Chromosomal Radiosensitivity in South African Breast Cancer Patients Before and After Radiotherapy

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Declaration

I, Xanthene Eleonore Muller declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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(Signature of candidate)

_____ day of _____ , 2012

To my parents, sisters and my loving husband. Thank you for all your support!

Thank you Lord for walking with me on this trying road.

Publications and presentations arising from this thesis

Poster presentations:

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Muller, X., Papadopoulos, A., Mabhengu, T., van der Merwe, D., Cairns, A., Willem, P., Vral, A., Slabbert, J., Baeyens, A. Chromosomal Radiosensitivity in South African breast cancer population. The Molecular Biosciences Research Trust retreat. WITS, Johannesburg, November 2010.

Muller, X., Herd, O., Mabhengu, T., Cairns, A., Willem, P., Beukes, P., Vral, A., Slabbert, J., Baeyens, A. Chromosomal Radiosensitivity in South African breast cancer patients before and after radiotherapy. The Joint International Conference of the African and Southern African Societies of Human Genetics. Cape Town, March 2011.

Muller, X., Herd, O., Mabhengu, T., Cairns, A., Willem, P., Beukes, P., Vral, A., Slabbert, J., Baeyens, A. Chromosomal Radiosensitivity in South African breast cancer patients before and after radiotherapy. The 14th International Congress of Radiation Research. Warsaw, Poland; August 2011.

Oral presentation:

Muller, X. Radiosensitivity of breast cancer patients in South Africa. South African Association for Physics in Medicine and Biology. Johannesburg, August 2011.

Abstract

Introduction: Radiosensitivity (RS) of South African women with breast cancer was investigated as it has been studied in European women, but to date this has not been studied in South African women. The micronucleus assay was used to determine the amount of DNA damage on lymphocytes of breast cancer patients.

Materials and Methods: The first component to this study involved the collection of blood samples from breast cancer patients and healthy individuals. For the second component, blood samples from breast cancer patients were collected before and after the completion of radiotherapy (RT). A centromeric micronucleus assay using the Fluorescent *in situ* Hybridisation (FISH) pancentromeric probe was used to investigate the origin of the micronuclei (MN) and to distinguish between radiation-induced [centromere negative (CM-)] and spontaneous [centromere positive (CM+)] MN.

Results: Micronucleus frequencies were slightly higher in breast cancer patients than those observed in lymphocytes of healthy donors. This was noted for the different radiation doses and indicated a trend towards an enhanced chromosomal radiosensitivity in this cancer population. Results were compared before and after radiotherapy. The micronucleus scores for the 0 Gy (sham irradiated samples) were significantly higher ($p < 0.05$) post radiotherapy. This is an expected result as ionising radiation causes more damage. However, blood samples from post-therapy patients, were shown to have fewer MN after subsequent *in vitro* 2 Gy and 4 Gy irradiation respectively. When assessing the centromeric micronucleus assay results, a significantly ($p < 0.05$) higher number of CM- MN was observed than CM+ MN after RT, thereby indicating that ionising radiation causes more breaks in the chromosomes (clastogenic damage).

Discussion and Conclusion: This study demonstrates that a group of South African breast cancer patients have slightly higher micronucleus frequencies compared to a population of healthy women, indicating a trend towards a higher sensitivity to radiation.

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I would firstly like to thank my supervisor, Dr Ans Baeyens, for all her support, guidance and patience throughout these past 2 years. I really appreciate the fact that you were always available to help me – even on those busy days.

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β	beta
^{0}C	degrees celsius
%	percentage
bp	base pair
dH_2O	distilled water
Gy	Gray
L	litre
µg	microgram
µl	microlitre
µM	micromolar
ml	millilitre
mM	millimolar
M	molar
ng	nanogram
ng/µl	nanograms per microlitre
rpms	revolutions per minute
U	Unit
U/µl	unit per microlitre

List of abbreviations

AJCC	American Joint Committee of Cancer
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
BAX	Bcl-2–associated X protein
BC	Breast Cancer
BN	Binucleated
BSA	Bovine Serum Albumin
CDK	Cyclin-dependent kinase
Chk 1 / 2	Checkpoint Homolog 1 / 2
CM-	Centromere negative
CM+	Centromere positive
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CT	Chemotherapy
Cyto B	Cytochalasin B
DAPI	4', 6-diamidino-2-phenylindole

DCIS	Ductal Carcinoma *in situ*
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dsb	Double strand break
dsbs	Double strand breaks
EDTA	Ethylenediaminetetra Acetic Acid
FCS/ FBS	Foetal Calf/Bovine Serum
FISH	Fluorescent *in situ* Hybridisation
HR	Homologous recombination
IDC	Invasive / Infiltrating Ductal Carcinoma
ILC	Invasive / Infiltrating Lobular Carcinoma
IR	Ionising radiation
KCl	Potassium Chloride
$MgCl_2$	Magnesium Chloride
MN	Micronuclei
MV	Megavolts

$NaAc_3$	Sodium Acetate
NaCl	Sodium Chloride
NCR	National Cancer Registry
NHEJ	Non-homologous end joining
p53	Protein 53
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
Rad	Role of radiation protein
RNA	Ribonucleic acid
RPA	Replication protein A
RS	Radiosensitivity
RT	Radiation therapy/ Radiotherapy
SDS	Sodium dodecyl sulphate
ssbs	Single stranded breaks
SSC	Sodium Saline Citrate
TBE	Tris base, Boric Acid, EDTA
TNM	Tumour-Node-Metastasis

1. Introduction

We compared chromosomal radiosensitivity (RS) in a cohort of South African breast cancer (BC) patients with healthy (cancer free) participants by assessing their lymphocytic responses to radiation by using the micronucleus assay. To date this has not been studied on the genetically diverse South African population before. The effects of various factors on micronucleus frequencies, such as the clinical and social data of BC patients, were also investigated. Amongst patients at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), it has been observed that South African BC patients react differently to radiation, compared to European BC patients. We therefore investigated the micronucleus frequencies of BC patients before and after their therapy.

1.1 Radiobiology

Radiobiology is the study of the sequence of events that occur in an organism following the absorption of energy from ionising radiation (IR). Radiation is described as any process in which energy emitted by one body travels through a medium or space, ultimately to be absorbed by another body (LaTorre Travis, 2000). IR consists of subatomic particles or electromagnetic waves that have enough energy to detach electrons from atoms or molecules and ionise them and is frequently used in several medical and industrial applications (Joiner, van der Kogel & Steel, 2009). The important characteristic of IR is the localised release of large amounts of energy, which can break a strong chemical bond (Hall & Giaccia, 2012). Examples of IR are illustrated in figure 1.1.

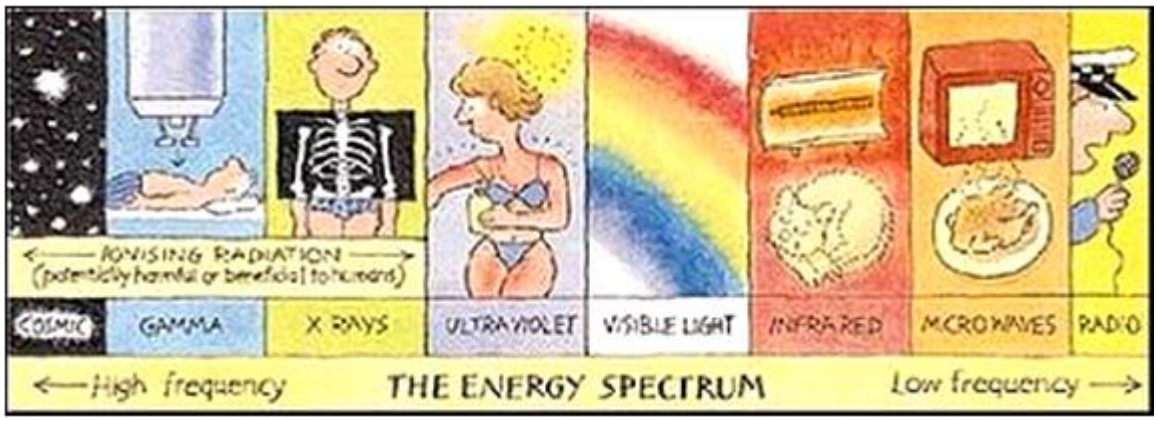


Figure 1.1: Examples of IR are gamma rays, X-rays and cosmic rays (World Health Organisation).

When ionising radiation interacts with a cell, it can either affect it directly or indirectly (Figure 1.2). Direct action occurs when an ionising particle interacts with, and is absorbed by, a biologic macromolecule (eg: DNA, RNA, proteins) within the cell. This absorption of energy results in ionisation and therefore damage of the biologic macromolecule. Indirect action occurs when the energy is absorbed through water, the medium surrounding the biologic macromolecules within the cell, resulting in the ionisation of water. This in turn induces chemical reactions such as ion pairs (H^+, OH^-) and free radicals ($H^\cdot$, $OH^\cdot$), which causes damage to the biologic macromolecules (LaTorre Travis, 2000).

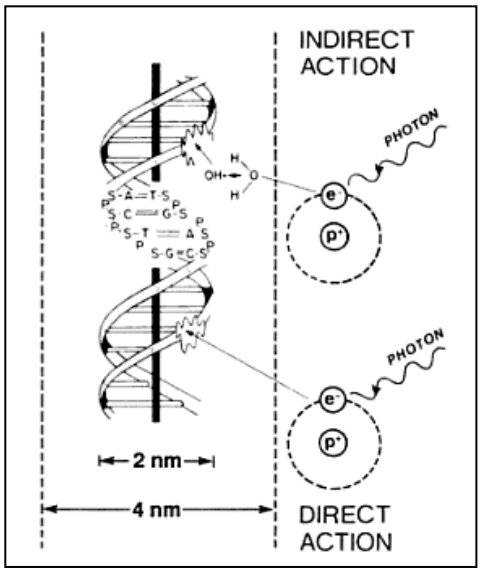


Figure 1.2: The two types of radiation damage on DNA: Direct action - direct damage to the biologic macromolecule; and Indirect action - damage to the biologic macromolecule through the ionisation of water (Hall & Giaccia, 2012).

1.1.1 DNA damage and repair

DNA is the most sensitive cellular target to radiation due to the fact that it carries genetic information and regulates all cellular activities, consequently having the most deleterious effect on the cell if it is damaged.

DNA radiation damage can be divided into 4 categories (LaTorre Travis, 2000):

1. Base damage: The loss or change of a base on the DNA strand.
2. Single-stranded breaks (ssbs): Breaks in one of the DNA strands.
3. Double-strand breaks (dsbs): Breaks in both backbones of the DNA strand.
4. Crosslinking: The production of crosslinks between two complementary strands or different DNA molecules.

Dsbs are the most significant type as they cause information to be lost in both DNA strands and can result in misrepairs and chromosomal aberrations, which in turn can lead to mutations or cell death.

1.1.1.1 DNA damage response

The biological effects caused by ionising radiation which have been found to be phase dependant within the cell cycle, may result in DNA repair, programmed cell death or cell cycle arrest. IR causes dsbs which can result in the amplification, deletion or rearrangement of genes and therefore alter gene expression (Turnbill, Mirugaesu & Eeles, 2006).

The DNA damage response (DDR) is governed by a number of signal transduction pathways that mainly consist of two components: one which involves the sensing and reporting of damage and another which involves the recruitment of molecules to fix the damage or induce apoptosis (Figure 1.3). After the sensor proteins are activated, the two main protein kinases, namely Ataxia Telangiectasa (AT) Mutated (ATM) and ATM+Rad3 related (ATR), are activated in the transducer pathway (Zhou & Elledge, 2002; Houtgraaf, Versmissen, & van der Giessen, 2006). Next ATM and ATR phosphorylate the mediator proteins (eg. P53 binding protein), which are specific to the type of damage as well as the point in the cell cycle. In the effector pathway, the serine/ threonine protein kinases Chk 1 and Chk 2 ultimately determine the fate of the damaged cell. This is either by cell repair, DNA transcription, cell cycle arrest or apoptosis.

To initiate cell cycle arrest, Chk 1 and Chk 2 together with ATM and ATR act to reduce cyclin–dependent kinase (CDK). This inhibition slows down or arrests

cell–cycle progression at the G1-S, intra-S and G2-M cell cycle checkpoints (Jackson & Bartek, 2009). These events allow sufficient time for DNA damage to be assessed and fixed. If the damage is too complex, then DDR signalling triggers cell death by apoptosis or cellular senescence (Jackson & Bartek, 2009).

Cellular senescence is defined as the permanent arrest of a cell whereby it loses its ability to divide. When this process is elicited by cellular stresses, in this case ionising radiation, it is termed 'premature' senescence. This process is characterised by a flattened cytoplasm and increase in granularity or biochemically by an increase in senescence-associated β-galactosidase expression. These processes are all activated by various molecular pathways in the DDR system, which are not yet clearly understood (Wouters, 2009).

The intrinsic apoptotic pathway, which is activated following the initiation of caspase 9, is triggered as a result of damage that occurs within the cell. The activation of caspase 9 precedes a cascade of proteins in or near the mitochondria that are activated following extensive DNA damage. Proteins involved include p53, BAX and cytochrome *c* (Wouters, 2009).

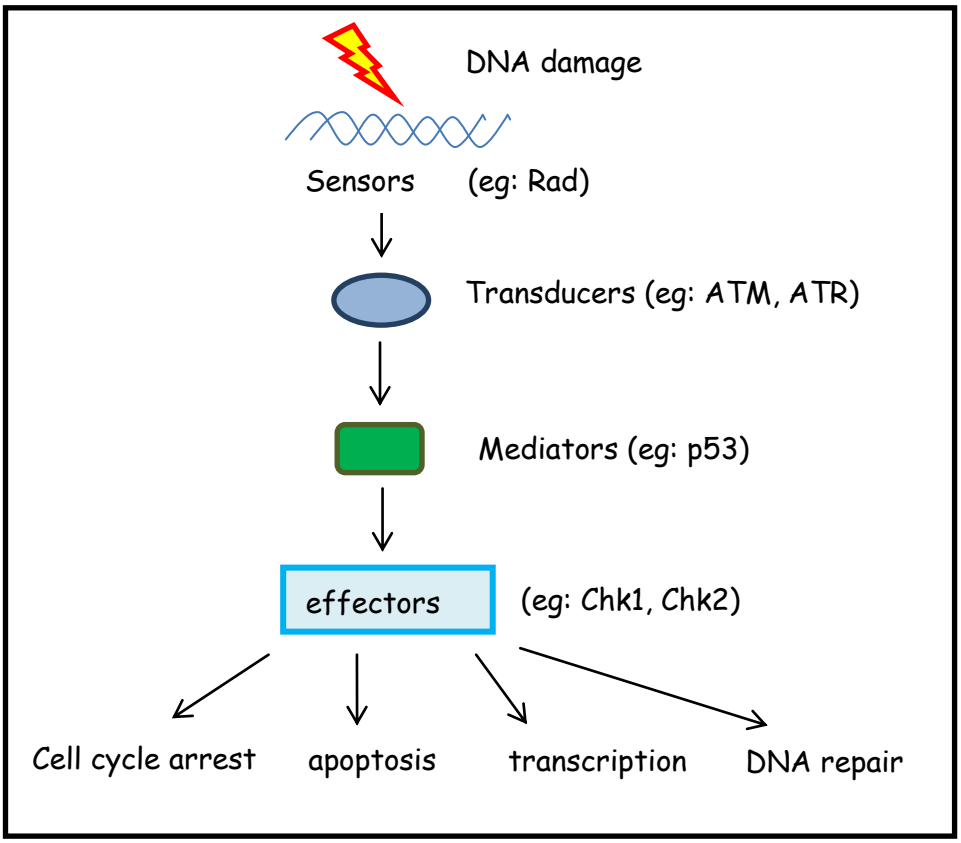


Figure 1.3: An outline of the DNA damage response signal-transduction pathway. This pathway consists of sensors, transducers, mediators and effectors. (Adapted from Zhou & Elledge, 2000).

1.1.1.2 DNA repair mechanisms

In the event of minor DNA damage, the DDR initiates cell repair. Various repair mechanism pathways are activated, which are in turn regulated by different genes. Damage caused by ionising radiation to the mammalian cell is repaired by 5 different repair mechanisms each depending on the type of damage done to the cell. These are base excision repair, nucleotide excision repair, mismatch repair, as well as homologous recombination and non-homologous end joining (Hoeijmakers, 2001) (Figure 1.4). Repair mechanisms help in the recovery process after radiotherapy (RT) and defective repair mechanisms can lead to radiosensitivity.

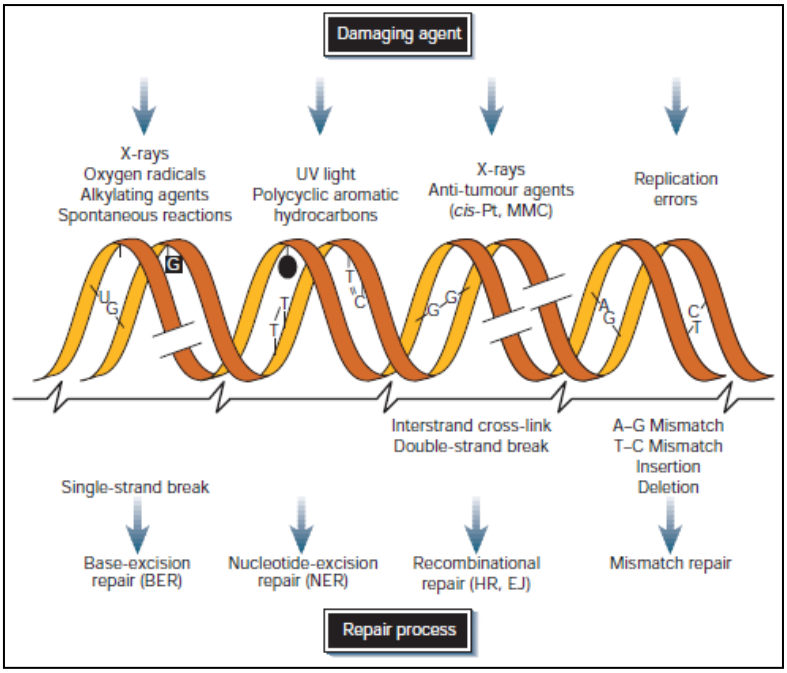


Figure 1.4: Different types of DNA repair mechanisms caused by ionising radiation and other agents (Hoeijmakers, 2001)

Double strand breaks are the most common type of radiation-induced damage. The two DNA repair mechanisms involved in repairing double strand breaks are non-homologous end joining and homologous recombination.

a) <u>Homologous recombination</u>

This process involves the use of homologous DNA sequences to repair the damaged double stranded DNA during the late S & G2 phase of the cell cycle (Figure 1.5). This repair process is mostly error free (Wouters & Begg, 2009). Each strand is cut back with an exonuclease or helicase to leave a 3' overhang from which the nucleotides will be added to, to form the new, repaired strand.

A nucleoprotein filament is formed through the polymerisation of the Rad 51 protein to the single-stranded DNA, which in turn searches for homologous DNA. In conjunction with Rad 51, other proteins such as Rad 52, Rad 54, Rad

55 / 57 and the single-strand DNA-binding proteins, replication protein A (RPA), are involved in the action of joining the homologous strands by forming a joint molecule. The DNA is then synthesised with DNA polymerase and ligase, the crossed strands fixed with resolvase and the result is two intact DNA strands (Kanaar, Hoeijmakers & van Gent, 1998).

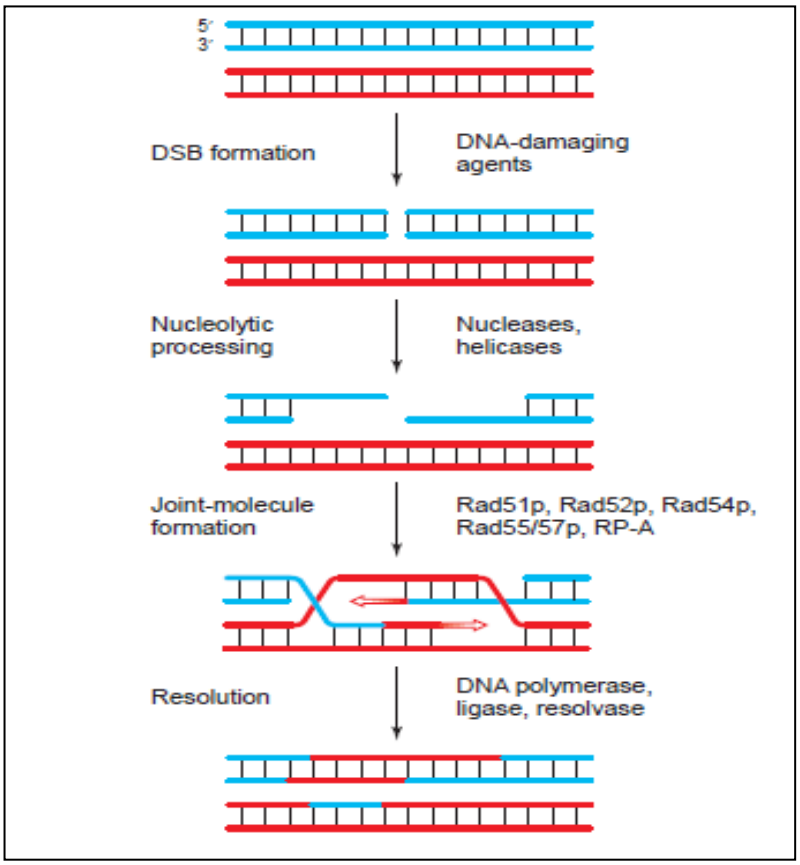


Figure 1.5: An overview of the process of Homologous Recombination where DNA is repaired using homologous strands (Kanaar, Hoeijmakers & van Gent, 1998).

b) Non-homologous end joining

With this process (illustrated in figure 1.6), which occurs during all stages of the cell cycle, two DNA strands are joined together without the need of a homologous template. This process is less accurate than HR and therefore the DNA strands will predominantly have more faults, which in turn can lead to mutations or apoptosis (Wouters & Begg, 2009). The KU heterodimer binds to the DNA ends and attracts DNA-PK_{CS}. This causes the phosphorylation of the DNA-PK_{CS} promoters, causing a structural change in the complex. The ends are processed by the RAD 50, MRE 11 and NBS 1 complex and lastly the DNA ligase IV XRCC4 complex re-joins the strands (Kanaar, Hoeijmakers & van Gent, 1998).

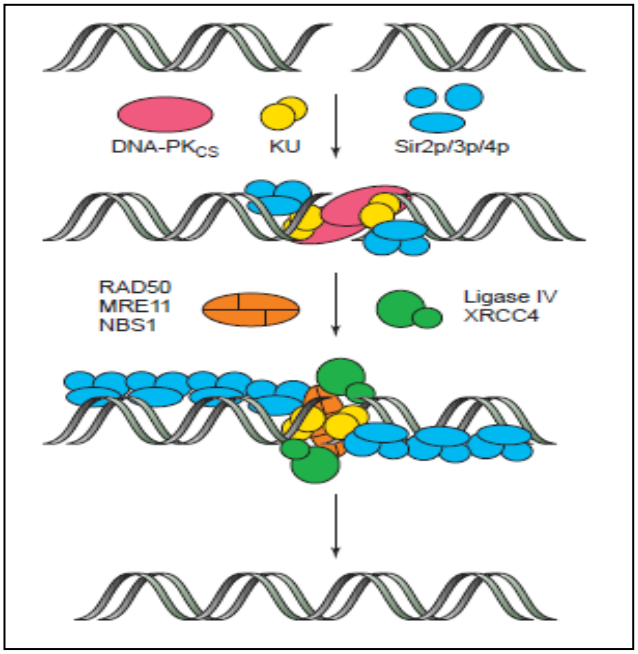


Figure 1.6: The process of NHEJ involves the use of various proteins to bind the two fragmented DNA strands together (Kanaar, Hoeijmakers & van Gent, 1998).

1.1.2 Chromosomal aberrations

Double and single strand breaks can lead to chromosomal aberrations. The process of how DNA breaks convert into visible chromosomal aberrations is still unclear. Different hypotheses are described in literature. The chromosomal aberrations can be divided in 2 groups: Chromosome and chromatid aberrations (Bryant, 1998; Bryant, Riches & Terry, 2010) (Figure 1.7), which include (LaTorre Travis, 2000):

Acentric fragments – fragments of chromosomes that do not contain centromeres.

Dicentrics – when the ends of two chromosomes that each contain a centromere join together.

Translocations – the transfer of a segment of one chromosome to another chromosome.

Deletions – the loss of a whole segment of a chromosome in one or both arms.

Inversions – the reversal and re-annealing of the same fragment. In this process the fragment is reversed and re-annealed to the chromosome at the same breakage site.

Rings – occur when the fragmented arms of the chromosome bind together, forming a ring.

Chromatid exchange – An exchange of segments between the sister chromatids of a chromosome.

Chromatid breaks – Breaks in one or both of the sister chromatids

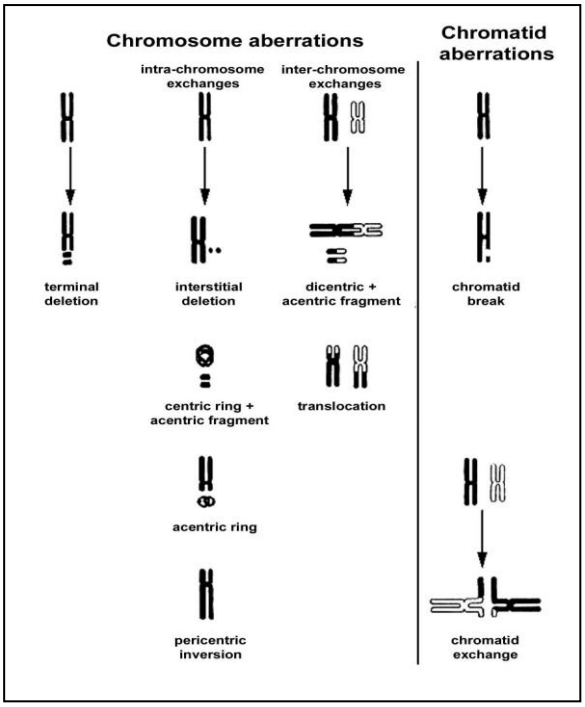


Figure 1.7: The different types of chromosomal aberrations, which include acentric fragments, dicentrics, translocations and ring formations (Tubiana & Dutreix, 1990).

1.1.3 Chromosomal radiosensitivity

Enhanced chromosomal RS was initially described in patients with inherited cancer prone syndromes such as Ataxia telangiectasia, Fanconi's anaemia and Nijmegen breakage syndrome (Sanford, et al., 1989). Later it became apparent that enhanced chromosomal RS is also present in significant proportions of patients with different cancers, such as head and neck, colorectal, prostate, cervix and lung cancer (Jones, et al., 1995; Parshad, et al., 1996; Riches, et al., 2001). Several studies confirmed chromosomal RS to be present in breast cancer patients (Scott, et al., 1994, 1998, 1999; Baeyens, et al., 2002). However, none of these studies were done on an African population.

Various studies have demonstrated that an increase in chromosomal radiosensitivity has been associated with an increase of late side effects of radiation therapy (West, et al., 2001; Hoeller, et al., 2003; De Ruyck, et al.,

2005). It was noted that due to these late side effects resulting from RT, the dose level and therefore a chance of cure is limited (Borgmann, et al., 2008). For this reason, RS biodosimetry may be a good early predictor for patient specific late side effects.

1.1.4 Mechanisms leading to radiosensitivity

Several articles reviewed by Scott et al., (1999) suggest that defects in the repair mechanisms of radiation-induced DNA damage could lead to cancer predisposition and led to the idea that chromosomal aberration assays may be used as biomarkers of cancer risk.

Various mechanisms have been found to contribute to RS. Some include defects in DNA repair pathways and cell cycle checkpoint control (Parshad, Sanford & Jones, 1983). Scott, et al. (1999) suggested that reasons for defective DNA repair pathways could be due to mutations of the downstream genes involved in the detection and/or regulation of DNA damage. Defective apoptotic pathways could also contribute to RS (Baeyens, 2005).

1.1.5 Cytogenetic testing

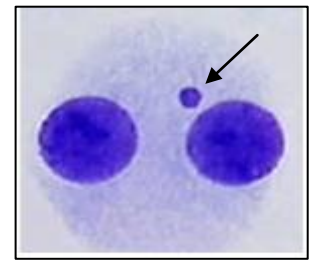
Cytogenetic testing involves the study of cell structure, especially human chromosome function and structure. This is done by assessing chromosomal aberrations that may have been caused by genetic or environmental factors (Darroudi, 2008). It is usually carried out using blood or bone marrow samples. Cytogenetic tests are also used for radiation biodosimetry and radiosensitivity studies. The different cytogenetic assays used in radiobiology include the G2 Assay, Micronucleus assay, Dicentrics assay, Comet assay and the Foci assay.

a) The Cytokinesis-block micronucleus (CBMN) assay

One of the assays to detect chromosomal aberrations is the Cytokinesis-block micronucleus assay. Micronuclei (MN) are small nuclear fragments that lag behind after nuclear division. MN can either occur spontaneously, through aneugenic events, whereby whole chromosomes lag behind during cell division or through clastogenic events, caused by agents such as ionising radiation, during which chromosomal breaks are created. These fragments or whole chromosomes are covered with a nuclear envelope at telophase, giving the micronucleus its structure (Fenech, 2000) (Figure 1.8).

To stimulate the latent lymphocytes to divide, Phytohaemagglutinin (PHA) is added. As MN can be seen in dividing cells and are usually lost after division, the cytoplasmic inhibitor Cytochalasin B (Cyto B) is added. This inhibits cellular division, resulting in a binucleated (BN) cell (Fenech, 2000). Also, it allows to discriminate between cells which have undergone nuclear division and cells which have not. The number of MN following radiation is a marker of radiation-

induced DNA damage. The micronucleus assay is a good biological dosimeter for radiation (Norppa & Falck, 2003).



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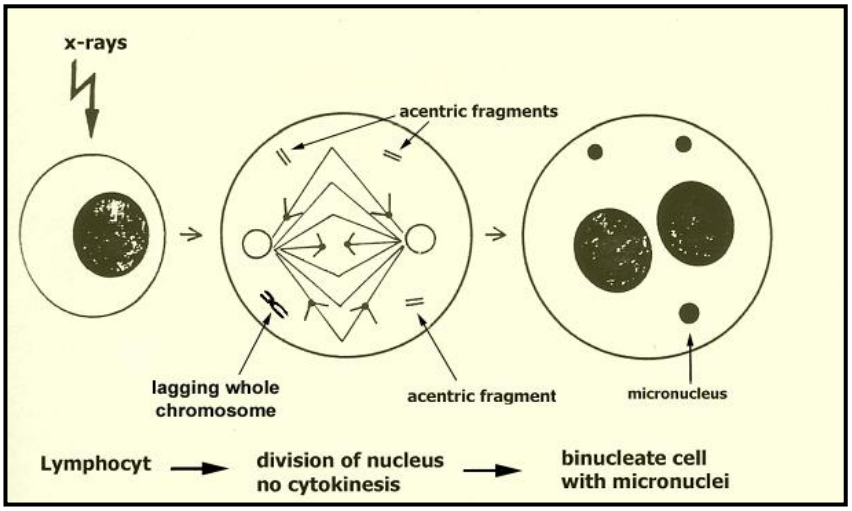


b

Figure 1.8: a) A BN cell containing a micronucleus. b) The process of micronucleus formation. (Baeyens, 2005).

The advantages of using the micronucleus assay include its practicality, the allowance for taking the proportion of cell division into account as well as the ability to assess chromosome breakage or loss by examination of acentric fragments or whole chromosomes (Baeyens, et al., 2002; Varga, et al., 2004; Baeyens, 2005). The main disadvantage of the micronucleus assay is the high variability in spontaneous micronucleus frequency.

Various factors have been discovered that could influence the number of MN produced. These include: age, sex (Thierens, et al., 2000), HIV status (Baeyens, et al., 2010), ethnicity (Amend, Hicks & Ambrosone, 2006), clastogenic medication, smoking and pre- or post-menopausal status (Dhillon & Dhillon, 1995; Ahmad, et al., 2000).

An automated microscopic system controlled by the Metafer 4 software program MetaSystems, which consists of a Zeis Axio Imager microscope with a camera attached, connected to a computer, can be used for automatic scoring of MN. The microscope has a motorised scanning stage able to hold 8 slides. The system scans the slide and takes pictures of each BN cell it finds as well as any micronucleus within the cytoplasmic boundary surrounding the nuclei, as specified on the system. These pictures are stored in a gallery on the computer for subsequent viewing (Schunck, et al., 2004).

b) The Micronucleus-centromere assay

The sensitivity of the micronucleus assay is restricted to a dose of 0.2 Gy. This is due to the high and variable spontaneous MN (Vral, Thierens & De Ridder, 1997). These high variable numbers of MN are mostly due to lagging whole chromosomes (Thierens, Vral & De Ridder, 1996). Radiation-induced MN will mainly be a result of clastogenic damage, consisting of acentric fragments that are centromere negative (CM-), while spontaneous MN will be centromere positive (CM+) (Thierens & Vral, 2009).

The combination of the Fluorescent *In Situ* Hybridisation (FISH) technique with a pancentromeric probe allows for the detection of the presence of a centromere in MN and allows discrimination between background MN and radiation-induced MN (Figure 1.9) Studies have shown spontaneous MN increase with age. This can be completely attributed to CM+ MN (aneugenic damage) reflecting an increased chromosome loss with age (Thierens, Vral & De Ridder, 1996).

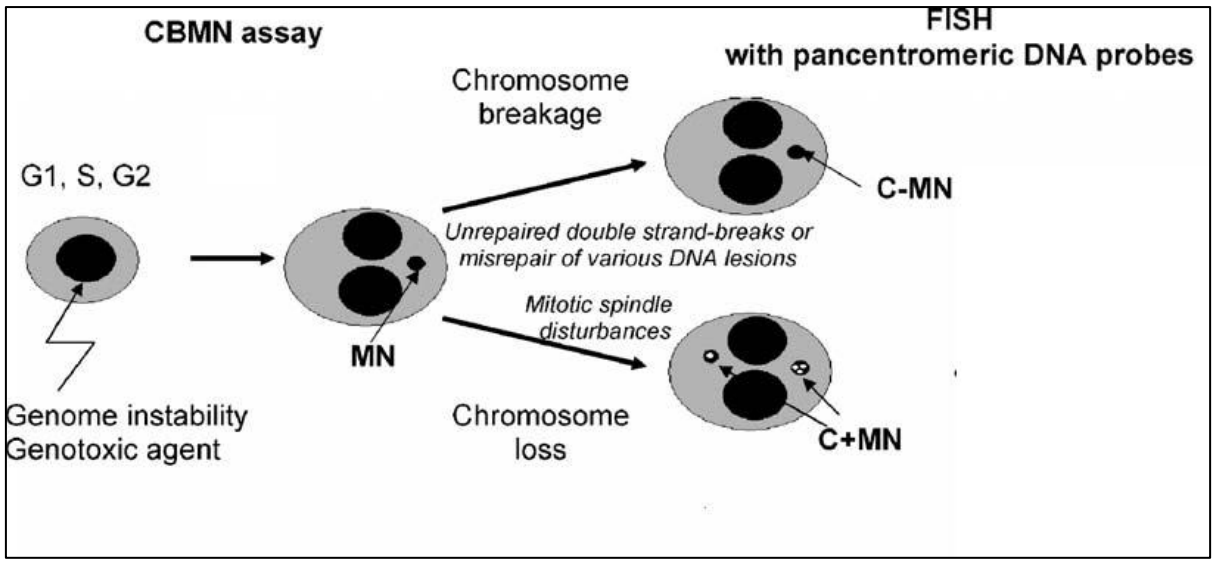
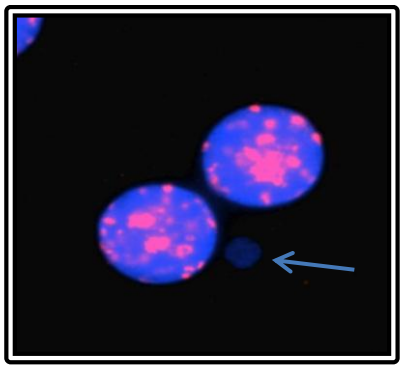


Figure 1.9: Using the FISH pancentromeric probe, centromere positive (CM+) MN can be distinguished from centromere negative (CM-) MN by looking for the positive fluorescent signals (Iarmarcovai, Botta & Orsiére, 2006).

The pancentromeric probe hybridises to all the centromeres of all the chromosomes in the main nuclei, as well as to any micronucleus containing centromeres (Figure 1.10).

This specific probe is commercially available (known as STAR FISH, SR Biosystem ©). However, for research purposes, an in-house probe can be made using the nick-translation method, which is more cost effective.

a) 

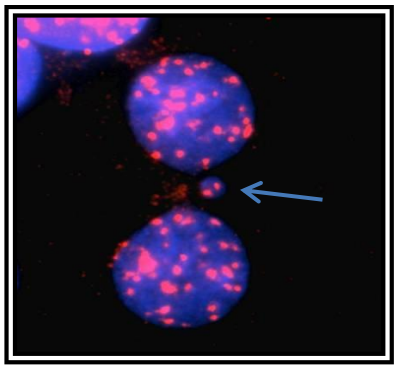
b) 

Figure 1.10: a) Binucleated cell with a centromere negative micronucleus;

b) Binucleated cell with a centromere positive micronucleus with 2 signals. Both MN are indicated by an arrow (Vral, Fenech & Thierens, 2011).

1.2 Breast Cancer

1.2.1 Background

Breast cancer is the leading cancer in females and accounts for approximately 18 % of all cancer cases in women worldwide (American Cancer Society, 2011). In South Africa, 1 in 29 women are diagnosed with BC each year. The 2000-2001 National Cancer Registry (NCR) Report shows that women have a lifetime risk of 1 in 8 of getting breast cancer (NCR, 2004).

The breast is made up of roughly 18 lobules of glandular tissue, which in turn consists of alveoli. These lobules of glandular tissue are surrounded by fat tissue, giving breasts their shape (Figure 1.11). Alveoli consist of cells that line the duct and are responsible for milk production. Breast cancer is a malignant tumour that originates from the cells lining the ducts or lobules of the breast. It can invade nearby breast tissue and metastasise through the lymph nodes to the rest of the body (Kopans, 2007).

Symptoms of breast cancer include (Jenkin, 2008):

- Lumps, dimples or swelling in the breast
- Nipple discharge, an inverted nipple or pain around the nipple.
- Tenderness of the breast
- Sudden prominent superficial veins.

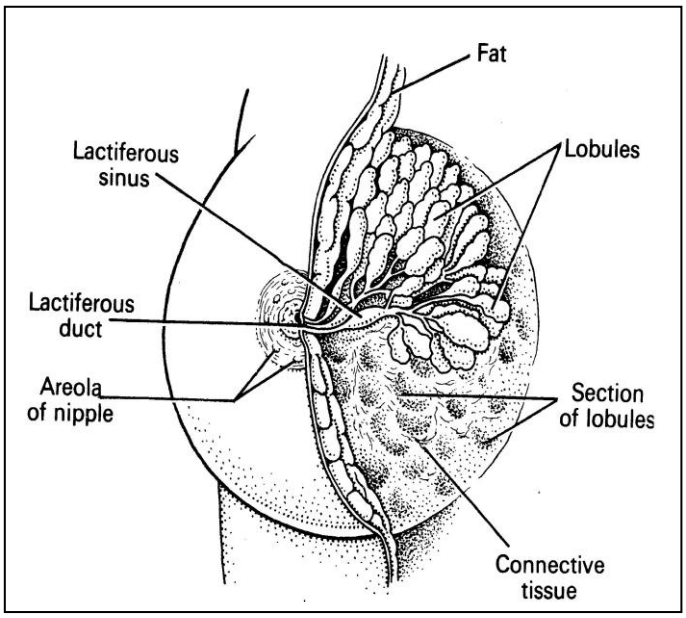


Figure 1.11: Anatomy of the breast (Ross & Wilson, 1981).

1.2.2 Risk factors

Risk factors that may influence the onset of breast cancer include gender, age, ethnicity, age at menarche (Constantino, et al., 1999) genetic risk factors, environmental factors, dense breast tissue, hormone replacement therapy and hormonal changes (menstrual cycle and menopause) (Song, Lee & Kang, 2010). Some other risks include obesity, alcohol consumption, diet, physical activity, oral contraceptives, nulliparity and late first full-term pregnancy (Chlebowski, et al., 2005; Song, Lee & Kang, 2010).

Breast Cancer genes 1 and 2 (*BRCA* 1 & 2) are two of the main genes involved in dsb repair by HR, transcriptional regulation (Barwell, et al., 2007), DNA recombination and cell-cycle checkpoint control and are classified as tumour suppressor genes (Tutt & Ashworth, 2002). Mutations of these genes lead to truncated proteins and missense mutations inactivating their products which results in the misrepair of dsbs. In the general population group, 3 - 5 % will

have one of these mutated BRCA genes (Gerhardus, et al., 2007) and women who have these mutated BRCA genes have up to an 85 % chance of getting breast cancer by the age of 70 years (Tutt & Ashworth, 2002).

1.2.3 Classification of tumours

BC can be classified into several types such as Lobular Carcinoma *in situ* (LCIS), Ductal Carcinoma *in situ* (DCIS), Infiltrating Ductal Carcinoma (IDC), Infiltrating Lobular Carcinoma (ILC) and Paget's disease of the nipple (Fraker, 2004). If it is non-invasive, the cancer stays in the milk ducts or lobules of the breast (its originating point). If it is invasive / infiltrating, the cancer has metastasised past its originating point into the surrounding breast tissues. Most breast cancers are the latter. In this study, the following breast cancer types were most commonly seen:

Infiltrating/ Invasive Ductal Carcinoma (IDC) - This is the most common type of breast cancer, accounting for approximately 80 % of all breast cancer cases. It is defined as cancer that has spread to the surrounding breast tissues through the milk ducts from where it originates (Fraker, 2004).

Infiltrating/ Invasive Lobular Carcinoma (ILC) -This is the second most common type of breast cancer and occurs in 10 % of breast cancer cases. ILC is diagnosed when the cancer has spread from the lobules to the rest of the breast tissues (Fraker, 2004).

Ductal Carcinoma *in situ* (DCIS) – DCIS is a non-invasive neoplastic proliferation of epithelial cells confined to the ductal-lobular system. This type accounts for approximately 10 % of all cases (Wiechmann & Keurer, 2008).

1.2.4 TNM Staging of tumours

In 2001 the Breast Task Force revised the American Joint Committee of Cancer's (AJCC) staging system for breast carcinoma. Consequently the tumour-node-metastasis (TNM) system was officially adopted in 2003. It is a system used to provide more details on the characteristics of the tumour. This is done by evaluating the extent of the primary tumor (T), regional lymph nodes (N), and distant metastases (M) and provides a ''stage grouping'' based on T, N, and M. The system is frequently updated due to technology always being upgraded (Edge, et al., 2010). The summary of the TNM system is shown in the table 1.1 below.

Table 1.1: Adaptation of the recent TNM stage grouping for BC from the AJCC (Edge, et al., 2010)

The final TNM stage grouping for BC			
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0	N1mi	M0
	T1	N1mi	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

1.3 Radiosensitivity and Breast Cancer

1.3.1 Radiotherapy

RT is the medical use of ionising radiation as part of cancer treatment to control local malignant cells (LaTorre Travis, 2000). The aim of radiotherapy is to inactivate the tumour cells while minimising damage to the normal surrounding cells. The difficulty of this mode of treatment involves deciding on the most appropriate dose and fractionation scheme because patients vary considerably in their tumour and normal tissue responses (Borgmann, et al., 2008).

For most patients at CMJAH radiation is delivered externally using high energy X-ray beams, which has an energy value measured in megavolts (MV). The international system of units of absorbed dose given to a patient is the gray (Gy) (World Health Organisation).

In some cases, depending on the characteristics of the tumour, RT can be given in combination with other treatments such as surgery and chemotherapy. RT can be given before or after the other treatments. At CMJAH, it is often given after surgery and chemotherapy. Neoadjuvant chemotherapy is given when the patient is diagnosed at an advanced stage (stages III and IV) and used for systemic control while RT is used to achieve local control of the breast tumour post-surgery.

1.3.2 Clinical radiosensitivity

Patients who display more side effects following RT may be classified as clinically radiosensitive. Side effects of radiation therapy can be acute or late effects. Acute RT effects include skin reactions, dyspnoea, difficulty swallowing, fatigue, nausea or loss of appetite. Mental side effects include insomnia, depression or concentration difficulties.

Telangiectasia, hyperpigmentation, ulceration, swelling of the soft tissue (edema) in the breast and or arm (Barber, et al., 2000; Yi, et al., 2009), change in appearance and or shrinkage of the breast, difficulty in raising or moving the affected arm are all late effects that occur about 90 days post treatment (Sjovall, et al., 2009).

1.4 Aim

This project was divided into two components. The first was to compare the chromosomal radiosensitivity between a group of breast cancer patients and healthy (cancer free) participants in a South African population.

The second component of the study was to assess chromosomal radiosensitivity in BC patients before and after completion of radiation treatment.

1.5 Research objectives

1. Compare chromosomal radiosensitivity in South African breast cancer patients with healthy (cancer free) individuals, using the micronucleus assay.
2. Correlate chromosomal radiosensitivity with clinical parameters, such as the tumour pathology and social parameters, such as age and ethnicity.
3. Compare the micronucleus frequency in breast cancer patients before and after radiotherapy.
4. Investigate the origin of MN seen in breast cancer patients before and after radiation therapy using the Micronucleus-centromere assay.

2. Materials and Methods

2.1 Sample collection

Heparinised blood samples of breast cancer patients were collected at the CMJAH. A total of 97 samples were collected: 64 were patient samples and 33 were from healthy individuals. The healthy control samples were obtained from student and staff members of the University of the Witwatersrand (WITS) Medical School and the CMJAH Radiation Oncology Department. The age range was between 28-82 years (mean = 56) for the BC patients and 21-62 (mean = 31) for the healthy individuals. Age matching of patients and controls was attempted but not achieved due to the reduced age of staff members used primarily as controls.

For the first component of the study, the radiosensitivity of 40 pre-surgery breast cancer patients, who had not received chemotherapy, was compared with that of 33 healthy individuals, who comprised the control group. Samples were collected at CMJAH and analysis was performed. For the second component of the study, radiosensitivity was compared between patients before RT and after completion of RT. Samples were collected and processed (MN Assay) at CMJAH and analysis was performed at a collaborative laboratory in the Department of Radiobiology, Ghent University in Belgium. In total, 24 patient samples were collected before and after the completion of their treatment. At the time of sampling, 8 of these patients had not had chemotherapy before, while the remaining 16 BC patients had.

Written informed consent was obtained from each volunteer. Social data such as age group, ethnicity, gender, smoking habits and monthly income was

obtained from each volunteer, by using a questionnaire that they completed. Clinical information on the tumour pathology, medication and treatments (radio- and/or chemotherapy) was obtained from the patient files.

The study was approved by the Human Research Ethics Committee, WITS; ethics number: M10372 (see Appendix).

2.2 Methods

2.2.1 The G0 Micronucleus assay

Heparinised blood (0.5 ml), (left at room temperature for at least 30 minutes to allow for mixing of blood with anti-coagulants), and pre-warmed complete RPMI medium (4.5 ml) (see Appendix) were added to a sterile culture flask. The blood-medium mixture was either irradiated or sham irradiated. For irradiation, the culture flasks were placed in a Phantom-water tank. The distance from the culture flasks to the radiation source was 100 cm at an angle of 90 degrees. The field size was 10:10 and the Energy Value 6 MV using the Linac X-ray machine (Siemens). Each sample was irradiated with 3 different doses and each dose was done in duplicate (6 cultures). Samples were irradiated at either 2 Gray (Gy) or 4 Gy respectively at a dose rate of approximately 1.33 Gy / min. A 0 Gy dose was used as a sham-irradiated control. The radiations were done at the Medical Physics Department in the Radiation Oncology Unit at CMJAH.

The dose of 2 Gy was chosen since this is the dose given per fraction during conventional radiotherapy. To improve the sensitivity of the test when comparing radiosensitivity of patients with healthy donors, a dose of 4 Gy was given as this yield a higher level of induced chromosomal aberrations.

Immediately after irradiating the samples, 100 µl (1M/ml) PHA was added to the blood to stimulate the lymphocytes to divide and incubated at 37 ^{o}C, 5% CO_2. To inhibit cytokinesis, 20 µl (1.5 µg/ml) Cyto B was added to the cultures 23 hours later which were incubated further. The cells were harvested 70 hours after adding PHA, with a cold (4^{o}C) hypotonic shock using 7 ml 0.075M KCl (see Appendix). This was done by transferring the culture to a 15 ml centrifuge tube and centrifuging for 8 minutes at 1000 rpms. The supernatant was taken off and KCl added slowly to the culture while vortexing. After centrifuging for another 8 minutes at 1000 rpms, the supernatant was discarded. The cells were fixed in methanol: acetic acid: Ringer solution (4:1:5) (see Appendix) to maintain the structure of the cells. The tubes with cells were stored overnight at 4 ^{o}C.

The next day, the cells were fixed again with a methanol: acetic acid (4:1) wash solution to increase the mechanical strength and stability of the cells. This step was repeated 2 - 3 times until the pellet was cleaned of remaining impurities. For slide preparation, the pellet was re-suspended in a few microlitres of methanol: acetic acid wash solution and 40 µl of the cell suspension dropped on a clean slide and allowed to spread across the slide.

a) For automatic scoring, a drop of DAPI was placed on a slide and covered with a cover slip. The cells were then scanned by the automated microscope from MetaSystems using the DAPI filter and a 10 x objective. With DAPI, only the nuclei were stained blue. Slides were scanned at iThemba LABS in Cape Town.

b) For manual scoring, in the second component of our study, the cells were stained with Acridine Orange. The slides were placed in the Acridine Orange work solution (see Appendix) in a Coplin jar for 1 minute, rinsed in distilled water and then placed in the Acridine Orange buffer in another Coplin jar (see Appendix) for another minute. The slides were then removed from the Coplin jar and about 20 µl of the Acridine Orange buffer was dropped onto the slides.

   To prevent the cells from drying, cover slips were placed on top of the slides and rubber cement placed around the edge of the cover slips to seal it.

   The slides were scored using the DAPI triple filter with a 20 x objective using a Zeis Axioskop microscope. Acridine orange stained the nuclei green and the cytoplasm orange.

Scoring criteria

For manual scoring, the criteria of Fenech, et al., (2003) were followed:

Only separate binucleated cells with nuclei in the same cytoplasmic boundary were counted (Figure 2.1 A). Overlapping nuclei had to be visibly separated by their nucleic boundary (Figure 2.1 B). The nuclei had to be approximately the same size and had to have a similar staining pattern and intensity. If the nuclei were joined by neoplasmic bridges (Figure 2.1 C, D), they could still technically be scored as binucleates, but in this study, they were excluded.

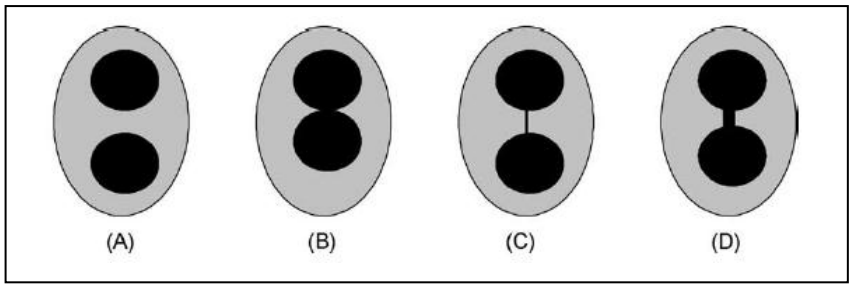


Figure 2.1: Different types of BN cells that can be scored (A - D). C & D contain neoplastic bridges that were excluded in this study (Fenech, et al., 2003).

The scoring criteria for the MN, which are usually round or oval, required that it be separate from the nuclei. If the micronucleus overlapped with the nuclei, a clear boundary of the micronucleus needed to be visible. MN could be between 1 / 16 to 1 / 3 the size of the main nuclei and each cell could contain one or more micronucleus. The staining intensity of the micronucleus had to be equal or greater than that of the main nuclei (Figure 2.2) (Fenech, et al., 2003).

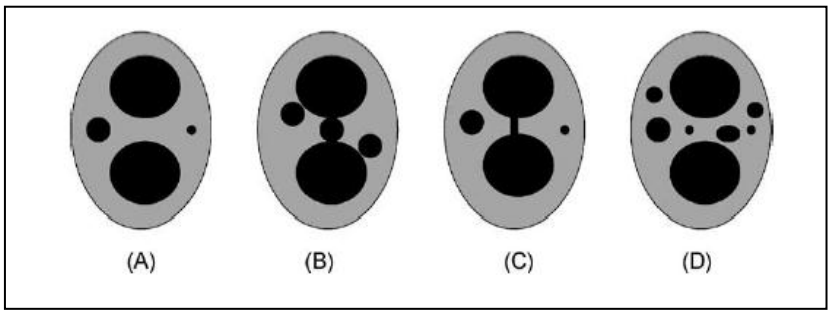


Figure 2.2: Different types of BN cells containing viable MN varying in sizes between a 1/16 to 1/3 of the main nuclei (A - D) (Fenech, et al., 2003).

For automatic scoring, the same criteria used for manual scoring were applied. The Metafer 4 software program (MetaSystems), which has an automated BN and MN scoring module (MNScore) was used. The software program has specific classifiers used to scan the slides and find BN cells containing similar sized nuclei. An additional classifier was also applied to search for MN with a

specific size, within a specified circular area surrounding the two nuclei (Schunck, et al., 2004). Images of each BN cell were taken using a digital camera attached to the microscope and placed in a gallery. Associated MN numbers were automatically recorded for each image, thus allowing a scorer to perform manual checks to ensure quality control.

Table 2.1: Classifier settings used for the Metafer software program to score BN cells and MN:

Classifier name: Ans MN Nov 2011

Nuclei			
Object threshold	15 %	Maximum distance	25 µm
Minimum area	80.00 µm^2	Max area asymmetry	80 %
Maximum area	1000.00 µm^2	Region of interest radius	30 µm
Max reference concavity depth	0.160	Max object area in ROI	35 µm^2
Max aspect ratio	1.370		
Imaging Processing Operations: Sharpen (3,4)			

Micronucleus			
Imaging Operations: Median V (3) Median H (3) Average (3,1) Sharpen (5,5)			
Object threshold	7 %	Max reference concavity depth	0.500
Minimum area	1.00 µm^2	Max aspect ratio	1.700
Maximum area	40.00 µm^2	Maximum distance	30 µm

AutoSeperate	
A – Concavity regression radius	10/10 µm
B – Concavity min contour angle	45 ^{0}
C – Min concavity distance	40 %

2.2.2 The Micronucleus-centromere assay

The micronucleus assay combined with the pancentromeric probe allows the differentiation between background MN and MN induced by radiation. For this study, the FISH pancentromeric probe was made using an optimised version of the nick translation method.

2.2.2.1 The pancentromeric probe production

1. DNA extraction

A blood sample was collected from a male donor in an EDTA tube in order to obtain sequences from both the X and Y chromosomes with the purpose of amplifying the centromeric region. DNA was extracted using the QIAamp mini DNA blood kit (QIAgen), in which DNA is absorbed onto a silica-gel membrane, which is washed to remove contaminants, and the DNA released from the column using an elution buffer. The manufacturer's protocol was followed:

The EDTA tubes were centrifuged at 1500 rpms for 10 minutes. Approximately 400 µl buffy coat was transferred into a 1.5 ml microcentrifuge tube containing 40 µl QIAGEN proteinase K. To this, 400 µl of buffer AL was added, mixed by vortexing for 15 seconds and then left to incubate for 10 minutes at 56 ^{0}C to lyse the cells. After incubation, 400 µl of 100 % ethanol was added and mixed by pulse vortexing.

The mixture was carefully applied to a QIAamp mini spin column and centrifuged at 8000 rpms for 1 minute. The collection tube was then discarded and the column placed in a new collection tube, to which 500 µl of wash buffer AW1 was added and the tube centrifuged again. The column was placed in a

new collection tube and 500 µl of wash buffer AW2 was added to the column and centrifuged at full speed (14000 rpms) for 3 minutes.

The column was placed in another collection tube and spun for 1 minute to remove any residual buffer. The column was then placed in a 1.5 ml Eppendorf tube and 200 µl of elution buffer was added to the column. This was left to incubate at room temperature for 1 minute and centrifuged. The concentration of the extracted DNA was then measured with the NanoDrop Spectrophotometer. The extracted DNA was stored at - 20 ^{0}C.

2. Polymerase Chain Reaction (PCR)

Centromeric DNA was amplified by PCR using primers that bind specifically to the centromeric DNA sequence. The whole procedure was done on ice.

Table 2.2: Reagent concentrations and volumes used for the PCR reaction. Adapted from Weier, et al., (1991)

Reagent	[Stock]	[Final]	Amount
DNA	X ng/µl*	250 ng	X µl *
Forward primer (Bioline) 5' –GAA GCT TAA CTC ACA GAG TTG AA -3'	100 µM	1.2 µM	1.2 µl
Reverse primer (Bioline) 5' -GCT GCA GAT CAC AAA GAA GTT TC -3'	100 µM	1.2 µM	1.2 µl
10x NH4 reaction buffer (Bioline)	10 x	1 x	10 µl
MgCl2 (Bioline)	10 mM	1.6 mM	16 µl
dNTP mix (Bioline)	10 mM	100 µM	1 µl
BioTaqpolymerase (Bioline)	5 U/µl	5 U	1 µl
dH20 (Sabax)			Make up to 100 µl
*DNA concentrations varied per sample		Total:	100 µl

PCR program: +95 ^{0}C, 10'

+96 ^{0}C, 1'
+45 ^{0}C, 1'
+72 ^{0}C, 1'
} 30 x

+72 ^{0}C, 5'

+4 ^{0}C, ∞

The PCR reaction was done using the Eppendorf Thermal Cycler.

To check if the PCR was successful, 5 µl of the PCR product was run on a 2 % agarose gel (see Appendix):

A 6x Orange DNA loading dye was mixed with the PCR product as well as with the O'GeneRuler 100 bp DNA ladder. The products were pipetted into separate wells and run at a potential of 90 V for about 30 minutes.

A successful PCR showed 2 bands with a monomeric fragment of 175 bp and a dimeric fragment of 345 bp when viewed under fluorescent light (Figure 2.3) (Weier, et al., 1991).

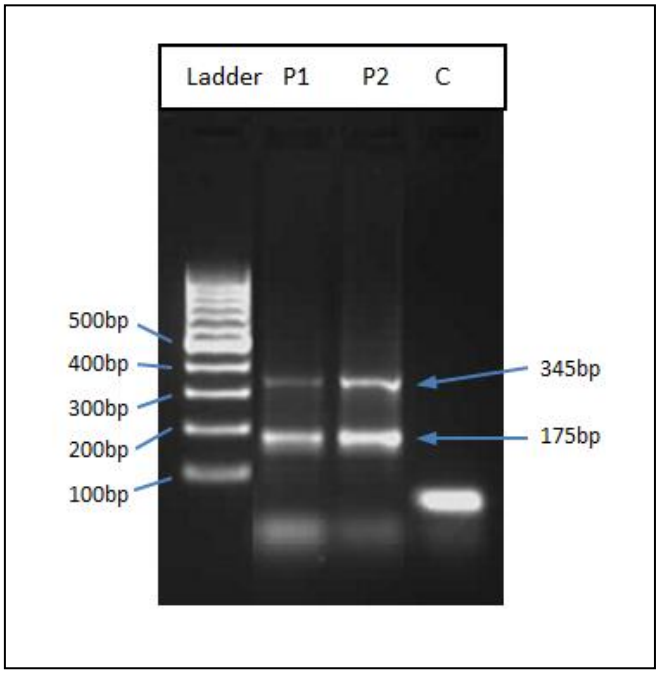


Figure 2.3: PCR showing centromeric sequenced products of 175 bp and 345 bp. P1 and P2 indicates the PCR products and C indicates the negative control. The bottom bands in all lanes indicate primer dimers.

3. PCR purification

The rest of the PCR product was purified, following a successful PCR reaction. This was done by using the Biospin PCR Purification Kit (BioFlux) and by following their protocol.

The rest of the purified PCR product was transferred to a 1.5 ml Eppendorf tube and twice the volume of binding buffer was added to the PCR product and briefly vortexed. This was then applied to a column and centrifuged for 1 minute at 7500 rpms. The flow through was discarded and the column was placed in a new collection tube. Next, 650 µl of wash buffer was added to the column and the column centrifuged for 60 seconds at 10600 rpms, the flow through discarded again and the step repeated. The column was placed in a new collection tube and centrifuged again for 1 minute at 10600 rpms. The column was then placed into a 1.5 ml Eppendorf tube and 50µl of dH_20 added to the column and incubated at room temperature for 1 minute. Thereafter, the tube was centrifuged for 1 minute at 10600 rpms and the concentration of the PCR product was checked using the NanoDrop Spectrophotometer.

4. Direct labelling of PCR product by nick translation method

The centromeric DNA was directly labelled using the nick translation method, in which the DNase 1 enzyme makes single stranded breaks in the double strands of DNA. Polymerase 1 then removes nucleotides and adds fluorescently labelled ones with its exonuclease and endonuclease activity, resulting in fluorescently labelled DNA fragments that can be hybridised to the region of interest.

Table 2.3: Reagent volumes for labelling method

Reagent	Volume
NT buffer (Bioline)	10 µl
β-mercaptoethanol (Sigma-Aldrich)	10 µl
dNTP mix (with spectrum orange; ENZO)	8 µl
DNase I (Bioline) (Working solution)	1 µl
DNA polymerase I (Bioline)	3 µl
DNA	1-2 µg
dH_20 (Sabax)	Make up to 100 µl
Total:	100 µl

*See Appendix for methods

The products were pipetted into a PCR tube and placed in a thermal cycler for 30 minutes at 15 ^{0}C and then immediately placed on ice.

To check the size of the probe, 8 µl was denatured using the Eppendorf Thermal Cycler at 96 ^{0}C for 3 minutes and then immediately placed on ice. The 8 µl of denatured probe was run on a 2 % agarose gel together with a 100 bp DNA ladder for about 30 minutes at 90 V. A smear of 200 – 500 bp was seen when viewed under fluorescent light (Figure 2.4). Fragments of this size are

small enough to penetrate the nuclear pore but large enough to avoid cross hybridisation during FISH.

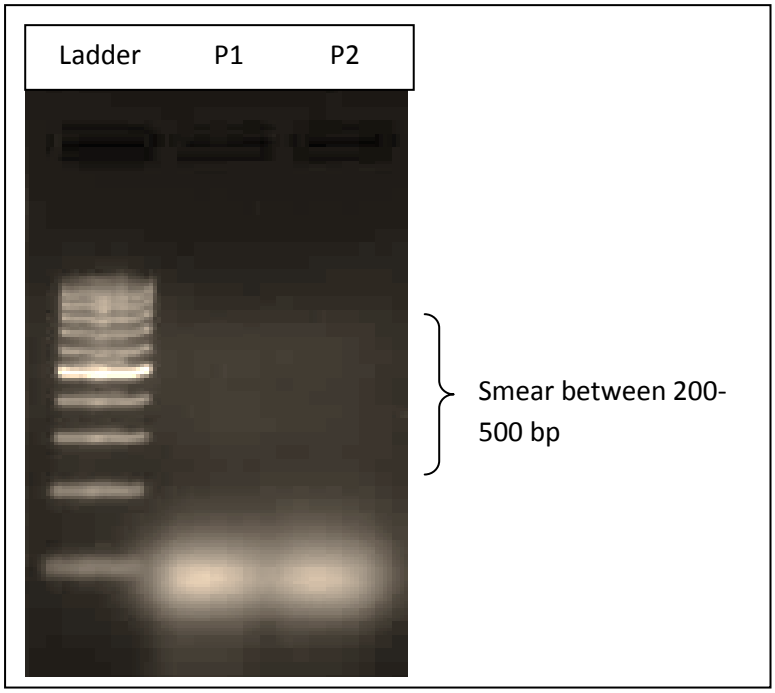


Figure 2.4: Direct labelling of the purified PCR products using nick translation, showing a smear between 200 – 500 bp. Bottom bands indicate unincorporated Spectrum Orange dUTPs

5. Probe precipitation

Enzymatic inactivation

The probe mixture was transferred to a 1.5 ml Eppendorf tube and 3 µl 0.5 M Ethylenediaminetetraacetic acid (EDTA) and 1 µl 10 % Sodium Dodecyl Sulfate (SDS) was added to the probe mixture and placed in a heating block at 65 ^{o}C for 15 minutes while kept in the dark. Then 2 µl of Herring sperm DNA (binds to repetitive DNA to reduce non-specific binding of the probe) was added to the mixture followed by 10 % total volume of probe mixture 3 M $NaAc_3$ and 2.5 X total volume of probe mixture ice cold 100 % ethanol and mixed well. The probe mixture was then stored at - 20 ^{0}C overnight.

The next day, the tube was removed from the freezer and spun at 13000 rpms for 30 minutes at 4 ^{o}C. The supernatant was poured off and 200 µl of ice cold 70 % ethanol was added to the tube to wash the pellet and centrifuged again for 13000 rpms for 10 minutes at 4 ^{0}C. The supernatant was poured off again and the pellet air dried for about 5 - 10 minutes while keeping it in the dark. The probe pellet was re-suspended in 60 µl of hybridisation buffer (see Appendix) and left on a shaker to allow the pellet to dissolve, after which it was stored at - 20 ^{0}C.

2.2.2.2 Fluorescent *in situ* Hybridisation (FISH) using the pancentromeric probe

Aging the slides

Before commencement of the FISH assay, the slides were first aged to remove excess fixative from the cells. This was done by placing the slides in a 70 % - 90 % - 100 % ethanol series for 5 minutes each in a Coplin jar and allowed to air-dry.

1. Hybridisation

The slides were denatured by placing them in the pre-heated denaturing solution (see Appendix) using a heat resistant Coplin jar, in a waterbath at 76 ^{0}C for 5 minutes. Next, the slides were hydrated by placing them in an ice cold ethanol series of 70 % - 90 % - 100 % for 5 minutes each while shaking. To denature the probe, 8 µl per slide was aliquoted into a 1.5 ml Eppendorf tube and placed in the waterbath at 76 ^{0}C for 5 minutes, keeping it in the dark. It was then placed on ice and placed aside (keeping it in the dark). Next, 8 µl of the probe was pipetted on a coverslip and the slide placed on top. It was then

sealed with rubber cement, placed in a beaker with moist tissue paper, covered with foil and then placed in an incubator at 37 ^{0}C overnight.

2. Washing the slides

After removing the rubber cement, the coverslips were removed by allowing the slides to soak in a 50 % formamide and 20 x SSC solution for about 5 minutes to loosen the coverslips. The slides were then washed in 3 different Coplin jars containing 50 % formamide and 20 x SSC solutions at 45 ^{0}C for 10 minutes each, followed by a 2 x SSC solution for another 10 minutes and lastly in a 2 x SSC + Tween solution for 5 minutes. The slides were counterstained by placing a drop of DAPI onto the slides and covered with a coverslip. The slides were viewed using the MetaSystems microscope.

Scoring criteria

The same criteria for finding BN cells were used for automatic scoring of the FISH pancentromeric probe. The scorer reviewed all the cells in the image gallery and selected only those BN cells with MN for re-scanning using the Metafer Classifier Autocapt. The TRITC filter was used for the purpose of viewing the fluorescent signals of the stained centromeres of both the nuclei and centromere-containing MN with a 40 x objective. Images of the MN were captured and stored in another image gallery for distinguishing between CM- and CM+ MN.

2.3 Statistical Analysis

GraphPad Prism 5 was used for statistical analysis. The Mann-Whitney and ANOVA one way tests were used at 95% confidence intervals.

The sample size of this pilot study was based on other similar studies done in European populations.

The Mann-Whitney test was used to compare micronucleus frequencies between the different groups of patients and healthy controls. This is a non-parametric, or distribution-free test that is suitable to compare groups with small sample sizes where no underlying distribution can be assumed.

The Wilcoxon test was used to compare the before and after RT micronucleus frequencies. This is a non-parametric test used to compare two related samples.

The ANOVA test was used to compare more than one clinical or social parameter such as the ethnicity groups or tumour staging with the micronucleus frequencies.

The coefficient of determination (R^2) was used to determine the strength of the correlation between the BC patients and the healthy controls and their age.

3. Results

For specific p values, see Appendix B

3.1 Micronucleus assay comparing the radiosensitivity of non-treated breast cancer patients to healthy individuals

For the first component of the study, the radiosensitivity of 40 pre-surgery breast cancer patients was compared with 33 healthy individuals, who comprised the control group. Not all the MN cultures set up from these 73 donors were successful. In 25 % of the breast cancer patients and 9 % of the healthy individuals, the yield of the BN cells was insufficient for microscopic analysis. Only those samples where 500 BN cells could be scored were included in the study. It should be noted that the number of BN cells scored was generally lower in the BC group than in the healthy individuals, due to patient medication or immunological conditions that could have influenced cell survival rates.

Figure 3.1 shows the average of the spontaneous MN per 1000 BN cells for both studied population groups. The mean spontaneous micronucleus count was significantly higher for the BC patients (33 MN/ 1000 BN) compared to the healthy individuals (20 MN/ 1000 BN) (Mann-Whitney, $p < 0.05$).

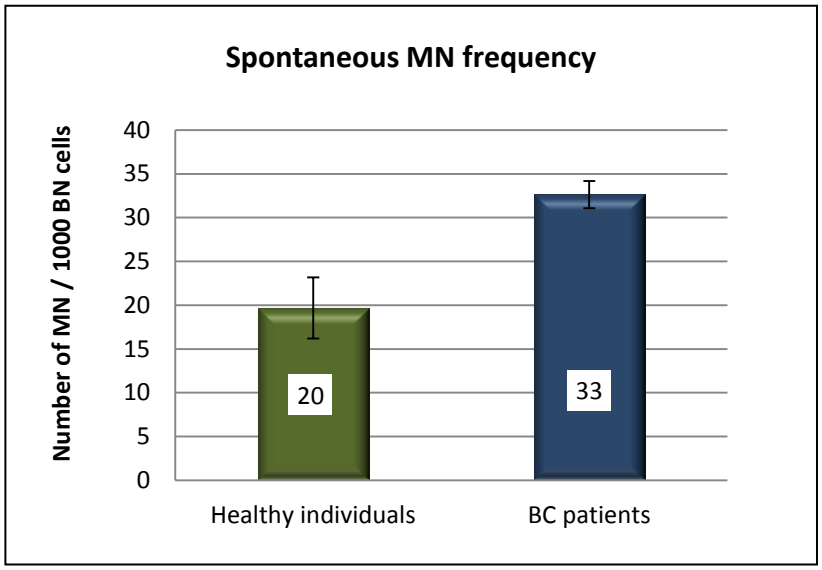


Figure 3.1: Mean of spontaneous micronucleus frequency of BC patients and the healthy control group (error bars =SEM). Values indicated = mean.

The radiation-induced micronucleus frequency was obtained by subtracting the spontaneous micronucleus frequency from the irradiated micronucleus frequency. No significant differences were noted when the radiation-induced micronucleus frequencies for the doses 2 Gy and 4 Gy were compared between BC patients [mean ± SEM (2 Gy: 193 ± 9; 4 Gy: 604 ± 30 MN/ 1000 BN)] and healthy individuals [mean ± SEM (2 Gy: 197 ± 9**;** 4 Gy: 575 ± 24 MN/ 1000 BN)] (Mann-Whitney, $p > 0.05$) (Figure 3.2). However, although not significant, the BC patients did show a slightly higher micronucleus frequency after 4 Gy.

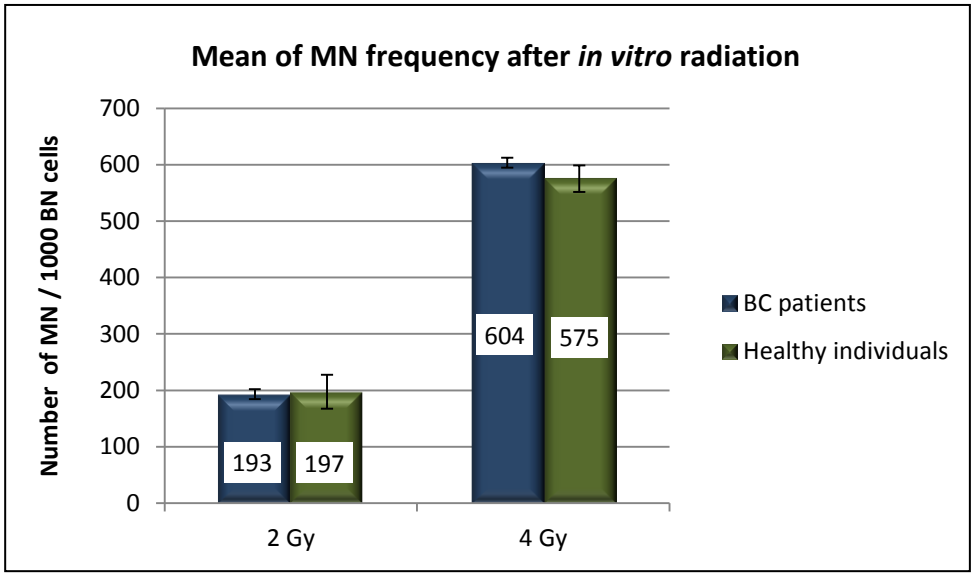


Figure 3.2: Mean of radiation-induced micronucleus frequency after 2 Gy and 4 Gy doses *in vitro*, when comparing BC patients with healthy individuals (error bars =SEM). Values indicated = mean.

To support the results observed in figure 3.2 where the BC patients were marginally more radiosensitive, the distribution of the 4 Gy micronucleus frequencies between the BC patients and healthy individuals were evaluated (Figure 3.3). No statistically significant difference was observed (Mann-Whitney, $p > 0.05$). It was observed that the micronucleus values of the BC patients lay in the higher micronucleus frequency range indicating a slight increase in total chromosomal damage caused by radiation.

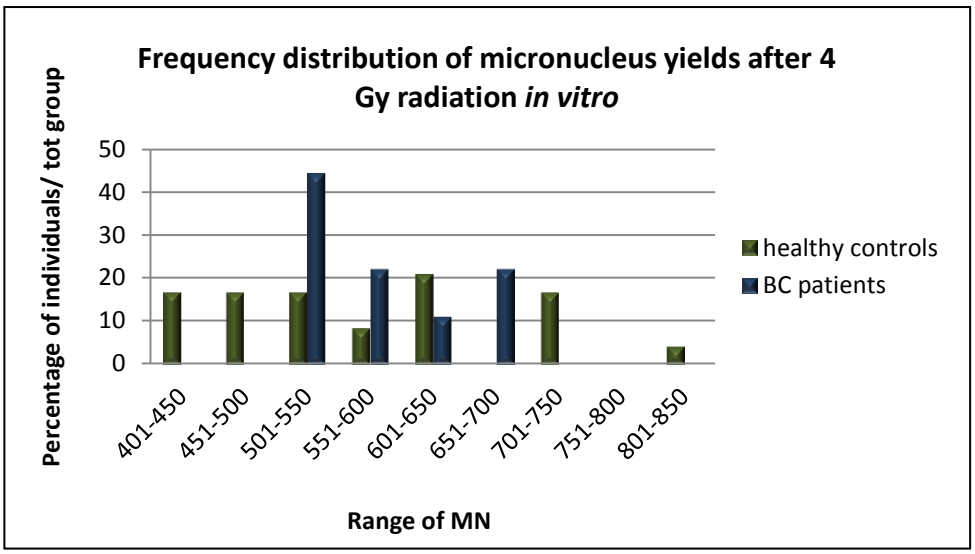


Figure 3.3: Distribution of the radiation-induced micronucleus frequency after 4 Gy of BC patients and healthy individuals.

3.2 Correlation between clinical parameters and chromosomal radiosensitivity

The age distribution of the BC patients used in this study is presented in figure 3.4. The majority of BC incidences in our study occurred between the ages of 41 and 80 years.

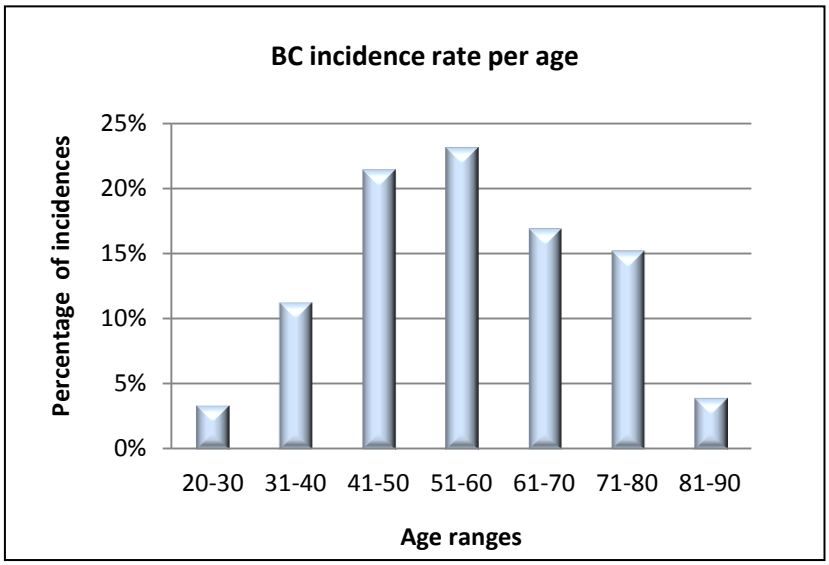


Figure 3.4: The BC incidence rate per age group of our study.

3.2.1 Correlation between spontaneous MN and age

No clear correlation between the spontaneous micronucleus frequency and age could be observed in the BC patients (R^2 [BC patients] = 0.13) or the healthy control group (R^2 [healthy controls] = 0.11) (Figure 3.5).

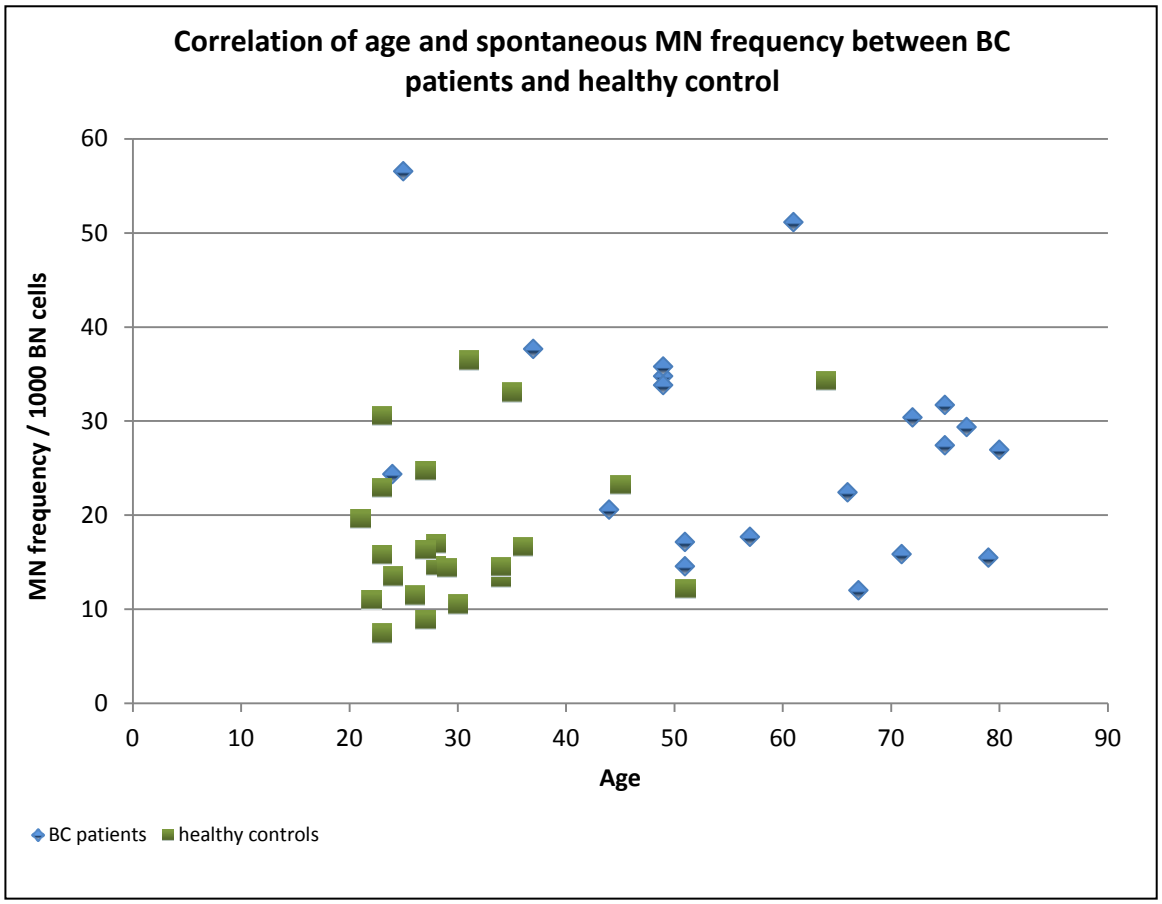


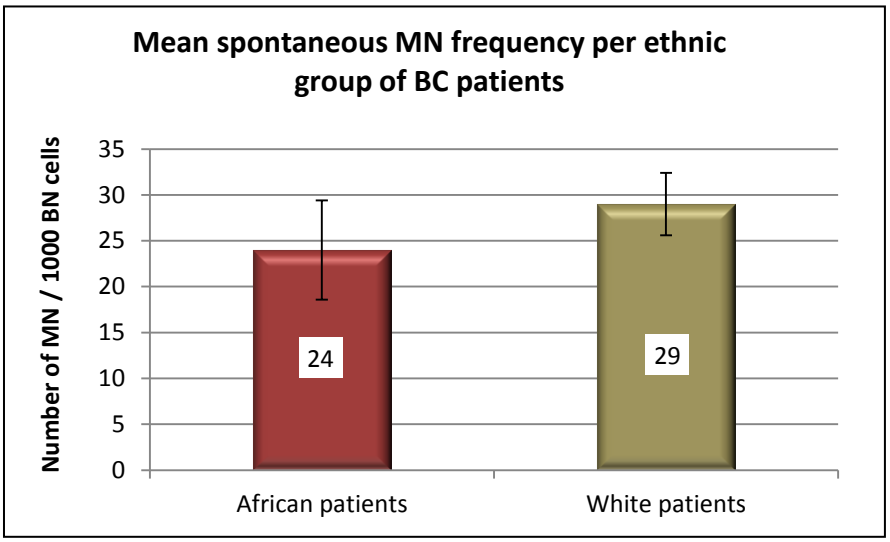
Figure 3.5: Correlation between the spontaneous micronucleus frequency and age in both BC patients and healthy controls.

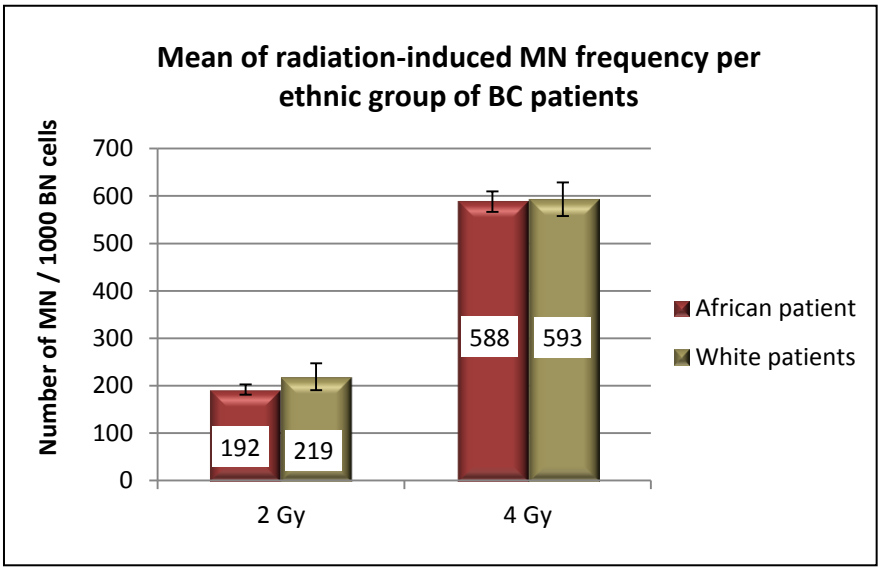
3.2.2 Association between spontaneous MN and ethnic group

The South African population is genetically very heterogeneous. The potential influence of ethnicity on the micronucleus frequency for both the BC patients and healthy participants was investigated. In the studied population group, most of the patients were either African (65 % of group) or white (24 % of group). The results showed that on average the white patients had a propensity towards a higher spontaneous micronucleus frequency [mean ± SEM (29 ± 3 versus 24 ± 5 MN/ 1000 BN)] (Figure 3.6 a) as well as a higher induced micronucleus frequency compared to the African patients [mean ± SEM (2 Gy: 219 ± 22 versus 192 ± 11**;** 4 Gy: 593 ± 35 versus 588 ± 29 MN/ 1000 BN)] (Figure 3.6 b), although the difference was not statistically significant (Mann-Whitney, $p > 0.05$).

Amongst the healthy control group, no significant differences in the spontaneous micronucleus frequency when correlating the ethnicity versus the micronucleus values were observed (Mann-Whitney, $p > 0.05$).

a





b

Figure 3.6: a) Mean of the spontaneous micronucleus frequency per ethnic group of BC patients.

b) Mean of the radiation-induced MN per ethnic group of BC patients (error bars =SEM). Values indicated = mean.

3.2.3 Association between spontaneous MN and menopause

The spontaneous micronucleus frequencies of pre- and post-menopausal patients were examined to determine if menopause may influence the micronucleus frequency (Pre (*n*) = 8; Post (*n*) = 32). It was noted that on average the post-menopausal patients had a lower spontaneous micronucleus yield (28 MN/ 1000 BN) than the pre-menopausal patients (37 MN/ 1000 BN) (Figure 3.7). However, this is not entirely conclusive due to the difference in group sizes.

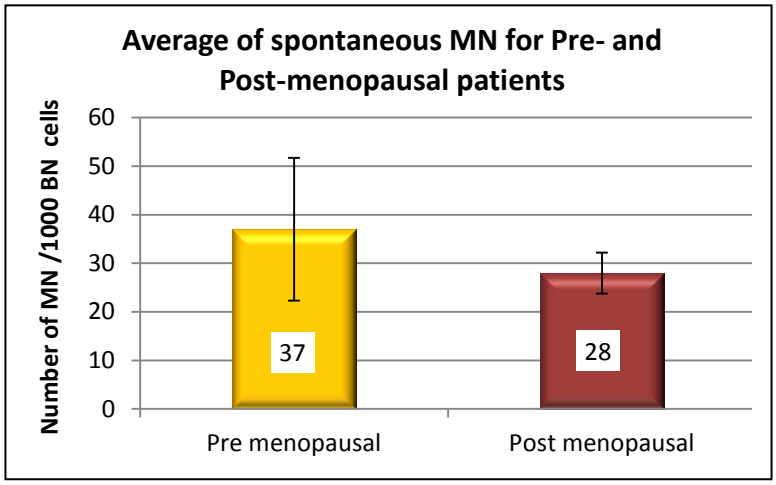


Figure 3.7: Comparison of the spontaneous micronucleus frequency between the pre- and post-menopausal patient group (error bars =SEM). Values indicated = mean.

3.2.4 Association of spontaneous MN and tumour pathology in BC patients

The potential influence of the tumour pathological factors on the micronucleus frequency was examined. No significant difference in the micronucleus frequency with respect to the type of tumour (eg DCIS, IDC), size of the tumours or the number of nodes present was observed (ANOVA, $p > 0.05$).

When the BC patients were divided into 3 groups according to the pathological staging of their tumours, a slight affinity towards an increased average of spontaneous MN with increasing stage was observed. In particular, it was noted that patients with a stage III tumour (23% of group) had a higher, but not statistically significant (ANOVA, $p > 0.05$), spontaneous micronucleus frequency (mean ± SEM = 34 ± 10) compared to patients with tumour stage I (27% of group; mean ± SEM = 24 ± 6) or stage II (17% of group; mean ± SEM = 24 ± 4) (Figure 3.8). There were no patients with stage 0 or IV.

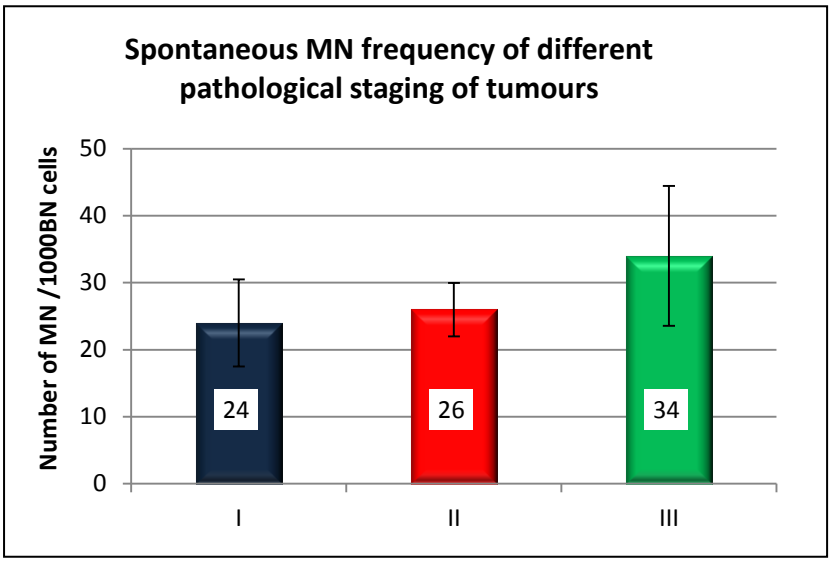


Figure 3.8: Comparison of the spontaneous micronucleus frequency between BC patient groups with different pathological tumour staging (error bars =SEM). Values indicated = mean.

No significant correlation could be found between the number of MN and the hormone markers, estrogen/ progesterone/ HER2 hormone receptor (ANOVA, $p > 0.05$). Only one patient in our group had triple negative BC. Interestingly this patient, despite being an isolated case, showed a higher frequency of spontaneous MN compared to the other BC patients with different hormonal marker receptor types.

3.3 The micronucleus frequency of BC patients before and after radiotherapy

Blood samples from 8 patients who underwent RT without prior chemotherapy were included in this part of the study. The first blood sample was taken before initiation of RT and the second blood sample was taken immediately after completing the last fraction of RT. An average total dose of 40.05 Gy in 15 fractions of approximately 2 Gy was given for an average of 3 weeks.

Slides for each sample were stained with Acridine Orange and scored manually. Not unexpectantly, the number of spontaneous MN (without giving an *in vitro* radiation dose), in patients after completion of their treatment was significantly higher than prior to their therapy (30 MN/ 1000 BN versus 131 MN/ 1000 BN; $p < 0.05$) (Figure 3.9).

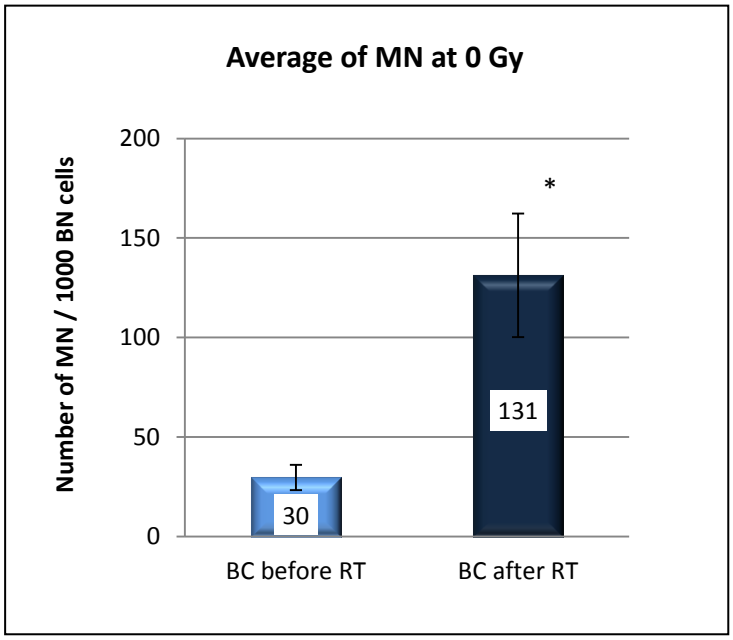


Figure 3.9: The total number of spontaneous MN in BC patients before and after RT (error bars= SEM). *Significantly different compared to before RT ($P < 0.05$). Values indicated = mean.

In addition, the before and after therapy samples were given an *in vitro* dose of 2 Gy and 4 Gy respectively. As expected, the number of MN after 4 Gy (mean ± SEM = 858 ± 16) was considerably higher than that of the 2 Gy (mean ± SEM = 291 ± 35) micronucleus results. It was noticed that there was a trend of a lower number of MN in the group that received RT (Figure 3.10), but this was not statistically significant (Wilcoxon, $p > 0.05$).

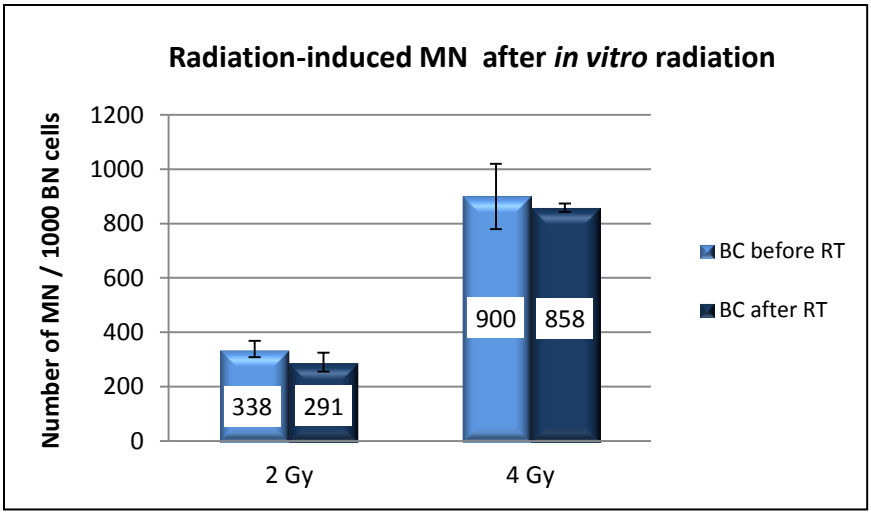


Figure 3.10: The number of radiation-induced MN after 2 Gy and 4 Gy doses that were delivered *in vitro* to patient blood samples, obtained before and after RT (error bars = SEM). Values indicated = mean.

3.4 The Micronucleus-centromere assay using the FISH pancentromeric probe for patients before and after RT

With this assay, the origin of the spontaneous MN before and after RT could be investigated by determining if they were caused by aneugens or clastogens. A total of 8 patients were investigated. The proportion of centromere negative (CM-) MN and centromere positive (CM+) MN was similar in patients before RT. After RT, although both centromeric micronucleus numbers had increased, the number of CM- was significantly higher (Wilcoxon, $p < 0.05$) than that of the CM+ MN (Figure 3.11).

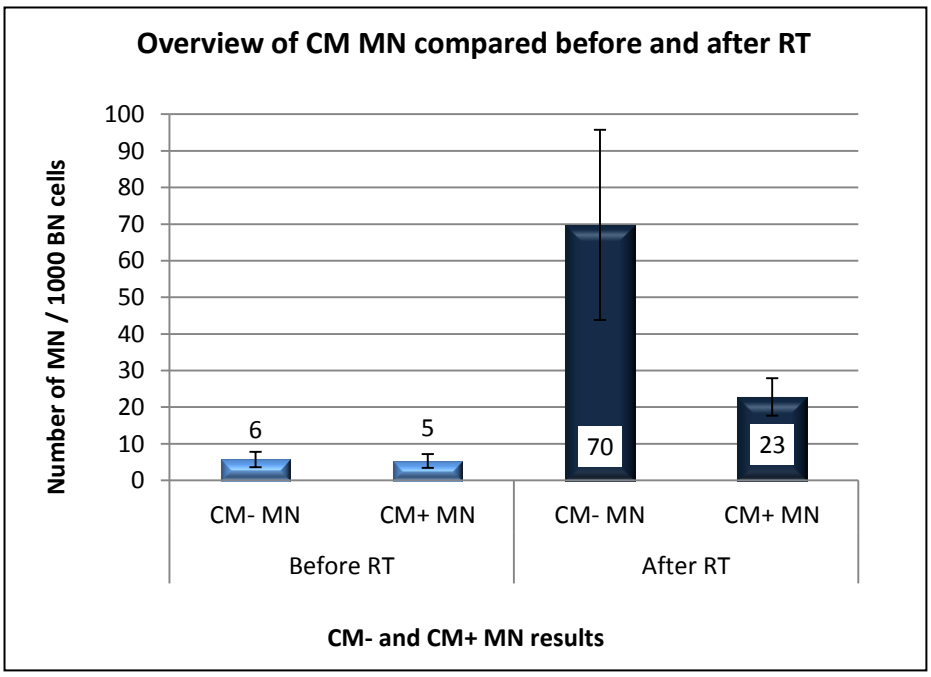


Figure 3.11: The mean CM- and CM+ MN obtained in lymphocytes of BC patients prior to and after RT (error bars =SEM). Values indicated = mean.

Figure 3.12 represents the number of centromeric MN in 3 individual patients to illustrate the high inter-individual variation in CM- MN and CM+ MN that can be seen amongst patients. These results could allow evaluation of individual radiosensitivity. Patients 1 and 3 showed higher micronucleus counts which could be interpreted as these patients being more sensitive to radiation. Patient 3 had the highest number of CM+ MN before RT, which may be due to a higher chromosomal instability. This patient also showed higher number of CM- MN after RT.

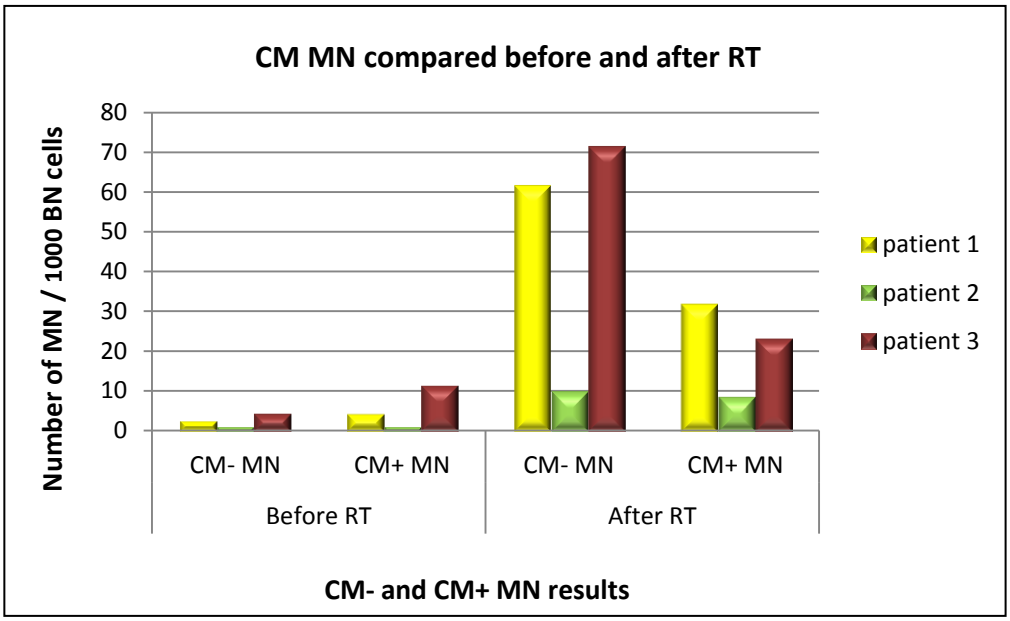


Figure 3.12: The number of CM- and CM+ MN for 3 individual BC patients before and after RT. Values indicated = mean.

The majority of patients had neoadjuvant CT at CMJAH. It was decided to evaluate whether or not chemotherapy would have any influence on the micronucleus results. Samples with a very low total BN cell count (ie < 250 BN) were excluded. In total, 13 of the 16 samples had a good BN cell count. It was noted that patients who had CT (Figure 3.13) displayed a similar spontaneous micronucleus count as that of patients who did not have CT (Figure 3.11).

Neoadjuvant CT patients displayed a slightly higher spontaneous CM+ MN count than CM- MN before therapy. After the completion of their therapy, similar results were observed as that of the non-neoadjuvant CT patients, which was an increase in the total number of MN. However, it was noted that the total micronucleus frequency, before and after RT, was on average less for the neoadjuvant CT patients (CM- + CM+: before RT = 8 CM MN/ 1000 BN**;** after RT = 70 MN/ 1000 BN) compared to the non-neoadjuvant BC patients (CM- + CM+: before RT = 11 CM MN/ 1000 BN**;** after RT = 93 MN/ 1000 BN). This was, however, not statistically significant (Wilcoxon, $p > 0.05$).

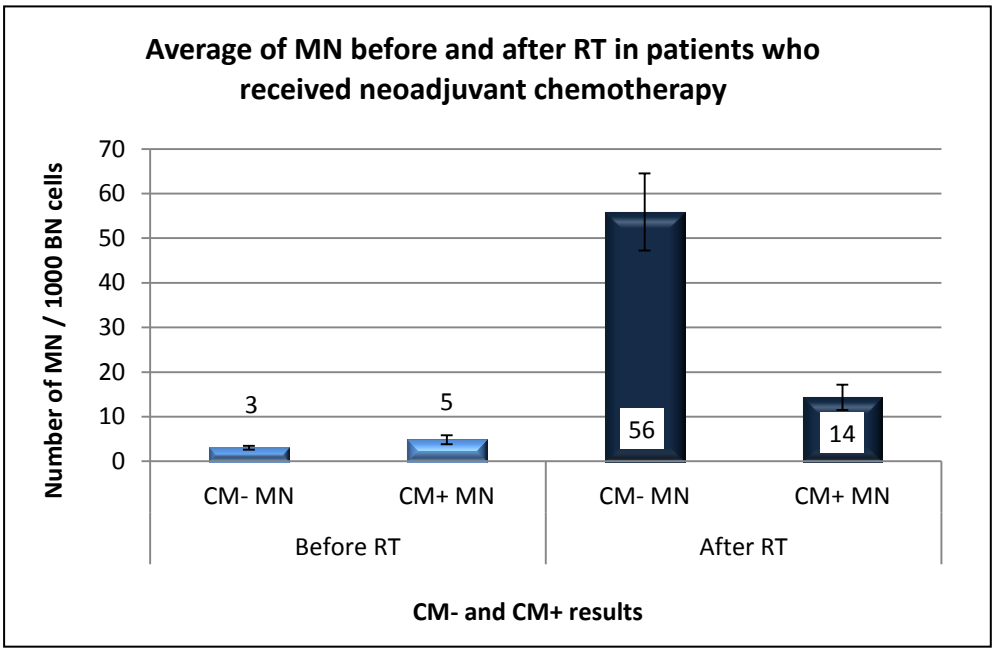


Figure 3.13: The average of the number of CM- and CM+ MN obtained in lymphocytes of BC patients who had received chemotherapy (error bars =SEM). Values indicated = mean.

4. Discussion

The main purpose of this study was to assess whether South African breast cancer patients are more radiosensitive than healthy individuals as has been observed in various European studies (Jones, et al., 1995; Thierens, Vral & De Ridder, 1996; Scott, et al., 1998; Hoeller, et al., 2003; Baeyens, et al., 2005). The micronucleus assay was used to assess the chromosomal damage in the lymphocytes as this assay is an easy, non-laborious and reliable test to assess chromosomal radiosensitivity (Scott, et al., 1999; Gamulin, et al., 2010).

We found that in our South African cohort, the mean spontaneous MN frequency for the BC patients was significantly higher than that of the healthy control group. This is in contrast to the work of Baeyens, et al., (2004) who did not find any significant difference in the spontaneous micronucleus frequency between European breast cancer patients and healthy controls. Our results did however coincide with Jones, et al., (1995); Scott, et al., (1998); Hoeller, et al., (2003) and Baeyens, et al., (2005) who observed similar results.

The higher average number of the spontaneous micronucleus frequency in BC patients in our study could be explained by the difference in average age between the group of breast cancer patients and healthy individuals (BC patients mean = 56 yrs; healthy controls mean = 31 yrs). This is a limitation in our study. Thierens, et al., (2000) showed an age-dependant increase of 0.58MN / year for healthy women. Gamulin, et al., (2010) also found a significant correlation between the age and the increase in occurrence of MN in breast cancer patients. Unfortunately we could not confirm a correlation when

we plotted the spontaneous MN versus age in our cancer or healthy control groups as the R^2 values were too low to deduce conclusive results (Figure 3.5).

When considering the age and ethnicity in the South African population, African BC patients were on average younger than the other ethnic groups studied. This could explain their lower spontaneous micronucleus values. The white BC patient group had the highest spontaneous micronucleus frequency. They were on average older, therefore this could explain their higher spontaneous micronucleus frequency.

The *in vitro* induced micronucleus (2 Gy & 4 Gy) results showed no statistically significant difference between the BC patients and healthy individuals. This is in contrast to the enhanced chromosomal radiosensitivity observed in BC patients in European studies (Scott, et al., 1998, 1999; Terzoudi, et al., 2000; Riches, et al., 2001; Baeyens, et al., 2004, 2005). Although not significant, there was a trend toward the BC patients having higher micronucleus values. Possible reasons for the higher spontaneous micronucleus frequency and somewhat higher radiation-induced micronucleus frequency observed in blood lymphocytes of the BC patients could be as a result of defective cellular repair processes (Parshad, et al., 1996; Sterpone, et al., 2010) or a delay in the cell cycle checkpoint (Lavin, et al., 1994). This is similar to what Hu, et al., (2002) noticed, i.e. a delay in the G2 phase of lymphocyte cell cycles after irradiation of the BC patients.

Studies that showed an enhanced chromosomal radiosensitivity in BC patients suggested that BC patients containing defective DNA repair mechanisms generally have more DNA damage after radiation and therefore could be more

prone to developing cancer. In support of this, Rigaud, et al., (1990 a, b) reported that a lower DNA repair efficiency was associated with an increased number of acentrics.

The lack of significant differences between the *in vitro* radiation-induced MN of BC patients and that of healthy controls in our study could be explained by the genetic diversity of the South African population. To support this hypothesis of genetic diversity, the micronucleus values of the patients were correlated with the ethnicity of the patient. The white BC patients showed on average a higher micronucleus frequency than the African patients, suggesting a higher sensitivity to radiation, although this was not statistically significant. The healthy control group was also examined and no significant differences were detected between the different ethnic groups.

The small cohort that was used for this study limits the detection of any small differences between the micronucleus frequency and the different South African ethnic groups. Therefore, a larger cohort will have to be collected and the micronucleus frequency investigated to determine any conclusive differences between the ethnicity groups (African, White, Coloured, Indian), especially as no previous studies on this subject could be found.

The histology of the tumours was also examined. There was no significant difference in the spontaneous micronucleus frequency when comparing the different types of tumours (IDC, DCIS), grading of the tumours (1, 2, 3) or tumour size. There was, however, a slight correlation between the spontaneous micronucleus frequency and the tumour stages (I, II, III).

With regard to the pathological staging, patients with stage III tumours had a higher spontaneous micronucleus frequency than patients with tumour stages I or II. This was consistent with the results obtained from Scott, et al., (1999) who observed similar results.

The potential influence of the hormone receptor markers on the micronucleus frequency was investigated and we observed that the triple negative BC patient (ER-, PR, HER2-), although an isolated case, had a higher spontaneous micronucleus frequency than the triple positive (ER+, PR+, HER2+) or positive / negative (eg: ER+, PR+, HER2-) BC patients. Given that triple negative BC is considered to be a more aggressive tumour, is more difficult to treat and has a higher rate of recurrence and mortality (Dent, et al., 2007; Reis-Filho & Tutt, 2008), it is possible that these patients may be more genetically unstable, which may thus result in a higher spontaneous micronucleus frequency. A bigger sample size of triple negative BC patients will have to be collected to deduce conclusive results as this is solely a speculative assumption.

When dividing the BC patients into pre- and post-menopausal groups, we found that the average spontaneous micronucleus frequency of the pre-menopausal BC patients was on average 25 % higher than that of the post-menopausal BC patients. In literature there are some hypotheses that estradiol, which is present in higher concentrations in pre-menopausal women, might act as a direct clastogenic agent and this may explain the higher micronucleus frequency in pre-menopausal women (Dhillon & Dhillon, 1995; Ahmad, et al., 2000). Jung (2001) found that post-menopausal females had a lower risk of getting breast cancer due to an enhanced rate of cell death caused by aging and a reduced

cell proliferation rate. Therefore, a decrease in RS would be suspected as cells that may contain more DNA damage would have been killed off, resulting in less chromosomally unstable cells. This could explain our results as well as that of the correlation between age and the micronucleus frequency discussed earlier. A bigger cohort would however need to be acquired to deduce any conclusive results.

When comparing the spontaneous micronucleus frequency in patients before and after radiotherapy, the results showed that the number of MN after RT was much higher than before therapy. Despite the small sample size, this confirms the study of Rigaud, et al., (1990 a), which demonstrated that RT induces chromosome aberrations such as dicentrics and acentric fragments in circulating lymphocytes. After the samples were given an additional 2 Gy or 4 Gy dose *in vitro*, it was noted that the number of BN cells as well as MN after therapy was slightly lower than the pre-therapy samples for both doses although these differences were not statistically significant. These results were in agreement with those reported by Rigaud, et al., (1990 b) who found a decreased number of acentric fragments post RT. Possible reasons could be due to the saturation effect, cells undergoing necrosis / apoptosis due to irreparable damage or the adaptive response which involves the selective killing of lymphocytes with reduced DNA repair efficiency.

The combination of the FISH with a pancentromeric probe with the micronucleus assay was done to distinguish between aneugenic and clastogenic damage. An increase in the spontaneous MN formed by whole chromosomes (CM+ MN) could be the result of chromosomal instability. When

comparing the average number of spontaneous MN with the pancentromeric probe in patients before radiation therapy, a similar number of CM+ MN and CM- MN were observed. Yet when we looked at the individual patient values, it was noted that one patient (patient 3; Figure 3.12) had a higher percentage of CM+ MN. This could indicate a greater chromosomal instability for this patient, which can be supported by the observation that the total micronucleus frequency after RT was also the highest in this patient. When comparing all the samples, the CM+ micronucleus frequency had increased after RT, but the CM- micronucleus frequency had increased more significantly. Our CM- MN results after RT compared well with the study of Vral, Thierens & De Ridder (1997), which showed that radiation induces more acentric fragments and therefore more CM- MN.

In our population group, many BC patients went for chemotherapy prior to surgery, because the majority was diagnosed with late stage, large tumours and node positive BC. The number of centromeric MN was studied in patients who had neoadjuvant chemotherapy to see if CT influenced radiosensitivity assessment. It was noted that lymphocyte stimulation was less successful in this group of patients possibly due to prior damage caused by CT. No statistically significant difference was observed but the total number of MN scored was 26 MN/ 1000 BN less compared to the non-chemo group. Samples obtained prior to therapy, had on average slightly more CM+ MN than CM- MN compared to the patients who did not go for CT, but no significant differences were observed. The higher number of spontaneous CM+ MN is thought to be due to the genotoxicity of CT. Attia, et al., (2009) observed that although

chemotherapeutic agents to a larger part had clastogenic properties, it also, to a lesser extent, had aneugenic properties.

According to Rigaud, et al., (1990 b), the accumulation of damage in the cells, due to the genotoxic effects of RT as well as CT could have led to apoptosis or to an adaptive response. A possible weakening of lymphocytes by CT could have induced a preferential killing or a reduced ability to respond to PHA stimulation due to greater irreparable damage after RT, resulting in the cells' inability to divide, which may explain the lower total number of stimulated lymphocytes (Rigaud, et al., 1990 a).

Conclusion

From the results obtained, it appears that breast cancer patients in South Africa show a trend to be more sensitive to radiation than the healthy control group. However, their sensitivity was not significant possibly due to their diverse genetic background. This however needs to be confirmed with a larger study group.

Regarding the number of MN before and after RT, it was found that on average there was a higher micronucleus frequency in specimens collected after RT. This was expected as the radiation would cause damage to the cells. When the centromeric micronucleus assay with the FISH pancentromeric probe was used to look at the type of damage done to the chromosomes, it was noted that these patients had a higher number of CM- MN than CM+ MN after RT. This showed that they had obtained more clastogenic damage, which thus indicated that radiation causes more CM- MN. In the group of BC patients who received chemotherapy, similar results were observed as that of the non-chemo patients. However, the total number of cells cultured was on average less and the average number of CM+ MN (before RT), although not significant, was slightly higher, which can be explained by the aneugenic action of chemotherapy.

Future studies:

Due to the difficulty in obtaining samples from patients who did not have chemotherapy prior to RT and due to lymphocyte stimulation problems, further research will have to be done to assess RS using a bigger cohort to measure the micronucleus frequency immediately after the patient had completed her treatment and over a period of time post-therapy (3, 6 or 12 months after RT). It may also be of interest to look at the repair mechanisms of breast cancer patients in order to assess if the South African population might have different repair mechanisms, given its genetic diversity, compared to a European population. With regard to the radiosensitivity versus ethnicity of BC patients, it would be interesting to look at the micronucleus frequency, especially in coloured BC patients as this is the third biggest ethnic group in South Africa. A bigger cohort of different ethnic groups will have to be collected. Additionally, a bigger cohort of triple negative BC patients will have to be collected to establish if these BC patients are more RS compared to the other BC sub-types.

Appendix A

List of products:

Micronucleus assay:

Culture flasks:	Greiner bio-one
Phytohaemagglutinin (Sigma):	25 mg PHA + 25 ml dH_2O
Cytochalasin B (Sigma):	5 mg Cyto B + 3.3 ml DMSO
Complete medium:	500 ml RPMI-1640 with L-glutamine (Gibco) 5 ml penicillin/ streptomycin (10 000 U/ml +10 000 µg/ml) (Invitrogen) 75 ml Foetal Calf Serum (FCS) (Gibco)
Ringer:	9 g NaCl (Merck) 0.42 g KCl (Merck) 0.24 g $CaCl_2$ (Merck) 1L dH_2O
KCl:	5.6 g KCl (Merck) 1L dH_2O
Fixative for harvesting:	Methanol/ Acetic acid/ Ringer (4/1/5, 4 ^{0}C) (Merck)

Fixative for washing:	Methanol/ Acetic acid (4/1, 4 ^{0}C)
	(Merck)

Acridine Orange Staining:

Stock solution:	0.1 g acridine orange stain
	100 ml dH_2O
Acridine Orange Buffer:	1 buffer tablet
	1L dH_2O
	pH = 6.8
Acridine Orange work solution:	0.4 ml stock solution
	+ 40 ml Acridine Orange buffer
Acridine Orange buffer:	40 ml Acridine Orange buffer
Rubber cement	Fixogum (Marabu)

DAPI Staining:

DAPI (4',6-diamidino-2-phenylindole):	Vectashield

Pancentromeric probe method:

A: PCR

$MgCl_2$ (10 mM): 10 µl 50 mM $MgCl_2$

40 µl dH_2O

dNTP mix:

Reagent	Volume
dATP (10 mM)	1.5 µl
dCTP (10 mM)	1.5 µl
dGTP (10 mM)	1.5 µl
dTTP (10 mM)	0.75 µl
dH_2O	24.75 µl
Total:	**30 µl**

PCR Purification kit: Biospin PCR Purification Kit (BioFlux)

B: Labelling of PCR by nick translation & probe precipitation

Nick translation (NT) buffer: 500 µl 1M Tris-HCl

100 µl 0.50M $MgCl_2$

50 µl 10 mg/ml BSA

350 µl dH_2O

Total: 1000 µl

β-mercaptoethanol (0.1M): 0.1 ml β-mercaptoethanol (Sigma-Aldrich)

14.4 ml dH_2O

dNTP mix (Spectrum Orange):

Reagent	Volume
dATP (10 mM) (BioRad)	1.5 µl
dCTP (10 mM) (BioRad)	1.5 µl
dGTP (10 mM) (BioRad)	1.5 µl
dTTP (10 mM) (BioRad)	0.75 µl
Spectrum Orange dUTP (1 mM)	7.5 µl
dH_2O	17.25 µl
Total:	**30 µl**

Spectrum Orange (1 mM) (ENZO): 50 mMol + 50 µl dH_2O

DNase I stock solution (stored at -20 ^{0}C): 3 mg DNase I (Roche)

0.5 ml 0.3 M NaCl

0.5 ml glycerol

DNase working solution (always made fresh): 0.5 µl of DNase I stock solution

in 1 ml of ice cold dH_20

Hybridisation buffer (25 ml): 12 ml deionised formamide (100 %;

Merck)

(stored at -20 ^{0}C) 2.5 ml 20 x SSC

2.5 g dextran sulphate

0.195 g sodium dihydrogen

Orthophosphate (Merck)

dH_2O up to 25 ml

pH = 7

EDTA (0.5 M):	186.1 g EDTA Disodium Salt Dihydrate (Merck) 1L dH_2O pH = 8
SDS (10 %):	10 g SDS (Merck) 100 ml dH_2O
$NaAc_3$ (3 M):	24.609 g Sodium acetate (Merck) 100 ml dH_2O
Herring Sperm DNA (Promega):	10 mg/ml
10 x TBE buffer:	108 g Tris (Merck) 55 g boric acid (Merck) 40 ml 0.5 M EDTA (pH = 8) 1L dH_2O
1 x TBE buffer:	100 ml 10x TBE + 900 ml dH_2O
Agarose gel (2 %):	1.2 g agarose (1 g/100 ml 1 x TBE) (Invitrogen) 60 ml 1 X TBE 6 µl GelRed (1 µl /10 ml) (Biotium)

O’Gene Ruler 100 bp ladder (Fermentas)

C: FISH technique

20 x SSC:	87.5 g NaCl (Merck) 44.1 g triNaCitrate (Merck) 500 ml dH2O pH = 7
Deionised formamide (per 100 ml):	1 spatula Analytical grade mixed bed Resin (BIORAD) to formamide (Merck). Stir for 2 hrs and filter with no. 1 filter paper. Store at 4 ^{0}C.
Denaturing solution:	35 ml deionised formamide 5 ml phosphate buffer 5 ml 20 x SSC 5 ml dH_2O pH = 7

Phosphate buffer:

Solution A (acid):	KH_2PO_4 (Merck) - 4.54 g / 500 ml
	pH 4.51
Solution B (base):	Na_2PO_4 (Merck) – 5.94 g / 500 ml
	pH 8.97
Phosphate buffer (in 100 ml):	41.3 ml solution A + 58.7 ml solution B
	pH = 7
50 % formamide (per 150 ml):	15 ml 20 X SSC
	65 ml dH_2O
	70 ml formamide (Merck)
	pH = 7
Washing solution:	5 ml of 20 X SSC + 45 ml dH_2O
Tween washing solution:	5 ml of 20 X SSC + 45 ml dH_2O +
	25 µl Tween 20 (Merck)

Appendix B

List of p values for compared micronucleus frequencies

Sample	Statistical test	P value (95% confidence interval)
Healthy participants vs BC patients (0 Gy)	Mann Whitney	0.048
Healthy participants vs BC patients (2 Gy)	Mann Whitney	0.1010
Healthy participants vs BC patients (4 Gy)	Mann Whitney	0.7464
African vs white BC patients	Mann Whitney	0.2346
African vs white BC patients (2 Gy)	Mann Whitney	0.68
African vs white BC patients (4 Gy)	Mann Whitney	1
Pre vs Post-menopausal	Mann Whitney	0.5370
Path. Stages (I, II, III)	One-way ANOVA	0.5890
Before vs after RT	Wilcoxon	0.0381
Before vs after RT (2 Gy)	Wilcoxon	0.2316
Before vs after RT (4 Gy)	Wilcoxon	0.9969
CM – before vs after RT	Wilcoxon	0.0007
CM + before vs after RT	Wilcoxon	0.0249
CT vs non CT	Mann Whitney	0.5614

Appendix C

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Xanthene Muller

CLEARANCE CERTIFICATE	**M10372**
PROJECT	Chromosomal Radiosensitivity in South Africa Breast Cancer Patients befoore and after Radiotherapy (Under approval M091023)
INVESTIGATORS	Xanthene Muller.
DEPARTMENT	Department of Radiation Sciences
DATE CONSIDERED	26/03/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 26/03/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr P Willem

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

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