20, the blots were incubated for 1 hour at room temperature in goat anti-mouse antibodies conjugated to horse radish peroxidase (R & D systems, cat. HAF007). The bound antibodies were detected using enhanced chemiluminescence. This was done by washing three times with TBS-0.1% Tween-20, immersion of the membrane in Lumiglo chemiluminescent substrate system (KPL, cat. 54-61-00) and incubated for 5 - 15 minutes until the optimal intensity was attained. Optimization of this assay is described in appendix C.

2.5.8 Imaging and analysis

Images were developed using UVTEC alliance imaging system (UVTEC-Cambridge). The western blot images were analysed using the ImageJ software (national institute of health) as described in ([http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-withimage-j/]). This software enables density profiles `measurement as well as peak heights and peak intensity or band volume. The first lane of each sample (0Gy) was used as a reference point to which the relative change of the other lanes (2Gy and 8Gy) was compared (e.g. density of 2Gy and 8Gy was divided by density of 0Gy lane). This process was used to calculate Bcl-2, BAX and GAPDH relative density in each treated sample. The relative density of Bcl-2 and Bax was divided by the relative density of GAPDH (the loading control) for the same dose in order to calculate the ratio for each dose compared to GAPDH.

2.6: Statistical analysis

Statistica 12 (StatSoft, Southern Africa) was used to analyse the data. The Shaprio Wilks W test was used to determine normality of distribution and the.one-way ANOVA was used to determine statistically significant differences between groups in the variables tested, using median and percentiles. Spearman correlation (R and P-values)

was used to determine significant correlations between appropriate variables and a P-value of < 0.05 was considered to be statistically significant.

CHAPTER 3: RESULTS

3.1: Patient sample

Heparinised whole blood samples were collected from 42 healthy donors with a median age of 43 years (25% to 75% percentile range of 31-53). Twenty five of the participants were males and 17 female (table 3.1).

3.2: Radiation induced apoptosis in CD4+ and CD8+ T-cells

After being irradiated at a dose of 2Gy and 8Gy, flow cytometric analysis using the leucocyte apoptotic assay demonstrated that both CD4+ and CD8+ lymphocytes isolated from male donors had a relatively higher apoptotic response when compared to the female donors. This difference however, was not statistically significant and is shown in Table 3.1.

As expected, both CD4 and CD8 positive lymphocytes demonstrated a dose dependent RIA response (Figures 3.1 and 3.2 and table 3.2). CD4+ helper lymphocytes, irradiated at a dose of 8Gy, had significantly increased percentages of cells undergoing apoptosis (median: 10.22%; range 7.12-18.42%) when compared to those exposed to only 2Gy of radiation (median: 3.00% (range 1.02-6.10%; P= < 0.0001).

Analysis of the CD8+ lymphocytes showed that the percentage of cells undergoing apoptosis was higher than the CD4+ subset (p=0.0027) (see fig 3.2). However, similar to the CD4+ T-cells, a significant difference was observed between the lymphocytes exposed to 8Gy and those receiving only 2Gy of irradiation (median: 27.59% (range 16.98-37.63%) vs. 8.88% (range 4.45-12.87%); P= < 0.0001). These results are depicted in Figs 3.1 and 3.2).

Further analysis of the results revealed a significant correlation between the radiation induced apoptosis incurred by CD4 lymphocytes exposed to 2Gy and CD4 lymphocytes exposed to 8Gy (R =f 0.5628 and P-value= 0.0001.

Table3.1: Donor information and RIA for both CD4 and CD8 lymphocytes

	All	**Male**	**Female**	**P-value**
	N42	**N25**	**N17**	
Age	43 (31,53)	44 (31,53)	35 (31,50)	0.4164
% RIA CD4 2Gy	3.00 (1.02,6.1)	4.22 (1.31,6.49)	2.23 (0.90,4.45)	0.1764
% RIA CD4 8Gy	10.2 (7.1,18.4)	11.9 (7.7,17.9)	7.8 (7.0,21.2)	0.4019
% RIA CD8 2Gy	8.88 (4.45,12.87)	9.27 (5.63,12.46)	8.49 (3.87,12.87)	0.5094
% RIA CD8 8Gy	27.6 (17.0,37.6)	28.6(15.6,37.6)	22.1 (18.4,38.5)	0.9596

Table3.2: Interdose comparison of radiation induced apoptosis (RIA) between 2Gy and 8Gy in CD4 and CD8 lymphocytes

Total, N42			
Cell type and dose		**R**	**P-value**
CD4 RIA			
% RIA CD4 2Gy	% RIA CD4 8Gy	0.5628	0.0001
CD8 RIA			
% RIA CD8 2Gy	% RIA CD8 8Gy	0.6823	<0.0001
CD4 and CD8 RIA			
% RIA CD4 2Gy	% RIA CD8 2Gy	0.2065	0.1894
% RIA CD4 8Gy	% RIA CD8 8Gy	0.4511	0.0027

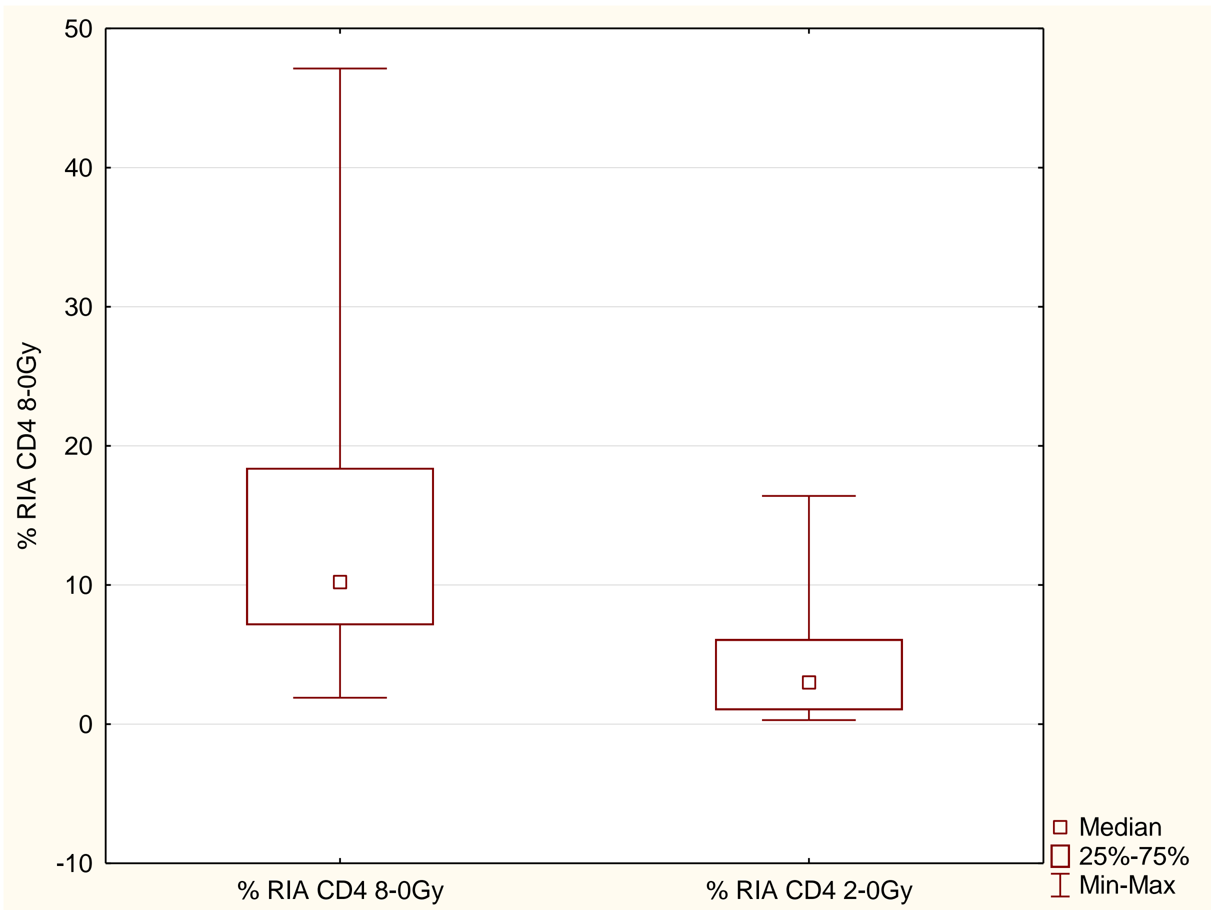

Figure 3.1: The % RIA CD4 at 8Gy was significantly higher than the % RIA CD4 at 2Gy. Median (percentiles) for % RIA CD4 8Gy: 10.22 (7.12, 18.42); % RIA CD4 2Gy: 3.00 (1.02, 6.10); P < 0.0001.

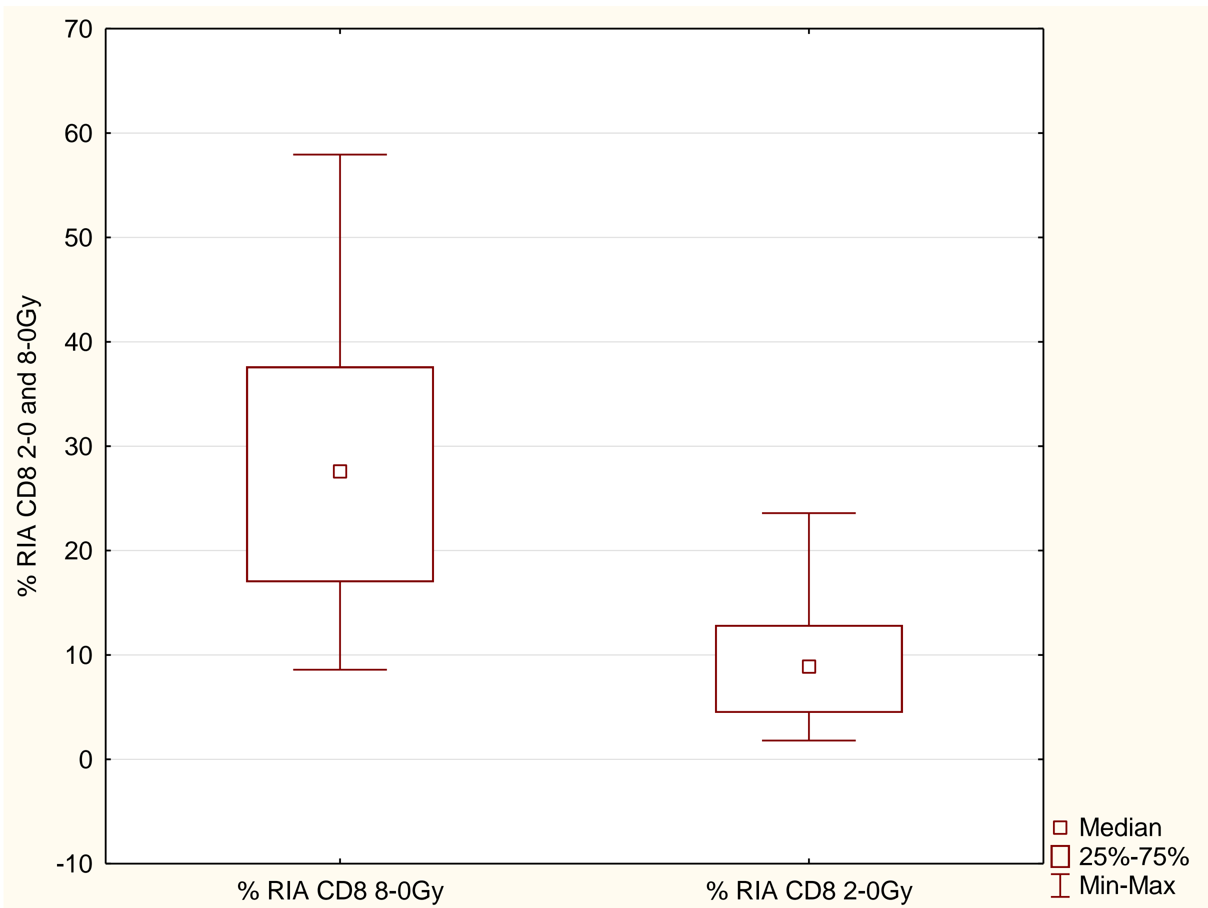

Figure 3.2: The % RIA CD8 at 8Gy was significantly higher than the % RIA CD8 at 2Gy. Median (percentiles) for % RIA CD8 8Gy: 27.59% (16.98, 37.63); % RIA CD8 2Gy: 8.88% (4.45, 12.87); P < 0.0001.

3.2.1: Age and radiation induced apoptosis.

As expected a negative correlation was observed between the age of the donor and the radiation induced apoptosis of the CD4+ lymphocytes after exposure to 2Gy of irradiation (R = -0.3277 and P = 0.0341). Although not statistically significant a near significant correlation was detected after 8Gy (R= -0.2916 and P = 0.0610). These results are shown in Fig 3.3 and 3.4. In contrast, although a significant negative correlation was also observed between age and radiation induced apoptosis in CD8 lymphocytes, after 8Gy exposure (R= -0.3378 and P = 0.0287), no correlation could be detected after 2Gy. (R = 0.1246 and P = 0.4318). See figures 3.5 and 3.6.

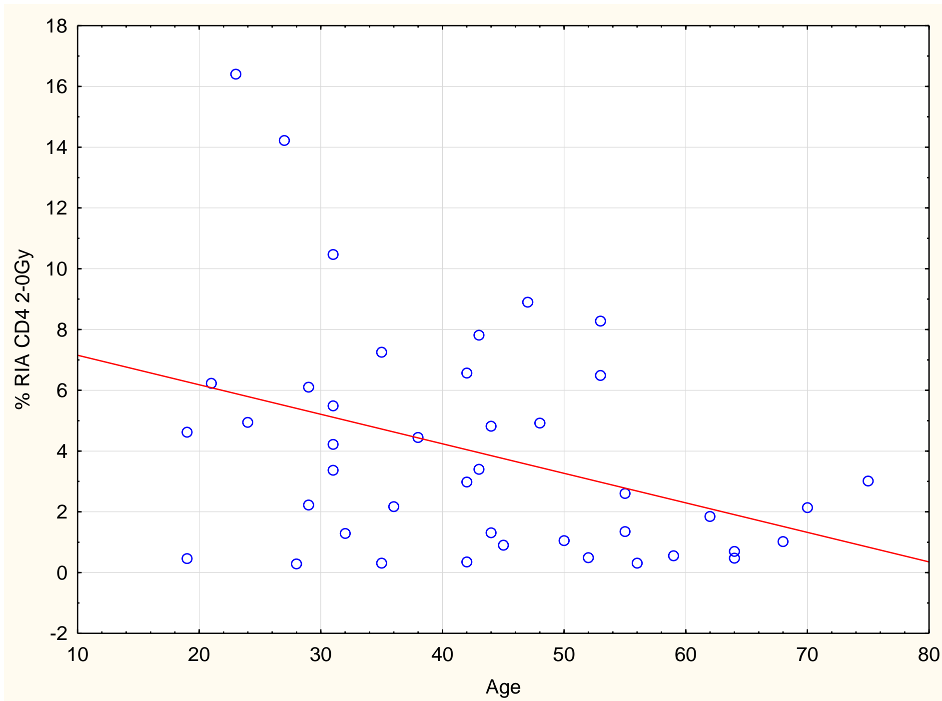

Figure 3.3: Correlation of radiation induced apoptosis (RIA) in CD4 lymphocytes following 2Gy exposure and age, regression line is indicated by the solid line. There was a significant correlation between age and % RIA CD4 2Gy; R = -0.3277; P = 0.0341.

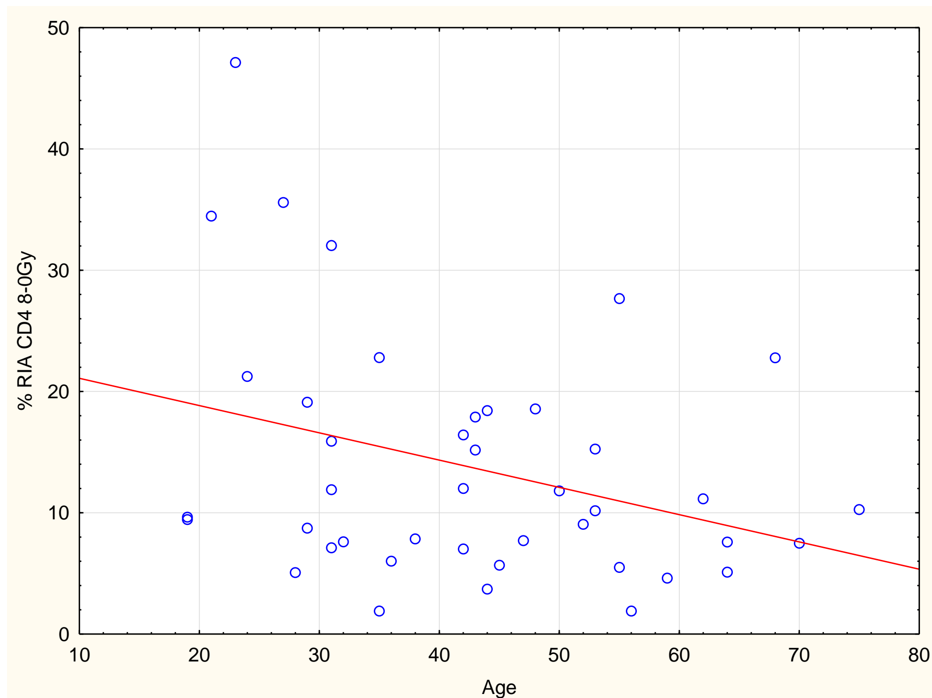

Figure 3.4: Correlation of radiation induced apoptosis (RIA) in CD4 lymphocytes following 8Gy exposure and age, regression line is indicated by the solid line. There was a near significant correlation between age and % RIA CD4 2Gy; R = -0.2916; P = 0.0610.

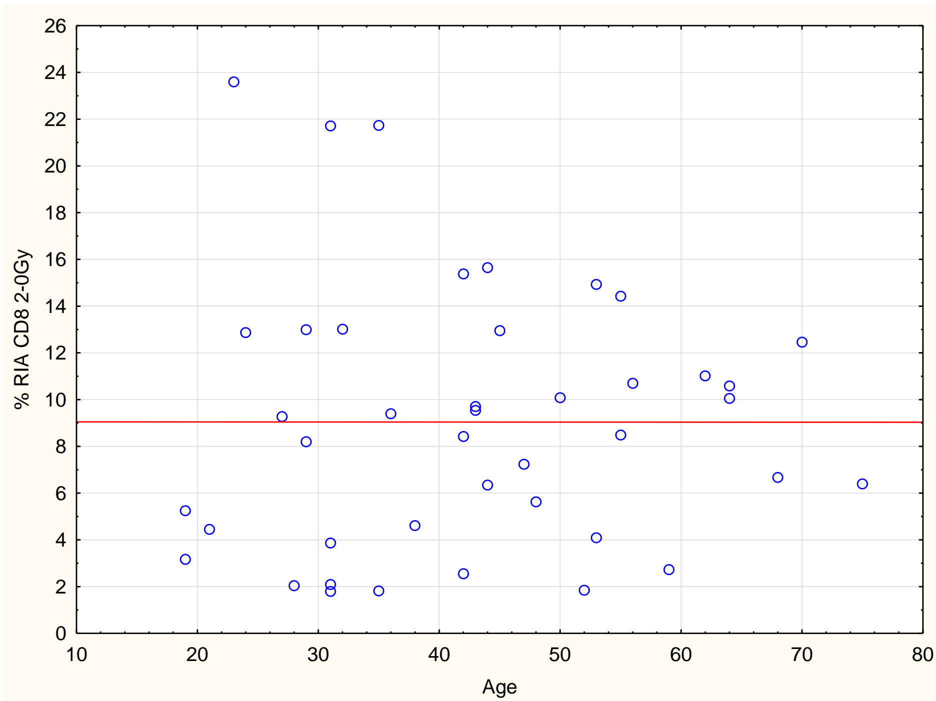

Figure 3.5: Correlation of radiation induced apoptosis (RIA) in CD8 lymphocytes following 2Gy exposer and age, regression line is indicated by the solid line. There was no significant correlation between age and % RIA CD8 2Gy; R = 0.1246; P = 0.4318

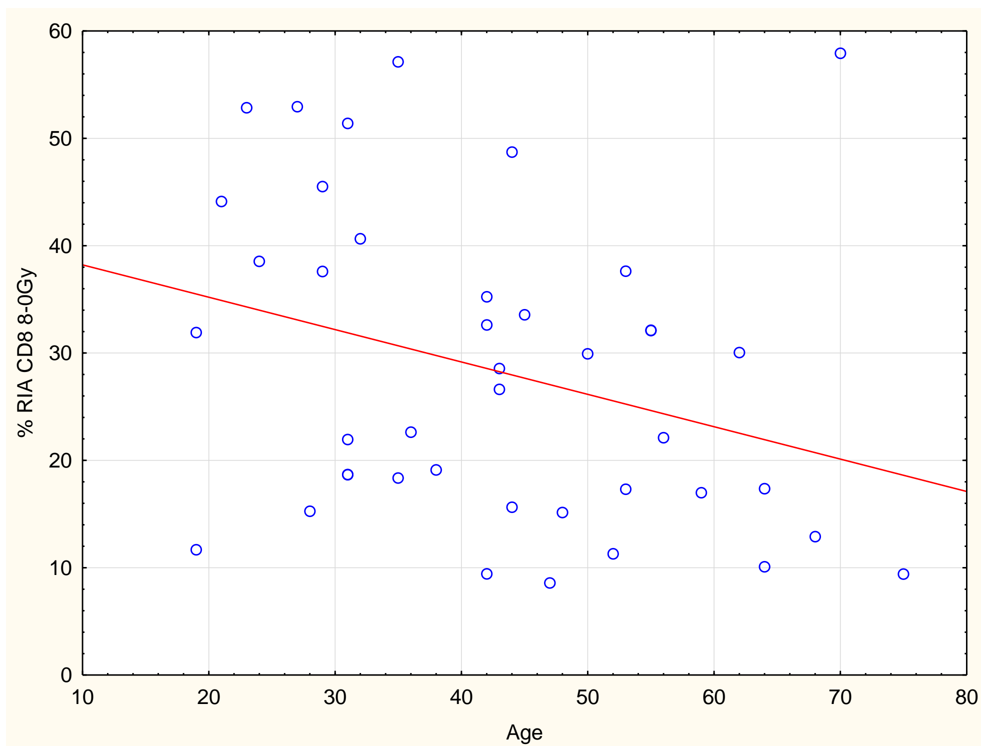

Figure 3.6: Correlation of radiation induced apoptosis (RIA) in CD8 lymphocytes following 8Gy exposer and age, regression line is indicated by the solid line. There was a significant correlation between age and % RIA CD8 8Gy; R = -0.3378; P = 0.0287.

3.3: Western blot analysis

To explore the role of Bcl-2 and Bax protein expression on individual radiosensitivity, lymphocytes from 42 donors were exposed to different doses of radiation (0Gy, 2Gy and 8Gy), however only samples from 25 of the 45 donors yielded sufficient protein concentration for Western blot analysis. Western blot analysis was employed in samples from 25 donors order to compare protein expression levels between treated and control samples. After analysis, protein bands of 23kDa were identified as Bax, 26kDa as Bcl-2 and 37kDa as GAPDH (figure 3.7).

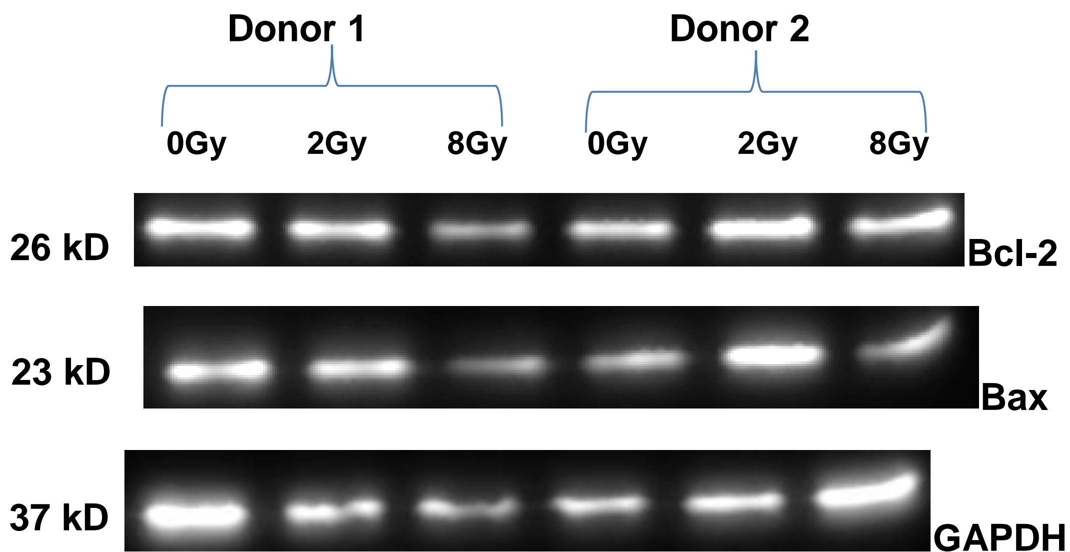

Figure 3.7: Bcl-2, Bax and GAPDH Western blots of samples exposed to 0, 2 and 8Gy

3.3.1 Bcl-2 expression

No statistical significant difference in the protein expression of Bcl-2 was detected between the various dose points. (0Gy: median 1.0296 (range 0.8284-1.2925), 2Gy: median 0.9897 (range 0.9103, 1.3386) and 8Gy: 0.9725 (range 0.7669-1.0950) P-value=1.000). Bcl-2 protein expression in lymphocytes after 0Gy, 2Gy and 8Gy exposure are shown in figures 3.8.

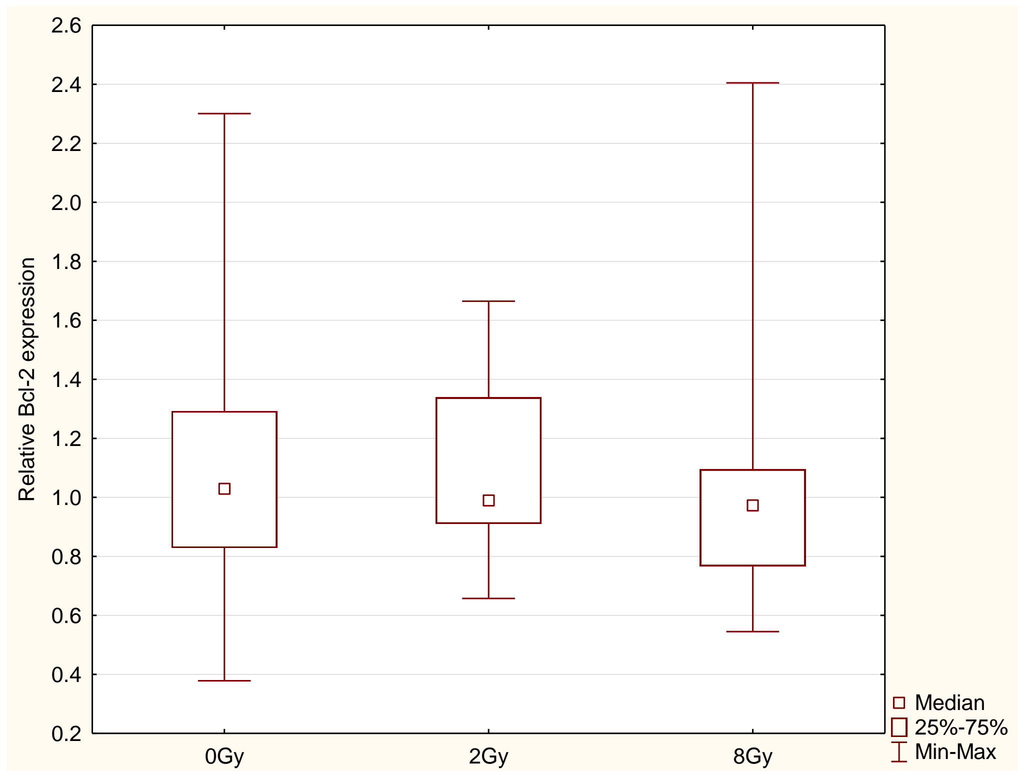

Figure 3.8 There was no significant difference in the relative Bcl-2 expression between 0Gy, 2Gy and 8Gy (P = 1.000). Median (percentiles) for 0, 2 and 8Gy respectively: 1.0296 (0.8284, 1.2925), 0.9897 (0.9103, 1.3386); 0.9725 (0.7669, 1.0950).

3.3.2 Bax expression

In contrast to BCL-2, a significant difference was observed in Bax expression between lymphocytes exposed to 0Gy (median: 0.7220; range 0.3882-1.155) and 8Gy (median: 1.0051; range 0.7536, 1.663; P= 0.0361) while a near significant difference was observed between those exposed to 2Gy (median: 1.0051; range 0.7536, 1.663) and 8Gy (P= 0.0924). No statistical significant difference could be detected between 0Gy and 2Gy (p=1.000). The Bax protein expression in lymphocytes after 0Gy, 2Gy and 8Gy irradiation exposure is shown below in figure 3.9.

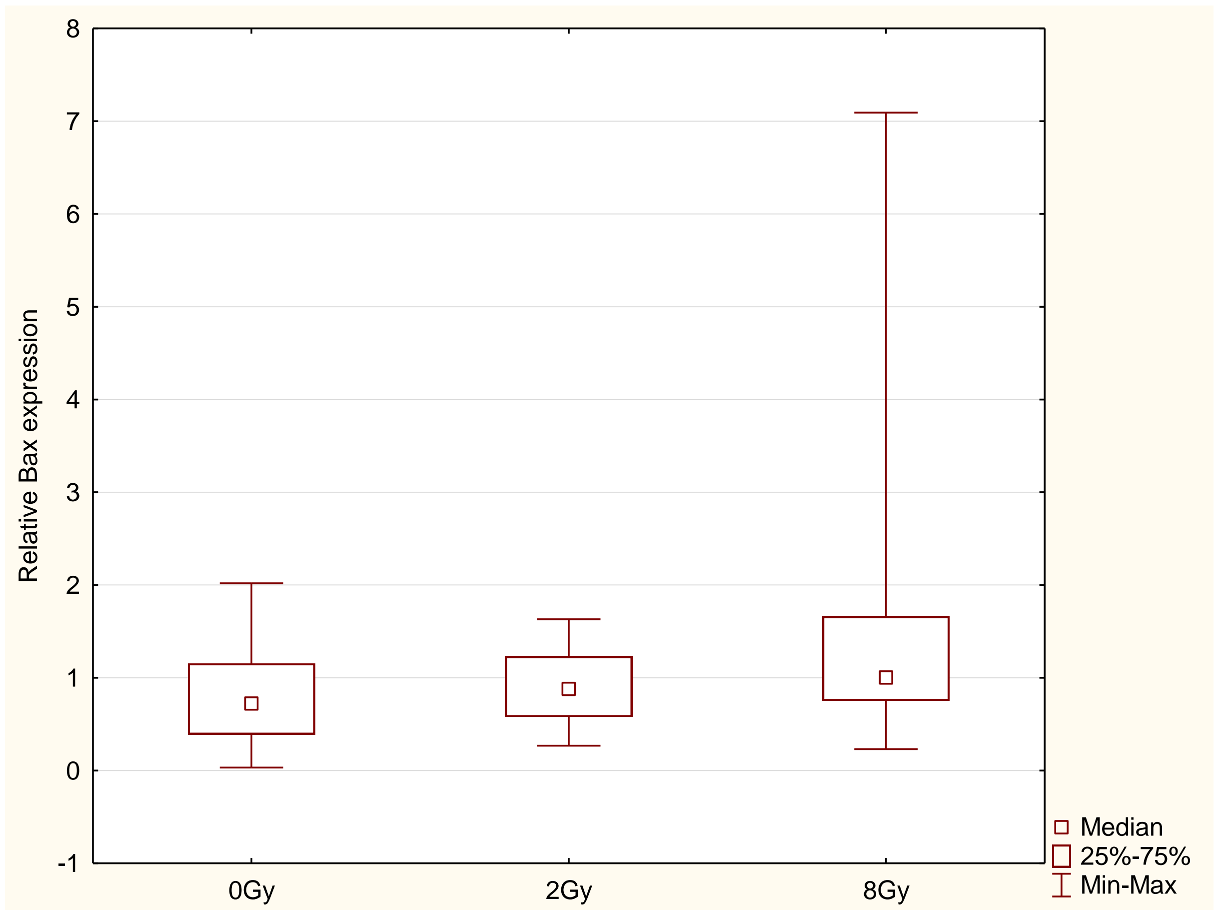

Figure3.9 There was a significant difference in the relative Bax expression between 0Gy and 8Gy (P = 0.0361), a near significant difference between 2Gy and 8Gy (0.0924) and no difference between 0Gy and 2Gy (P = 1.000). Median (percentiles) for 0, 2 and 8Gy respectively: 0.7220 (0.3882, 1.155), 0.8789 (0.5793, 1.231); 1.0051 (0.7536, 1.663).

3.3.3 Bax: Bcl-2 ratio

A significant increase in the Bax: Bcl-2 ratio (P = 0.0182) was observed in lymphocytes which had been exposed to 8Gy of radiation (median: 1.0671, range 0.8131- 1.5331) when compared to 0Gy (median: 0.7598, range 0.3122, 1.0866). The ratio at 8Gy was also significantly increased (P = 0.0469) when compared to 2Gy (median: 0.9107, range 0.6205 - 1.1090). However, there was no significant difference between the Bax: Bcl-2 ratio of lymphocytes exposed to 2Gy of radiation when compared to lymphocytes that were untreated (P = 1.000). The Bcl-2 and Bax expression ratio after exposure to 0, 2 and 8Gy in lymphocytes is shown below in figure 3.10.

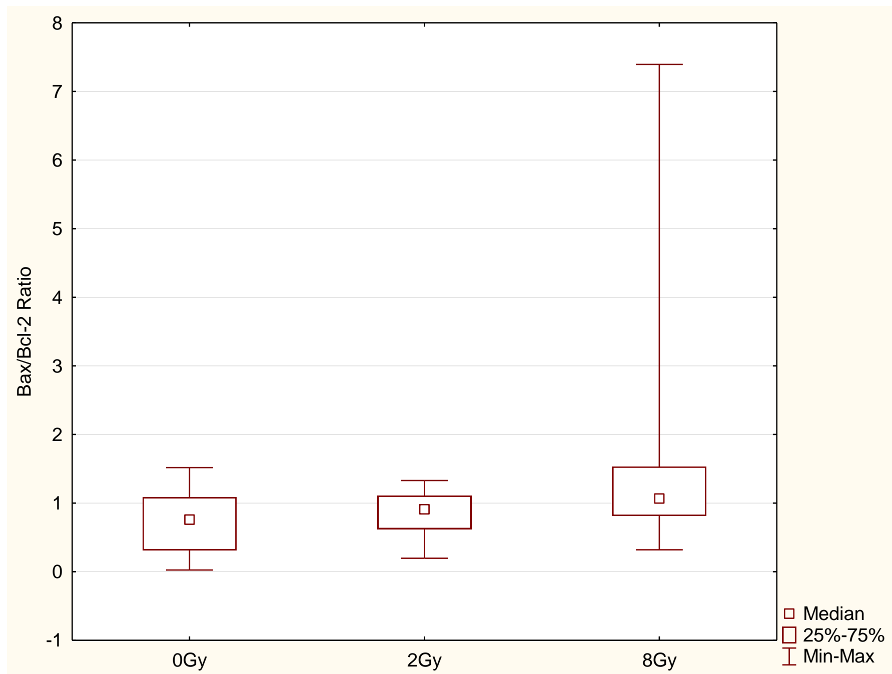

Figure 3.10: There was a significant difference in the Bax/Bcl-2 ratio between 0Gy and 8Gy (P = 0.0182), between 2Gy and 8Gy (P= 0.0469) and no difference between 0Gy and 2Gy (P = 1.000). Median (percentiles) for 0, 2 and 8Gy respectively: 0.7598 (0.3122, 1.0866), 0.9107 (0.6205, 1.1090); 1.0671 (0.8131, 1.5331).

3.4: Correlation between BCL-2 and Bax expression with the LAA. Analysis of the CD4+ lymphocytes demonstrated a significantly higher RIA after 8Gy radiation exposure when compared to 2Gy (Median (percentiles) respectively: 10.22% (7.12%-18.42%).and 3.00% (1.02%-6.10%) with P-value < 0.0001). The analysis of the CD8+ lymphocytes was also significantly higher after 8Gy when compared to 2Gy, (Median (percentiles) respectively: 27.59% (16.98%-37.63%) and 8.88% (4.45%-12.87%) with P-value < 0.0001). There was a significant difference in the Bax expression between 0Gy and 8Gy (P = 0.0361), (Median (percentiles) for 0, 2 and 8Gy respectively: 0.7220 (0.3882, 1.155), 0.8789 (0.5793, 1.231); 1.0051 (0.7536, 1.663) and a near significant difference between 2Gy and 8Gy (0.0924). However no significant difference was detected between 0Gy and 2Gy. There were no significant differences in the expression of Bcl-2 between 0Gy, 2Gy and 8Gy. The expression of BCL-2 and BAX could not be correlated with the results obtained using the LAA.

CHAPTER 4: DISCUSSION

The aim of this project was to investigate the role of Bcl-2 and Bax protein expression on individual radiosensitivity. In order to do this the Leucocyte Apoptosis Assay (LAA) was utilised to measure radiation induced apoptosis in CD4 and CD8 positive lymphocytes after exposure to increasing doses of radiation and Bcl-2 and Bax protein expression was measured using Western Blot analysis.

4.1: Radiation induced apoptosis in lymphocytes

The lymphocytes demonstrated a dose dependent radiation induced apoptotic response, with CD8+ lymphocytes having a significantly higher apoptotic response when compared to CD4+ lymphocytes. Similar to these observations were results reported by Ozsahin et al (2005). In this study radiation induced apoptosis (RIA) was

assessed in CD4 and CD8 lymphocytes after 8 Gy X-rays exposure of fresh blood samples collected from 393 patients on radiotherapy for a variety of cancers. The authors reported a median radiation induced apoptosis of 12.5 (mean 13.6; SD, 7.0; range, -3.6 to 45.5) for CD4 lymphocytes and 20.7(mean, 22.3; SD, 11.3 range 3.4 to 70.8) for CD8 lymphocytes (Ozsahin et al, 2005). In another study, examining the peripheral blood of 45 healthy donors (30 males and 15 females), irradiated at 0, 2 and 8Gy, higher RIA was observed in the CD8+ lymphocytes when compared to the CD4+ lymphocytes. The mean RIA in the CD4 subset was 4.33, 10.06 and 15.22 for 0, 2 and 8 Gy respectively while in contrast the mean RIA in the CD8+ lymphocytes was 4.20, 14.55 and 23.35 for 0, 2 and 8Gy respectively (Ozsahin et al, 1997).

Even though the R value was less than 0.4 (R= 0.3277 for CD4 2Gy-0; R= 0.2916 for CD4 8Gy-0; R=0.1246 for CD8 2Gy-0 and R= 0.3378 for CD8 8-0), a negative correlation between the age of the donors and the radiation induced apoptosis was a consistent trend observed for both cell types in the current study. This observation is similar to those described by Ozshahin et al who reported a decreased ability to elicit radiation induced apoptosis with increasing age in 45 healthy donors (R=0.32 for CD4 2Gy-0; R= 0.30 for CD4 8Gy-0; R=0.18 for CD8 2Gy-0 and R= 0.20for CD8 8-0) (Ozsahin et al, 1997). This was confirmed by the findings of Crompton and colleagues who reported on two cohorts of participants from two different cities. One hundred and five healthy donors from Zurich and 48 cancer patients from Basel were found to be normal RIA responders. This study also showed that with each 10 years of life there was 6.5% less radiation induced apoptosis in T-lymphocytes exposed to 9Gy X-rays (Crompton et al, 1999). This suggests that as individuals age T-lymphocytes become less competent in eliciting apoptosis in response to radiation damage.

Although not significantly different, when compared with females, in the current study on average males had higher levels of radiation induced apoptosis in both CD4+ and CD8+ lymphocytes at all dose levels. In contrast to this result, Ozsahin et al reported no significant differences between males and females, however similar findings were observed by Solomon who described a significant difference between the two genders (Ozsahin et al, 1997 and Solomon, 2009). These differences could be due to the fact that in this study and the one by Ozsahin et al there were fewer participants, 42 and 45 respectively, whereas the study by Solomon had 300 participants.

Lymphocytes are immunocompetent cells that are extremely sensitive to radiation and demonstrate a radiation dose dependent increase in apoptosis. Therefore the measurement of radiation induced apoptosis (RIA) in lymphocytes could offer more information about individual radiosensitivity (Crompton and Ozsahin, 1997). In exploring the leukocyte apoptosis assay as a tool to determine individual radiosensitivity, Ozsahin and colleagues studied the interdonor radiation induced apoptosis variation of 45 healthy donors and 5 children which included a patient with ataxia telangiectasia (AT). As expected, the results of the boy with ataxia telangiectasia proved to be radiosensitive with compromised radiation induced apoptosis in both CD4+ and CD8+ lymphocytes as his RIA results were lower than expected for his age (Ozsahin et al, 1997). Ataxia telangiectasia is a genetic disorder associated with the inability to execute a DNA damage response thus these patients are highly radiosensitive (O'Driscoll and Jeggo, 2006).

Furthermore, a retrospective study of twelve hypersensitive cancer patients who displayed toxicity due to radiotherapy and nine patients with ataxia telangiectasia homozygotes showed lower radiation induced apoptosis than expected for their age.

The leukocyte apoptosis assay was utilised in this study and the results demonstrated that eight of the patients developed early toxicity; three displayed late toxicity and one patient experienced both types of side effects. The results were compared to that of 153 normal radiation induced apoptosis responders which included 105 healthy donors and 48 cancer patients. A substantial difference in the radiation sensitivity of CD4+ and CD8+ lymphocytes from the hypersensitive patients was observed. All twelve patients had less apoptosis than the expected average of the 153 normal responders. Once again, as expected, the nine ataxia telangiectasia patients displayed low apoptosis (Crompton et al, 1999).

In 2005 a prospective study was performed on 399 cancer patients receiving curative doses of radiotherapy. A toxicity assessment was done during therapy and up to six weeks following treatment for early effects. Late effects were assessed every three months for up to two years following treatment. The examination of 330 of these patients demonstrated an inverse correlation between the late effects and CD4+ and CD8+ radiation induced apoptosis. These results therefore confirmed that the Leucocyte apoptosis assay could significantly predict if patients would develop grade 2 and 3 late effects (Ozahin et al, 2005).

With the above information, individuals with a high percentage of RIA were defined as being resistant to radiation as they have a good ability to trigger apoptosis. They are therefore able to endure high doses of radiation as they can rapidly rid the body of toxicity (Ozsahin et al, 1997). Individuals with low percentages of RIA were defined as being sensitive to radiation with a poor ability to trigger apoptosis which could result in a more severe pathological reaction. In radiotherapy these individuals should be treated with lower doses or alternative treatment, as radiation toxicity could remain

within their bodies for longer periods of time with the consequent development of side effects (Crompton et al, 2001 and Ozsahin et al, 2005).

4.2: Bcl-2 and Bax protein expression

The ability of T-lymphocytes to induce apoptosis after exposure to radiation has been shown to differ among individuals of different ages, race and genders (Ozsahin et al, 1997; Crompton et al, 1999 and Solomon, 2009). The reason for these differences remain unknown however, it can be postulated that they could be dependent on the relative expression of proteins that either promote (eg BAX) or inhibit (eg Bcl-2) the process of apoptosis. As the cell contains both pro- and anti-apoptotic proteins, it is important to investigate if the expression of these proteins such as Bax and Bcl-2 play a role in the regulation of radiation induced apoptosis. These studies could explain why there is a variation in radiosensitivity among individuals and understanding the cellular mechanisms involved in stimulating apoptosis could be important in the development of individual radiotherapy protocols.

The current study demonstrated no significant difference in the expression of the BCL-2 protein between all doses of radiation (0, 2 and 8Gy). This result has been supported by Ohno et al (1998) who also reported no significant correlation between apoptosis and BCL-2 protein expression in twenty cervical carcinoma patients before and after 9Gy fractionated radiotherapy. In this study patients received a total dose of 9Gy and tissue biopsies from the tumour sites were evaluated for apoptotic cell index both before and after treatment. The results showed that before treatment the apoptotic index was 0.22% and that this significantly increased post therapy to 1.20% (P= 0.0004). Despite this increase there was no significant change in BCL-2 expression. BCL-2 protein expression was detected in 15% (3 of 20 patients) prior to treatment and

after 9Gy of radiotherapy 25% (5 of 20 patients) had increased expression (Ohno et al, 1998).

Consistent with these findings, Kokawa et al (1999) also found no significant difference in BCL-2 expression before and after radiation treatment in seven patients with invasive cervical carcinoma. Radiation induced apoptosis and BCL-2 protein expression was assessed four hours after different doses of fractionated radiotherapy (0, 900, 1980, 3960, 6300cGy). The apoptotic index was significantly increased at 900cGy compared to 0Gy but there was no significant change in BCL-2 expression between the two doses (Kokawa et al, 1999). In contrast, Azimian et al, in a study that analysed Bcl-2 and Bax gene expression in freshly isolated human peripheral blood mononucleated cells,at 48 hours following 20, 50 and 900mGy radiation exposure, reported up-regulation of Bcl-2 with the subsequent down-regulation of Bax gene expression at all doses (Azimian et al, 2015).

Although not significant, in our study, Bax protein expression was increased at 2Gy when compared to 0Gy implying that there is a directly proportional relationship to radiation induced apoptosis. Importantly, there was a significant increase of Bax protein expression at 8Gy when compared to 0Gy which suggests that as the dose of radiation increases there is an up-regulation of Bax protein production which subsequently leads to an increase in apoptosis. Similar to these observations Ohno et al (date) also reported a significant increase in Bax protein expression after treatment with a total of 9Gy gamma radiation when compared to samples taken from the same sites prior to treatment. They described an increase in the percentage (60%) of patients expressing the Bax protein (12 of 20 patients) after radiation compared to 15% (3 of 20 patients) before treatment (Ohno et al, 1998).

These observations are similar to Kokawa et al who reported an increase in the apoptotic index (52.0 ± 9.6) in cells exposed to 900cGy compared to cells which had no exposure (9.4 ± 3.5). In addition, this study also reported that there was an increase in Bax protein expression, which rose from (3.2 ± 0.8) before treatment to (50.1 ± 7.1) after radiation (Kokawa et al, 1999).

However, in contradiction to these findings, Guida and colleagues investigated the radioprotective effects of thymoquinone on irradiated rats and reported different results. Whole body irradiation at 4Gy was performed on three groups of rats consisting of 8 per group. These included a non-irradiated group; irradiated group and an irradiated group which received a thymoquinone supplement. CD4+ and CD8+ T-lymphocytes were evaluated for Bax, Bcl-2 expression and apoptosis. The results showed a significant increase in apoptosis in both treated groups when compared to the control group. The irradiated group, however demonstrated significantly increased Bcl-2 expression with decreased Bax expression (Guida et al, 2016).

In the current study there was no correlation between Bcl-2 and Bax expression and the LAA, however a significant increase in Bax was demonstrated after 8Gy exposure. It is important to note that apoptosis is a complex process with multiple interconnected regulatory pathways that may act independently and which might explain why the expression of these proteins do not correlate with the end stage of cell death as measured by the LAA. Thus, understanding the processes and mechanisms involved in radiation induced apoptosis will require the analysis of all the proteins and molecules involved in both the inhibition and promotion of programmed cell death. These should include the expression of p53, cytochrome c and the caspases and also involve the use of assays which target different stages of the apoptotic pathway. P53, for example

is an important role player in radiation induced apoptosis. After activation, P53 inhibits BCL-2 allowing the cell to undergo programmed cell death (Lima et al, 2011). This action could explain why BCL-2 is not increased after irradiation, however further research is required in order to confirm this theory. The detection of elevated Bax protein expression at 48 hours following 8Gy radiation exposure, suggested that radiation induced cell death could be initiated due to an increase of Bax expression relative to BCL-2 at this time point. Bax promotes apoptosis and when stimulated moves into the mitochondria, forms homodimers and attaches to the outer mitochondrial membrane. This action leads to the formation of membrane channels and consequently the release of cytochrome C which results in the activation of the effector proteins of the apoptotic cascade (Grosse et al, 1999).

BCL-2, an inhibitor of apoptosis, did not increase and remained constant at all doses of irradiation. This could present an opportunity for future work investigating the role of the BCL-2 family of proteins and their interaction with other molecules and effectors in radiation induced apoptosis. These experiments should investigate the relationship with all apoptotic proteins and should also include an analysis of the up-regulation and expression of genes involved in these processes.

4.3: Limitations and future studies

This study of 42 normal donors has shown that increased levels of apoptosis in healthy individuals at high doses of irradiation could be linked to Bax protein up-regulation rather than BCL-2 expression. This is a significant finding, as most studies have investigated patients undergoing treatment for cancer rather than healthy individuals (Kokawa et al, 1999; Ohno et al, 1998 and Guida et al, 2016). It is clear from these articles however that cells are able to alter their cellular activity after radiation insult

and therefore future studies examining protein expression both prior to irradiation and at a variety of time points up until 48 hours post irradiation could be useful.

In a previous study by Solomon, donors of African descent showed an increased apoptotic response compared to donors of mixed descent and Caucasian origin (Solomon, 2009). However the low number of African descent donors recruited in the current study did not allow statistical analysis to be performed between different population groups. Although male donors demonstrated higher levels of radiation induced apoptosis when compared to females, this was not significant and therefore the results of Solomon (2009) could not be confirmed. . The reason for these differing results could be the low numbers of participants (42) in our study compared to the 300 in the work done by Solomon (Solomon, 2009).

A further limitation of this study was that it was not possible to categorize donors as sensitive or resistant using the z-score due to the low numbers of participants. The aim of future work would be to increase the number of donors and compare the expression of BCL-2 and BAX between those with a high and those with a low z-score.

4.4: Conclusion

In an attempt to explore the role of BCL-2 and Bax protein expression in individual radiation sensitivity, the current study used flow cytometry and western blotting techniques to quantify cell death and to investigate the expression of BCL-2 and Bax proteins in lymphocytes after exposure to gamma radiation. The study showed a significant dose dependent radiation induced apoptosis in lymphocytes. There was no correlation between BCL-2 expression and radiation induced apoptosis at all doses and no correlation between Bax expression and apoptosis at 0Gy and 2Gy. This finding

suggests the presence of alternative cellular regulatory mechanisms at low doses of radiation.

The current study did however demonstrate a significant increase in the expression of the pro-apoptotic protein Bax after 8Gy radiation exposure. This finding strongly suggests that the Bax protein may play an important role in radiation induced apoptosis particularly at higher doses. In conclusion, these results imply that Bax protein expression, but not BCL-2 expression may play a role in radiation induced apoptosis.

CHAPTER 5: REFERENCES

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Appendix A: Ethics certificates

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)

Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
•Tel: +27 21 959 6352 • Fax +27 21 953 8490
Email: danielso@cput.ac.za

2 May 2013
CPUT/HW-REC 2013/H27

Faculty of Health and Wellness Sciences
Biomedical Sciences Department

Dear Mr Nkosikho Sogwagwa

APPLICATION TO THE HW-REC FOR ETHICAL CLEARANCE

Approval was granted on 24 April 2013 by the Health and Wellness Sciences-REC to Nkosikho Sogwagwa for your application. This approval is for research activities related to an MTech: Biomedical Technology at this institution. This ethics approval is subject to the student supplying the supervisor with a letter of consent from the research facility. The student may not start the research before this approval letter has been received.

TITLE: The role of *Bcl-2* and *Bax* gene expression on individual radiosensitivity

Internal Supervisor: **Dr G Davison**
Internal Co-supervisors: **Mrs W Solomon & Prof S Khan**

Comment:
Approval will not extend beyond 27 June 2014. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

Note:
The investigator(s) should understand the conditions under which they are authorized to carry out this study and they should be compliant to these conditions. **It is required that the investigator(s) complete an annual progress report that should be submitted to the HW-REC in December of that particular year, for the HW-REC to be kept informed of the progress and of any problems you may encounter.**

Kind Regards

Zuleika Nortje
CHAIRPERSON – ETHICS RESEARCH COMMITTEE
FACULTY OF HEALTH AND WELLNESS SCIENCES

Cape Peninsula
University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

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06 October 2014
CPUT/HW-REC 2014/H09

Faculty of Health and Wellness Sciences – Biomedical Sciences Department

Dear Mr Sogwagwa

YOUR APPLICATION TO THE HW-REC FOR EXTENSION

Approval was granted by the Health and Wellness Sciences-REC on 02 October 2014 to Mr. Nkosikho Sogwagwa for ethical clearance. This approval is for research activities related to your MTech Biomedical Technology at CPUT.

TITLE: The role of BCL-2 and BAX gene expression on individual radiosensitivity

SUPERVISOR: Dr. GM Davison
CO-SUPERVISER: Prof. S Khan

Comment:
Approval will not extend beyond 07 October 2015. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the conditions under which they are authorized to carry out this study and they should be compliant to these conditions. **It is required that the investigator(s) complete an annual progress report that should be submitted to the HW-REC in December of that particular year, for the HW-REC to be kept informed of the progress and of any problems you may encounter.**

Kind Regards

MR. NAVINDHRA NAIDOO
CHAIRPERSON – ETHICS RESEARCH COMMITTEE
FACULTY OF HEALTH AND WELLNESS SCIENCES

Appendix B: Participant information sheet

The role of Bcl-2 and Bax protein expression on individual radiosensitivity

Dear Sir /Madam,

You are hereby asked to participate in a study to determine an individual's response to radiation exposure. The study is based on measuring the apoptotic (cell death) response of individual following exposure of your blood to radiation.At this stage there has been no way to predict the sensitivity to radiation and the potential long term side effects which differ from patient to patient. Tests that will be utilized within this study may prove valuable in measuring the radiosensitivity of the individual patient. It may also allow doctors to better predict and adapt the dose of radiation for future cancer patients according to the results obtained. In order to conduct this study we need 1× 4ml heparinized and 3 × 6ml EDTA blood samples taken at the time of your routine blood donation. This will be a once off requirement. If needed, the specimen and data from this study could be used to further the investigation into individual radiosensitivity.

Voluntary participation

Participation in this study is entirely 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 (Ref: CPUT/HW-REC 2013/H27). Information regarding your age, gender and ethnic/race group may be 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 principle investigator.

Thank you for your valuable participation.

Mr N. Sogwagwa

Tel: 021 9596902

e-mail : SogwagwaN@cput.ac.za

CONSENT FORM

The role of Bcl-2 and Bax protein expression on individual radiosensitivity

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 4 ml heparinised and three 6 ml EDTA blood samples to be taken during my routine blood donation for inclusion in the above-mentioned 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 Phlebotomist …………………………………………………

Appendix C: Western Blot Optimization

1: Western blotting

To explore the role of Bcl-2 and Bax expression on individual's sensitivity to radiation, lymphocytes were isolated from blood samples of 25 donors from western province blood transfusion service clinics as explained in **2.2**, exposed to deferent doses of radiation (0Gy, 2Gy and 8Gy). Western blot analysis was employed on lymphocyte lysate to compare protein expression levels of treated and control samples. Total protein concentration on each sample was quantified using Bradford protein assay (see **2.5.2**). Western Blot analysis was then performed using lysates volume adjusted for a total protein concentration of 20 µg.

The western blot images were developed using UVTEC alliance imaging system (UVTEC-Cambridge). Protein bands were identified at 23kDa as Bax, 26kDa as Bcl-2 and 37kDa as GAPDH (figure 3.9). The first lane of each sample (0Gy) was used as a reference point to which the relative change of the other lanes (2Gy and 8Gy) was compered to. This process was calculated for Bcl-2, BAX then GAPDH to obtain relative density for each treated sample. The relative density of Bcl-2 and Bax was divided by relative density of GAPDH for the same dose to calculate the ratio for each dose compared to GAPDH, the loading control.

Donors with relative expression between the 25th and 75th percentiles were selected for comparison. The data was analysed with statistical package software STATISTICA, one-way ANOVA test with a Tukey post-hoc test was used with a P-value ≤ 0.05 considered to be statistically significant.

2: Validation of the Western Blotting technique

To ensure optimal protein transfer from the SDS-PAGE to PVDF membrane, two methods were used namely PageBlue™ Protein Staining and Ponceau S staining. PageBlue™ Protein Staining Solution (fermentas, cat. R0571) was used to check how much protein was left on the gel after transfer onto the PVDF membrane and to check if gel ran successfully. Illustrated in Figure 1 is a picture of SDS-PAGE gel stained with PageBlue™ Protein Staining Solution after protein transfer step. Two black lines were drawn over the approximate area where GAPDH, Bcl-2 and Bax proteins would be. It

was noticed that there were no protein bands remaining in this area, however some bands remaining higher up in the gel can be seen. This is due to the fact that during the transfer high molecular weight proteins transfer slower thus remains in the gel.

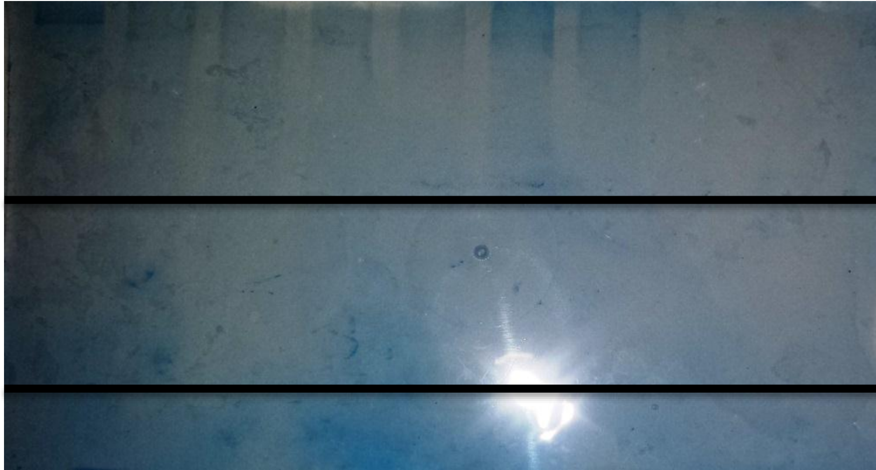
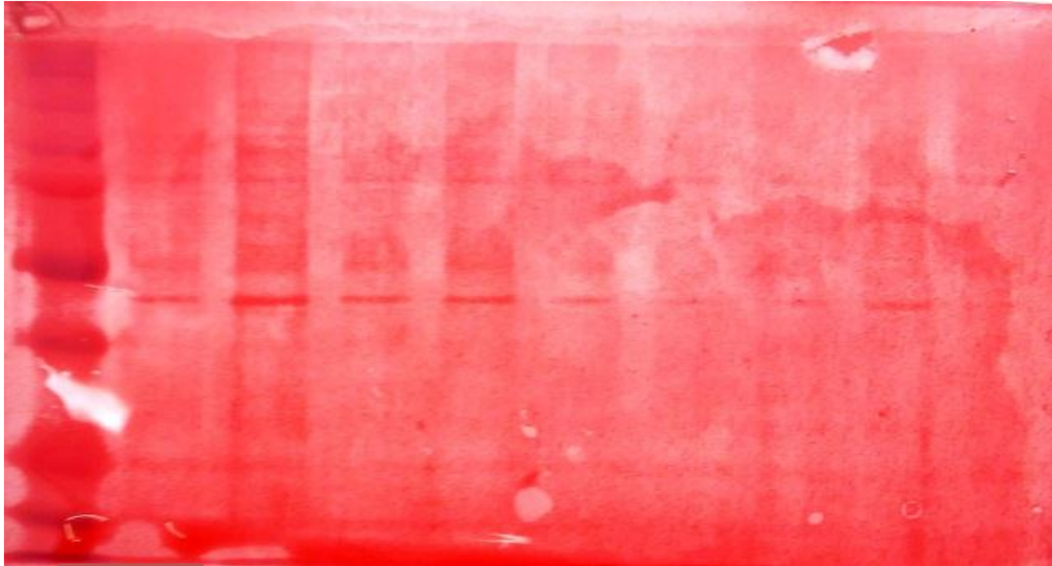


Figure 1: SDS-PAGE gel stained with PageBlue™ Protein Staining Solution after protein transfer step.

To verify protein transfer from SDS-PAGE to the PVDF membrane, two PVDF membranes were used in each transfer. Figure 2a illustrate PVDF membrane which was in direct contact with the gel stained with Ponceau S, red bands are proteins transferred from SDS-PAGE and indicate that transfer was successful. In Figure 2b is an image of a second PVDF membrane which was placed behind the first one, to check if there are proteins transferring through the first membrane. It is clear that protein transfer protocol adequately transferred target proteins from the SDS-PAGE to the first PVDF membrane not beyond that.



(a)

(b)

Figure 2: Two PVDF membranes were used in each protein transfer.