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Analysis of radiosensitivity in South African cervical and breast cancer patients

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ANALYSIS OF RADIOSENSITIVITY IN SOUTH AFRICAN CERVICAL AND BREAST CANCER PATIENTS

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree Doctor of Philosophy.

Johannesburg, 2015

Declaration

I, Olivia Herd, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination at this or any other university.

Signed_____ Date_____

Abstract

Introduction: Ionising radiation can cause DNA double strand breaks (DSB), that result in chromosomal aberrations if un- or mis-repaired. Individuals with compromised DNA damage repair mechanisms display increased chromosomal radiosensitivity. The G0-micronucleus assay (MN assay) and the γ-H2AX assay are two assays used in radiobiology to study DNA DSB and repair.

Breast cancer is the leading cancer amongst South African women, with a lifetime risk of 1 in 34. Since most cancer patients in South Africa present with late-stage disease, chemotherapy and radiotherapy are commonly-used treatments. Several international studies have shown breast cancer patients to be more chromosomally radiosensitive than healthy controls. These studies have not been confirmed on a cancer population living in South Africa.

Cervical cancer is the second most common cancer in South Africa; however, it is the leading cancer amongst black women with a lifetime risk of 1/35 compared to 1/82 in white women. Studies show a genetic link to cervical cancer susceptibility and DNA damage repair genes. International studies on radiation-induced DNA damage in lymphocytes of cervical cancer patients remain inconclusive and have never been performed on a South African population. Cervical cancer is caused by infection with the Human Papilloma Virus (HPV). Human Immunodeficiency Virus (HIV), HPV and cervical cancer are epidemiologically linked. Due to the high rate of HIV in South Africa, a significant proportion of cervical cancer patients receiving radiotherapy treatment will be HIV-positive. Studies show an effect of HIV on chromosomal radiosensitivity, however this has not been confirmed on a cancer population. The MN assay on the biopsies and exfoliated cervical cells of cervical cancer

patients could be used as a predictive test for response to radiotherapy. The overall aim was to study chromosomal radiosensitivity in South African cervical and breast cancer patients.

Materials and methods: Chromosomal radiosensitivity of lymphocytes of cervical and breast cancer patients was examined using the MN assay with the Metafer 4 of Metasystems. Different scoring methods for the Metafer system were compared to each other. The effect of HIV, HPV, ethnicity, clinical parameters and age on micronuclei (MN) values in lymphocytes was investigated. The MN assay was attempted on cells from cervical biopsies and exfoliated cervical cells. The γ-H2AX was performed on the lymphocytes of a group of cervical cancer patients.

Results: A new scoring method for the Metafer 4 system that is more reliable in patients with late-stage disease was introduced. Cervical cancer patients had significantly higher MN values with HIV patients having the highest values. HPV, clinical parameters and age had a limited effect on MN values. The MN assay was unsuccessful on biopsies and exfoliated cervical cells of cervical cancer patients. There was no difference in double strand break induction and repair between cervical cancer patients and controls. In breast cancer patients, ethnicity had an effect on MN values, with only white breast cancer patients having significantly higher MN counts.

Conclusion: The study showed increased chromosomal radiosensitivity in cervical cancer and white breast cancer patients. Results highlight how such studies are important within the South African context, where factors like HIV, disease stage and ethnicity can have an effect on chromosomal radiosensitivity and where unique genes/polymorphisms may play a role in cancer risk.

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List of abbreviations

α	alpha
APE 1	Apurinic/Apyrimidinic endonuclease
ARV	Antiretroviral treatment
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
β	beta
BER	Base excision repair
BN	Binucleated cells
bp	Base pairs
BRCA1/2	Breast cancer 1/2
BSA	Bovine serum albumin
CHEK1/2	Checkpoint kinase 1/2
CSA	Cockayne syndrome group A
CSB	Cockayne syndrome group B
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
DAPI	4,6-diamidino-2-phenyldole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase catalytic subunit
DSB	Double strand breaks
EDTA	Ethylene-diamine-tetra-acetate
ELISA	Enzyme-linked immunosorbent assay
EME1	Crossover junction endonuclease EME1
ERCC1/2/4	Excision repair cross-complementing protein 1/2/4
ER	Estrogen receptor
FBS	Foetal bovine serum
FA	Fanconi Anemia
FFPE	Formalin-fixed paraffin-embedded
GGR	Global genome repair

Gy	Gray
HER2	Human epidermal growth factor receptor 2
H2AX	Histone subtype H2A isoform X
HIV	Human immunodeficiency virus
HNSCC	Head and neck squamous cell carcinomas
HPV	Human papilloma virus
HR	Homologous recombination
IC	Internal control
IHC	Immunohistochemistry
KGM	Keratinocyte growth medium
LET	Linear energy transfer
MLH1	MutL homolog 1
MMR	Mismatch repair
MN	Micronuclei
MN assay	G0-Micronucleus assay
MRE11	Meiotic recombination 11 homolog
MSH	MutS homolog
MUS81	MUS81 structure-specific endonuclease subunit
NBS1	Nijmegen breakage syndrome 1
NDI	Nuclear division index
NER	Nucleotide excision repair
NHEJ	Non-homologous endjoining
PALB2	Partner and localizer of BRCA2
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PHA	Phytohaemagglutanin
PIKK	Phosphatidylinositol-3-kinase-related kinase
PMS2	Post meiotic segregation increased 2

PR	Progesterone receptor
RAD50	RAD50 homolog
RAD51	RAD51 recombinase
RAD52	RAD52 homolog
RAD54	RAD54 homolog
RAM-TRITC	Rabbit-anti-mouse tetramethyl rhodamine
RBE	Relative biological effect
RNA	Ribonucleic acid
RPA	Replication protein A
RPMI	Roswell Park Memorial Institute
rpm	Revolutions per minute
RT	Room temperature
SPSS	Statistical Package for Social Sciences
SSB	Single strand breaks
TCR	Transcription-coupled repair
TGF-β	Transforming growth factor beta
Vpr	Viral Protein R
XPC/E/F/G	Xeroderma pigmentosum complementation group C/E/F/G
XRCC1/2/3/4	X-ray cross complementing 1/2/3/4
γ	gamma

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Herd O, Francies F, Cairns A, Muller X, Slabbert JP & Baeyens A (2014). Ethnical differences in breast cancer characteristics in a South African population. The Breast Journal, accepted for publication June 2014.

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1 Introduction

1.1 Ionising radiation and radiobiology

1.1.1 Types of ionising radiation

Ionising radiation is radiation that causes a molecule to lose an electron upon absorption because its energy exceeds the energy of the intra-molecular bindings of the molecule. Ionising radiations can be divided into electromagnetic and particle radiations. X-rays and gamma (γ) rays are two types of electromagnetic radiations. Examples of particle radiations include electrons, protons, neutrons and α-particles. Ionising radiation can be further classified based on linear energy transfer (LET). LET is the amount of energy deposited along the path of the ionising particle per unit of path length. Since X- and γ-rays have ionisations that are sparse and infrequent, they are considered low LET radiation. Low LET radiations are commonly used in medical applications. Particle radiations like α-particles and neutrons are considered high LET radiations as they cause frequent ionisations over a short distance. The same dose of radiation with different LET do not produce the same biological effect. Relative biological effect (RBE) is the term used to define the biological effects produced by different types of radiation. For example, a dose of 1 Gray (Gy) of X-ray results in approximately 1000 single and isolated ionisation tracks, whereas 1 Gy with α-particles results in approximately 4 tracks, but the ionisations are more dense, complex and damaging, causing α-particles to have a higher RBE.

1.1.2 Effect of ionising radiation on the cell

The effect of ionising radiation on a cell can be direct or indirect. Direct action is when the energy of the radiation is deposited directly into a critical biological macromolecule such as DNA, RNA or proteins. Indirect action is when ionisations occur elsewhere in the cell but still

affect critical targets. Indirect action primarily occurs through ionisation of H_2O which forms H atoms and hydroxyl radicals. These radicals are highly reactive and account for 70% of DNA damage induced by radiation (Jeggo and Lavin, 2009).

Damage to DNA can have serious consequences as it regulates all cellular activity. 5 major categories of DNA damage can occur when a cell is targeted by ionising radiation: 1) Base damage: change or loss of a base on the DNA strand; 2) Crosslinks: links are formed between two complementary strands of DNA; 3) Destruction of sugars: pentose sugars forming the backbone of DNA; 4) Single strand breaks (SSB): breaks on one strand of DNA; and 5) Double strand breaks: breaks in both strands of DNA. These different types of damage can occur separately or together which results in complex DNA damage. Once DNA damage occurs in a cell, a number of proteins detect the damage (sensors) and initiate a series of responses (signalers). These responses include apoptosis, alterations of gene expression, changes in cell cycle check points and DNA damage repair.

1.1.3 DNA damage repair

There are 5 main types of DNA damage repair that can occur in mammalian cells depending on the type of damage, cell type and phase of the cell cycle. These include 1) Base excision repair (BER); 2) Nucleotide excision repair (NER); 3) Mismatch repair (MMR); 4) Homologous recombination (HR) and 5) Non-homologous endjoining (NHEJ).

BER: A damaged base is recognised and removed by DNA glycosylases. Apurinic/Apyrimidinic endonucleases (e.g. APE1) incise the DNA strand next to the baseless sugar residue and it is replaced with a new nucleotide by DNA polymerase β. The nick is ligated by the ligase 3/ X-ray cross complementing 1 (XRCC1) complex (Georgakilas, 2008).

Mutations in this pathway have been shown to be associated with colon cancer (Karahalil et al., 2012).

NER: There are 2 NER pathways: global genome repair (GGR) which removes DSB across the whole genome; and transcription-coupled repair (TCR) which only removes DSB in transcribed genes. These methods are the same except for recognition and involve the following: 1) recognition of the lesion by Xeroderma pigmentosum complementation group C (XPC) - Xeroderma pigmentosum complementation group E (XPE) protein complex in GGR or RNA polymerase with Cockayne syndrome group A (CSA) and Cockayne syndrome group B (CSB) in TCR; 2) DNA incision by Xeroderma pigmentosum complementation group G (XPG) and Xeroderma pigmentosum complementation group F (XPF) - Excision repair cross complementing protein 1 (ERCC1) that bracket and remove the lesion of 24-32 nucleotides in length; 3) Filling in of the gap by polymerases and Proliferating cell nuclear antigen (PCNA) followed by DNA ligation. Mutations in this pathway lead to disorders such as Xeroderma Pigmentosum (Hall and Giacci, 2012).

MMR: Replication errors can result in mismatched base pairs. Proteins MutS homolog (MSH) recognise and bind to the mismatched pairs, which results in recruitment of MutL homolog 1 (MLH1) and Post meiotic segregration increased 2 (PMS2). The damaged region is cleaved and removed by exonucleases. The single strand gap is then filled in by DNA polymerase α (Houtgraaf et al., 2006)**.** Mutations in this pathway are associated with hereditary nonpolyposis colorectal cancer and Lynch syndrome, where there is an increased risk for colon and other cancers (Kaz and Brentnall, 2006, Goodenberger and Lindor, 2011)**.**

HR: This pathway is involved in the repair of DSB. It is mostly error free as it relies on undamaged templates to restore any lost sequence information. It only functions in the late

S and G2 phase of the cell cycle when a sister chromatid is available. Sensors detect the DNA double strand break and Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3-related (ATR) (protein kinases belonging to the phosphatidylinositol-3-kinase-related kinase (PIKK family)) are recruited to the damaged site. ATM recruits Breast cancer 1 (BRCA1) by phosphorylating a protein, Histone subtype H2A isoform X (H2AX), which in turn regulates the Nijmegen breakage syndrome 1 (NBS1)/ Meiotic recombination 11 homolog (MRE11)/RAD50 homolog (RAD50) complex. MRE11 resects the DNA resulting in a 3' single stranded DNA that serves as a binding site for RAD51 recombinase (RAD51). BRCA2, which is recruited to the site by BRCA1, facilitates RAD51 being loaded onto single strand overhangs coated with Replication Protein A (RPA) which keeps the single DNA strand from binding to itself. RAD52 homolog (RAD52) is also recruited to the site to prevent any exonucleolytic degradation. RAD54 homolog (RAD54) uses its ATPase activity to unwind the double-stranded molecule. The two invading ends act as primers for DNA synthesis and thereafter holiday junctions (interwound DNA strands) are resolved by Crossover junction endonuclease EME1 (EME1) and MUS81 structure-specific endonuclease subunit (MUS81) (Shibata and Jeggo, 2014). Mutations in the ATM gene of this pathway cause the syndrome Ataxia Telangiectasia, a neurodegenerative disorder where patients show increased susceptibility to cancer and high toxicity to radiation therapy (Jeggo and Lavin, 2009). The HR pathway is illustrated in figure 1.1.

NHEJ: This pathway, also involved in double strand break repair, is more error-prone than HR as it does not rely on a sister chromatid to act as a template for homologous recombination. The first step is the recruitment of the Ku heterodimer (70-kDA and 83-kDA subunits), which has a high affinity for DNA, to the damaged site. The bound heterodimer

recruits DNA-dependent protein kinase catalytic subunit (DNA-PK), causing assembly of DNA-PK at the ends of the DNA double-strand break. DNA-PK (in complex with another protein Artemis) is large and bridges the gap between the two broken DNA ends so they are in close proximity. Artemis, when phosphorylated, can activate its endonuclease activity and deal with 5' to 3' overhangs and hairpins. More proteins are recruited to the site and the final ligation step of the two processed DNA ends is performed by the X-ray cross complementing 4 (XRCC4) - DNA ligase IV complex (Hall and Giacci, 2012). Mutations in this pathway can lead to syndromes like Ligase IV syndrome which is characterized by acute sensitivity to radiation and immunodeficiency (Chistiakov, 2010). The NHEJ pathway is illustrated in figure 1.1.

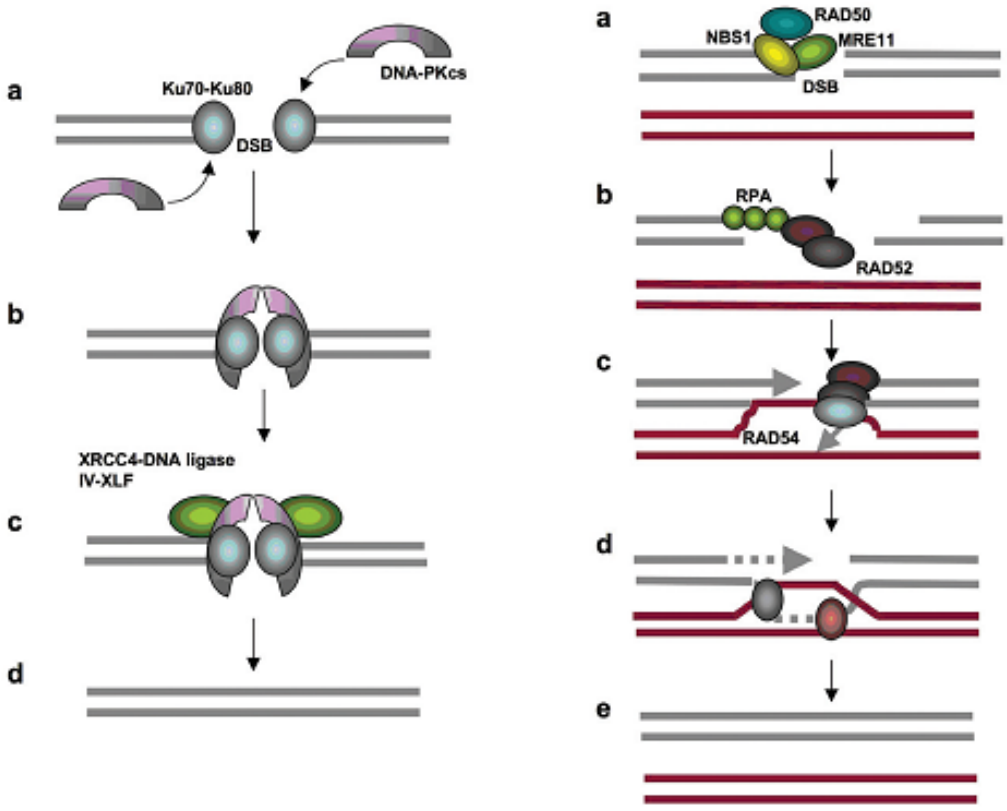

Figure 1.1: Pathways of DNA double strand repair and some of the proteins involved. Left panel shows NHEJ where broken ends are joined directly. Right panel shows HR where homology of a sister chromatid is used to repair the break (IARC, 2008).

DNA DSB that are not adequately repaired can result in chromosomal aberrations. Chromosomal aberrations can be in the form of acentric fragments, dicentrics, rings, translocations, inversions and deletions. These aberrations can result in genomic instability and subsequent carcinogenesis. If irradiation occurs before DNA synthesis, damage is replicated and occurs in both chromatids, resulting in chromosomal aberrations. If cells are irradiated after DNA synthesis, damage occurs in one chromatid arm only and are called chromatid aberrations.

1.2 Radiosensitivity

Clinical radiosensitivity is the display of adverse normal tissue side effects after exposure to ionising radiation for cancer treatment. *In vitro* chromosomal radiosensitivity is increased sensitivity of cells to the DNA-damaging effect of ionising radiation at the chromosome level. Increased *in vitro* chromosomal radiosensitivity has been shown in patients with different cancers, including breast, head and neck and prostate cancer (Parshad et al., 1983, Jones et al., 1995, Riches et al., 2001, Baeyens et al., 2002). This elevated chromosomal radiosensitivity is believed to be the result of inherited mutations in DNA repair genes that not only lead to increased chromosomal radiosensitivity in these patients but may also predispose them to cancer (Jeggo and Lavin, 2009). The first indication for a possible inherited basis for radiosensitivity came from patients with rare genetics syndromes such as Ataxia Telangiectasia and Nijmegen breakage syndrome (Jeggo and Lavin, 2009). These patients were shown to display both clinical and *in vitro* chromosomal radiosensitivity (Huo et al., 1994). Patients with these syndromes have germline mutations in genes involved in DNA damage repair and are also predisposed to many cancers. Studying chromosomal

radiosensitivity and its underlying mechanisms can further elucidate the link between inherited mutations in DNA damage repair genes and predisposition to cancer.

1.3 Biomarkers of radiation-induced DNA damage

Chromosomal damage induced by ionising radiation can be tested through a variety of assays. The traditional assay for radiation research is the analysis of dicentric chromosomes. While this technique is very sensitive (doses as low as 0.1 Gy can be detected) (Vaurijoux et al., 2009), one of its major disadvantages is it being time consuming and requiring highly skilled cytogeneticists. As alternatives, MN and γ-H2AX-foci have become two well-established biomarkers of radiation-induced DNA damage. Recent automation of both these techniques has made them especially attractive because of increased speed and reduced subjectivity.

1.3.1 The G0-Micronucleus assay

The MN assay is a test that measures chromosomal aberrations, one of the major effects of ionising radiation. MN are small nuclei that form in the cytoplasm when whole chromosomes or chromosome fragments are not incorporated into the daughter nuclei subsequent to cell division (Norppa and Falck, 2003). In the MN assay, lymphocytes are irradiated in the G0 phase of the cell cycle. MN are the result of mis-repaired or non-repaired DNA damage so they can be used as a biological marker to assess radiosensitivity.

The MN assay is a well-established, robust assay in radiation research. It is performed on lymphocytes which can be easily obtained through venepuncture. After irradiation, cells are stimulated to divide with the addition of the mitogen, phytohaemagglutanin (PHA). To ensure that MN are scored in cells that have undergone one nuclear division, cytochalasin B

is added to block cytokinesis by preventing actin formation, resulting in binucleated cells (BN) (figure 1.2).

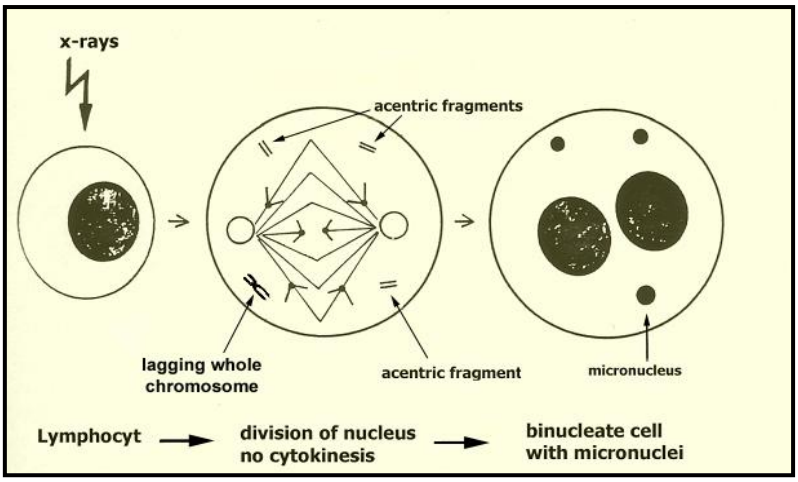

Figure 1.2: Overview of the micronucleus assay. Lymphocytes which complete one nuclear division appear as BN (Baeyens, 2005)

Introduction of image analysis systems using advanced computer algorithms have allowed the scoring of MN to become automated. The aim of automated MN scoring is faster detection of MN, improved accuracy and reduced need for highly trained personnel (Fenech et al., 2013). The Metafer 4 system of Metasystems (Altlussheim, Germany), an automated scoring platform for various cytogenetic tests, was first introduced in 2004 (Schunck et al., 2004). The MNScore software module of the system identifies BN and MN based on a set of defined parameters called a classifier (figure 1.3). BN are detected by identifying two nuclei of similar size and shape. Detection of MN, in a defined area around the BN, is based on a series of morphology criteria. BN (with and without MN) are displayed in an image gallery at a 10x magnification. Studies have shown that staining of the cells with 4,6-diamidino-2-phenydole (DAPI) results in the best fluorescence (Varga et al., 2004). MN in the image gallery can be checked manually by a scorer to correct for false positives and false negatives (figure 1.4). The Metafer has been widely documented in biodosimetry research studies and

in a few cancer research studies (Varga et al., 2005, Varga et al., 2006, Willems et al., 2010, Baeyens et al., 2011, Fenech et al., 2013, Thierens et al., 2014).

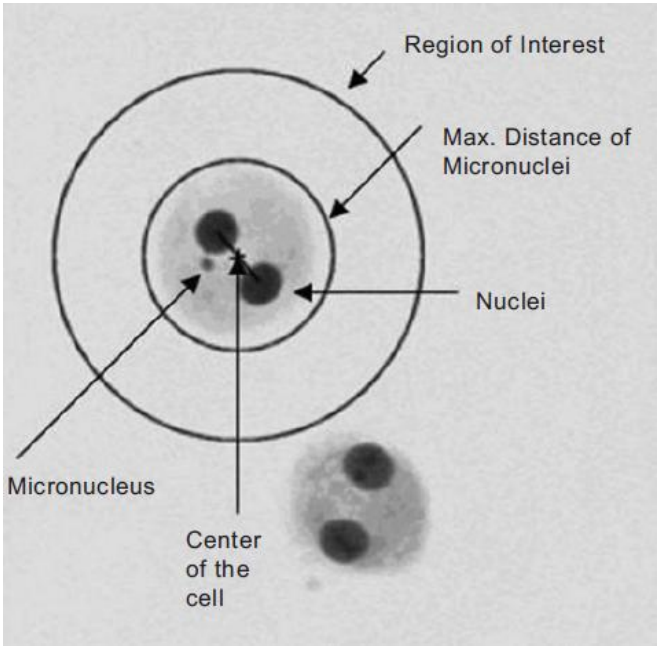

Figure 1.3: Schematic representation of some of the parameters MNScore software uses to identify BN and MN (Varga et al., 2004).

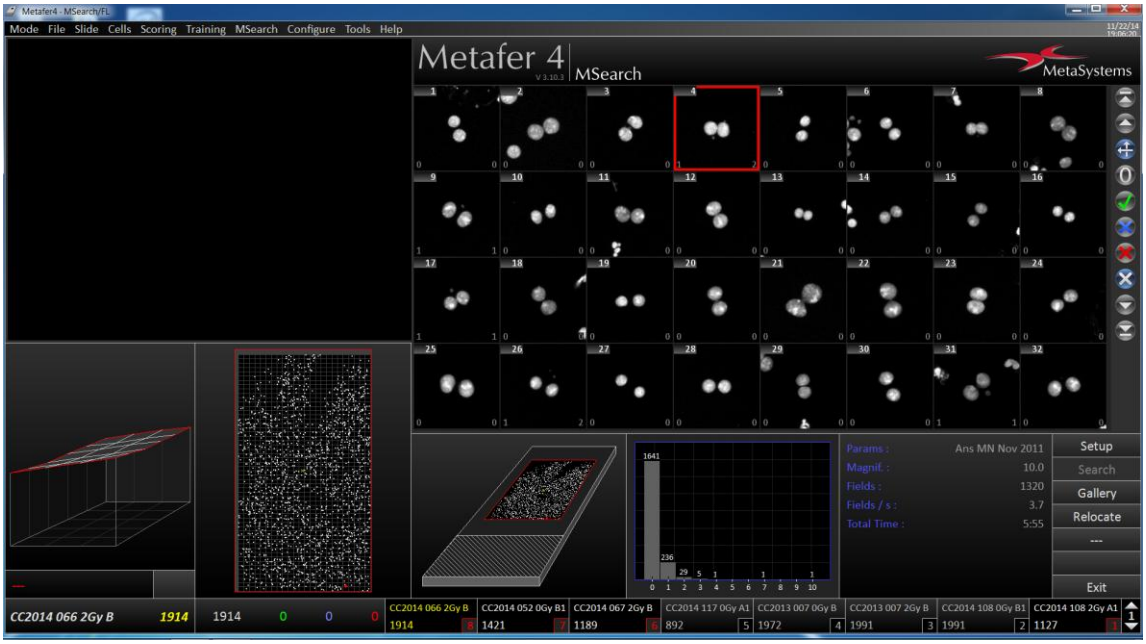

Figure 1.4: The MNScore module of the Metafer 4 system showing BN with MN. In the red box, the bottom left corner indicates the MN score as determined by the system while the right bottom corner shows the MN count corrected by a scorer.

1.3.2 The γ-H2AX foci assay

Nucleosomes, around which DNA is wrapped, are the basic building block of chromatin. They consist of octamers made of two copies of H2A, H2B, H3, H4 with the linker histone H1 (Fernandez-Capetillo et al., 2004). H2AX is a variant of H2A. It varies from H2A as it has a highly-conserved COOH terminal tail. H2AX accounts for about 2-25% of the total H2A pool in mammalian cells (Rogakou et al., 1998). When a double strand break occurs, the serine 139 within the COOH terminus of H2AX is phosphorylated by the protein ATM. Phosphorylation of this protein, referred to as γ-H2AX, rapidly spreads over an extensive region surrounding the double strand break leading to the formation of foci that can be detected microscopically with labeled phospho-specific antibodies (Rogakou et al., 1999). H2AX can also be phosphorylated in the absence of ATM by DNA-PK, resulting in similar levels of foci, however at a slightly slower rate (Stiff et al., 2004). In most cases the proteins work together in a redundant, overlapping manner to phosphorylate H2AX (Stiff et al., 2004).

The linear increase of γ-H2AX foci with increasing dose has made it a widely-used biomarker in radiobiology (Pilch et al., 2003, Beels et al., 2010). Foci are microscopically visible in G0 lymphocytes which have low levels of background foci, making this a sensitive test (Rothkamm and Horn, 2009). Foci formation increases rapidly after exposure to radiation, reaches a maximum at 30 minutes to 1 hour later, and then decreases rapidly following the kinetics of double strand break repair (Rothkamm and Horn, 2009). Foci disappearance over time has been shown to follow double strand break rejoining and cells with impaired DNA damage repair pathways display reduced foci-loss (Rothkamm and Horn, 2009, van Oorschot et al., 2014).

Automated slide scanning and foci scoring on the Metafer 4 platform with the Metacyte software module of Metafer has been described in Vandersickel et al., (2010). This module recognises a cell nucleus based on the parameters of a classifier and captures it as a DAPI image at 40x magnification. In a second step, the filter is changed to a TRITC filter that detects fluorescent signals in a nucleus as a z-stack. Z-stacking allows a microscope to combine multiple images taken at different focus planes in a cell. DAPI-stained nuclei with fluorescent signals representing foci are displayed in the image gallery with an automated foci count that can be corrected by a scorer (figure 1.5). Automated counting of spots is based on 2 approaches: 'direct spot count' counts the number of fluorescent signals in a nucleus. However, as radiation dose increases, foci melt in to larger foci and 'direct spot count' will yield an underestimated count (Rothkamm and Horn, 2009). Therefore, the 'corrected foci count' considers the 3D structure of the spots, and will result in a more accurate foci count (Vandersickel et al., 2010). While fully-automated foci scoring has been shown to be useful with the Metafer system (Vandersickel et al., 2010), manual scoring of foci allows for consideration of staining quality and can be more accurate at higher doses where foci are more complex (overlapping foci edges). It is also more accurate at lower doses where it is important to differentiate between true foci and background noise. Manual scoring of foci with the Metafer 4 system is the preferred method in current literature (Depuydt et al., 2013, Vandersickel et al., 2014).

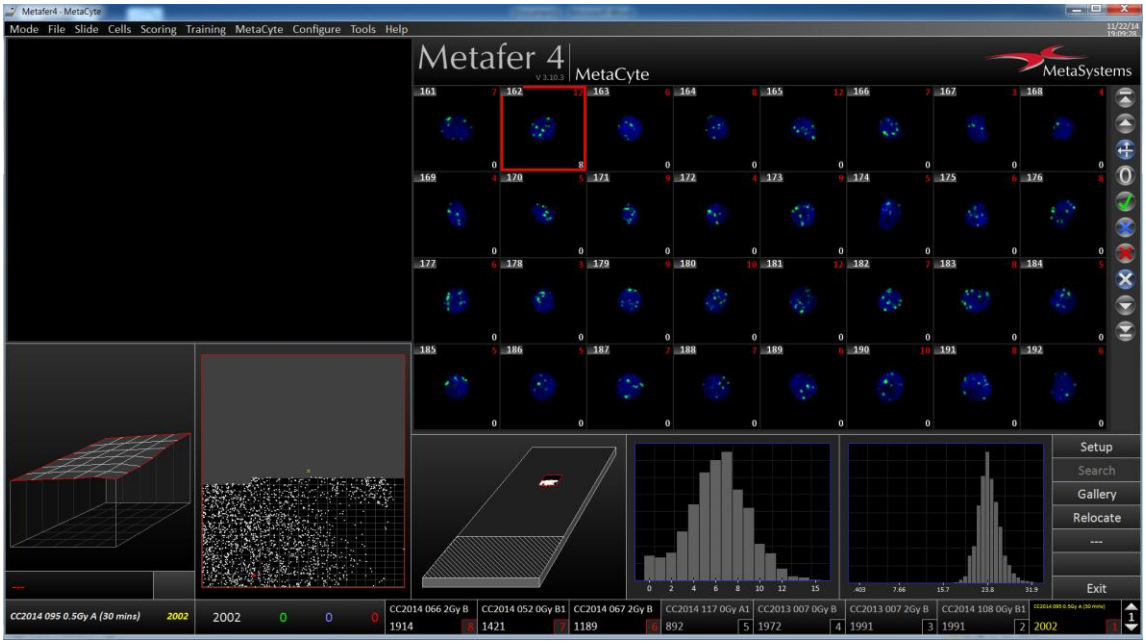

Figure 1.5: The Metacyte module of the Metafer 4 system showing lymphocytes with γ-H2AX foci. The top right corners show the foci count as determined by the system while the bottom right corner indicates the foci count as determined by a scorer (red box).

1.4 Cervical cancer

1.4.1 General

Cervical cancer is the most common cancer amongst women in Sub-Saharan Africa, with an age-standardised incidence rate of 34.8 per 10^5 females (IARC, 2012). It is also the leading cause of cancer-related deaths amongst women in this region (IARC, 2012). In South Africa, cervical cancer is the second cancer after breast cancer; however, it is the leading cancer amongst black South African women with a lifetime risk of 1/35 in compared to 1/82 in white women, according to the most updated national cancer registry available in South Africa (NCR, 2008). The high incidence of cervical cancer in South Africa is likely due to a combination of factors. These include lack of awareness of the disease and its causes, challenges in implementing regular screenings and limited access to HPV vaccinations, which were only recently introduced into the South African health care system (Anorlu, 2008, Sitas et al., 2008, Denny et al., 2013, DOHSA, 2014).

The female lower genital tract consists of three different compartments: endocervix, ectocervix and vagina (figure 1.6). The endocervix is covered by mucin-secreting simple columnar epithelium. The vagina and ectocervix are covered by stratified squamous epithelium, which acts as a physical barrier to pathogens. Squamous epithelial cells in the ectocervix grow as stratified epithelium. The basal layers divide as stem cells and after division, one of the daughter cells migrates upwards and undergoes terminal differentiation while the other daughter cell remains in the basal layer maintaining the self-renewing population (Narisawa-Saito 2007) (figure 1.7, figure 1.8). As the cells mature, they produce greater amounts of keratin and these squamous cells are often referred to as cervical keratinocytes. Invasive cervical cancers can be broadly divided into two groups according to their histology: these are non-keratinising, which are normally poorly differentiated, and keratinising, which are normally moderately to well differentiated.

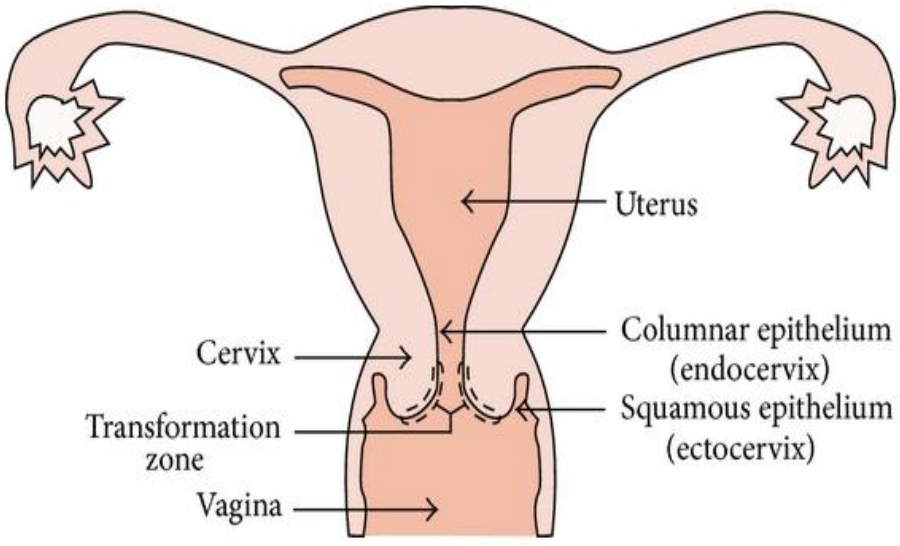

Figure 1.6: Anatomy of the female reproductive tract including endocervix, ectocervix and vagina (Bengtsson and Malm, 2014).

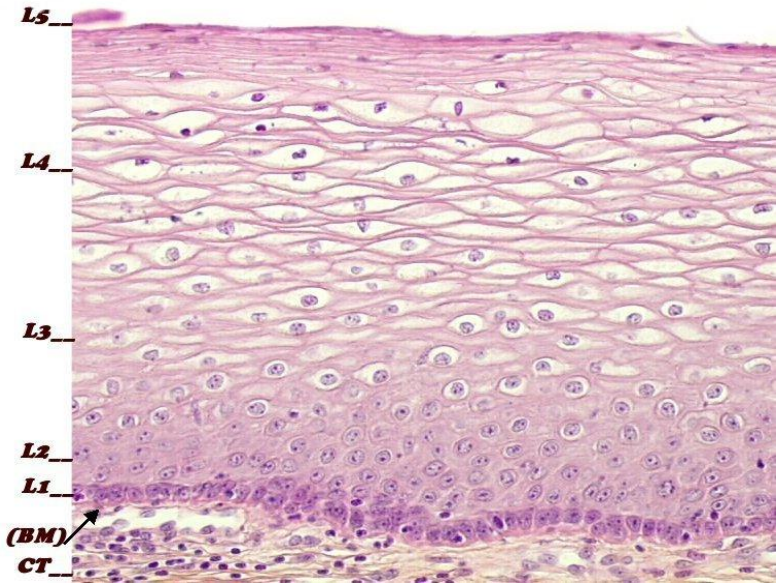

Figure 1.7: Structure of the ectocervix. CT = connective tissue, BM = basement membrane, L1 = basal cells, L2 = parabasal cells, L3 = intermediate cells, L4 = superficial cells, L5 = exfoliating cells (IARC, 2014).

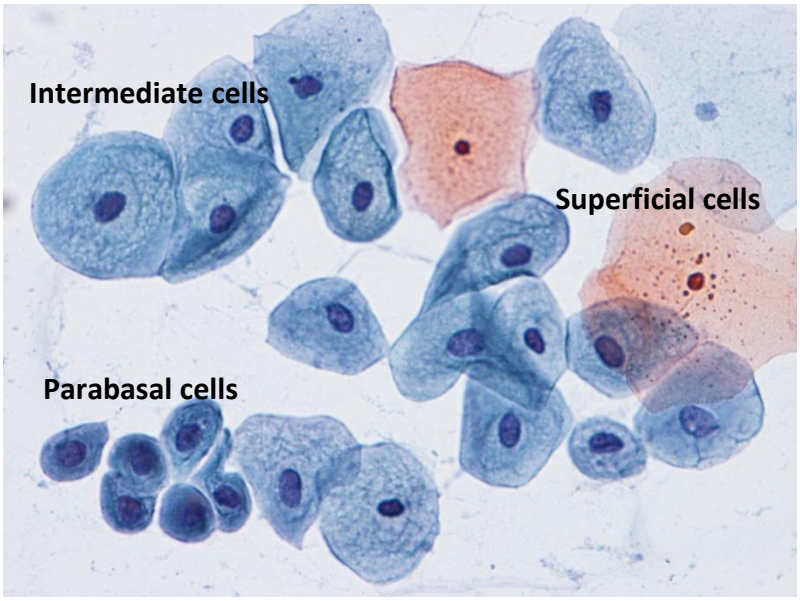

Figure 1.8: Different cells types found in the ectocervix (icytology, 2011)

1.4.2 Cervical cancer and HPV

Cervical cancer is caused by infection with oncogenic HPV (Walboomers et al., 1999, Bosch et al., 2002). Over 100 HPV have been classified based on sequences of the L1 gene of the virus which is highly conserved and codes for its capsid (figure 1.9) (de Villiers et al., 2004). The two main HPV genera are the alpha (α) and beta (β) genera which are further divided

into species (de Villiers et al., 2004). HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 are considered as high-risk for developing cervical cancer. HPV can be detected through a number of assays based on cell morphology (example: morphology of Pap smear cells); detection of HPV proteins (example: western blots or immunohistochemistry); detection of HPV genomes (example: polymerase chain reaction (PCR)) or detection of anti-HPV antibodies (example: Enzyme-linked immunosorbent assay (ELISA)) (Lina Villa and Denny, 2006). Local studies have shown that there are HPV-type distributions that are unique to the South African population (Moodley et al., 2010, De Vuyst et al., 2012, Denny et al., 2014).

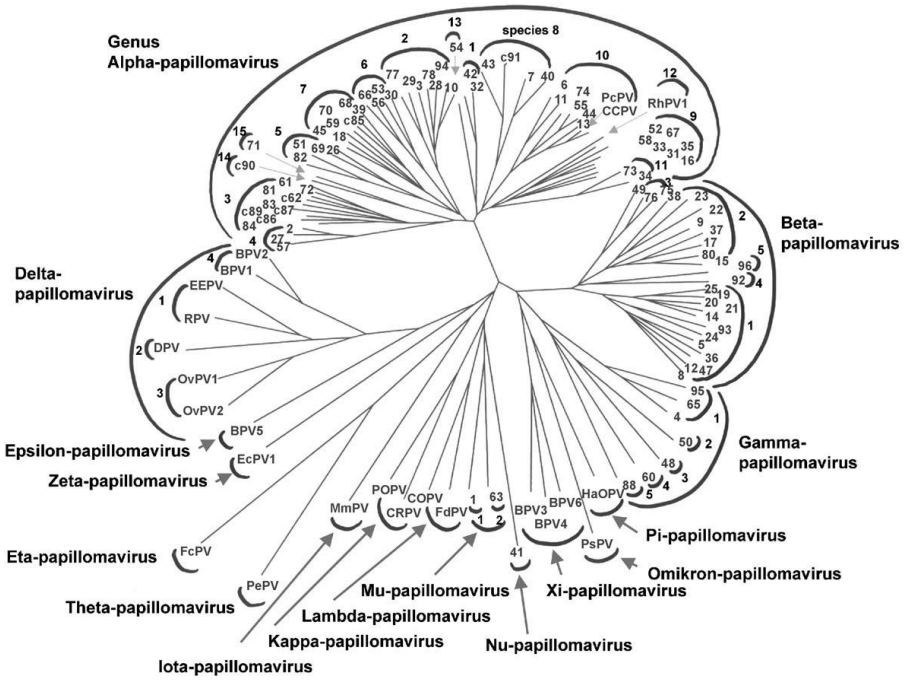

Figure 1.9: Phylogenetic tree of HPV subtypes(de Villiers et al., 2004).

Infection with HPV occurs through microwounds in the transformation zone (border between endocervix and ectocervix) that allow the HPV virus to access the basal cells (Doorbar et al., 2012). As infected daughter cells migrate to the upper layers of epithelium,

viral genes are activated, resulting in high-level amplification of the viral genome (Narisawa-Saito and Kiyono, 2007). In the outer layer of the epithelium, viral DNA is packaged into capsids and virions are released to reinitiate infection (figure 1.10). In most cases, viral infections are cleared by the body. However, if the virus integrates into the host genome, carcinogenesis can occur. Upon integration, the viral gene E2 becomes deactivated which results in the expression of viral genes E6 and E7 (Narisawa-Saito and Kiyono, 2007). Expression of E6 and E7 interfere with host p53 and pRb, two important proteins in tumour suppression and cell cycle regulation. E6 forms a stable complex with a ubiquitin-protein ligase called E6-AP. This complex binds to p53 and induces multi-ubiquitination. The ubiquitinated p53 is then recognized by a large multi-subunit protease complex, called the 26S proteasome, which degrades it into small peptides (Scheffner, 1998). Degradation of pRb by E7 is also mediated by the ubiquitin-proteasome pathway (Boyer et al., 1996).

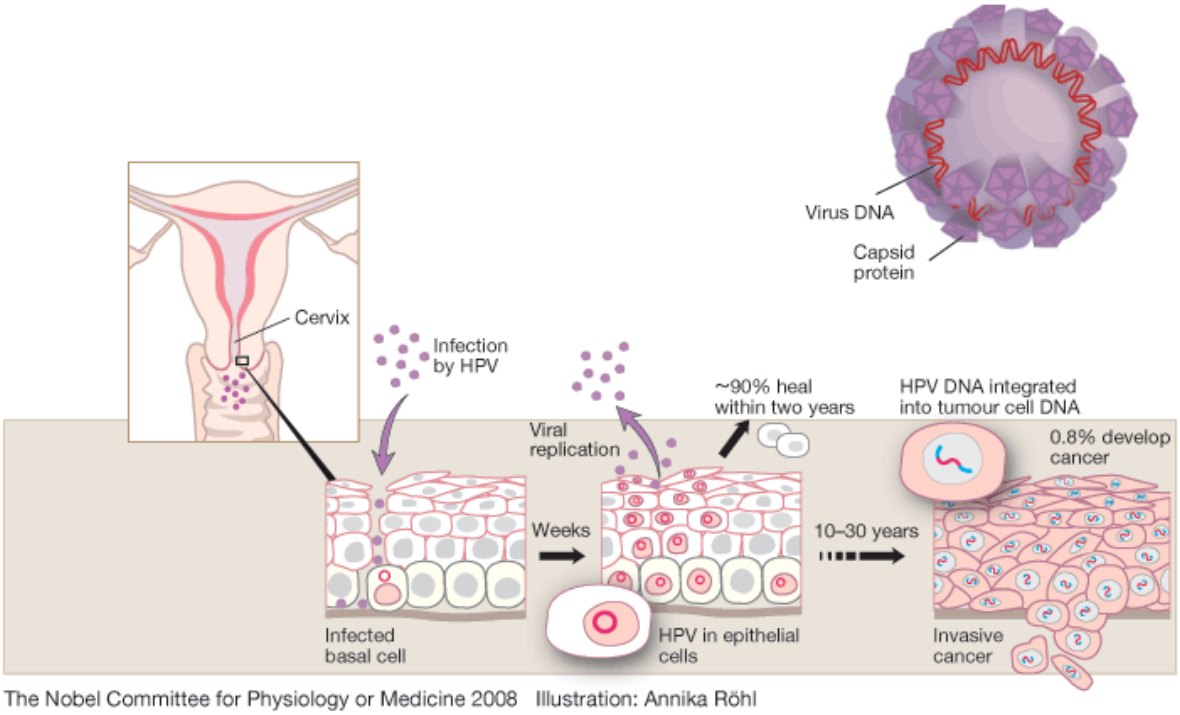

Figure 1.10: Life cycle of HPV in the squamous cells of the ectocervix (Nobelprize.org, 2008).

The reason why some women infected by HPV develop cervical cancer while others don't is still unknown (Woodman et al., 2007, Jaisamrarn et al., 2013). Heritability studies have shown that there is a genetic link to cervical cancer susceptibility and that the disease shows familial clustering (Magnusson et al., 1999, Magnusson et al., 2000, Zelmanowicz Ade et al., 2005). While many heritability studies on cervical cancer have focused on genes involved in immune response (Chen et al., 2013, Jiang et al., 2013, Chen et al., 2014), recent reports have shown an association between cervical cancer and genes involved in DNA damage repair. These include genes such as APE1, XRCC2, XRCC3, ERCC1, ERCC2, ERCC4, ATM (Oliveira et al., 2012, Bajpai et al., 2013, Perez et al., 2013, Wang et al., 2013).

1.4.3 Cervical cancer and HIV

By mid-2014, there were 5.5 million people in South Africa infected with HIV (STATSSA, 2014). HIV, HPV and cervical cancer are epidemiologically associated and in 1993 Invasive cervical carcinoma was classified as an AIDS-defining illness by the United States Centers for Disease Control and Prevention (CDC, 1993). Studies have shown that infection with HPV is higher in HIV-positive than in HIV-negative women, even after taking into account potential confounding factors such as age and sexual behaviour (Sitas et al., 2008, Adler, 2010). Chances of HPV infection increase with decreasing CD4 cell count (Firnhaber et al., 2010, Mbulawa et al., 2010). HIV-positive women have a higher incidence of cervical lesions compared to HIV-negative women (Denslow et al., 2014). In 2012, there were more than 2 million South Africans on Antiretroviral treatment (ARV) (UNAIDS, 2013). Although potent ARV clearly benefit the patient's immunity in terms of increasing CD4 counts, this does not seem sufficient to resolve high-risk HPV persistence and the role of ARV in cervical cancer incidence remains unclear (De Vuyst et al., 2008, Adler, 2010, Denslow et al., 2014).

1.4.4 Cervical cancer and chromosomal radiosensitivity

The *in vitro* chromosomal radiosensitivity of lymphocytes of cervical cancer patients has been investigated using a variety of cytogenetic assays, however results have been unclear (Baria et al., 2001, Ban et al., 2004, Bozsakyova et al., 2005, Gabelova et al., 2008). Conflicting studies related to treatment of cervical cancer indicate that the MN assay has no value in predicting clinical radiosensitivity or clinical outcome (Slonina et al., 2000, Gabelova et al., 2008, Slonina et al., 2008), while others show the MN assay to have predictive value (Widel et al., 1999, Widel et al., 2001, Widel et al., 2003). The HPV virus has been shown to potentially play a role in radiosensitivity of cervical cancer tumours (Vozenin et al., 2010). Tumours positive for the α7 HPV subtypes (HPV 18, 39, 45, 68, 70) show reduced radioresponsiveness and a worse prognosis than α9 HPV subtypes (HPV 16, 31, 33, 35, 52, 58) after radiotherapy treatment (Hall et al., 2013). Patients with HPV-positive squamous cell carcinomas of the head and neck (HNSCC) display improved survival outcome to their HPV-negative HNSCC counterparts after radiotherapy (Kimple et al., 2013). This has been confirmed by studies showing increased *in vitro* radiosensitivity of HPV-positive HNSCC cell lines compared to HPV-negative ones (Rieckmann et al., 2013). Despite the high incidence of cervical cancer in South Africa, and the extensive use of radiotherapy to treat it, there are no data on the chromosomal radiosensitivity of South African cervical cancer patients.

Due to the high rate of HIV in South Africa and its association with cervical cancer, it is likely that a significant proportion of cervical cancer patients receiving radiotherapy as part of their treatment will be HIV-positive. Baeyens et al., (2010) showed that individuals infected with HIV are more chromosomally radiosensitive than uninfected controls, however this has not been confirmed on a cancer population.

1.5 Breast Cancer

1.5.1 General

Breast cancer is the leading cancer amongst South African women, with a lifetime risk of 1 in 34 (NCR, 2008). South Africa is a country consisting of citizens from diverse ethnic groups. These include: black/African (80.2%), white/Caucasian (8.5%), mixed/coloured (8.9%) and Indian/Asian (2.4%) (STATSSA, 2014). The lifetime risk of breast cancer differs according to ethnicity with 1/52 in black women, 1/18 in white women, 1/22 in coloured women and 1/19 in Indian women (NCR, 2008). It is well known that African populations have more genetic diversity than other populations. While the lifetime risk of developing the disease is lower in African women than in white, coloured and Indian women, it is rising due to increased life expectancies and urbanisation which leads to lifestyle changes that elevate exposure to known risk factors for breast cancer such as change in diet, delayed and decreased parity, reduction in breast feeding and exercise (Porter, 2008, Porter, 2009, Knaul et al., 2012). The mortality rate of existing South African breast cancer patients is high, owing to limited access to hospitals in rural areas, diagnosis at more advanced stages, lack of awareness, poor socio-economic status, and limited screening of breast cancer markers (Igene, 2008).

Breast cancer can be sporadic or familial. Mutations in highly penetrant genes such as BRCA1/2, which normally occur in familial cases, only account for about 5% of all breast cancer patients (Nathanson et al., 2001). A substantial amount of patients may be predisposed to breast cancer through mutations in low penetrance genes. Genes other than BRCA1/2 involved in DNA damage repair, for example, Partner and localizer of BRCA2 (PALB2) and Checkpoint kinase 2 (CHEK2), have shown to be good candidates for breast

cancer predisposition (Lina Villa and Denny, 2006, Bau et al., 2007, Smith et al., 2008, Willems et al., 2008, Ricks-Santi et al., 2011, Vral et al., 2011).

The two main categories of breast cancer are Ductal and Lobular, depending on where in the breast the abnormal cells originate (figure 1.11). Breast cancer is considered *in situ* before abnormal cells invade surrounding tissue and invasive once cancerous cells infiltrate into other parts of the breast. Hormone receptor status (estrogen receptor (ER), progesterone receptor (PR)), Human epidermal growth factor receptor 2 (HER2) status, tumour grade and stage are important features determining treatment and prognosis in breast cancer.

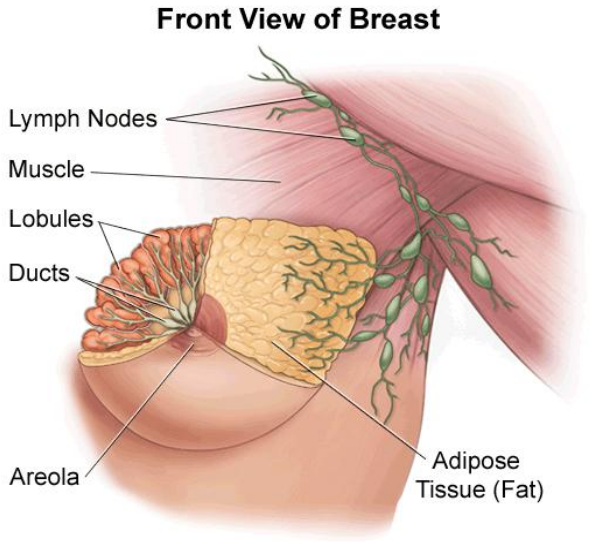

Figure 1.11: Anatomy of the breast.(MUSCHealth, 2014)

1.5.2 Breast cancer and chromosomal radiosensitivity

Many studies, using a range of cytogenetic assays, have shown unequivocally that breast cancer patients are more sensitive to ionising radiation than healthy individuals. Until now, these studies have only been performed on European, Asian, American and African-American populations (Scott et al., 1994, Terzoudi et al., 2000, Riches et al., 2001, Baeyens

et al., 2002, Varga et al., 2006, Poggioli et al., 2010, Ryabchenko et al., 2012, Djuzenova et al., 2013). Chromosomal radiosensitivity has never been investigated in a South African breast cancer cohort.

2 Aims and rationale

The overall aim of the study was to investigate the chromosomal radiosensitivity of South African cervical and breast cancer patients. Investigating the chromosomal radiosensitivity of these patients can lead to insights on both predisposition to the diseases, as well as on the radiotherapy treatment of these patients. Two tests were used to measure different biological endpoints, induction of DSB after radiation (γ-H2AX foci assay) and chromosomal damage after radiation (MN assay).

2.1 Cervical cancer research objectives

1. Investigate the chromosomal radiosensitivity, using the MN assay with the Metafer4 platform, of South African cervical cancer patients in a case-control study design. Concurrently evaluate the quality of this assay on these patients and validate different scoring methods available for the MN assay with the Metafer 4 platform.

Recent automation of the MN assay with the Metafer 4 of Metasystems has made this an attractive assay for biodosimetry and radiosensitivity studies. Two scoring methods have previously been described with this platform. The 'fully-automated' scoring method (Willems et al., 2010) and the 'semi-automated' scoring method (Baeyens et al., 2011, Bolognesi et al., 2011, Thierens et al., 2014). In this study, a second 'semi-automated' scoring method was introduced as an extra validation**.** The three scoring methods were compared on the lymphocytes of cervical cancer patients and healthy controls. To evaluate the quality of this assay on these patients, rate of success of cultures was determined and the average nuclear division index (NDI) was calculated for the different groups.

2. Compare the MN values of HIV-negative cervical cancer patients, HIV-positive cervical cancer patients and healthy controls.

Comparing the chromosomal radiosensitivity of HIV-negative patients to controls investigates a potential link between chromosomal radiosensitivity and predisposition to cervical cancer. Baeyens et al. (2010) showed HIV-positive individuals to have higher chromosomal radiosensitivity compared to HIV-negative individuals. This has never been confirmed on a cancer cohort. The epidemiological link between cervical cancer and HIV in South Africa means that many of these patients seeking treatment, often radiotherapy, will be HIV-positive. To determine if there is an effect of HIV on the chromosomal radiosensitivity of the cervical cancer patients, the group of cervical cancer patients was divided into HIV-positive and HIV-negative patients and the MN values of these were compared to healthy controls.

3. Investigate if there is any correlation between chromosomal radiosensitivity of South African cervical cancer patients and clinical parameters of disease stage and histology. Also investigate if there is any correlation between chromosomal radiosensitivity and age of onset of the disease.

Patients were divided into groups according to these parameters and age and MN values were compared between the subgroups.

4. Perform the γ-H2AX foci assay on the lymphocytes of HIV-negative cervical cancer patients and healthy controls.

This assay looks at a different biological end-point to the MN assay. It gives information on DNA double strand break induction after exposure to radiation and residual DSB after

repair, while the MN assay measures chromosomal damage resulting from mis/non-repaired DSB after exposure to radiation. This assay does not require dividing cells, which was seen to be challenge with the MN assay. γ-H2AX foci were measured 30 mins and 24 hrs after exposure to radiation. To measure the amount of repair undergone by each sample, a 'repair factor' was calculated.

5. Compare repair factors with MN values in lymphocytes of HIV-negative cervical cancer patients and healthy controls.

The MN assay was performed on the same set of samples as above. Since the two assays measure different endpoints, foci and MN values cannot be directly compared, however the repair factors can be compared to MN values as they both give information on residual damage.

6. Develop a MN assay that can be performed on cervical biopsy tumour cells and exfoliated cervical cells of cervical cancer patients and compare MN values in lymphocytes, cervical tumour biopsy cells and exfoliated cervical cells to determine if there is concordance in the number of MN and chromosomal radiosensitivity across the three cell types.

Cervical cancer tumour biopsies and exfoliated cervical cells can be obtained with fairly non-invasive procedures. This gives the unique opportunity to obtain tissue that will be directly affected during radiation treatment. Performing the MN assay on these cells could lead to a predictive radiosensitivity screening test to determine how normal and tumour tissue of cervical cancer patients may respond clinically to radiotherapy.

7. Develop and optimise an in-house PCR that can be used to genotype HPV. Determine the HPV subtypes in DNA extracted from patient tumour biopsies and correlate this with MN values of lymphocytes.

HPV has been shown to play a role in radiosensitivity. Cervical cancer risk may be a result of interaction between the HPV and host genes involved in DNA damage repair. Chromosomal radiosensitivity may indicate susceptibility to certain HPV subtypes.

2.2 Breast cancer research objectives

1. Perform the MN assay on lymphocytes of South African breast cancer patients and investigate the differences in chromosomal radiosensitivity of breast cancer patients from different ethnic groups.

European and American studies have shown that breast cancer patients are more radiosensitive than controls at the chromosome level (Scott et al., 1994, Terzoudi et al., 2000, Riches et al., 2001, Baeyens et al., 2002, Varga et al., 2006, Poggioli et al., 2010, Ryabchenko et al., 2012, Djuzenova et al., 2013). This has never been performed on a population living in South Africa. South Africa has a diverse population with women from different ethnic backgrounds including African, European, Indian and Mixed-race. This unique setting allows the chromosomal radiosensitivity of different ethnic groups to be measured and compared.

2. Investigate if there is any correlation between chromosomal radiosensitivity of South African breast cancer patients and clinical parameters and age of onset of the disease.

Patients were divided into groups, positive or negative for hormone receptors estrogen and progesterone and the HER2 receptor. They were also divided into groups based on tumour size, stage, and histology. MN values between subgroups were compared. The influence of age was also investigated.

3 Materials and Methods

3.1 Study populations

3.1.1 Cervical cancer

Cervical cancer patients (n = 147) were recruited from Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), a public hospital in Johannesburg, where they were undergoing curative hysterectomies or starting their treatment. Both HIV-positive and HIV-negative patients were recruited. Only patients with squamous cell carcinoma were included, as approximately 80-90% of cervical carcinomas amongst South African women are of this histological subtype (Lomalisa et al., 2000, Denny et al., 2014). Exclusion criteria included: 1) adenocarcinomas; 2) recent blood transfusion; 3) previous cancers; 4) prior chemotherapy or radiotherapy and 5) CD4 counts below 300 cells/mm^2 in HIV-positive patients (not optimal for lymphocyte culturing according to Baeyens et al., (2010)). Heparin and Ethylene-diamine-tetra-acetate (EDTA) blood tubes were collected from all patients. Cervical smears and biopsies (fresh tissue or formalin-fixed paraffin-embedded (FFPE)) were also collected. FFPE were obtained from the Division of Anatomical Pathology, School of Pathology, University of Witwatersrand. For patients undergoing hysterectomies, a tumour and an adjacent piece of healthy tissue was collected to optimise cell culturing techniques.

Patient clinical and biographical information was obtained through questionnaires (Appendix B) and hospital files. Clinical information obtained from patient files included: 1) tumour histology type; 2) stage of cancer; 3) HIV status, CD4 count and if patient was on ARV. Biographical information obtained from questionnaires included 1) ethnicity and birthplace; 2) income; 3) education; 4) information on smoking and other illnesses. All donors signed informed consent and ethical approval for the study was obtained through

the Human Research Ethics Committee, University of Witwatersrand, Johannesburg, South Africa (M110230) (Appendix B).

3.1.2 Breast cancer

Heparinised blood samples were collected from breast cancer patients (n = 137) recruited from both CMJAH and University of the Witwatersrand Donald Gordon Medical Centre, a private hospital in Johannesburg. Exclusion criteria included: 1) previous cancers; 2) prior chemotherapy or radiotherapy. Patient clinical and biographical information was obtained through questionnaires (Appendix B) and hospital files. Clinical information obtained from patient files included: 1) tumour histology type; 2) stage of cancer; 3) grade of cancer; 4) size of tumour; 5) Estrogen, progesterone and HER2 receptor status and 6) HIV status and CD4 count. The same biographical information was obtained in the questionnaires as in the cervical study, except an extra question on status of menopause was included. All donors signed informed consent and ethical approval for the study was obtained through the Human Research Ethics Committee, University of Witwatersrand, Johannesburg, South Africa (M110248) (Appendix B).

3.1.3 Healthy controls

Heparinised blood samples from healthy controls (n = 91) were also collected. These were female students and staff members at CMJAH. The age and ethnicities of these controls were matched to patients. Twenty six exfoliated smear samples were also collected from healthy women attending a colposcopy clinic at CMJAH to optimise cell culturing techniques.

3.2 Micronucleus assay on peripheral blood lymphocytes

The MN assay was performed to investigate the *in vitro* chromosomal radiosensitivity of both South African cervical and breast cancer patients. MN values were also used to determine if there is an influence of clinical parameters and age on chromosomal radiosensitivity.

3.2.1 Irradiation and blood cultures

Chromosomal radiosensitivity was measured by irradiating blood *in vitro* with doses of 2 Gy and 4 Gy. A dose of 2 Gy was used as this is the fractionated-dose given during conventional radiotherapy treatment. A dose of 4 Gy was also administered to increase the sensitivity of the test. A 0 Gy dose was included as a sham-irradiated control. Radiations were done in the Radiation Oncology Unit at CMJAH. Cultures were stimulated into division immediately after irradiation with PHA and incubated at 37°C. Cytochalasin B was added after 23 hrs to block cytokinesis. After 70 hrs cells were harvested with hypotonic shock and fixed in methanol:acetic acid. Duplicate slides were made and stained with vectashield containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, USA). Two co-cultures were set up for each dose, accounting for experimental variability. See Appendix C for a detailed protocol of the MN assay.

3.2.2 Slide scanning and scoring

Slides were scanned on the Metafer 4 platform connected to a motorised Zeiss AxioImager M1 microscope. The settings for the classifier were based on Willems et al. (2010). Three different scoring methods (involving varying degrees of visual validation of automated scores) were compared and validated. The first was the 'fully-automated' scoring method in which MN counts are based directly on those obtained by the MNScore module (Willems et

al., 2010). The second is a 'semi-automated' scoring method which has been validated and published elsewhere (Baeyens et al., 2011, Bolognesi et al., 2011, Thierens et al., 2014). In this method, referred to here as 'semi-automated A', only false positive MN are corrected by the scorer. For this study, a third scoring method, referred to as 'semi-automated B' was introduced as an extra validation**.** In this method, every BN cell is checked by the scorer and both false positive and false negative MN are corrected. Using cervical cancer samples and healthy controls as the cohort, 'fully-automated', 'semi-automated A' and 'semi-automated B' MN scores were obtained. For the breast cancer patients, results were based on the 'semi-automated B' MN scores only. Radiation-induced MN values were obtained by subtracting background MN values for each dose point. Any data points with less than 500 BN per dose were excluded. In general, between 500-2000 BN were scored. All results were normalised to a MN frequency in 1000 BN.

3.2.3 Nuclear division index

To assess the proliferative status of lymphocytes of cervical cancer patients, the NDI was calculated on a sub-set of HIV-negative, HIV-positive cervical cancer patients and healthy controls. The formula was NDI = (N1+2N2+3N3+4N4)/Ntotal, where N1-N4 is the number of cells with 1-4 nuclei and Ntotal is the total number of cells scored (Ntotal = 500) (Fenech, 2007). For this, slides from the MN assay were stained with acridine orange (Appendix D) (Sigma-Aldrich, St Louis, MO, USA).

3.3 γ-H2AX Foci assay on peripheral blood lymphocytes

The γ-H2AX assay was performed on the lymphocytes of HIV-negative cervical cancer patients and healthy controls at 30 mins after exposure to radiation, to allow for maximal foci formation (Rothkamm and Horn, 2009), and 24 hrs after exposure, the time widely used

in γ-H2AX foci studies to investigate DNA damage repair (Beels et al., 2010, Djuzenova et al., 2013, van Oorschot et al., 2014, Vandersickel et al., 2014).

3.3.1 Lymphocyte isolation

Peripheral blood was collected in tubes with EDTA which has good anti-coagulation properties for separation of cells. Lymphocytes were isolated from whole blood using low density gradient centrifugation. See Appendix C for a detailed protocol.

3.3.2 Irradiation and cultures

For the γ-H2AX foci assay, isolated lymphocytes were irradiated with the same set-up as for the MN assay. Since doses of 2 Gy and 4 Gy, used in the MN assay, could result in foci per cell too numerous to be accurately counted by eye (Scherthan et al., 2008, Rothkamm et al., 2013), lymphocytes were irradiated with 0.5 Gy and 1 Gy doses. A non-irradiated control of 0 Gy was included. For each dose point, two co-cultures were set-up. Lymphocytes were placed in a waterbath for 30 mins at 37°C immediately after irradiation. Thereafter, cells were placed on ice for 20 mins to arrest DNA damage repair. After time on ice, 250 µl cell suspensions were spun onto Poly-L-lysine coated slides (Thermo Scientific, Massachusetts, USA) at 500 revolutions per minute (rpm) for 5 mins in a Cytospin, which facilitates the attachment of cells to the slides. Cells were fixed onto slides by placing them in 3% Paraformaldehyde (PFA) (Merck, Darmstadt, Germany) (Appendix D) for 15 mins at room temperature (RT), followed by 0.5% PFA (Appendix D) overnight at 4°C. Tubes with remaining cells were transferred from the waterbath to a 37°C pre-gassed (5%CO_2/95% air) incubator and the procedure was repeated at 24 hrs.

3.3.3 γ-H2AX immunostaining

After overnight fixation, slides were immunostained with primary antibody anti-phospho-histone H2AX (Biolegend, San Diego, USA) followed by secondary antibody Rabbit-anti-mouse tetramethyl rhodamine isothiocyanate (RAM-TRITC) (Dako, Glostrup, Denmark) which results in a fluorescent signal that can be viewed microscopically. See Appendix C for a detailed protocol.

3.3.4 Slide scanning and scoring

Slides were scanned on the Metafer 4 platform connected to a motorized Zeiss AxioImager M1 microscope using the Metacyte module. The settings for the classifier were based on Vandersickel et al. (2010), except 3 focal planes instead of the 10 were used in the z-stack. This reduces the time taken to scan a slide and still yields reliable foci scores. Quality of immunostaining was checked across slides before scoring. Since manual scoring is more accurate than automated scoring for both high (foci overlap) and low (background noise) radiation doses, only manual foci scorings were used for the analysis. Foci in 100 cells per condition were counted to give a result of the mean number of foci/cell. Cells with apoptotic morphologies or bright nuclei (complete coverage by immunostain) were excluded from the analysis.

3.4 Micronucleus assay on cervical biopsy tumour cells and exfoliated cervical cells

In order to perform the MN assay on these cells, *in vitro* cultures needed to be established as the MN assay requires dividing cells. Immunohistochemistry (IHC) on these cultures with Anti-pan Cytokeratin Antibody AE1+AE3 from abcam® (Cambridge, England, United Kingdom) ensured that the correct cells, cervical keratinocytes (cervical epithelial cells transformed by HPV), were cultured.

3.4.1 Tumour biopsy cultures

A protocol for culturing tumour biopsies was established in-house by combining and adapting the protocols of Widel et al., (1999); Brink et al., (2002); Heymer et al., (2009); Darroudi et al., (2010) and Freshney, (2010). Fresh biopsies were placed in collection medium (Freshney, 2010) (Appendix D) at 4°C until processing (<4 hrs). Many biopsies, especially those of late-stage disease, consisted of disintegrating brownish tissue material, indicating severely necrotic tissue. These samples were not suitable to be cultured. The use of fine needle aspirates from tumours was attempted to obtain single-cell suspensions, but this yielded insufficient cells for culturing. Viable samples were minced with a sterile scalpel before digestion. Various steps for optimisation of digestion were tested (table 3.1).

Table 3.1: Optimisation of tumour digestion.

Digestion Enzyme	Manufacturer	Reference	Concentration	Digestion conditions	Outcome
Collagenase/Dispase	Roche Diagnostics	(Widel et al., 1999) (Darroudi et al., 2010)	1 mg/ml	37°C for ±1 hr, according to manufacturer instructions **Optimisations:** Increased digestion time up to 24 hrs and 48 hrs. 1X and 2X concentrations.	Inadequate digestion in all conditions
Collagenase	Sigma-Aldrich	Digestion protocol of intestinal tumours from colleagues in another department.	1 mg/ml	37°C for ±1 hr	Inadequate digestion
Liberase Research Grade Purified Enzyme Blend	Roche Diagnostics	(Heymer et al., 2009)	0.12 mg/ml	37°C for ±1 hr, according to manufacturer instructions **Optimisations:** Increase digestion time to ±12 hrs.	Adequate digestion

Once biopsies had been adequately digested, they were strained through a 70 µm Falcon mesh strainer (BD Biosciences, New Jersey, USA) and loose cells were washed in DMEM/F12 (BioWhittaker, Walkersville, USA) with 10% Foetal Bovine Serum (FBS) (Gibco-Invitrogen, New York, USA) to halt the activity of digestion enzymes. Cell viability was determined with trypan blue (Sigma-Aldrich) staining and cells were counted with a haemocytometer. To allow for attachment, cells were seeded in 24 well plates (Greiner Bio-One, Monroe, North Carolina, USA) at a density 1 X 10^6 cells/ml with 0.8 ml culture-initiation medium (Brink et al, 2002 with modifications) (Appendix D). Obtaining this density of viable cells was not possible in most cases, so all cells were seeded and cell concentration was monitored and adjusted by visual checking under an inverted light microscope (Light Microscope Prima Vert, Zeiss Gottingen, Germany). Table 3.2 shows the steps taken to improve attachment of keratinocytes.

Table 3.2: Optimisation of cell attachment.

Optimisation	Effect
Increase time in culture-initiation medium from 24 hrs to 48 hrs	No attachment
CellBind® (Corning) plates treated with oxygen to be more hydrophilic for optimal cell attachment	Average attachment
Treat plates with Poly-L-lysine (Appendix D), a synthetic amino acid that is positively charged and aids attachment of cells to plastic	Good attachment

After 48 hrs, culture-initiating medium was removed and replaced with specialised Keratinocyte Growth Medium (KGM) (Lonza, Basel, Switzerland). This is a serum-free medium that is supplemented with Singlequots (Lonza) containing growth factors, insulin, hydrocortisone, antibiotics, cytokines and other supplements such as bovine pituitary extract to stimulate keratinocyte division. Despite attachment, sufficient growth of cells did

not take place in cultures and confluence was not reached in any samples. Literature suggests that extracellular calcium concentrations exert effects on the morphology, proliferation and differentiation of keratinocytes in serum-free medium. Therefore, culture of cervical tissue was attempted in Keratinocyte growth medium supplemented with calcium to 0.4 mM (Fichorova et al., 1997).

3.4.2 Exfoliated cervical cell cultures

Exfoliated cervical cells were collected from healthy women attending a colposcopy clinic at CMJAH to optimise cell culture methods. Exfoliated cervical cells usually contained many red blood cells, which could hinder keratinocyte attachment. Therefore, a lysis step was implemented. Cells were resuspended in erythrocyte lysis buffer (Appendix D) for 5 mins at RT, washed in complete medium (Appendix D) twice and counted with a haemocytometer. As cervical smears consist mainly of superficial keratinocytes cells that become very flattened as they differentiate, transparent, viable ones were difficult to observe with trypan blue staining, therefore cells were stained with acridine orange instead. This fluorescent stain enhanced visualisation of keratinocytes under the microscope and improved viability checking. Many samples were discarded at this stage due to fungal infection and/or dead cells. In samples with healthy cells (good morphology), cells were planted in wells coated with Poly-L-lysine (Appendix D) in culture-initiation medium (Appendix D) to allow for attachment (as above). Once attachment occurred medium was replaced with KGM (Lonza) supplemented with calcium to 0.4 mM.

3.4.3 Immunohistochemistry to characterise cultured cells

Immunohistochemistry with Anti-pan Cytokeratin Antibody AE1+AE3 from abcam® was used to characterise keratinocytes in culture. Primary mouse monoclonal antibodies AE1 and AE3

bind to cytokeratin antigens that are specific markers of epithelial cell differentiation. The secondary antibody was biotinylated rabbit-anti-mouse antibody (Dako, Glostrup, Denmark) which can be visualised microscopically by adding labelled strepavidin (Dako) which has a strong affinity for biotin. The antibody was tested for specificity on five cervical biopsies (3 normal ectocervix and 2 tumour biopsies). See Appendix C for a detailed protocol.

3.5 HPV genotyping

HPV genotyping was done on the DNA from biopsies of cervical cancer patients.

3.5.1 DNA extraction

DNA was extracted from fresh biopsy tissue and FFPE tissue. Extraction of DNA from fresh biopsies was done with the QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands). For FFPE, DNA was extracted using the phenol-chloroform method. See Appendix C for detailed protocols. DNA concentrations were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA). Extracted DNA was stored at -20°C until HPV genotyping.

3.5.2 Multiplex PCR

A multiplex HPV genotyping PCR was established in-house based on the methods of Nishiwaki et al., (2008). The protocol allows for the simultaneous detection of multiple HPV types in a single-tube reaction using genotype-specific primers. The HPV types included 16, 58, 52, 51, 56, 31, 18, 39, 66, 59, 6, 33, 35, 45, and 11. These primer sets included HPV types 16, 18, 35, 45, common sub-types in the tumours of South African cervical cancer patients (De Vuyst et al., 2012, Denny et al., 2014). PCR products were separated by 2% agarose gel (Appendix D) electrophoresis and HPV type-specific amplicons were visually identified based on fragment size. Gels were stained with Gelred (Biotium, California, USA) and run at 80-100

Volts for 40 mins. See Appendix C for detailed protocol. The PCR was optimised with the following steps:

1. PCR primers (sequences from Nishiwaki et al. 2008) were obtained from Integrated DNA technology (Leuven, Belgium).
2. Positive and negative controls typed by a routine laboratory with the Abbott RealTi*m*e High Risk HPV assay, for clinical purposes, were obtained. This DNA was typed according to HPV 16 positive, HPV 18 positive or 'other' (High-risk HPV subtypes other than HPV 16 or HPV 18).
3. PCR consumables were from a Qiagen Multiplex PCR kit.

Component	**Volume/reaction**	**Final concentration**
2X Qiagen Multiplex PCR Mastermix	25 µl	1X
10X primer mix (primers at 2 µM)	5 µl	0.2 µM
DNA	variable	<1 µg
H_2O	variable	
Total Volume	**50 µl**	

4. PCR cycling conditions were initially optimised on controls amplifying the internal control (IC) only. Aminolevulinate deltasynthase, a commonly-used housekeeping gene, was the internal control and was included to test integrity of template DNA.

   PCR conditions were:

Step	**Time**	**T°**	
Initial activation step	15 min	95°C	
Denaturation	30s	94°C	Number of cycles = 40
Annealing	90s	60°C	
Extension	90s	72°C	
Final extension	10 min	72°C	

5. Once there was amplification of the internal control, the PCR was tested on positive controls for HPV 16 (primers for HPV 16 and the IC at 2 µM each). The number of cycles in the PCR was reduced to 35 cycles which resulted in 'neater' bands. The final PCR cycling conditions were as follows:

Step	Time	T°	
Initial activation step	15 min	95°C	
Denaturation	30s	94°C	Number of cycles = 35
Annealing	90s	60°C	
Extension	90s	72°C	
Final extension	10 min	72°C	

6. The PCR was then tested on positive controls for HPV 18. The cycling conditions used for HPV 16 (table above) were maintained. The primer mix of HPV 18 was optimized to concentrations of HPV 18 at 1 µM and IC at 3 µM.
7. When typing samples positive for HPV 18 and other HPV subtypes, the best results were obtained by reducing the concentration of HPV 18 primers to 0.5 µM. The same applied to HPV 16.
8. A screening of the DNA samples, typed with the Abbott kit revealed positive controls for all HPV subtypes in the protocol except 35, 56 and 58. Positive controls for 35, 56 and 58 were obtained from the WHO HPV LabNet HPV DNA Typing Proficiency Study (Eklund et al., 2014). New primers were required for HPV 58 which were based on those of Romero-Pastrana, (2012).
9. The amount of the PCR consumables were halved to make the test more cost-effective.

10. To do multiple typings in one tube, the primer sets were split into 4 mixes according to band size for easier detection on a gel. These 4 mixes are shown below from smallest gel band in base pairs (bp) to largest.

Mix 1					
HPV subtype	IC	59	45	16	11
Size (bp)	99	169	205	397	472
Primer concentration µM (10X primer mix)	3	2	0.5	0.5	3

Mix 2						
HPV subtype	IC	18	39	6	51	52
Size (bp)	99	187	229	263	299	517
Primer concentration µM (10X primer mix)	3	0.5	2	0.5	2	0.5

Mix 3				
HPV subtype	IC	66	56	58
Size (bp)	99	277	330	414
Primer concentration µM (10X primer mix)	2	3	3	3

Mix 4				
HPV subtype	IC	33	31	35
Size (bp)	99	139	360	434
Primer concentration µM (10X primer mix)	2	2	2	2

11. DNA from cervical patients was typed with the final 4 primer mixes. Cycling condition remained the same as in step 5.

3.6 Statistical analysis

Statistical analysis was performed with SPSS (IBM, version 22) and Graphpad Prism 6. Differences between means of MN counts in cervical, breast patients and controls were tested for significance with the Mann-Whitney U test. Differences in MN values between groups according to clinical parameters and age were also tested for significance with the Mann-Whitney U test. Differences in NDI scores between groups were also tested with the

Mann-Whitney U test. This statistical test was used as it is a non-parametric, distribution-free test that is suitable to compare groups with small sizes where no underlying distribution can be assumed. Correlations were tested with Pearson's correlation coefficient. Differences in means of age and tumour size in breast cancer patients were tested using ANOVA. Differences in characteristics of breast cancer patients were tested for differences with the Chi-squared test.

4 Results

4.1 Cervical cancer patients

4.1.1 Study population

A total of 147 cervical cancer patients were recruited during the study. Table 4.1 shows the biographical and disease characteristics of the study population. The majority of patients were black (91%). Only 54% could be considered to be 'educated' (high school and tertiary school). Most of the patients were of low-income status with only 3% in an income bracket above R5000/month. The majority (47%) of patients presented with late-stage disease. Forty-two percent of the patients were HIV-positive. The average age of patients was 47 (range = 27-79). The average age of the HIV-positive group was 42 (range = 27-60), compared to 52 (range = 29-79) average age of the HIV-negative group. The average CD4 count in the HIV-positive group was 493 cells/mm^3. Since CD4 counts are not normally measured in HIV-negative cervical cancer patients in the clinical setting, these were not available for this group of patients.

Table 4.1: Characteristics of cervical cancer patients.

Race	n	%
Black	133	90.5
White	7	4.8
Coloured	7	4.8
Indian	0	0
Education		
Primary school	68	46.3
High school	70	47.6
Tertiary school	9	6.1
Income		
None	90	61.2
less than R500	1	0.7
R500-R1000	4	2.7
R1000-R2000	11	7.5
R2000-R5000	17	11.6
more than R5000	5	3.4
unknown	19	12.9
HIV status		
Negative	83	56.5
Positive	62	42.2
unknown	2	1.4
ARV treatment		
ARV	14	22.6
No ARV	17	27.4
unknown	31	50.0
Disease stage		
Early (1-2B1)	57	38.8
Late (2B2-4B)	69	46.9
unknown	21	14.3
Tumour Histology		
Non-keratinising (poorly differentiated)	18	12.2
Keratinising (Moderately-well differentiated)	65	44.2
Squamous cell carcinoma (histology unspecified)	64	43.5

4.1.2 Micronucleus assay on peripheral blood lymphocytes of cervical cancer patients and controls

4.1.2.1 Success rate of blood cultures and nuclear division index

Across all collected patient samples, 45% (n = 66) had lymphocyte cultures that were successful for the MN assay (data for all doses or for the 0 Gy and 2 Gy dose). Reasons for samples not being included in the final analysis were:

1. Poor lymphocyte proliferation (<1000 BN for 0 Gy) and/or high percentage (>25%) of misidentified BN (= apoptotic/dead cells) in 2 Gy and 4 Gy. Apoptotic cells were those that were small, dense and brightly stained (n = 55) (37%).
2. Technical issues, such as problems with irradiations, problems with CO_2 incubator or small BN. Small cells were later found to be a result of low humidity during dry seasons in Johannesburg and could be overcome by making slides in a humidified area (n = 26) (18%).

The success rate of blood lymphocyte cultures for each group were as follows:

HIV-negative patients (n = 83):

- Successful cultures: n = 42 (51%)
- Poor lymphocyte proliferation and apoptotic/dead cells: n = 23 (28%)
- Technical issues: n = 18 (21%)

HIV-positive patients (n = 62):

- Successful cultures: n = 24 (39%)
- Poor lymphocyte proliferation and apoptotic/dead cells n = 32 (52%)
- Technical issues: n = 6 (10%)

***For two samples HIV status was not known**

Controls (n = 91):

- Successful cultures: n = 72 (79%)
- Poor lymphocyte proliferation and apoptotic/dead cells: n = 6 (7%)
- Technical issues: n = 13 (14%)

To evaluate the quality of the MN assay, the NDI was calculated in a subset of randomly selected samples from each group (including both successful and unsuccessful cultures). The healthy controls had NDI of 2.170; 2.073 and 1.668 for the 0, 2 and 4 Gy doses. The HIV-negative patients had NDI of 2.160; 1.842 and 1.529, while the HIV-positive patients had NDI of 2.074; 1.912 and 1.516 respectively. The patients combined had statistically significant lower NDI than controls at both 2 Gy and 4 Gy ($p = 0.0091$ and $p = 0.0182$ respectively).

4.1.2.2 MN values in cervical cancer patients and controls and comparison of 3 scoring methods based on the MNScore software (Metasystems)

The MN values of 35 cervical cancer patients (mean age = 46) were compared to 20 healthy controls (mean age = 41) using three different scoring methods. Fifteen of the patients were infected with HIV (mean age = 43) and 20 were negative for HIV infection (mean age = 49). Scoring methods included: 1) 'Fully-automated' - MN counts are based directly on those obtained by the MNScore module; 2) 'Semi-automated A' - BN with MN are checked for quality and false positive MN are corrected; 3) 'Semi-automated B' - the quality of every BN cell is checked and both false positive and false negative MN are corrected. Radiation-induced MN values were calculated by subtracting background (0 Gy) MN values from MN values observed in irradiated cells. Background MN values and radiation-induced values for 2 Gy and 4 Gy are listed in Table 4.2. Using the 'fully-automated' and 'semi-automated A' scoring method, no significant differences were detected between patients and controls. With the 'semi-automated B' scoring method, patients had significantly higher MN values at

both the 2 Gy (p = 0. 0075) and 4 Gy (p = 0.0059) radiation dose. The coefficient of variation was also lowest in the ‘semi-automated B’ method.

Table 4.2: MN values for controls and cervical cancer patients with 3 scoring methods. *significantly different from controls (Mann-Whitney test p<0.05). SD = standard deviation. SEM = standard error of the mean. CV = coefficient of variation.

	AUTOMATED				SEMI-AUTOMATED A				SEMI-AUTOMATED B			
		0Gy	2Gy	4Gy		0Gy	2Gy	4Gy		0Gy	2Gy	4Gy
CONTROLS	**N**	20	20	20	**N**	20	20	20	**N**	20	20	20
	MEAN	56	125	323	**MEAN**	10	115	317	**MEAN**	13	155	454
	SD	37	36	56	**SD**	5	22	47	**SD**	5	28	41
	SEM	8	8	13	**SEM**	1	5	11	**SEM**	1	6	9
	CV (%)	67	29	17	**CV (%)**	50	19	15	**CV (%)**	38	18	9
PATIENTS	**N**	35	35	35	**N**	35	35	35	**N**	35	35	35
	MEAN	66	144	320	**MEAN**	12	124	327	**MEAN**	14	*179	*506
	SD	40	56	81	**SD**	8	26	56	**SD**	8	33	67
	SEM	7	9	14	**SEM**	1	4	10	**SEM**	1	5	12
	CV (%)	61	39	25	**CV (%)**	67	21	17	**CV (%)**	57	18	13

4.1.2.3 MN values in HIV-negative cervical cancer patients, HIV-positive cervical cancer patients and healthy controls

To assess the effect of HIV on chromosomal radiosensitivity and determine the chromosomal radiosensitivity in HIV-negative patients without HIV as a confounding factor, the patients were divided into HIV-positive (n = 15, mean age= 43) and HIV-negative (n = 20, mean age = 49) groups. Based on the results of table 4.2, the MN values were determined using the semi-automated B' method. There were no differences in background MN values when comparing the HIV-negative (mean MN = 17) or HIV-positive patients (mean MN = 10) with the controls (mean MN = 13). Figure 4.1 shows the frequency distributions of the groups according to MN values for both the 2 Gy and 4 Gy doses. For 2 Gy, there was no significant difference between the mean MN values for HIV-negative cancer patients versus controls ($p = 0.060$), however there was a clear shift in the frequency distributions towards patients having higher MN values. The difference in mean MN values between HIV-negative patients and controls was significant at 4 Gy ($p = 0.037$).

In HIV-positive patients, there were 2 samples with insufficient BN at the 4 Gy dose and were thus excluded at this dose point from the analysis. Although not statistically significant, the HIV-positive patients had higher MN values than the HIV-negative patients. 73% of these patients were on ARV treatment but these did not have significantly higher MN values than those not on ARV treatment. The HIV-positive patients had MN values that were statistically higher than healthy controls at both the 2 Gy ($p = 0.006$) and 4 Gy ($p = 0.008$) dose.

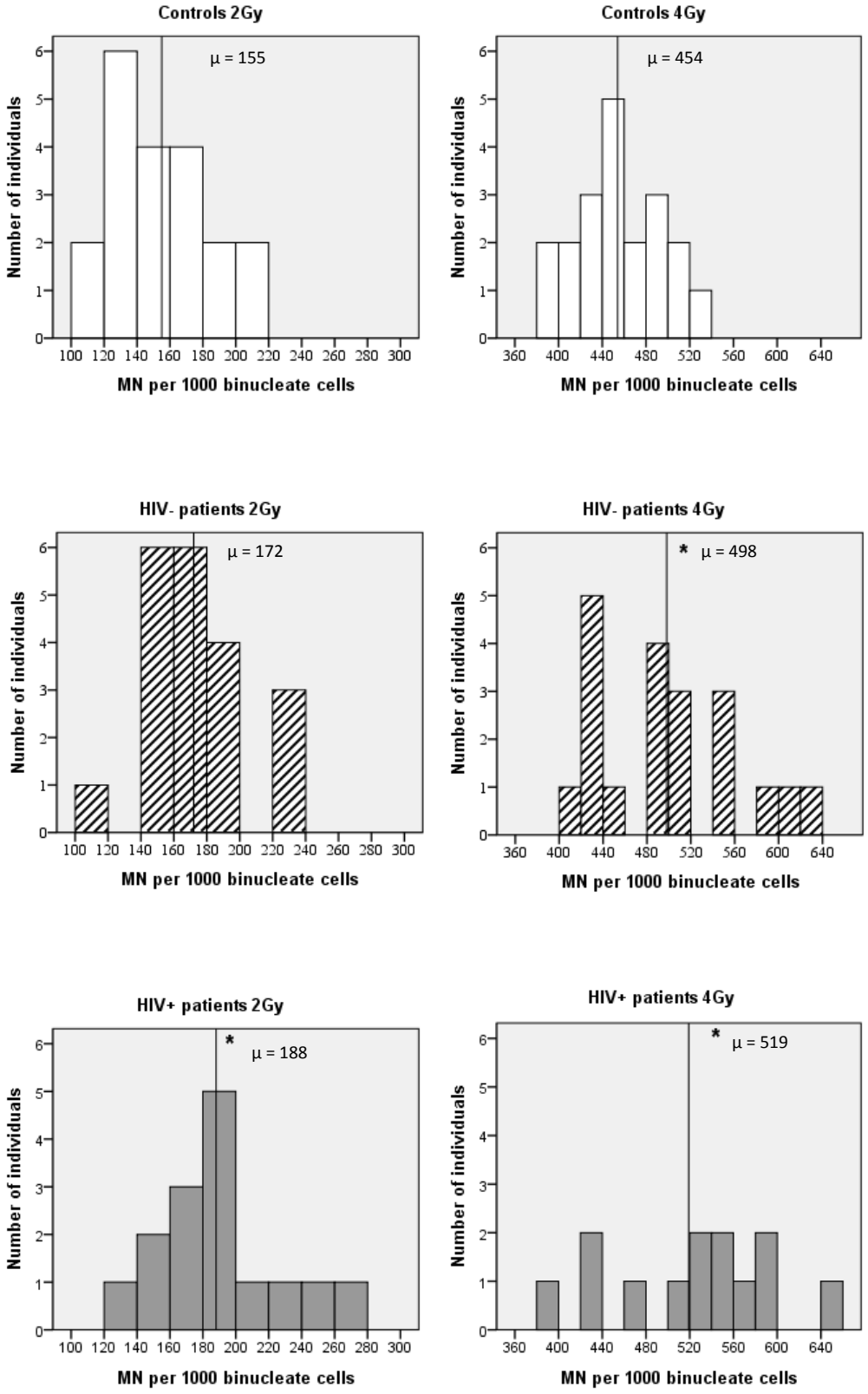

Figure 4.1: Radiation-induced MN values for controls, HIV-negative and HIV-positive patients after 2 Gy and 4 Gy dose of irradiation. Vertical line shows the mean MN value for each group. *Significantly different to controls (Mann-Whitney p<0.05).

4.1.2.4 Correlation between MN values in cervical cancer patients and clinical parameters and age of onset

To assess if there was a correlation between clinical parameters and chromosomal radiosensitivity, cervical cancer patients were divided into groups according to cancer stage and histology. The staging divisions were stage 1 (5 patients- 2 HIV+, 3 HIV-), stage 2 (11 patients- 5 HIV+, 6 HIV-) and stage 3 (17 patients- 7 HIV+, 10 HIV-). The mean MN scores were not significantly different for any of the doses when groups were compared to each other (Mann-Whitney test: $p>0.05$). Patients were divided into 2 histology categories defined as non-keratinising (poorly differentiated) and keratinising (moderately and well differentiated). There were no differences in MN values between the groups at the 0 Gy, 2 Gy or 4 Gy dose (Mann-Whitney test: $p>0.05$).

The age of onset was significantly correlated with the background MN values of the patients (Pearson's correlation, $p = 0.021$) No significant correlation could be found between radiation-induced MN and age of onset. For a second analysis to assess if age of onset had an influence on chromosomal radiosensitivity, patients were divided into subgroups of: 1) patients <40 years old (13 patients), 2) patients between 41 and 50 years old (7 patients), 3) patients between 51-60 years old (11 patients), 4) patients >61 years old (4 patients). There was a significant difference in radiation-induced MN values at the 4 Gy dose between the <40 years old group and the >61 years old group ($p = 0.030$), and <40 years old group and 41-50 age group ($p = 0.029$), the young group having higher MN values. In the younger group 7/13 were HIV-positive, which may explain the higher MN values. There were no differences in MN values amongst the other age groups (Mann-Whitney: $p>0.05$).

4.1.3 γ-H2AX Foci assay on peripheral blood lymphocytes of cervical cancer patients

The γ-H2AX assay was performed on the lymphocytes of 13 HIV-negative cervical cancer patients (mean age = 58) and 13 healthy controls (mean age = 46). Lymphocytes were irradiated with 0 Gy, 0.5 Gy and 1 Gy doses. Foci values were measured at 30 mins which shows initial number of induced DSB after irradiation. The number of DSB remaining after allowing repair for 24 hrs were also measured.

Figure 4.2 shows individual data points of the patients and controls at each dose and time point. In terms of baseline foci (0 Gy), the patients had higher foci values than controls after 30 mins but the difference was not significant. After 24 hrs, the patients had significantly higher baseline foci than the controls ($p = 0.039$). This significant difference was not reached if the outlier of 0.98 (seen in figure 4.2) was excluded from the dataset ($p = 0.068$).

Radiation-induced foci values (mean/cell) were obtained by subtracting baseline foci values (mean/cell). After 30 mins, at the 0.5 Gy dose, the controls had a mean foci radiation-induced value of 6.03 while patients had a mean foci value of 6.40 foci/cell. There was no significant difference. After 30 mins, at the 1 Gy dose, the controls had a radiation-induced mean foci value of 8.77, while patients had a mean foci value of 9.36 foci/cell. The difference was not significant.

After 24 hrs, at the 0.5 Gy dose, controls and patients had a mean radiation-induced foci value of 0.39 and 0.40 foci/cell respectively. After 24 hrs, at the 1 Gy dose, controls had a mean foci value of 0.71 foci/ cell while patients had 0.86 foci/cell, the difference not being significant.

To determine the level of repair undergone by each sample, we calculated a 'repair factor'. This was calculated with:

$$\frac{\text{Initial foci at a given dose - residual foci at the same given dose}}{\text{Initial foci at the same given dose}} \times 100$$

Higher factors mean better repair. Controls and patients had a repair factor of 93.4% and 93.8% at 0.5 Gy respectively. Controls had a repair factor of 91.7%, while patients had a repair factor of 90.7% at 1 Gy. No significant difference was found.

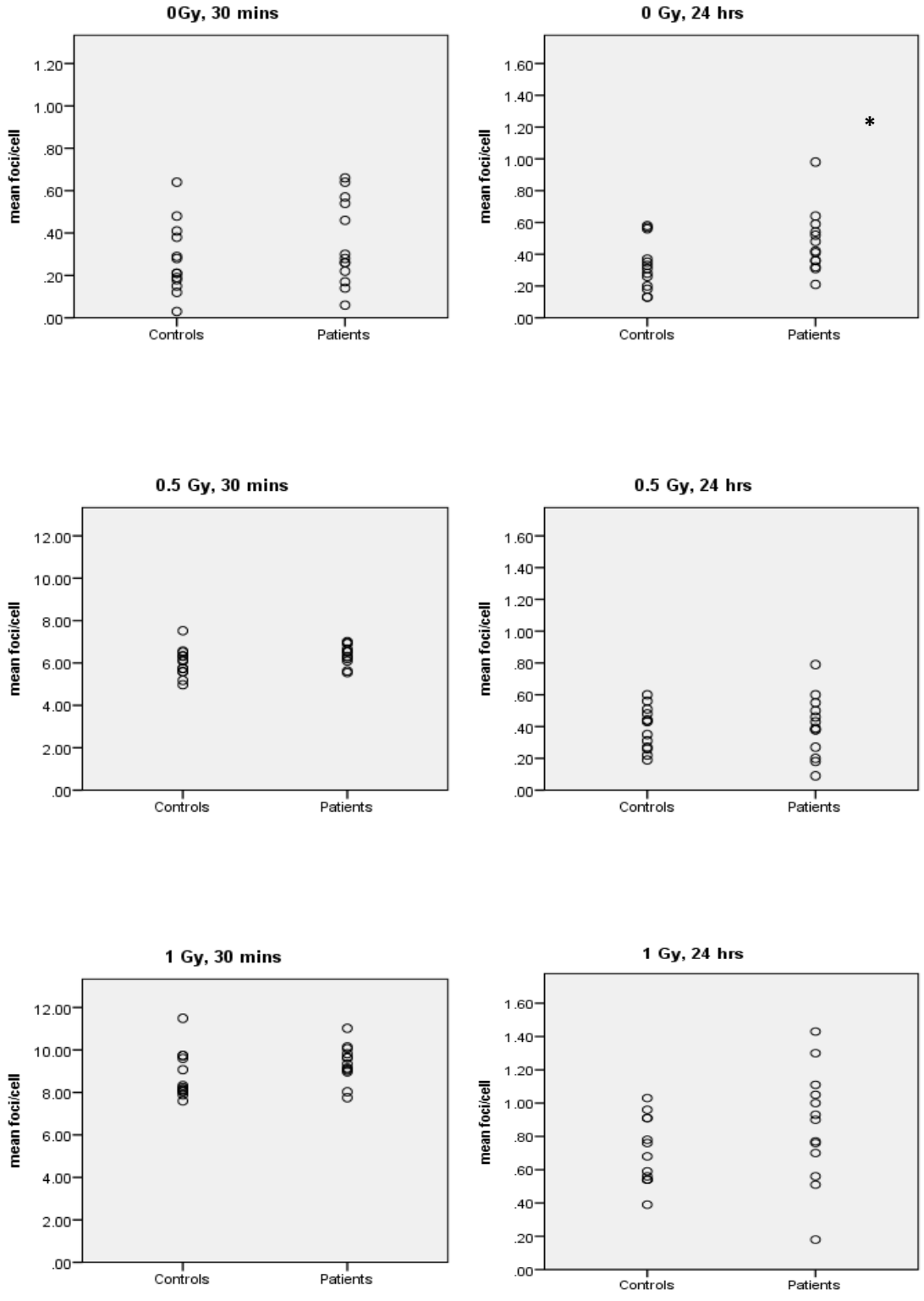

Figure 4.2: Foci values for 13 patients and matching controls. The foci for the 0.5 Gy and 1 Gy are radiation induced. *significant from controls at same dose and time (Mann-Whitney p<0.05).

4.1.4 Comparison of γ-H2AX repair factor and MN values in peripheral blood lymphocytes of cervical cancer patients

The MN assay was performed on the same set of samples and controls. The repair factors were compared to MN values as they both give information on residual damage. Table 4.3 shows the repair factors, MN values and NDI. There was no correlation between repair factors and MN values for controls and patients at any of the doses. Unlike the MN assay, there was zero attrition of cervical cancer samples with the γ-H2AX assay. While the MN values were higher in patients than controls, no significant difference could be reached due to high attrition in the patient samples. The NDI were significantly higher in patients than controls at the 2 Gy and 4 Gy dose.

Table 4.3: Comparison of repair factor in controls and HIV-negative patients with MN values and NDI.

Healthy control	Age	repair factor %		MN data			NDI		
-	-	0.5 Gyi	1 Gyi	0 Gy	2 Gyi	4 Gyi	0 Gy	2 Gy	4 Gy
Control 1	25	96.54	96.61	11	132	438	2.4	2.2	1.9
Control 2	47	95.08	94.38	7	112	388	2.2	2.0	1.7
Control 3	53	91.54	86.41	15	150	539	2.0	2.1	1.6
Control 4	30	94.91	93.94	4	137	455	1.7	2.1	1.8
Control 5	62	92.42	88.67	20	93	296	2.1	2.0	1.6
Control 6	56	93.23	87.83	4	149	497	2.3	2.0	1.6
Control 7	61	93.92	91.95	11	114	336	2.1	1.8	1.5
Control 8	51	91.70	92.50	10	118	428	2.4	2.2	1.9
Control 9	28	97.06	90.73	8	141	569	2.3	2.1	1.5
Control 10	40	89.26	88.96	8	130	390	2.2	1.9	1.9
Control 11	35	91.12	93.28	2	121	363	2.4	2.0	1.6
Control 12	57	96.17	93.21	17	113	475	1.9	1.6	1.3
Control 13	57	91.70	93.29	14	154	604	1.8	1.7	1.4
MEAN	**46**	**93.4**	**91.7**	**10**	**128**	**444**	**2.1**	**2.0**	**1.6**
STDEV		**2.4**	**3.0**	**5**	**18**	**92**	**0.2**	**0.2**	**0.2**
CV%		**2.6**	**3.2**	**53**	**14**	**21**	**10.8**	**9.1**	**11.5**
Patient	**Age**	**repair factor %**		**MN data**			**NDI**		
-	-	0.5 Gyi	1 Gyi	0 Gy	2 Gyi	4 Gyi	0 Gy	2 Gy	4 Gy
Patient 1	37	96.44	98.37	10	144	438	2.1	2.0	1.7
Patient 2	65	94.56	94.27	14	NS	NS	1.9	1.6	1.5
Patient 3	53	92.21	88.45	13	193	NS	2.1	1.7	1.4
Patient 4	67	94.34	94.32	8	110	NS	2.2	2.0	1.4
Patient 5	50	90.80	92.27	6	NS	NS	2.1	1.9	1.6
Patient 6	47	92.75	89.60	8	156	488	2.2	1.9	1.5
Patient 7	56	90.99	86.12	10	110	409	2.2	1.8	1.5
Patient 8	50	95.65	84.16	16	191	461	2.5	2.1	1.7
Patient 9	68	87.88	90.79	14	174	NS	2.0	1.6	1.4
Patient 10	61	97.33	89.57	NS	NS	NS	1.7	1.3	1.3
Patient 11	70	98.49	90.03	9	105	NS	2.0	1.8	1.4
Patient 12	64	93.45	90.49	26	NS	NS	1.7	1.4	1.1
Patient 13	63	93.99	90.16	20	177	427	2.1	1.9	1.5
MEAN	**58**	**93.8**	**90.7**	**13**	**151**	**445**	**2.1**	**1.8***	**1.5***
STDEV		**2.9**	**3.6**	**6**	**36**	**31**	**0.2**	**0.2**	**0.2**
CV%		**3.1**	**4.0**	**44**	**24**	**7**	**10.0**	**13.7**	**11.2**

NS = not suitable for reliable MN counts (insufficient BN and/or apoptotic cells) * significantly different from controls (Mann-Whitney p<0.05)

4.1.5 Micronucleus assay on cervical biopsy tumour cells and exfoliated cervical cells

4.1.5.1 Tumour biopsy cultures

Optimisation of tissue digestion showed the best results with Liberase Research Grade Purified Enzyme Blend for 4 - 12 hours. Tissue digested from endocervical biopsies showed cells in the squamous epithelial layers of the endocervix, particularly parabasal and superficial keratinocytes. Viability checking with trypan blue showed many of the cells to be viable after digestion. After optimisation of attachment, cells showed good attachment and could be cultured for up to 2 weeks before dying. Despite culturing cells in medium with growth factors and bovine pituitary extract which stimulates keratinocyte division, there was never sufficient proliferation of the cells to perform the MN assay. Figure 4.3 shows the attached cells under the inverted light microscope.

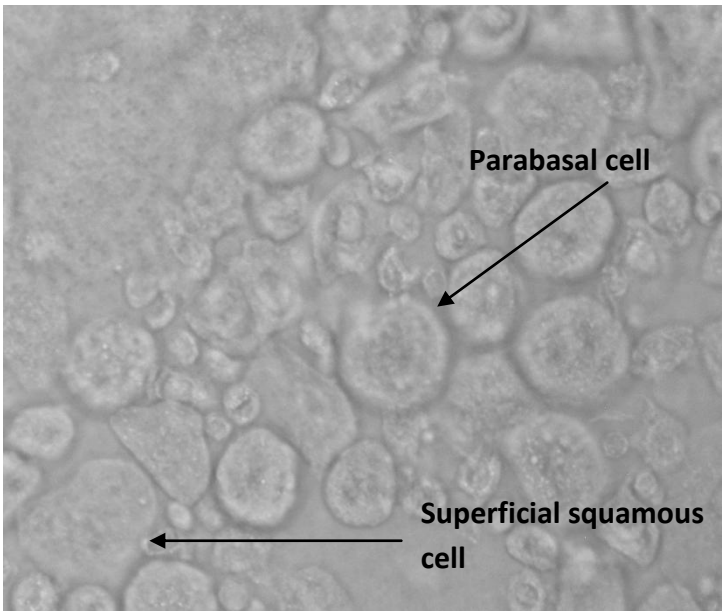

Figure 4.3: Cervical biopsy cells in culture. Arrows indicate the squamous cells from different layers of the ectocervical epidermis (40x objective, phase contrast light microscopy).

4.1.5.2 Exfoliated cervical cell cultures

Cell culture of exfoliated cervical cells was attempted on smears obtained from healthy women attending a colposcopy clinic. Samples were checked and any with bacterial/fungal infection were discarded. Many specimens had many blood cells that could hinder growth of cervical cells in culture (figure 4.4). Adding an erythrocyte lysis step reduced the number of red blood cells without affecting morphology of exfoliated cervical cells (figure 4.4). Due to the thin morphology of exfoliated cervical cells, viability checking with trypan blue was difficult, therefore cells were stained with acridine orange (figure 4.5). In culture, cells showed attachment and could be maintained in culture for up to 2 weeks before dying (figure 4.6). Despite adding medium with growth factor and bovine pituitary extract, sufficient proliferation to perform the micronucleus assay could not be achieved. Many superficial keratinocyte cells showed pyknotic nuclei (figure 4.5) that is indicative of the beginning of apoptosis, which may explain the low proliferation yield.

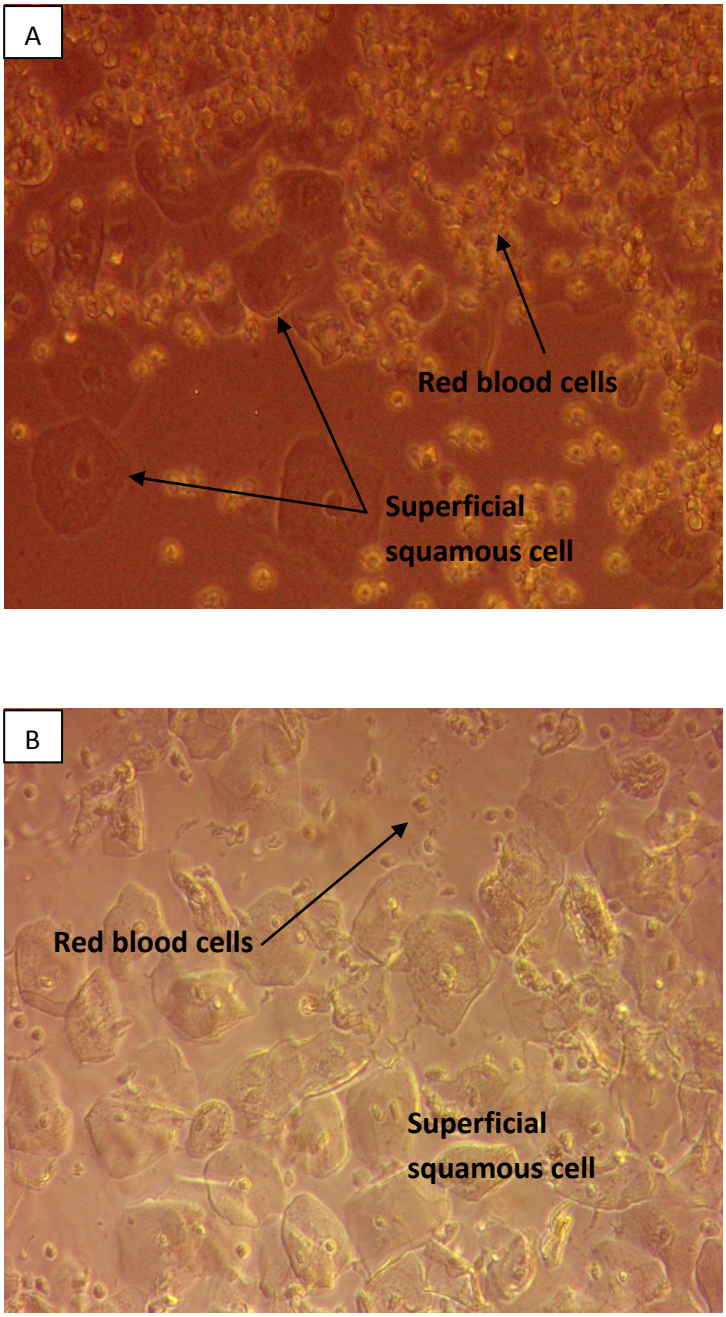

Figure 4.4: Exfoliated cervical cells before and after lysis step. Red blood cells hindered exfoliated cervical cell growth in cervical smears (A). A lysis step reduced the number of red blood cells (B) (40x objective, phase contrast light microscopy).

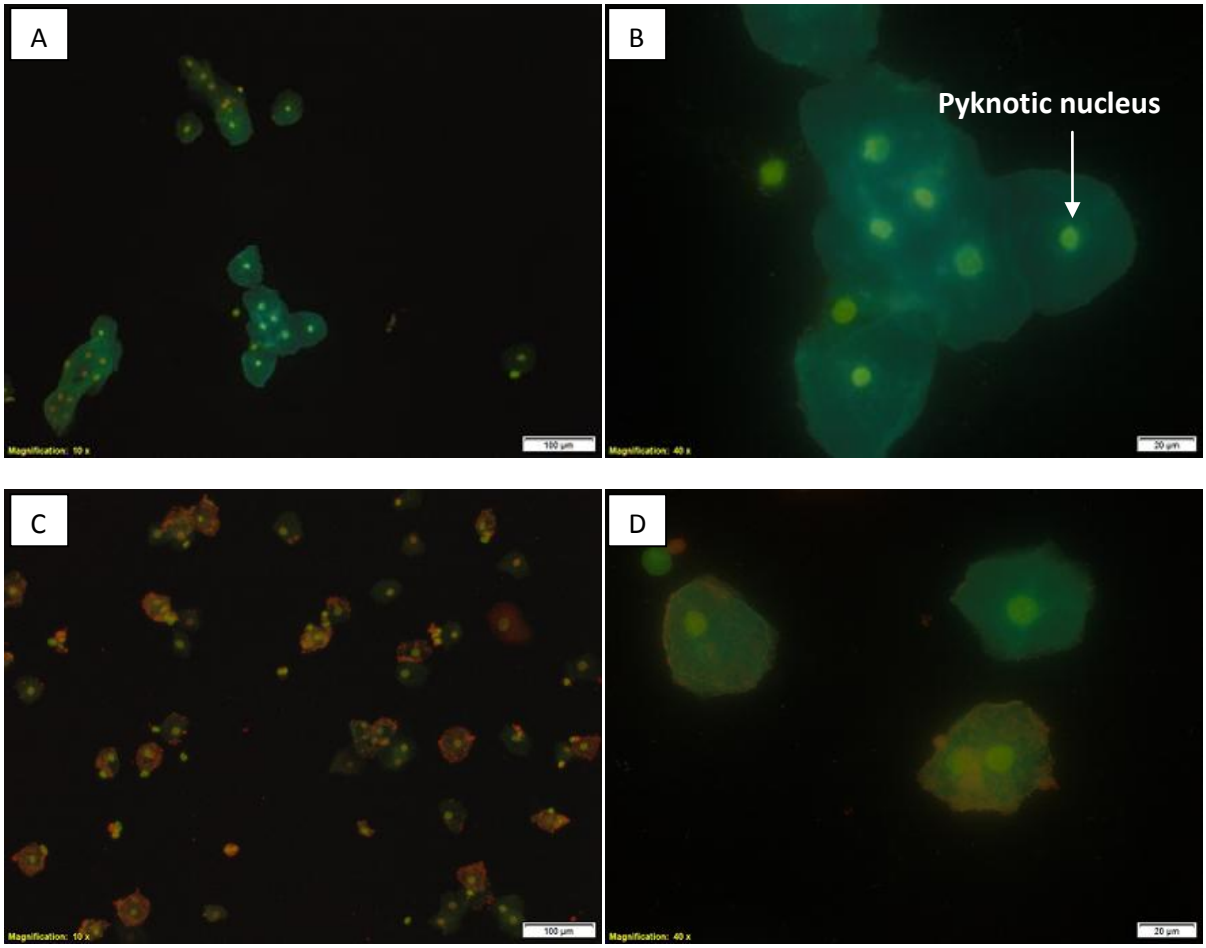

Figure 4.5: Viability staining of exfoliated cervical cells with acridine orange.A (100X original magnification) and B (400X original magnification) show viable exfoliated cervical cells, however nuclei are pyknotic indicating early apoptosis. C (100X original magnification) and D (400X original magnification) show non-viable exfoliated cervical cells with degraded cytoplasms (10x and 40x objective).

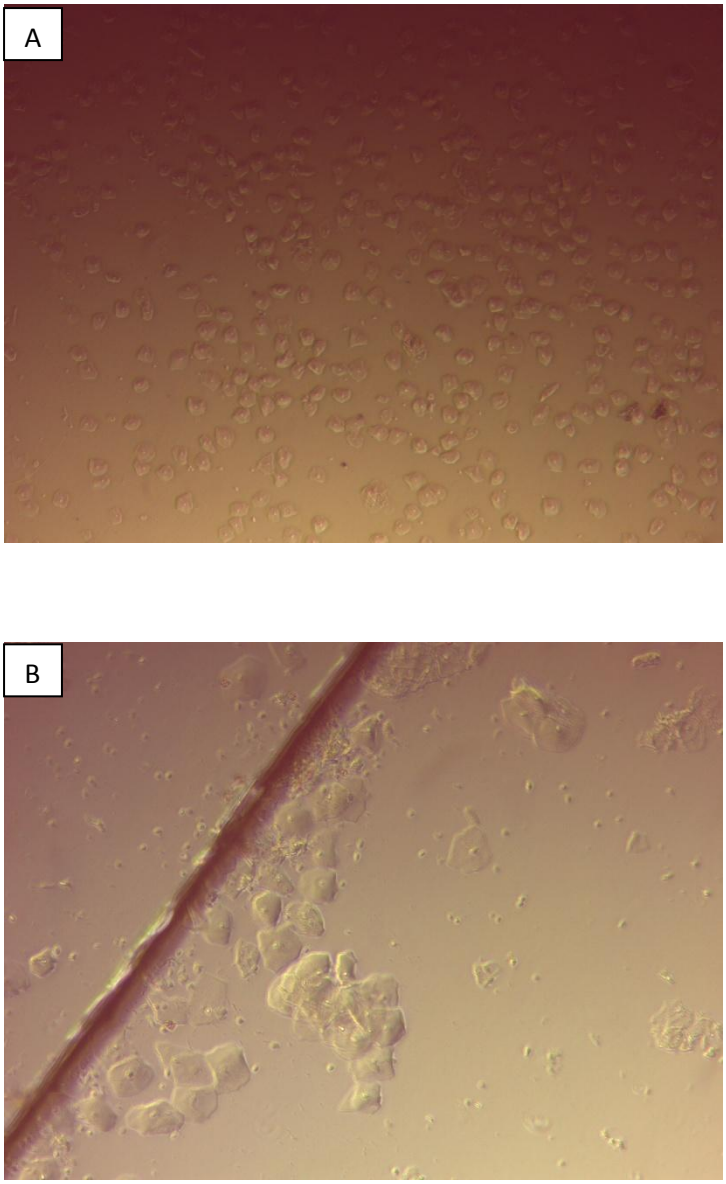

Figure 4.6: Exfoliated cervical cells in culture. A and B show exfoliated cervical cells attached to cell culture plates (10x and 20x objective, phase contrast light microscopy).

4.1.6 Immunohistochemistry to characterise cultured cells

Immunohistochemistry with Anti-pan Cytokeratin Antibody AE1+AE3 from abcam® was performed to characterise Keratinocytes in culture. The antibody was tested for specificity on five cervical biopsies (3 X normal ectocervix and 2 X tumour biopsies). Optimal staining was achieved with a citric acid buffer pre-step that allowed antigens to be better reached (antigen-retrieval). The antibody was shown to be specific to cervical keratinocytes (figure 4.7).

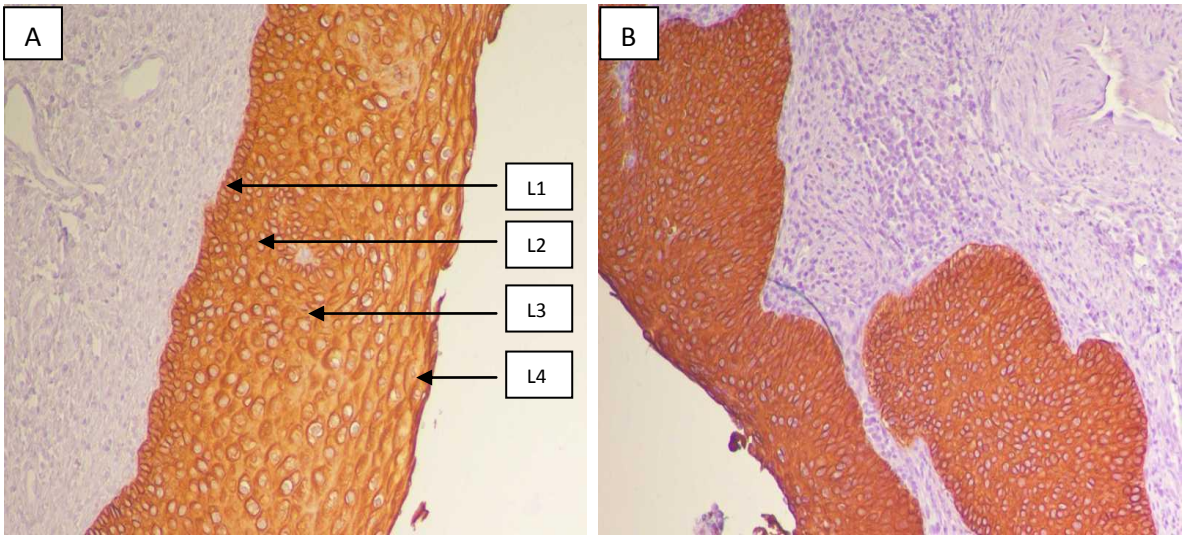

Figure 4.7: Stratified squamous epithelium of cervical ectocervix samples. A shows stratified squamous epithelium of a normal cervical ectocervix sample. The layers include: the basal layer (L1), parabasal layer (L2), intermediate layer (L3), superficial cells/exfoliating cells (L4). B shows stratified squamous epithelium of an invasive cervical cancer sample. Atypical cells from the basal layers have proliferated and spread into the underlying connective tissue (12.5x objective, bright field light microscopy).

4.1.7 Correlation of chromosomal radiosensitivity with HPV virus

HPV typing was performed on 49 patient biopsies. Positive and negative controls typed by a routine laboratory with the Abbott RealTi*m*e High Risk HPV assay, for clinical purposes, were obtained and included in every PCR. Figure 4.8 shows examples of agarose gels with positive and negative controls and patient samples on which HPV typing was performed. 78% of biopsies were HPV positive. The most common HPV subtype was HPV 16 (52%) (figure 4.9). Multiple HPV subtypes in 1 sample were only present in 2 patients.

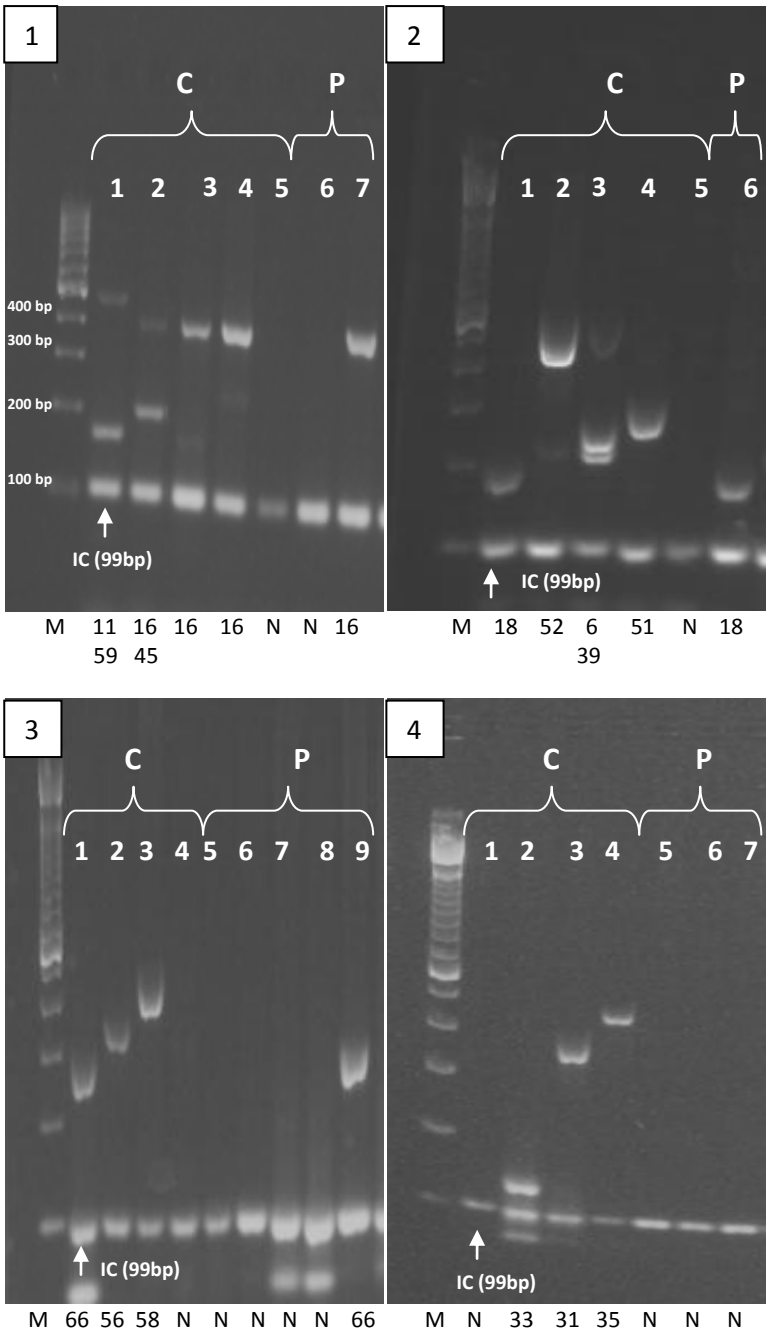

Figure 4.8: Agarose gel electrophoresis showing HPV genotyping with four HPV mixes.
Numbers represent the 4 HPV typing mixes. Lanes are numbered at the top of each gel. M = marker/DNA ladder (0.1 to 1.5kb). C = controls. P = patient samples. N = negative for any HPV type. The internal control (test for DNA integrity) is at 99bp in every lane.
1) Mix 1. Controls in lane 1= HPV 11 (472 bps) and HPV 59 (169 bps); lane 2 = HPV 16 (397 bps) and HPV 45(205 bps); lane 3 = HPV 16; lane 4 = HPV 16; lane 5 = negative control. Lanes 6 and 7 are patient samples. The patient in lane 7 is positive for HPV 16 (397 bps).
2) Mix 2. Controls in lane 1 = HPV 18 (187 bps); lane 2 = HPV 52 (517 bps); lane 3 = HPV 6 (263 bps) and HPV 39 (229 bps); lane 4 = HPV 51 (299 bps); lane 5 = negative control. Lane 6 shows a patient sample that is positive for HPV 18 (187 bps).
3) Mix 3. Controls in lane 1 = HPV 66 (277 bps); lane 2 = HPV 56 (330 bps); lane 3 = HPV 58 (414 bps); lane 4 = negative control. Lanes 5-9 are patient samples. Lane 9 shows a patient sample that is positive for HPV 66 (277 bps).
4) Mix 4. Controls in lane 1 = negative control; lane 2 = HPV 33 (139 bps); lane 3 = HPV 31 (360 bps); lane 4 = HPV 35 (434 bps). Lanes 5-7 are patient samples, none are positive for an HPV type.

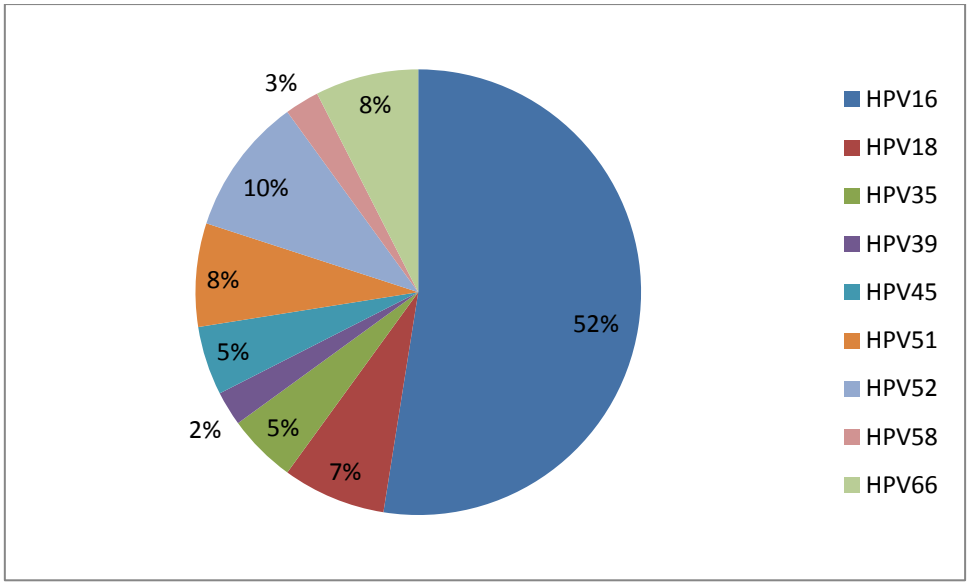

Figure 4.9: Percentages of HPV subtypes in biopsies of cervical cancer cohort.

MN data were available for 32 of these patients. For every sample there were data for the 0 Gy and 2 Gy dose, however 4 Gy data was only available for 22 patients. There was no difference in the average radiation-induced MN values of lymphocytes of patients positive for HPV (n = 22; 0Gy =12; 2Gy = 178, 4Gy = 471) compared to those negative for HPV (n = 10; 0Gy = 13; 2Gy = 186, 4Gy = 500). There was also no significant difference in the radiation-induced MN values of patients infected with subtypes 16 and 18 (most high risk subtypes), others combined, or negative (figure 4.10). There was also no difference in MN values between the α7 and α9 HPV subtypes (Hall et al., 2013).

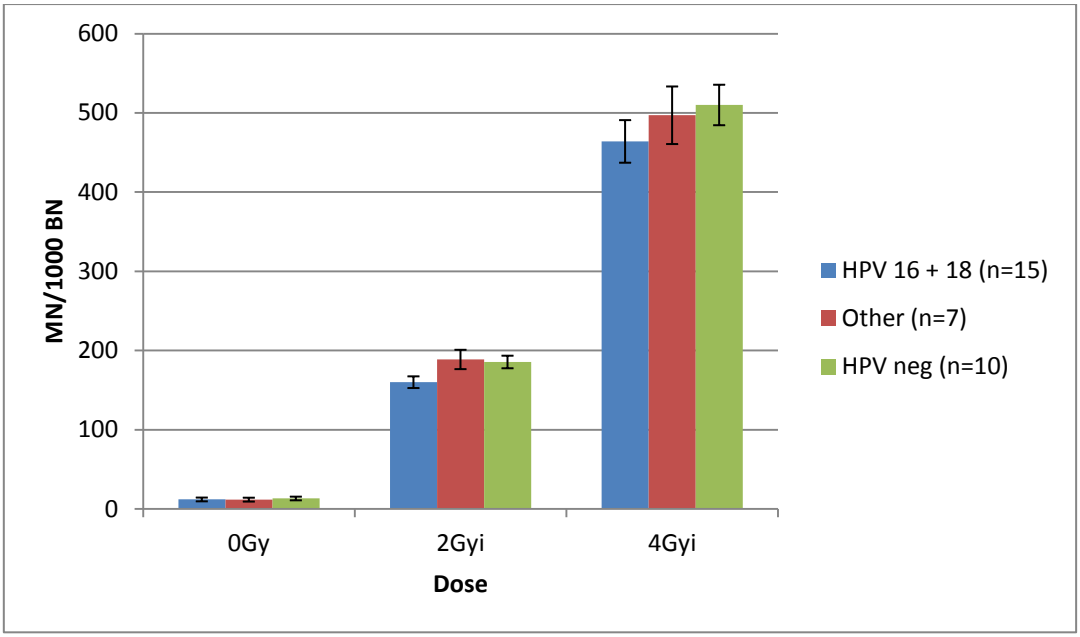

Figure 4.10: MN values in patients according to HPV subtypes. Error bars = SEM

4.2 Breast cancer patients

4.2.1 Study population

One hundred and thirty seven breast cancer patients were recruited during the study. Table 4.4 shows the biographical characteristics of this cohort. Black breast cancer patients showed the earliest age at diagnosis and were significantly younger than white and Indian patients. Coloured patients were also significantly younger than white patients. Black and coloured patients also had the highest level of 'pre-menopausal' breast cancer. The highest level of education and income was seen in white and Indian patients. HIV positivity was only seen in black and coloured patients.

Table 4.5 summarises the tumour characteristics of the patients. Black patients presented with the largest tumours, and along with coloured patients had significantly larger tumours than white patients. There was no difference in histology amongst the groups, ductal carcinoma being the commonest in all. Black patients had the highest percentage of stage 3 tumours. Coloured and Indian women had the highest percentage of estrogen-negative tumours, while coloured and black had the highest percentage of progesterone-negative tumours. Coloured patients also had the highest percentage of HER2-negative tumours. While there were differences in percentages of receptors, the difference was not significant (Chi-squared $p>0.05$).

Table 4.4: Characteristics of breast cancer patients.

Characteristic	Black (%)	White (%)	Coloured (%)	Indian (%)	p-value
Number of patients	65 (47.4)	54 (39.4)	5 (3.6)	13 (9.5)	
Age at diagnosis	48	62	60	55	p<0.001*
Menopause					
Pre-menopausal	31 (48.4)	5 (9.4)	3 (60)	4 (30.8)	P<0.001**
Post-menopausal	30 (46.9)	44 (83)	2 (40)	7 (53.8)	
Hysterectomy	3 (4.7)	4 (7.5)	0	2 (15.4)	
unknown	1	1	0	0	
Education					
Primary school	14 (21.9)	2 (3.9)	0	3 (23.1)	P=0.008**
High school	36 (56.3)	28 (54.9)	5 (100)	9 (69.2)	
Tertiary school	14 (21.9)	21 (41.2)	0	1 (7.7)	
unknown	1	3	0	0	
Income					
None	30 (48.4)	17 (38.6)	3 (60)	7 (53.8)	p=0.049**
<5000 ZAR	18 (29.0)	6 (13.6)	1 (20)	0	
>5000 ZAR	14 (22.6)	21 (47.7)	1 (20)	6 (46.2)	
unknown	3	10	0	0	
HIV-status					
Positive	11 (20)	0	1 (25)	0	p=0.008**
Negative	44 (80)	40 (100)	3 (75)	11 (100)	
unknown	10	14	1	2	

Percentages calculated without taking missing data (unknown) into account
*ANOVA statistical test
** Chi-squared statistical test

Table 4.5: Tumour characteristics of breast cancer patients according to ethnicity

Characteristic	Black (%)	White (%)	Coloured (%)	Indian (%)	p-value
Tumour size mm (average)	29	20	37	24	P=0.025*
Histology (%)					
Ductal	38 (84.4)	32 (76.2)	4 (100)	7 (63.6)	P> 0.05**
Lobular	3 (6.7)	3 (7.1)	0	2 (18.2)	
In situ Ductal/Lobular	3 (6.7)	7 (16.7)	0	2 (18.2)	
Other	1 (2.2)	0	0	0	
unknown	20	12	1	2	
Disease stage (%)					
0	1 (2.4)	2 (5.9)	0	1 (11.1)	p>0.05**
1	4 (9.5)	8 (23.5)	1 (25)	2 (22.2)	
2	22 (52.4)	18 (52.9)	3 (75)	5 (55.6)	
3	15 (35.7)	6 (17.6)	0	1 (11.1)	
4	0	0	0	0	
unknown	23	20	1	4	
Tumour grade (%)					
1	8 (18.2)	9 (22.5)	0	3 (30)	p>0.05**
2	21 (47.7)	14 (35)	3 (75)	4 (40)	
3	15 (37.4)	17 (42.5)	1 (25)	3 (30)	
unknown	22	14	1	3	
Estrogen receptors (%)					
Positive	39 (84.8)	35 (87.5)	3 (75)	8 (72.7)	p>0.05**
Negative	7 (15.2)	5 (12.5)	1 (25)	3 (27.3)	
unknown	19	14	1	2	
Progesterone receptors (%)					
Positive	29 (63)	31 (79.5)	3 (75)	7 (63.6)	p>0.05**
Negative	17 (37)	8 (20.5)	1 (25)	4 (36.4)	
unknown	18	15	1	3	
HER2 receptors (%)					
Positive	10 (22.2)	8 (22.9)	0	4 (36.4)	p>0.05**
Negative	35 (77.8)	27 (77.1)	4 (100)	7 (63.6)	
unknown	20	19	1	2	

Percentages calculated without taking missing data (unknown) into account
*ANOVA statistical test
** Chi-squared statistical test

4.2.2 Micronucleus assay on peripheral blood lymphocytes of breast cancer patients and controls

The radiation-induced MN values of 49 breast cancer patients (mean age = 49) and 52 healthy controls (mean age = 37) were compared. The MN values were determined with the 'Semi-automated B' method based on the results obtained for the cervical cancer patients. There was no significant difference between patients and controls at 0 Gy, 2 Gy and 4 Gy ($p = 0.068$). The patients and controls were split into groups according to ethnic groups which included: 1) Black group (20 patients - mean age = 48; 18 controls - mean age = 35). 2) Coloured group (4 patients - mean age = 45; 5 controls - mean age = 34). 3) Indian group (8 patients - mean age = 45; 5 controls- mean age = 35). 4) White group (17 patients - mean age = 59; 24 controls -mean age = 39). Figure 4.11 shows the radiation-induced MN yields for each ethnicity at 2 Gy and 4 Gy doses. There was no significant difference in radiation-induced MN between controls and patients except in the white group at the 4Gy dose ($p = 0.027$).

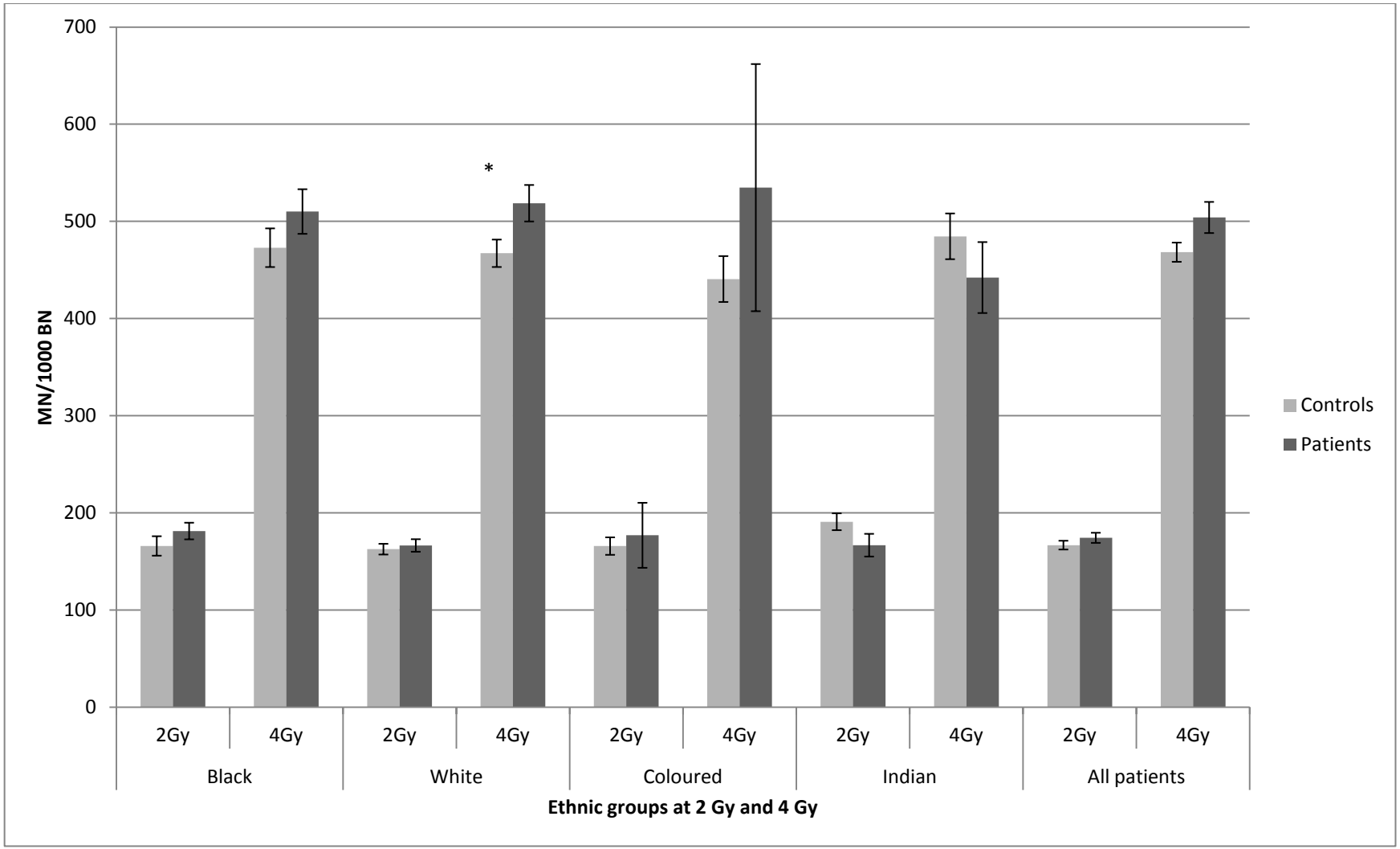

Figure 4.11: Comparison of radiation-induced MN frequencies between patients and controls across the 4 ethnic groups. * significantly different from controls (Mann-Whitney p<0.05). Error bars = SEM.

4.2.3 Correlation between MN values in breast cancer patients and clinical parameters and age of onset

No correlation was found between age and MN values ($p>0.05$, Pearson's correlation). For a further analysis, all breast cancer patients were divided into subgroups. These were the same groups as for the cervical cancer patients. 1) patients <40 years old (10 patients), 2) patients between 41 and 50 years old (12 patients), 3) patients between 51-60 years old (14 patients), 4) patients >61 years old (12 patients). There were no significant differences in radiation-induced MN values between the different age groups at any dose points (Mann-Whitney test $p>0.05$).

All the breast cancer patients were also split into groups according to clinical parameters and MN values in these groups were compared. There was no difference in MN values according to menopausal status of women. There was also no difference between groups with different tumour histology types. For tumour size, groups were split into groups (>2cm and <2cm). No differences were found in MN values between the groups. There was also no difference in MN values according to tumour stage and HER2 receptor. At the 2 Gy dose, there was an effect of estrogen receptor positivity ($p = 0.039$) and progesterone receptor positivity ($p = 0.028$). Those positive for the receptors having higher MN values.

5 Discussion

5.1 Cervical cancer patients

One hundred and forty seven cervical cancer patients with squamous cell carcinomas were recruited throughout the study. Major exclusion factors were previous treatment with chemotherapy or radiotherapy and blood transfusions, which are often given to cervical cancer patients to treat anaemia. Baeyens et al., (2010) showed that samples with CD4 counts below 300 cells/mm^3 were not suitable for the performance of the micronucleus assay so this was another exclusion factor for HIV-positive patients. The characteristics of this cohort in this study are similar to what has previously been described in other South African studies (Lomalisa et al., 2000, Moodley et al., 2001, Nyongesa et al., 2006, Denny et al., 2014), especially regarding the majority of patients presenting with late-stage disease and HIV-positive patients being 10 years younger than HIV-negative patients (Lomalisa et al., 2000, Moodley et al., 2001). A striking contrast in our cohort was the high level of HIV positivity amongst patients. Forty-two percent of patients were HIV-positive, compared to Lomalisa et al., (2000) who saw 7.2%, Moodley et al., (2001) 21% and Denny et al., (2014) 24.6%. This cannot be explained by increased HIV-positivity in the overall South African population, which was 10.2% in 2014 (STATSA, 2014).

The first aim of the study was to perform the MN assay on lymphocytes of cervical cancer patients and controls and validate the different scoring methods with the MNScore software of the Metafer (Metasystems). The Metafer has been used in two similar population studies on breast and prostate cancer (Varga et al., 2005, Varga et al., 2006). Using automated MN scores only, Varga et al. (2005, 2006) were able to distinguish clearly between breast cancer patients and controls but not between prostate cancer patients and controls. In our study, a

significant difference in MN after both 2 Gy and 4 Gy was only seen between patients and controls with the 'semi-automated B' scoring method which requires every BN selected by the Metafer system to be checked by a scorer. We noticed that factors hindering the other two scoring methods included apoptotic cells, missing of MN if there were many in a cell and cell debri.

Apoptotic cells, cell debri and a wide cell size range have been mentioned in other studies as factors impairing the efficiency of the Metafer system (Bolognesi et al., 2011, Fenech et al., 2013). The cancer population in this study was of mostly late-stage disease, which can contribute to the presence of apoptotic cells. We noticed that two apoptotic cells of similar size in close proximity were often mistaken as a BN cell by the MNScore software. These apoptotic cells could be identified by the scorer as they were smaller, denser and more brightly staining than nuclei of non-apoptotic cells. Adjusting the classifier may have ameliorated this problem but the classifier used also for the breast cancer samples was maintained so both cancer groups could be compared with the same controls. Two apoptotic cells in close proximity were sometimes misidentified as BN and were rejected when scoring with the 'semi-automated B' method. Samples with a high level of apoptosis (>25% rejection of cells with 'semi-automated B' scoring in 2 and 4 Gy) were excluded from the final analysis. In samples with moderate apoptosis, checking every BN and rejecting misidentified BN allowed for more reliable MN counts.

It has been noted in another study, using automated scoring only, that the system may fail in detecting all MN in a cell if they are close to or attached to the main nuclei and that the fully automated scoring may fail to count all MN if there are many MN in the same cell (Fenech et al., 2013). The *in vitro* doses administered in our study resulted in multiple MN,

which were missed with the 'fully-automated' and 'semi-automated A' scoring method but could be counted with the 'semi-automated B' method. Cell debri on slides was also noticed as a factor hindering the 'fully-automated' scoring due to many false positive MN, however these could be corrected with the 'semi-automated B' method. While 'semi-automated B' scoring on the Metafer does take longer than the other two methods, it is still quicker than scoring manually under a microscope. An additional benefit of the automated system is the image gallery that can be archived as a 'virtual slide' and re-analysed later. The 'semi-automated B' method may be the more suitable/reliable method when performing radiosensitivity studies in late-stage disease cancer populations.

The next aim was to compare chromosomal radiosensitivity in HIV-positive and HIV-negative cervical cancer patients with healthy controls. In the HIV-negative group, at a 2 Gy dose, the difference was not significant but there was a clear shift in the frequency distributions of MN counts being higher in patients than controls. The difference between patients and controls became significant at 4 Gy. Previous studies on chromosomal radiosensitivity in lymphocytes of HIV-negative cervical cancer patients with the MN assay have been inconclusive. In a study by Ban et al. (2004), patients were found to have lower radiation-induced MN frequencies than controls. However, blood samples in a large proportion of the patients were taken after or during radiotherapy treatment. This resulted in high spontaneous MN values which affect radiation-induced MN values. It was also suggested by the authors that the lower MN values seen in cervical patients may be the result of "an adaptive-response like phenomenon" from the *in vivo* therapy. A study similar to ours was performed by Encheva et al. (2011), where MN values in lymphocytes in 40 gynaecological patients (23 cervical and 17 endometrial combined into one group) were compared to those

obtained in 10 healthy controls. They found similar MN values for the combined gynaecological patients and the controls after exposure to an *in vitro* dose of 1.5 Gy. The inclusion of 17 patients with endometrial cancer, however, makes it difficult to draw conclusions on the chromosomal radiosensitivity of cervical cancer patients alone.

The increased MN values of HIV-negative cervical cancer patients compared to controls in our study suggests increased chromosomal radiosensitivity. Despite cervical cancer being caused primarily by infection with HPV, inherited genes are believed to play a role in cervical cancer susceptibility as only a fraction of women with HPV progress to disease. The disease also shows familial clustering (Magnusson et al., 1999). While immune response genes have been of focus in cervical cancer research, (Chen et al., 2013, Jiang et al., 2013, Chen et al., 2014), recent reports have shown associations between DNA damage repair genes and cervical cancer risk (Wang et al., 2010, Oliveira et al., 2012, Bajpai et al., 2013, Perez et al., 2013, Wang et al., 2013). Additionally, studies have shown Fanconi Anemia (FA) patients (patients with deficient DNA damage repair mechanisms) to be more susceptible to HPV-positive head and neck squamous cell carcinomas compared to non-FA patients with the same lesions (Kutler et al., 2003). Park et al. (2010) showed that there is an interplay between HPV and the FA pathway. The exact role of DNA damage repair genes and cervical cancer is still unclear. Evidence from studies using retroviruses shows that when a virus integrates into the host genome, a double strand break is formed, causing a DNA damage response similar to that seen when cells are exposed to ionising radiation (Skalka and Katz, 2005). If these DSB are not repaired and the integration process is not properly regulated, it can result in genomic instability which can lead to carcinogenesis (Oliveira et al., 2012). Research has also shown that HPV's preferential targets of integration are fragile sites

(Thorland et al., 2003). DNA damage repair genes like ATR, BRCA1, Checkpoint kinase 1 (CHEK1), have been shown to play a role in fragile site stability (Casper et al., 2002, Arlt et al., 2004, Glover et al., 2005, Durkin et al., 2006). There may be a link between compromised DNA damage repair, fragile sites stability and susceptibility to HPV integration and subsequent carcinogenesis.

HIV-positive cervical cancer patients had higher MN values than HIV-negative cervical cancer patients, despite the difference not being significant. The HIV-positive patients had MN values that were significantly higher than the healthy controls at 2 Gy and 4 Gy, which confirms the results of Baeyens et al. (2010). Our results highlight that when doing studies in developing countries, factors like HIV should be considered. Evidence suggests that these patients suffer from increased side-effects after radiotherapy but further investigation is required to confirm this (Shrivastava et al., 2005, Gichangi et al., 2006, Housri et al., 2010). The reason for higher MN counts in HIV-positive individuals is not known. Studies have shown HIV proteins like Viral Protein R (Vpr) and Integrase to interact with host DNA damage repair proteins (Bouhamdan et al., 1996, Ha et al., 2001, Mulder et al., 2002, Cooper et al., 2013), however this hypothesis is not yet fully clear (Ariumi et al., 2005). Vpr is also known to play a role in cell cycle regulation and can arrest cells in the G2 phase which can influence DNA damage repair (Amini et al., 2004). It has been shown recently that antiretroviral treatment influences MN counts in cancer-free HIV-positive individuals (Herd et al., 2014b). Since 73% of the HIV-positive patients included in the MN analysis were on ARV, it is possible that this had an influence on the MN results. Patient files were primarily focused on cervical cancer treatment, therefore information on the type and duration of antiretroviral treatment was often not specified. Interesting future studies would be to

compare chromosomal radiosensitivity in HIV-positive cervical cancer patients with and without ARV to determine the effect of ARV on radiosensitivity in these patients.

A challenge encountered in our study was to efficiently stimulate the lymphocytes of cervical cancer patients to perform the MN assay. In many samples, there were not enough BN, or there was a high level of apoptosis by which these samples could not be included in the final analysis. This occurred in both HIV-negative and HIV-positive patients but more so in the latter group. When comparing the NDI of a set of samples from the HIV-negative, HIV-positive and control groups, patients (HIV-negative and HIV-positive) had significantly lower NDI's than controls after irradiation, with HIV-positive patients having the lowest NDI's, illustrating the decreased proliferation capacity of patient lymphocytes. This was likely due to arrest in G1 as the cells were irradiated in the G0 cell cycle and it would appear that the doses administered resulted in a high level of DNA damage that caused the cells to enter into apoptosis (Saini et al., 2012). Cervical cancer cells produce a cytokine, Transforming growth factor beta (TGF-β), that has been shown to inhibit lymphocyte proliferation (Alcocer-Gonzalez et al., 2006, Diaz-Benitez et al., 2009, Lopez-Munoz et al., 2013). The production of this cytokine increases with increasing malignancy (Peghini et al., 2012). Production of TGF-β is related to HPV infection, as cervical cancer cell lines transformed with HPV express this cytokine at greater levels than those without HPV (Alcocer-Gonzalez et al., 2006, Diaz-Benitez et al., 2009). Lopez-Munoz et al., (2013) showed that CD4 lymphocytes from healthy people cultured in the same medium that had previously been used to culture cervical cancer cell lines (conditioned medium), had reduced proliferation after addition of phytohaemagglutinin. The study also showed that the conditioned medium induced apoptosis of the lymphocytes. Diaz-Benitez et al., (2009) also saw reduced

proliferation after phytohaemagglutinin in lymphocytes of cervical cancer patients compared to controls. A study by Jain et al., (1990) found a significant decrease in CD4 lymphocytes in patients with increasing cervical lesions. Since many of the patients in our study were of late-stage disease, expression of immunosuppressive cytokines like TGF-β by cervical cancer cells is a possible explanation for the low level of stimulation of lymphocytes, especially in the HIV group who already have compromised CD4 cells. The anti-proliferative and apoptotic effect of TGF-β on CD4 cells described in the studies of Lopez-Munoz et al., (2013) and Diaz-Benitez et al., (2009) could not be demonstrated for CD8 cells. The same was noted by Jain et al., (1990) who saw an increase in the CD8 subset, resulting in lower CD4/CD8 ratios with increasing cervical cancer malignancy. This is important to keep in mind in radiobiology studies as different T-lymphocyte sub-populations exhibit different radiosensitivities and CD8 cells have been found to be the most sensitive (Wuttke et al., 1993, Wilkins et al., 2002a, Wilkins et al., 2002b, Vokurkova et al., 2006). This is particularly relevant when interpreting the MN data in HIV-positive patients who are likely to have the lowest CD4/CD8 ratios due to the combined effect of HIV and TGF-β on CD4 cells. Since only CD4 counts are tested in patients at our hospital, the ratio of CD4/CD8 at the time of taking blood could not be determined. Radiotherapy has been shown to decrease CD4 counts in both HIV-negative and HIV-positive individuals (Sankatsing et al., 2013). The high doses given in this study could have affected CD4 counts and may be a contributing factor to high level of apoptosis and 'fall-out' at the 4 Gy dose. Another aspect to mention with regard to HIV-positive patients and lymphocyte proliferation is HIV Vpr protein causing G2/M cell cycle arrest (Amini et al., 2004). This could be a contributing factor to the difficulty in stimulating the lymphocytes of these patients (Baeyens et al., 2010).

The third aim of the study was to assess if chromosomal radiosensitivity was influenced by clinical parameters and age of onset of the disease. Despite the groups being small, our results show that there were no differences in MN values between groups according to tumour stage and histology. While correlations have been observed between MN values and response to treatment (Slonina and Gasinska, 1997), literature on correlation between MN values in cervical cancer patients and clinical parameters is scarce. In our study we observed a difference in MN values between the youngest (<40 year old) age group and age groups 41-50 and >60, however the higher MN values in the young group may have been influenced by the high percentage of HIV-positivity in this group. There was also a correlation between patient age and background MN values (0 Gy), which has been well-established in many studies (Fenech and Bonassi, 2011).

Besides the MN assay, we also performed the γ-H2AX foci assay on lymphocytes of 13 HIV-negative cervical cancer patients and controls. This test, performed 30 mins and 24 hrs post irradiation, measures DNA double strand break induction and residual damage after repair, while the MN assay measures chromosomal damage resulting from mis/non-repaired DSB. Elevated γ-H2AX foci counts have been detected in lymphocytes of lung cancer (1 hr post-irradiation, 2.5 Gy dose) and bladder cancer (1 hr post-irradiation, 2.5 Gy dose) cohorts compared to controls after *in vitro* irradiation (Fernandez et al., 2013, He et al., 2013). A study performed on breast cancer patients showed a significantly higher number of γ-H2AX foci in patients compared to controls at 0.5 Gy after 30 mins and at 2 Gy after 24 hrs (Djuzenova et al., 2013). To our knowledge, γ-H2AX foci have not been investigated in a case control-study design in lymphocytes of cervical cancer patients. In our study, this was performed on HIV-negative patients only, as HIV was shown to have an effect on

chromosomal radiosensitivity in the MN assay. Regarding baseline foci, there was no difference between patients and controls after 30 mins of incubation. Patients showed a trend of higher mean foci values after 24 hrs incubation, however, the difference was not significant after removing one outlier. The reason for the higher level of DNA double strand breaks in this outlier is not known. The number of radiation-induced foci measured 30 mins after irradiation was similar between patients and controls with no significant difference between both groups at both doses. Foci were measured 24 hrs post-irradiation to compare residual foci in patients and controls after allowing for repair. There was no significant difference at both doses. There were also no difference in the repair factors between the groups.

The outcome of this assay was not in agreement with the MN assay performed on 35 patients where there was a clear difference between patients and controls. There was also no correlation between the repair factors and MN values performed on the same set of samples. Vandersickel et al., (2010) failed to see increased residual foci values in a repair-deficient breast cancer cell line compared to a repair-proficient one, while a significant increase in MN values was observed. The authors suggested that mis-rejoined breaks will not be detected by the γ-H2AX foci assay. Mis-rejoined breaks, which would cause foci to disappear, could result in micronuclei. This may be a possible explanation for the different outcomes between the two assays. Other studies have also found a lack of concordance between MN values and γ-H2AX foci (Yoshikawa et al., 2009), while some show agreement between the different endpoints of the 2 assays (Eastham et al., 2001, Scarpato et al., 2011). Another reason for not detecting differences between the groups is that at low doses like 0.5 Gy and 1 Gy the number of remaining DSB become too low to see significant differences.

Interesting further studies would be to repeat what was done here using 2 Gy and 4 Gy doses. At 30 mins, γ-H2AX foci could not be counted at such high doses but could give more information on differences in remaining damage after allowing for repair. This may also result in a better correlation between repair factors and MN values as the radiation doses would be the same. Additionally, repair kinetics studies, in which foci are counted at several different time points after irradiation may give more information on differences in repair. When comparing the practicalities of the two tests, unlike the MN assay, there was zero attrition of samples with the foci assay, and an advantage of the assay is that it does not require dividing cells which overcomes the problem of low stimulation and apoptotic cells in cervical cancer lymphocytes. However, disadvantages of this is it is more labor intensive and costly than the MN assay and places a greater burden on the Metafer 4 system (±1.5 hrs per slide compared to ±8 mins per slide for 2000 cells). The fluorescent stain also fades with time, so slides need to be scanned shortly after the immunostaining and cannot be stored long-term.

Another aim of the study was to develop a MN assay that could be performed on cervical biopsy tumour cells and exfoliated cervical cells of cervical cancer patients. Cell cultures from cervical tissue are traditionally established using 3T3 feeder cells (Coleman et al., 1993, Freshney, 2010), however, a few studies have successfully established cervical cell lines without feeder cells (Koopman et al., 1999, Widel et al., 1999, Brink et al., 2002, Darroudi et al., 2010). Widel et al., (1999) was able to perform the MN assay on irradiated biopsies, which was shown to have predictive value. The protocol of our study was modelled on that of Widel et al., (1999), Brink et al., (2002) and Darroudi et al., (2010) with some added modifications according to Freshney et al., (2010). These protocols were tested and

optimised on our samples. Despite achieving adequate cell digestions and cell attachment, and using mediums suggested by the above-mentioned protocols, cells could be maintained in culture for up to two weeks, but there was never sufficient proliferation to allow for enough BN to perform the MN assay. A major challenge encountered in this part of the work was the high number of late-stage disease patients. Many tumours from patients consisted of necrotic tissue and there was a high level of apoptosis and cells with poor morphology which hindered culturing and proliferation of the cells.

In some studies baseline micronuclei have been measured in un-stimulated exfoliated cervical cells and in general a correlation is observed between MN frequency and cervical cancer lesions (Leal-Garza et al., 2002, Samanta et al., 2011). Since the aim of this part of the study was to perform the MN assay in exfoliated cervical cells, we did not measure MN in un-stimulated cells. To our knowledge, exfoliated cervical cells have never been cultured. Like the cervical biopsies, despite many optimisations resulting in successful attachment and maintenance in culture, there was not enough division of these cells to allow for the MN assay. A possible reason is that these cells, being exfoliating cells, are very well differentiated with pyknotic nuclei (characteristic of apoptosis) when they reach the outer layers of the ectocervix. This may hinder their ability to proliferate in culture.

The final aim of our study performed on cervical cancer patients was HPV typing on patient tumour biopsies to correlate with MN data. Seventy eight percent of biopsies were positive for the HPV virus. This is lower than in other studies on South African cohorts, (Moodley et al., 2010, Denny et al., 2014) but this group is small and not representative of the overall population due to selection criteria of the study, for example, exclusion of patients who underwent a blood transfusion or patients with low CD4 counts. It was also only performed

on a sub-set of patients who agreed to give a biopsy for research purposes. Different HPV subtypes have been shown to produce proteins that have differing affinity for host p53 protein (Lechner and Laimins, 1994). If there is a link between DNA damage repair genes and susceptibility to HPV and cervical cancer, it is reasonable to hypothesise that compromised DNA repair may be correlated to HPV positivity or potentially to specific HPV subtypes. Our results suggest that MN values are not correlated with HPV infection or subtype or α7 and α9 subtypes. There was also no difference in background MN values suggesting no link between chromosomal instability, as determined with the MN assay, and susceptibility to HPV integration.

5.2 Breast cancer patients

In our study, similar trends were seen in the characteristics of the breast cancer cohort to what has previously been described in South African breast cancer populations (Cubasch et al., 2013, Herd et al., 2014a). The data in this study showed how characteristics of breast cancer can differ amongst different ethnic groups. For example, black women were significantly younger than other groups and had a significantly higher proportion of pre-menopausal patients. This trend also occurs in studies done on American breast cancer patients of different ethnicities (Clarke et al., 2012). Black and coloured women had significantly larger tumour sizes and a higher proportion of patients presenting in stage 3. Both tumour size and stage are linked with poorer disease outcome (Chagpar et al., 2011). The differences between the ethnic groups points to differences in the underlying biology of the disease and led to the idea of comparing chromosomal radiosensitivity in different ethnic groups.

Although many studies have shown breast cancer patients to be more chromosomally radiosensitive than controls (Scott et al., 1994, Terzoudi et al., 2000, Riches et al., 2001, Baeyens et al., 2002, Varga et al., 2006, Poggioli et al., 2010, Ryabchenko et al., 2012, Djuzenova et al., 2013), chromosomal radiosensitivity has never been assesed in a population residing in Sub-Saharan Africa. In our study, chromosomal radiosensitivity was analysed in 49 South African breast cancer patients and 52 healthy controls. The patients showed higher MN values at both the 2 Gy and 4 Gy dose but the difference was not significant for either dose. There was also no difference in spontaneous MN values. When dividing the patients into different groups based on ethnicity, higher MN values were observed in the black patient group, but the difference was not significant at any dose. In the white patient group, patients had higher MN values than the controls and the difference was significant at 4 Gy. The coloured patients had the highest MN values. Interestingly, the Indian patients had lower MN values than controls. These last two groups, however, were very small so no conclusions can be drawn based on these data. Our results in the white patient group showed elevated chromosomal radiosensitivity which confirms those of other studies on white patients. It is interesting that the same was not seen in black breast cancer patients. In a study performed by Wang et al., (2012) looking at chromatid breaks in young breast cancer patients, a similar trend was observed. They found significantly higher chromatid breaks in white and Mexican American women but no difference in Africa-American women. African breast cancer patients are known to have aggressive tumour phenotypes compared to European women and a higher prevalence of triple negative and premenopausal breast cancers. Understanding the underlying mechanisms in this group is of importance. Results indicate that in terms of DNA damage repair genes and breast cancer,

there may be a difference between women of European and African descent, however further studies are needed to validate this.

The effect of clinical parameters on chromosomal radiosensitivity was also investigated. We found no effect of menopause status on MN values of all breast cancer patients combined. The study of Riches et al., (2001) also did not detect an effect of menopause status on chromatid breaks in lymphocytes in the G2 phase. However, Baeyens et al., (2005a) found that pre-menopausal women were more chromosomally radiosensitive than post-menopausal women . A similar trend was noted by Ryabchenko et al., (2012). There was an effect of estrogen and progesterone receptor status on MN values at the 2 Gy dose, with receptor positive women having significantly higher MN values. A similar trend was observed in the study of Riches et al., (2001) where patients with increased G2 radiosensitivity had a higher proportion of ER receptor positive tumours. In the study of Baeyens et al., (2005b) no effect on MN values of *in vivo* and *in vitro* estrogen levels were observed . Other studies, however, have shown that hormones can affect chromosomal radiosensitivity (Ricoul et al., 1997). Since we did not measure the level of blood estrogen/progesterone in patients, it is not possible to comment if it is changes in these hormones, related to receptor status, that may be affecting the MN counts. No relationship was seen between chromosomal radiosensitivity and HER2. There was also no effect of tumour histology or tumour size or stage on MN yields. Also no difference was observed in MN values between age groups, this in agreement with Baeyens et al., (2005a).

5.3 Conclusion

The overall aim of the study was to investigate the chromosomal radiosensitivity of South African cervical and breast cancer patients. Our results suggest increased chromosomal

radiosensitivity in cervical cancer patients with the MN assay. Using the γ-H2AX assay, no difference was seen between patients and controls showing a lack of agreement between the endpoint of these two assay. Further studies are needed to unravel the link between chromosomal radiosensitivity, DNA damage repair genes and the underlying mechanisms of susceptibility to HPV-induced carcinogenesis. These could lead to a marker for increased cancer risk in women with HPV infections and could assist in prioritising those needing regular Pap smears which is helpful in resource-limited countries such as South Africa. Our findings confirm that cervical cancer patients seeking radiotherapy who are HIV-positive may form a distinct group that require individualised treatments. Our study also showed the benefits and challenges of using the MN and γ-H2AX foci assay on lymphocytes of late-stage disease patients. The scoring method introduced in this study for the automated MN assay may be of benefit to future research projects on late-stage cancer populations.

Results on the breast cancer patients confirmed those of overseas studies on women of European descent. However, the same was not seen in women of other ethnicities. This study highlights how different genetic mechanisms may underlie breast cancer risk in women from different genetic/ethnic backgrounds. Further studies are needed on African populations to further delineate unique genes/polymorphisms at play in breast cancer risk.

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7 APPENDIX A: Copy of publications emanating from this thesis

Ethnical differences in breast cancer characteristics in South African population

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Breast cancer is the most common cancer amongst South African women, with the National Cancer Registry of South Africa estimating a 1 in 32 lifetime risk of developing the disease (1). South Africa is a country with diverse ethnic groups and the lifetime risk of developing breast cancer differs according to ethnicity. The lifetime risk is 1/53 in black women, 1/15 in white women, 1/21 in coloured women and 1/20 in Indian women (1). The incidence of breast cancer is on the rise in South African women. This is typically due to increased life expectancies and urbanisation that leads to lifestyle changes that elevate exposure to known risk factors for breast cancer (2). The mortality rate of existing South African breast cancer patients is high owing to limited access to diagnostic centers in rural areas, lack of awareness, lower standards of healthcare facilities and limited screening (3).

Studies elucidating the characteristics and underlying markers of breast cancer are of importance as they can guide breast cancer prevention strategies, patient management and the development of novel treatments. These types of studies are especially important in countries like South Africa for the following reasons:

1) Women of African ancestry are shown to have more aggressive tumour phenotypes compared to European women and triple negative and premenopausal breast cancers are believed to be more prevalent

2) South Africa also has a large mixed-race ('coloured') population about which, in terms of breast cancer characteristics, little is documented

3) Breast cancer in South Africa occurs within the backdrop of a high prevalence of HIV infection. The exact role that HIV plays in breast cancer incidence, underlying tumour biology or patient response, is not yet entirely clear.

The socioeconomic factors and biological characteristics of 233 South African breast cancer patients from Johannesburg in relation to ethnicity were investigated (Table 1 and Table 2). The Chi square test was used to compare the differences in characteristics between the 4 ethnic populations.

The cancer cohort consisted of 55% black, 30% white, 6% coloured and 9% Indian women. The black women were statistically significantly younger than the other 3 groups. 34% of black women were pre-menopausal compared to 10% of white women, 23% of coloured women and 15% of Indian women. These findings are consistent with studies done on American breast cancer patients with different ethnicity (4).

Large distributions of income and education levels amongst patients were noticed and income / education levels were significantly associated with the age at diagnosis and menopausal status regardless of ethnicity.

The black and coloured women had the significantly ($p<0.001$) largest tumour sizes. As Chagpar et al. (5) suggested that tumour size is a driving force of poorer disease outcome,

improved and early screening is especially important for the black and coloured South African women. The majority of patients presented with moderate/poorly differentiated tumours (grade 2 -3) and this was similar over all the ethnical groups. However, 46% of the black patients were diagnosed in stage III or IV compared to 27% or less in women from other groups. Late stage disease is linked with a worse prognosis. In this study the late stage of the disease was significantly linked with lower income level ($p<0.001$) across the different ethnical groups.

Another factor influencing prognosis and treatment is the presence of hormone receptors. Black and coloured women presented with a higher incidence of ER- and PR- negative tumours. On the contrary, Her2- tumours occurred mostly in white and coloured women.

18% of our black breast cancer patients were HIV positive but their tumour characteristics did not differ significantly with those of the HIV negative patients.

Our study showed that ethnical differences occur in South African breast cancer patients. No factor could be exclusively responsible for the observed differences but income and education levels clearly attribute to the disease pattern. These findings suggest further improvement in the detection of the disease through awareness and education. Further explorations of the racial factors influencing the breast cancer characteristics are needed, as there may be relevant implications for prevention and treatment planning.

Table 1. Patient Characteristics according to ethnicity

Characteristic	Black	White	Coloured	Indian	p-value **
Number of patients (%)	129 (55,4)	71 (30,5)	13 (5,5)	20 (8,6)	
Age at diagnosis, mean (SD)	50,6 (11,8)	63,7 (12,0)	56,6 (9,6)	57,8 (11,0)	<0,001
Menopause (%)*					
pre -menopausal	44 (34,4)	7 (10,0)	3 (23,1)	3 (15,0)	<0,001
post menopausal	77 (60,1)	60 (85,7)	10 (76,9)	17 (85,0)	
hysterectomy	7 (5,5)	3 (4,3)	0	0	
missing data	1	1	0	0	
Highest eduction level completed (%)*					
Primary school	37 (32,5)	2 (3,0)	2 (15,4)	5 (27,8)	<0,001
High school	57 (50,0)	44 (65,7)	11 (84,6)	12 (66,7)	
Tertiary school	20 (17,5)	21 (31,3)	0	1 (5,5)	
missing data	15	4	0	0	
Income (%)*					
None	56 (51,9)	28 (46,7)	10 (83,3)	13 (81,3)	<0,001
< 5000 ZAR	39 (36,1)	12 (20,0)	1 (8,3)	0 (0)	
> 5000 ZAR	13 (12,0)	20 (33,3)	1 (8,3)	3 (18,7)	
missing data	21	11	1	4	

* percentages were calculated without taking the missing data into account

** Chi square tests for differences in non-missing proportions between 4 racial groups

Table 2.Tumour Characteristics according to ethnicity

Characteristic	Black	White	Coloured	Indian	p-value **
Tumour size, mm, mean (SD)	32,9 (20,6)	25,4 (19,4)	35,6 (26,1)	23,4 (14,1)	< 0,05
Histology (%)*					
Ductal	100 (84,0)	53 (82,8)	8 (72,7)	15 (78,9)	0,316
Lobular	3 (2,5)	6 (9,4)	1 (9,1)	3 (15,8)	
In situ ductal/lobular	11 (9,2)	5 (7,8)	1 (9,1)	1 (5,3)	
other types	5 (4,2)	0	1 (9,1)	0	
missing data	10	7	2	1	
Pathological stage					
0	4 (3,5)	4 (6,5)	1 (9,1)	1 (5,5)	<0,05
I	11 (9,7)	10 (16,4)	2 (18,2)	3 (16,7)	
II	47 (41,6)	33 (54,1)	5 (45,4)	10 (55,5)	
III	49 (43,4)	14 (23,0)	3 (27,3)	4 (22,2)	
IV	2 (1,8)	0 (0)	0 (0)	0 (0)	
missing data	16	10	2	2	
Tumour grade (%)*					
1	11 (10,3)	11 (18,3)	0 (0)	3 (15,8)	0,589
2	56 (52,3)	25 (41,7)	5 (55,5)	10 (52,6)	
3	40 (37,4)	24 (40,0)	4 (44,4)	6 (31,6)	
missing data	22	11	4	1	
Oestrogen receptors					
positive	88 (75,2)	54 (85,7)	7 (63,6)	16 (84,2)	0,212
negative	29 (24,8)	9 (14,3)	4 (36,4)	3 (15,8)	
missing data	12	8	2	1	

Progesteron receptors					
positive	68 (58,1)	46 (74,2)	6 (54,5)	12 (63,2)	0,181
negative	49 (41,8)	16 (25,8)	5 (45,5)	7 (36,8)	
missing data	12	9	2	1	
HER 2					
positive	47 (41,6)	16 (26,7)	1 (9,1)	7 (41,2)	0,058
negative	66 (58,4)	44 (73,3)	10 (90,9)	10 (58,8)	
missing data	16	11	2	3	

* percentages were calculated without taking the missing data into account

** Chi square tests for differences in non-missing proportions between 4 racial groups

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Chromosomal radiosensitivity of HIV positive/negative cervical cancer patients in South Africa

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Abstract

Cervical cancer is the second most common cancer amongst South African women and is the leading cause of cancer related deaths in this region. Several international studies on radiation-induced DNA damage in lymphocytes of cervical cancer patients remain inconclusive. Despite the high incidence of cervical cancer in South Africa, and the extensive use of radiotherapy to treat it, there is were no data on the chromosomal radiosensitivity of South African cervical cancer patients. Since many of these patients are HIV-positive, the effect of HIV infection on chromosomal radiosensitivity was also investigated. Blood samples from 35 cervical cancer patients (20 HIV-negative and 15 HIV-positive) and 20 healthy controls were exposed to *in vitro* doses of 6MV X-rays of 2 and 4 Gy. Chromosomal radiosensitivity was assessed with the micronucleus (MN) assay. MN scores were obtained with an automated microscopic system, Metafer 4 platform (Metasystems). Three scoring methods used with the MNScore module of Metafer, Metasystems were compared. Cervical cancer patients had higher MN values than healthy controls, with HIV-positive patients having the highest MN values. Differences between groups were significant when using a scoring method that corrects for both false positive and false negative MN. This study suggests increased chromosomal radiosensitivity in South African cervical cancer patients.

Introduction

Cervical cancer is the most common cancer amongst women in Sub-Saharan Africa, with an age-standardised incidence rate of 34.8 per 10^5 females (1). Cervical cancer is also the leading cause of cancer-related deaths amongst women in this region, with 22.5 per 10^5 women dying from the disease annually (1). It is well-established that infection with oncogenic Human Papilloma Viruses (HPV) is the main cause of cervical cancer (2, 3). The high incidence of cervical cancer in Africa is likely due to a combination of factors. These include lack of awareness of the disease and its causes, as well as challenges in implementing regular HPV Papanicolaou (Pap)-smear screenings and HPV vaccinations, which have only recently been introduced into the South African health care system (4-6) While many women are infected with the HPV virus, not all go on to develop cervical cancer (7). Heritability studies have shown that there is a genetic link to cervical cancer susceptibility (8) and recent reports have shown an association between cervical cancer and genes involved in DNA damage repair. These include genes such as APE1, XRCC2, XRCC3, ERCC1, ERCC2, ERCC4, ATM (9-12).

Enhanced chromosomal radiosensitivity is related to defects in genes involved in DNA damage repair (13). The first indication for a possible inherited basis for radiosensitivity came from patients with rare genetics syndromes such as Ataxia Telangiectasia and Nijmegen breakage syndrome (13). These patients were shown to display not only clinical, but also *in vitro* chromosomal radiosensitivity (14). Patients with these syndromes have germline mutations in genes involved in DNA damage repair and also displayed predisposition to many cancers. Their increased chromosomal radiosensitivity led to studies that showed an enhanced *in vitro* chromosomal radiosensitivity in cancer patients, including

breast, head and neck and prostate cancer (15-18). Mutations in DNA repair genes not only lead to increased chromosomal radiosensitivity in these patients but may also predispose them to the disease (13, 17, 19, 20). The *in vitro* chromosomal radiosensitivity of lymphocytes of cervical cancer patients has been investigated using a variety of cytogenetic assays, however results have been unclear (21-24).

A well-established method to measure chromosomal radiosensitivity is the Micronucleus (MN) assay, which quantifies residual chromosome damage resulting from mis-or non-repaired double strand breaks after exposure to radiation. This assay can be performed on lymphocytes, which are an attractive model for radiosensitivity studies as they are easily obtainable through venepuncture. MN scoring in this assay can be automated with an MN scoring module for the Metafer 4 platform (MetaSystems, Altlussheim, Germany). The MNScore micronucleus software module allows automatic screening of binucleate cells and the subsequent scoring of MN in these cells (25) (Schunck et al., 2004). With the system, different scoring methods can be utilized that involve varying degrees of visual validation of automated scorings to correct for false positive and false negative MN and reject unsuitable cells (26, 27). The benefits and challenges of using the MN assay with the Metafer 4 platform have been documented elsewhere (26-32).

A study performed by our group (33) has previously shown that individuals infected with Human Immunodeficiency Virus (HIV) are more chromosomally radiosensitive than uninfected people. In South Africa there are approximately 5.7 million people living with HIV (34). HIV, HPV and cervical cancer are epidemiologically associated and in 1993 invasive cervical carcinoma was classified as an AIDS-defining illness by the United States Centers for Disease Control and Prevention (35). Rates of HPV infection increase with decreasing CD4

cell count (36) and studies have shown that HPV infection still persists in a high proportion of patients receiving HAART treatment (37). Due to the high rate of HIV in Africa and its association with cervical cancer, it is likely that a significant proportion of cervical cancer patients seeking treatment will be HIV-positive.

The aim of this study was to investigate the *in vitro* chromosomal radiosensitivity in South African cervical cancer patients by means of the MN assay using a case-control study design. Concurrently, we evaluated different scoring methods when using the MN assay with the Metafer 4 platform. Due to the high rate of HIV in South Africa and its association with cervical cancer, the effect of HIV on chromosomal radiosensitivity of patients was also considered.

Materials and methods

Study population:

Blood samples were obtained via venepuncture from a total of 35 cervical cancer patients (mean age 46) and 20 healthy female controls (mean age 41). 15 patients were infected with HIV (mean age 43) and 20 were negative for HIV infection (mean age 49). Patients were recruited from Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), a public hospital in Johannesburg, South Africa, where they were undergoing curative hysterectomies or attending a radiotherapy clinic. Information about patients was obtained from questionnaires and hospital files. None of the patients had received chemotherapy or radiotherapy prior to sample collection. All patients had squamous cell carcinoma tumours. The majority of patients had late-stage disease. Only five were early-stage disease and disease stage was unknown for one patient. The healthy controls were staff members at

CMJAH. All blood donors signed informed consent and the study was approved by the Human Research Ethics Committee, University of Witwatersrand, Johannesburg, South Africa (M110230).

Irradiations and Micronucleus assay:

Lymphocyte cultures were set up by adding 0.5 ml of heparinised blood to 4.5 ml of Roswell Park Memorial Institute 1640 (RPMI) medium (BioWhittaker, Walkersville, USA) in tissue culture flasks (25 cm2) that was supplemented with 13% foetal bovine serum (Gibco-Invitrogen, New York, USA) and antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin; Gibco-Invitrogen). The medium was pre-warmed to 37°C and gassed (5%CO2/95% air). The radiations were done in the Radiation Oncology Unit at CMJAH. Culture flasks were placed in a Phantom-water tank at room temperature and irradiated with X-rays using a 6 MV photon beam from a medical linear accelerator (Siemens Healthcare, Erlangen, Germany). The distance from the culture flasks to the radiation source was 100 cm at an angle of 90 degrees. The field size at the depth of the sample was 10X10 cm. Samples were irradiated with 2 Gray (Gy) and 4 Gy at a dose rate of approximately 1.33 Gy/min. A 0 Gy dose was used as a sham-irradiated control. For each dose point, 2 co-cultures were set up. Immediately after irradiation the lymphocytes were stimulated with 100 µl of phytohaemagglutinin (stock solution 1mg/ml, Sigma-Aldrich, St Louis, MO, USA) and 23h later 20 µl cytochalasin B (stock solution of 1.5mg/ml, Sigma-Aldrich) was added to block cytokinesis. Cells were harvested at 70h after stimulation using a cold (4°C) hypotonic shock with 7ml 0.075M KCl (Merck, Darmstadt, Germany). This was followed by fixation in methanol:acetic acid:Ringer (0.9% NaCl) solution (4:1:5) (Merck) at 4°C overnight. Thereafter cells were fixed another three times with methanol:acetic acid (4:1)(Merck). Cell

suspensions were dropped on coded slides and stored at 4°C overnight. Slides were mounted with vectashield containing DAPI (4,6-diamidino-2-ohenylindole; Vector Laboratories, Burlingame, USA) before being scanned automatically with the Metafer 4 (28, 32).

Scoring:

Microscopic analysis was performed with the Metafer 4 platform connected to a motorised Zeiss AxioImager M1 microscope. MNScore software module of Metasystems identifies BN and displays them in an image gallery with a MN count per cell. Details of how this system works have been described previously (28, 32). We used the parameters of the classifier of Willems et al. (2010) (32) with minor adjustments. Three different scoring methods can be employed involving varying degrees of visual validation of automated scores. The first is the 'fully-automated' scoring method in which MN counts are obtained directly by the MNScore module (32). The second is a 'semi-automated' scoring method which has been discussed in other publications (26, 27, 38). In this study, we referred to this method of scoring as semi-automated A, where only false positive MN are corrected. As an extra validation, we introduced a third scoring method, referred to as semi-automated B. In this method, every BN cell (with and without MN) was checked in the image gallery and false negative MN were also corrected, in addition to the false positive MN corrected in semi-automated A. For each sample, 'fully-automated', 'semi-automated A' and 'semi-automated B' MN scores were obtained by 2 experienced scorers. The average number of BN per data point was 1600 BN. Data points with less than 500 BN were not included. All results were normalised to a MN frequency in 1000 BN.

The nuclear division index (NDI) was also calculated according to the formula: NDI = (N1+2N2+3N3+4N4)/ Ntotal, with N1-N4 is the number of cells with 1-4 nuclei and Ntotal the total number of cells scored (Ntotal = 500).

Statistical analysis:

Statistical analysis was performed with Graphpad Prism 6. Differences between means of MN counts in the various groups were tested for significance with the Mann-Whitney test. This statistical test was used as it is a non-parametric, distribution-free test that is suitable to compare groups with small sizes where no underlying distribution can be assumed. Significance was set at $p<0.05$.

Results

MN Values of cervical cancer patients vs healthy controls obtained with 3 scoring methods:

MN frequencies of cervical cancer patients were compared to healthy controls using the three different scoring methods of the Metafer system. Radiation-induced MN yields were calculated by subtracting spontaneous MN yields from MN yields in irradiated cells. Spontaneous MN values and radiation-induced values for 2 Gy and 4 Gy are listed in Table 1. Using the automated and 'semi-automated A' scoring method, no significant differences were detected between patients and controls. With the 'semi-automated B' scoring method, patients had clearly higher MN values compared to controls for all radiation dose points, the difference between the groups being significant at 2 Gy ($p = 0.0075$) and 4 Gy ($p = 0.0059$).

MN values of HIV-positive cervical cancer patients compared to HIV-negative cervical cancer patients and healthy controls:

To investigate if HIV has an influence on the chromosomal radiosensitivity of the cervical cancer patients, the group of cervical cancer patients was split in HIV-positive and HIV-negative patients. Based on the results shown in Table 1, to compare HIV-positive and HIV-negative patients to controls, we used the 'semi-automated B' scoring method only. The MN values of healthy controls, HIV-positive patients and HIV-negative patients are presented in Figure 1. The HIV-negative patients had clearly higher MN compared to controls but the difference was only significant at 4 Gy ($p = 0.037$). For 2 Gy, there was no significant difference between HIV-negative cancer patients and controls ($p = 0.060$), however there was a clear shift towards higher MN values for the patients (figure 1).

In HIV-positive patients at 4 Gy there were 2 samples with insufficient BN and were thus excluded from the analysis. Although not statistically significant, the HIV-positive patients had higher MN values than the HIV-patients. The HIV-positive patients had statistically significant higher radiation induced MN values than healthy controls at both 2 Gy ($p = 0.006$) and 4 Gy ($p = 0.008$).

Nuclear division index (NDI) of cervical cancer patients and healthy controls:

To evaluate the quality of the MN assay in the different groups, we calculated the nuclear division index. The healthy controls had NDI for 0, 2 and 4 Gy of respectively 2.170; 2.073 and 1.668, while the cancer patients had NDI for 0, 2 and 4 Gy of respectively 2.117; 1.883 and 1.535. The differences in NDI were statistically different between both groups for the 2

and 4 Gy ($p = 0.0091$ and $p = 0.0182$). The HIV-positive cancer patients had the lowest ranges of NDI.

Discussion

The aim of this study was to compare the chromosomal radiosensitivity of South African cervical cancer patients with healthy controls using the micronucleus assay. The development of the MNScore software module by Metasystem has allowed the automation of MN scoring (25). The system has been documented in several studies on biodosimetry and cancer research and allows for different scoring methods that involve varying degrees of visual validation of automated scores (26-32). The presence of apoptotic nuclei and a wide cell size range have been mentioned as factors impairing the efficiency of the Metafer system (26, 29). Another challenge noted is that the system "may fail to identify all MN if they are close or attached to the main nuclei or if there are more than one MN in the same BN cell" (29). The cancer population group in this study was of late-stage disease, which can contribute to apoptotic and varying cell sizes and the *in vitro* doses we administered can result in multiple MN. For these reasons we included a 'semi-automated B' scoring method as an extra validation step to correct for false positive and false negative MN in all BN detected. While 'semi-automated B' scoring on the Metafer does take longer than 'semi-automated A' scoring, it is still quicker than scoring manually under a microscope. The system also adds additional benefits in that the BN screening is less subjective and the full image gallery can be archived as a 'virtual slide' that can be re-analysed whenever necessary. The Metafer has been used in two similar population studies on breast and prostate cancer populations (30, 31). Using the automated scores only, Varga et al. (30, 31) were able to distinguish clearly between breast cancer patients and controls but not

prostate cancer patients and controls. The Metafer scoring has not previously been used to investigate a cervical cancer population. When comparing cervical cancer patients to controls using the 'fully-automated' and 'semi-automated A' method, no differences were seen between patients and controls (Table 1). With the 'semi-automated B' method, patients had significantly higher MN at 2 and 4 Gy. At the 4 Gy dose, many BN have multiple MN which will be missed in the 'fully-automatic' and 'semi-automatic A' scoring methods. Another reason is the high level of cell debri that was noted on the slides of the cancer patients, which may be a result of increased apoptosis, due to elevated radiosensitivity or other cellular stress associated with late-stage disease. The lower yield of BN in the cancer group, which also may be a results of apoptosis or cellular stress, was reflected in the significantly lower NDI compared to the healthy control group. These issues can result in a number of false positive MN that can affect the automated scoring.

Our results suggest increased chromosomal radiosensitivity in patients compared to controls. Previous studies on the chromosomal radiosensitivity of lymphocytes of cervical cancer patients have been inconclusive. In a study by Ban et al. (22), using the micronucleus assay, patients were found to have lower MN frequencies than controls. Blood samples, however, were taken in a large proportion of the patients after or during radiotherapy treatment. This resulted in high spontaneous MN values which affect radiation induced values. It was also suggested by the authors that the lower MN values seen in cervical patients may be the result of "an adaptive-response like phenomenon" from the in vivo therapy. A similar study to ours was performed by Encheva et al. (39), where MN values from lymphocytes of 40 gynaecological patients (23 cervical and 17 endometrial combined into one group) were compared to 10 healthy controls. They found similar MN values for

the combined gynaecological patients and the controls after exposure to an *in vitro* dose of 1.5 Gy. The inclusion of 17 patients with endometrial cancer makes it difficult to draw conclusions on the chromosomal radiosensitivity of cervical cancer patients alone. Our study was conducted on a cohort with late-stage disease and unique genetic background.

It is now accepted that inherited genes play a role in cervical cancer susceptibility as only a fraction of women with HPV infection progress to disease, and the disease shows familial clustering (40). HPV-induced cervical carcinogenesis is a complex, multi-faceted process that is not yet well understood (7). While many studies on cervical cancer have focused on genes involved in immune response (41-43), genes involved in DNA damage repair and cervical cancer are also an area of interest. Recent reports have shown associations with genes involved in DNA damage repair and cervical cancer risk (9-12, 44). Additionally, studies have shown Fanconi Anemia (FA) patients (patients with deficient DNA damage repair mechanisms) to be more susceptible to HPV-positive head and neck squamous cell carcinomas compared to non-FA patients with the same lesions (45). Park et al. (46) showed that there is an interplay between HPV and the FA pathway. Defects in DNA damage processing genes affect chromosomal radiosensitivity. The increased chromosomal radiosensitivity observed in our study suggests deficient DNA damage processing, which may play a role in increased susceptibility to cervical cancer. The exact role of DNA damage repair genes and cervical cancer is still unclear. Evidence from retroviruses show that when a virus integrates into the host genome, a double strand break is formed, causing a DNA damage response similar to that seen when cells are exposed to ionising radiation (47). If these DSB are not repaired and the integration process is not properly regulated, it can result in genomic instability which can lead to carcinogenesis (12). Research has also shown

that HPV's preferential targets of integration are fragile sites (48). DNA damage repair genes like ATR, BRCA1, CHK1, have been shown to play a role in fragile site stability and expression (49-52). There may be a link between compromised DNA damage repair, fragile sites stability and susceptibility to HPV integration and subsequent carcinogenesis.

Our research group previously showed HIV-positive individuals to be more radiosensitive than non-infected individuals (33). HIV is widespread in Africa and the epidemiological link between HIV and cervical cancer means that many women seeking treatment (often radiotherapy) for the disease will be HIV-positive. For this reason, we added a group of HIV-positive cervical cancer patients to our cohort. In 2 HIV-positive patients, we couldn't obtain enough BN after the 4 Gy dose. This is likely due to HIV infection which compromises CD4 counts, in combination with the late-stage disease causing cells to not withstand the cytotoxicity of a dose as high as 4 Gy. HIV-positive cervical cancer patients had higher MN values than HIV-negative cervical cancer patients, despite the difference not being significant (figure 1). This could suggest an additional effect of HIV on the radiosensitivity of cervical cancer patients that are infected with HIV. The HIV-positive patients had MN values that were significantly higher than the healthy controls at 2 Gy and 4 Gy. This confirms the results of Baeyens et al. (33).

Our results show when using the Metafer system on cancer populations with advanced disease or HIV, the 'semi-automated B' scoring method yields the most reliable results. This technique seems to be useful to investigate the radiosensitivity of other cancer population groups. Our results also showed cervical cancer patients to have higher MN values than controls, suggesting increased chromosomal radiosensitivity. Our findings confirm that cervical cancer patients seeking radiotherapy who are HIV-positive may form a distinct

group that require individualized treatments. It would be interesting to follow up the *in vitro* data with the clinical response of HIV cervical cancer patients undergoing radiotherapy. Evidence suggests that these patients suffer from increased radiation side-effects but further investigation is required to confirm this (53-55). Our results highlight that when doing studies in developing countries, factors like HIV should be considered. Further studies are needed to unravel the link between chromosomal radiosensitivity, DNA damage repair genes and the underlying mechanisms of susceptibility to HPV-induced carcinogenesis. These could lead to a marker for increased cancer risk in women with HPV infections and could assist in prioritizing those needing regular pap-smears which is helpful in resource-limited countries such as South Africa.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1: Summary of spontaneous and radiation - induced MN values for controls and cervical cancer patients with 3 scoring methods. *Significantly different from controls (Mann-Whitney test $p<0.05$)

	AUTOMATED			SEMI-AUTOMATED A				SEMI-AUTOMATED B			
	0Gy	2Gy	4Gy		0Gy	2Gy	4Gy		0Gy	2Gy	4Gy
CONTROLS	**N**	20	20	20	**N**	20	20	20	**N**	20	20	20
	MEAN	56	125	323	**MEAN**	10	115	317	**MEAN**	13	155	454
	SEM	8	8	13	**SEM**	1	5	11	**SEM**	1	6	9
PATIENTS	**N**	35	35	35	**N**	35	35	35	**N**	35	35	33
	MEAN	66	144	320	**MEAN**	12	124	327	**MEAN**	14	*179	*506
	SEM	7	9	14	**SEM**	1	4	10	**SEM**	1	5	12

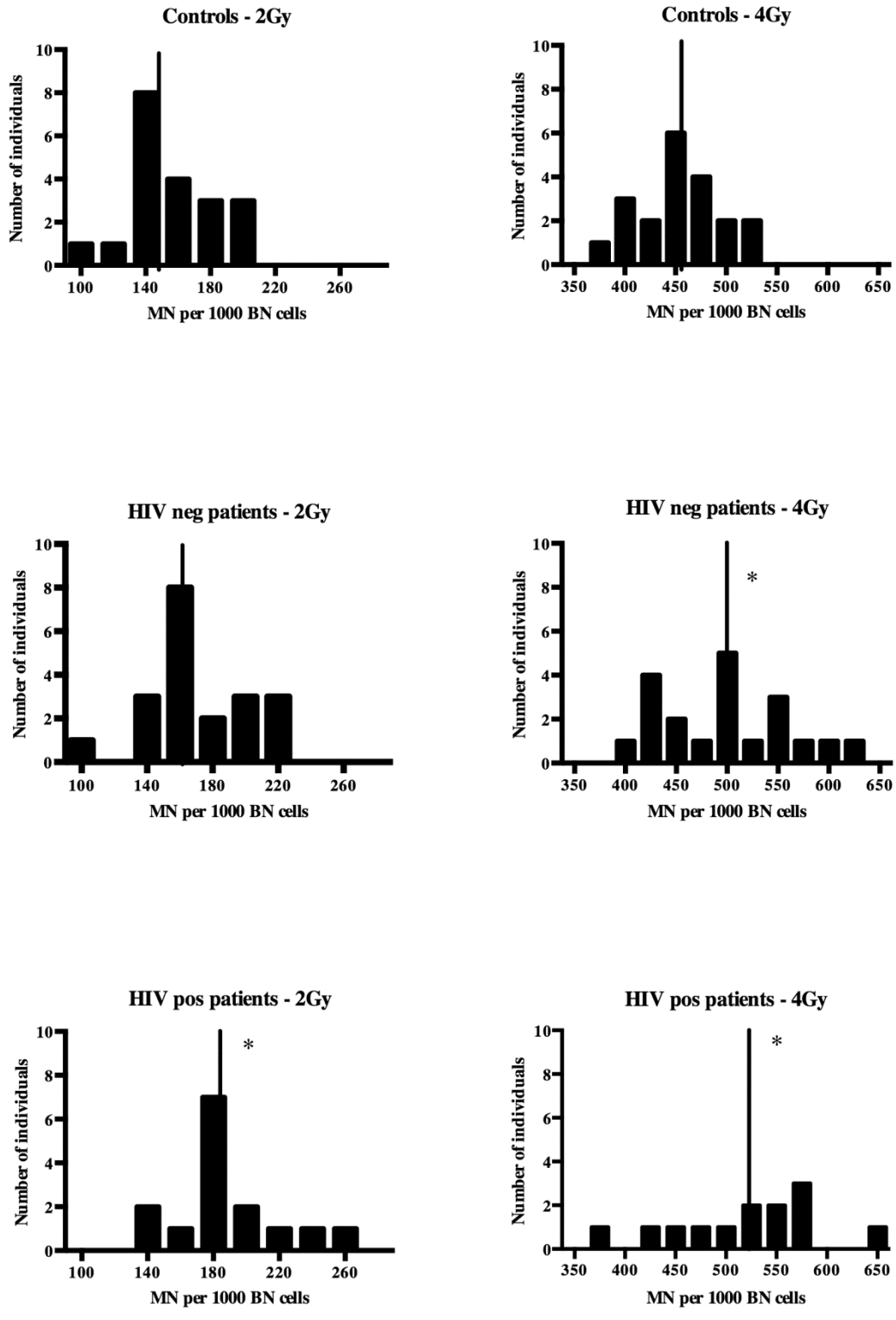

Figure 1: Radiation – induced MN yields after 2 Gy and 4Gy irradiations for cancer patients and controls. Midline = mean MN yield of the group. * Significantly different from controls ($p<0.05$)

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The Effect of HIV and Antiretroviral Therapy on Chromosomal Radiosensitivity

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Abstract

Introduction: Antiretroviral Treatment (ART) has led to an improvement in survival of HIV infected individuals. Some of them will develop cancer during the course of their infection and will require radiation therapy. HIV positive cancer patients have presented with adverse side effects of radiotherapy and elevated chromosomal radiosensitivity. This study investigated if ART has an influence on chromosomal radiosensitivity of HIV positive individuals.

Methods and materials: Blood samples from 60 HIV positive individuals were in vitro exposed to doses of X-rays of 0, 2 and 4Gy and chromosomal radiosensitivity was assessed with the micronucleus assay. The micronucleus assay was also performed on lymphocytes of a group of non HIV-infected health care workers taking prophylactic post-exposure ART to measure the effect of these ART drugs on chromosomal radiosensitivity without HIV as a confounding factor.

Results: All HIV patients (those on ART and without ART) had significantly higher radiation induced Micronuclei (MN) than healthy controls. The MN yields increased in the HIV patients taking ART compared to HIV patients not on treatment. The evaluation of chromosomal radiosensitivity of health care workers on ART revealed no effects of ART.

Conclusions: HIV positive individuals show an increased chromosomal radiosensitivity. Antiretroviral treatment given to HIV positive individuals can lead to enhanced chromosomal radiosensitivity and therefore impose higher risks for radiotherapy side effects in these patients.

Keywords: Antiretroviral treatment; HIV; Chromosomal radiosensitivity

Introduction

In 2013, there were 5.6 million people living with Human Immunodeficiency Virus (HIV) in South Africa [1]. Antiretroviral Therapy (ART) has led to the improvement in health and survival of patients with HIV [2]. South Africa has one of the largest ART roll-outs in the world and in 2013 over 2 million South Africans were on ART [2]. The 2006 World Health Organisation's guidelines for treatment of HIV infection in adults recommended a first-line regimen of two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) combined with one Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) [3]. Due to their cost effectiveness, Stavudine (d4T) and Zidovudine (AZT) were the most commonly prescribed NRTIs in low and middle income countries [4], however due its toxicity, the WHO updated guidelines recommended using Tenofovir (TDF) instead of d4T in first-line therapy [5]. In 2010 the South African Department of Health changed it guidelines to recommend TDF+lamivudine (3TC)/emtricitabine (FTC)+efavirenz (EFV) as first-line therapy for all new HIV patients and for those on d4T + 3TC/FTC+EFV without toxicity to remain on that regimen [6]. According to the WHO 2013 guidelines [7] all patients should be switched to the TDF-based regimen and the transition of patients to a fixed-dose combination one-pill ART regimen containing TDF+FTC+EFV began in South Africa in 2013 [8].

ART is not only used in treatment of HIV but also in prevention. ART is administered prophylactically to non-infected persons after occupational exposure to HIV. A study in Health Care Workers (HCWs) has shown post-exposure prophylaxis to be protective after exposure to the HIV virus [9]. With the high incidence of HIV in South Africa, HCWs face constant risk of being exposed to HIV after being cut or pricked by sharps during routine patient care. A South African study showed that 69% of medical interns reported at least 1 percutaneous injury during their year-long internship [10]. The WHO guidelines recommend a combination of antiretroviral drugs for post-exposure prophylaxis based on two nucleoside reverse transcriptase inhibitors [11].

HIV infection is associated with a high risk of developing specific AIDS-related cancers such as non-Hodgkin lymphoma, Kaposi sarcoma and cervical cancer [12]. The prolonged survival of HIV infected individuals, largely owing to improved ART access, has also led to an increasing incidence of non-AIDS related cancers amongst these individuals [13,14]. Radiotherapy is a mainstay treatment for cancers in South Africa where cancer patients, many of which will be HIV positive, often present with advanced disease due to limited access to diagnostic centers in rural areas, lack of awareness and lower standards of healthcare facilities. As South Africa is also a country with

a prolific radiation industry and many radiation workers, the high number of HIV infected people and taking ART in the general South African population is also likely to be reflected in this workforce. The effect of HIV and ART on chromosomal radiosensitivity can have important implications for radiation protection in these workers.

A pilot study by Baeyens et al. has previously shown HIV positive patients to be more chromosomally radiosensitive than HIV negative controls [15]. Chromosomal radiosensitivity is increased vulnerability of cells to the DNA-damaging effects of ionizing radiation. It can be a result of impaired DNA damage repair. Associations between clinical response to radiotherapy and chromosomal radiosensitivity [16,17] and elevated radiotoxicity such as mucositis and neutropenia has been reported in some HIV patients receiving radiotherapy for several types of cancers (reviewed in [18]). As radiotherapy decreases CD4 cell counts [19], HIV positive people in clinical settings are advised to start ART before undergoing radiotherapy to minimise the risk of developing AIDS, particularly when the patient has low CD4 counts.

Since NRTIs and NNRTIs interact with host DNA, it is possible that they could have an impact on DNA damage repair and affect chromosomal radiosensitivity. AZT, 3TC and TDF have been shown to inhibit cell growth [20], to interfere with cell cycle arrest, induce cell death by apoptosis and inhibit telomerase activity [21-24]. Conflicting studies have shown AZT to act as a radioprotector or a radiosensitizer [25,26].

Chromosomal radiosensitivity can be tested through a variety of cytogenetic assays. A reliable and well-established method is the cytokinesis-block micronucleus assay (MN assay) that is commonly used in biological dosimetry and chromosomal radiosensitivity studies. Micronuclei (MN) are small nuclei that form in the cytoplasm when chromosomes or chromosome fragments are not incorporated into the daughter nuclei subsequent to cell division [27]. MN reflects the amount of DNA damage and DNA damage repair after exposure to radiation. The MN assay can be performed on lymphocytes, which are easily obtainable via venipuncture. The micronucleus assay is also considered a sensitive method to evaluate the cytotoxic potential of an active agent. The nucleoside reverse transcriptase inhibitors AZT, 3TC and d4T have been shown to produce an increase in MN in human lymphocyte cultures [28-30].

In this study the micronucleus assay on the lymphocytes of HIV patients not taking ART and of HIV patients on one of the 2 most commonly administered ART regimens in South Africa (d4T+3TC +EFV and TDF+3TC+EFV) was performed to assess the effect of HIV and these drugs on chromosomal radiosensitivity. The micronucleus assay on the lymphocytes of a group of non HIV-infected health care workers taking prophylactic post-exposure ART was also performed to measure the effect of these ART drugs on chromosomal radiosensitivity without HIV as a confounding factor.

Materials and Methods

Study population

Heparinised blood samples from 60 HIV positive individuals were obtained from the Themba Lethu HIV unit at the Helen Joseph Hospital in Johannesburg, South Africa. Twenty participants were HIV positive but not taking any ART medication. Twenty participants were on the d4T+3TC+EFV (30 mg/150 mg/600 mg) regimen and twenty participants were taking the TDF+3TC+EFV (300 mg/150 mg/600 mg) regimen. HIV patients were on ART for at least 6 months. Blood samples of 20 HIV-negative healthy individuals, age-matched with HIV positive individuals, were included as controls. These were staff members and students from Charlotte Maxeke Johannesburg Academic hospital (CMJAH) where the study was undertaken. Population characteristics and treatment data are shown in Table 1. The CD4 counts, measured on the same day as the MN assay, were extracted from clinical records.

			HIV ART	HIV ART	
	Healthy individuals	**HIV without ART**	**D4T/3TC/EFV**	**TDF/3TC/EFV**	**Health care workers**
Gender					
female/male	14/6	15/5	13/7	18/2	8/2
Age					
mean ± SD	30 ± 7	36 ± 10	37 ± 7	41 ± 6	31 ± 11
range (min-max)	23 - 52	18 - 55	29 - 54	34 - 53	22 - 56
CD4 cell counts					
mean ± SD		480 ± 164 **	623 ± 240	649 ± 204	
range	(500 - 1200)*	268 - 858	202 - 1081	302 - 933	
Duration of ART (months)					
mean ± SD			51 ± 26	51 ± 28	1
range			13 - 100	6 - 99	
* normal range of CD4 counts in a healthy population					
** significantly lower than HIV ART groups					

Table 1: Patient Characteristics.

For the second part of the study, heparinised blood samples of 10 health care workers on post-exposure ART prophylaxis were collected from CMJAH. All the HCWs were taking a combination of 2 NRTIs (300 mg AZT+ 150 mg 3TC or 200 mg FTC + 300 mg TDF) for 28 days. Blood samples of these HCWs were taken at 3 time points: The first was the day of the needle-stick or sharps injury, just before starting prophylaxis treatment (T1); the second was 3-4 weeks after T1, after completion of the ART course of medication (T2); the third was between 3 and 4 months after T1 (T3). All blood donors signed informed consent and the study was approved by the Human Research Ethics Committee, University of Witwatersrand, Johannesburg, South Africa (M110361 and M120270).

Micronucleus assay

The micronucleus assay was performed as described by Baeyens et al. [15]. In vitro chromosomal radiosensitivity was measured by scoring MN in lymphocytes irradiated with doses of 2 Gray (Gy) and 4 Gy. A 2 Gy dose is frequently used in radiotherapy fractionation schedules. A 0 Gy dose was used as a sham-irradiated control (spontaneous MN) and also allowed us to examine the cytotoxic effect of ART on lymphocytes without irradiation. Lymphocyte cultures were set up by adding 0.5 ml blood to 4.5 ml of prewarmed RPMI

(Roswell Park Memorial Institute) 1640 (BioWhittaker, Walkersville, USA) that was supplemented with 13% foetal bovine serum (Gibco-Invitrogen, New York, USA) and 0.01% antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin; Gibco-Invitrogen). The irradiations were done in the Radiation Oncology Unit at CMJAH. Culture flasks were placed in a Phantom-water tank at room temperature and irradiated with X-rays using a 6 MV photon beam from a medical linear accelerator (Siemens Healthcare, Erlangen, Germany). The distance from the culture flasks to the radiation source was 100 cm at an angle of 90 degrees. The field size at the depth of the sample was 10X10 cm and the dose rate was approximately 1.33 Gy/min. For each dose point, 2 co-cultures were set up. Subsequent to irradiation, lymphocytes were stimulated by adding 100 µl of phytohaemagglutinin (stock solution 1mg/ml, Sigma-Aldrich, St Louis, MO, USA). To block cytokinesis, 20 µl cytochalasin B (stock solution of 1.5 mg/ml, Sigma-Aldrich) was added to the cultures at 23 hrs. Cells were harvested 70 hrs after stimulation using a cold (4°C) hypotonic shock with 7 ml 0.075M KCl (Merck, Darmstadt, Germany). This was followed by fixation in methanol: acetic acid: Ringer (0.9% NaCl) solution (4:1:5) (Merck) at 4°C overnight. Thereafter cells were fixed another three times with methanol: acetic acid (4:1) (Merck). Cell suspensions were dropped on slides and stained for 1 minute with acridine orange stain (10 µg/ml) (Sigma-Aldrich, St Louis, MO, USA) and immersed for 1 min in acridine orange buffer (Sigma-Aldrich, St Louis, MO, USA). Duplicate slides were made of each sample, coded and scored blindly by 2 experienced scorers using a Zeiss Axioskop fluorescent microscope (400X) (Carl Zeiss, Gottingen, Germany). At least 500 binucleated cells (BN) were scored per slide, according to the criteria of Fenech [31]. The results of both scorers were combined and normalised to 1000 BN. The number of mononucleated (N_1), binucleated (N_2), trinucleated (N_3) and polynucleated (N_4) cells per slide was scored and the nuclear division index (NDI) was calculated according to the formula: NDI= $(N_1+2N_2+3N_3+4N_4)$/Ntot where Ntot is the total number of cells per slide counted (Ntot = 500 cells).

Statistical analysis

Statistical analysis was performed with Graph Pad Prism 6. The non-parametric Mann-whitney test was used to compare means of MN counts and NDI values between the different population groups. For HCWs, differences between MN counts at different time points were tested for significance with the non-parametric Wilcoxon test. Significance was set at $p<0.05$.

Results

Effects of ART on chromosomal radiosensitivity in HIV positive individuals

The results of the MN assay for the four groups (HIV patients not on treatment, HIV individuals on ART regimens TDF+3TC+EFV and D4T+3TC+EFV and healthy controls) are shown in Table 2. In unirradiated cells (0Gy), there were no significant differences in spontaneous mean MN yields between the groups. Radiation-induced MN yields were calculated by subtracting spontaneous MN yields from MN yields in irradiated cells. The radiation-induced MN yields were significantly higher in patients (both regimens and without ART) compared to healthy controls at both 2Gy and 4Gy. Although both patient groups on ART had higher MN yields than HIV patients not on treatment, patients on TDF+3TC+EFV had the highest average MN yields, the increase being significant at 4Gy. The HIV patients without ART had significantly lower CD4 counts compared to those on ART. There was no correlation between CD4 counts, time on ART treatment and MN yields (data not shown). The mean NDI values for the unirradiated (0Gy) cultures were similar for all the groups, but the mean NDI for the irradiated cultures (2Gy and 4Gy) were significantly lower in the patients on ART compared to the HIV patients without ART and the healthy controls ($p<0.005$).

				HIV ART	HIV ART
		Healthy individuals	HIV without ART	D4T/3TC/EFV	TDF/3TC/EFV
0Gy	N	20	20	19	20
	Mean	14	14	12	10
	SD	6.4	6.0	7.4	5.8
2Gy induced	N	20	19	18	18
	Mean	300	355	368	386
	SD	58.3	71.4	58.9	58.2
	p values vs controls		0.0125	0.001	<0.0001
	p values vs HIV without ART			0.5414	0.1531
4Gy induced	N	20	18	16	19
	Mean	842	969	1018	1076
	SD	151.6	134.6	157.8	146.6
	p values vs controls		0.0098	0.0017	<0.0001
	p values vs HIV without ART			0.3407	0.0277

Table 2: MN yields (per 1000BN) for the HIV positive individuals not taking ART, HIV positive individuals taking 2 different ART regimes and healthy individuals.

Effect of ART on chromosomal radiosensitivity of health care workers

The MN yields for the 10 health care workers on prophylactic ART before starting ART (T1), at the completion of ART (T2), or 3-4 months after the completion of ART (T3) are shown in Table 3. There were no significant differences between the mean spontaneous and radiation-induced MN yields at the 3 measured time points.

		T1	T2	T3
0 Gy	Mean	10.3	13.4	10.9
	SD	3.5	5.2	4.4
	p values		0.234	0.836
2 Gy	Mean	307.7	297.1	296.1
	SD	90.9	78.8	82.7

	p values		0.625	0.547
4Gy	Mean	826.2	842.0	834.0
	SD	146.2	206.0	192.2
	p values		0.943	0.844

Table 3: Comparison of MN yields (per 1000BN) of health care workers taking prophylactic post-exposure ART before starting ART (T1), after 3-4 weeks on ART (T2) and 3 months after ART (T3).

Discussion

The present pilot study investigated if HIV and antiretroviral medication has an influence on chromosomal radiosensitivity. This is one of the first studies looking at the effects of ART on radiosensitivity in HIV infected individuals and is potentially very important for many cancer patients and radiation workers in South Africa exposed to radiation that will be HIV positive and on ART. Since NRTIs interact with host DNA, inhibit mammalian DNA polymerases and affect the cell cycle [21], it is possible that they have an impact on DNA damage repair and affect chromosomal radiosensitivity. Some studies have shown antiretroviral drugs to be radioprotective while others have suggested a radiosensitising effect [25,26]. For the first part of this study we investigated the chromosomal radiosensitivity of HIV patients not on ART, HIV patients on two common ART regimens (d4T+3TC+EFV and TDF+3TC+EFV) and compared it to healthy uninfected controls.

Micronuclei are considered a biomarker of cytotoxicity. In unirradiated cells no difference in MN yields between the four groups was detected, suggesting no cytotoxicity of the ART in-vivo. These findings are in agreement with those of Robbins et al. [32] who detected no biologically relevant changes in lymphocyte chromosomal aberrations in 26 HIV-positive patients on combinations of ART (AZT, 3TC, d4T) across a number of time points. On the contrary, Shafik et al. [33] observed a high increase in chromosomal aberrations in AIDS patients treated with AZT and suggested that AZT induces genetic damage in lymphocytes of AIDS patients. None of the studied HIV positive patients were taking AZT, so this could explain the discrepancy between Shafik et al. [33] and the results presented here. Proliferation rates of lymphocytes from HIV infected individuals are known to be higher than HIV uninfected individuals [34], but this was not reflected in the NDI's calculated in this study. In lymphocytes irradiated with 2 and 4Gy, a difference in MN between the groups was noted that points to differences in chromosomal radiosensitivity. All HIV patients (those on ART and without ART) had significantly higher MN than healthy controls. This confirms earlier findings of Baeyens et al. [15]. The patients on the ART regimens had higher MN yields than HIV patients not on ART, with the TDF+3TC+EFV regimen presenting with significantly higher radiation-induced MN at 4Gy. The results suggest that substitution of d4T with TDF in the ART regimen of HIV patients leads to an enhanced chromosomal radiosensitivity. Cytotoxicity studies on TDF *in vitro* have been conflicting. Cihlar et al. [35] and Hecht et al. [36] have shown TDF to exhibit low cytotoxicity in various cell types, while a study by Bruning et al. [23] indicated that TDF induced DNA damage, reduced cell viability and induced cell cycle disturbances leading to apoptosis in cancer cells. This last statement could mean that TDF may be beneficial in radiosensitising tumour cells in HIV positive individuals, but its effect on normal tissue radiotoxicity, seen in the lymphocytes in this study, may be adverse. The significantly higher MN yields at 4Gy in the TDF group could reflect the un/misrepaired, severe DNA damage caused by the combination of HIV-infection, TDF and ionising radiation. Since studies have shown an association between clinical response to radiotherapy and chromosomal radiosensitivity [16,17], the effect of ART, especially TDF, on radiotoxicity requires further investigation.

For the second part of the study, we investigated the chromosomal radiosensitivity of 10 non-HIV-infected South African HCWs on the post-exposure prophylaxis ART to determine the effect of ART on chromosomal radiosensitivity without HIV as a confounding factor. All participants were on an ART regimen including two NRTIs. In unirradiated lymphocytes of these donors, no differences were detected in average MN yields before, during or after ART. The increase in chromosomal aberrations in AIDS patients taking AZT, seen in the study of Shafik et al. [33], was not noted in the HCWs taking AZT. The different results between both studies could suggest that not only AZT but the combination of HIV infection and AZT lead to increased chromosomal damage. The in vitro genotoxicity effects of AZT and 3TC seen in studies of Stern et al. [29], Lourenco et al. [28], and Gonzalez Cid et al. [30] were not confirmed in the current study. The fact that in these studies, ART was added in high concentrations for short terms in vitro to cell cultures, while in this study the ART was administered in vivo over a longer period, could explain the observed difference. No changes were noted in average radiation-induced MN yields of the HCWs at the different time points. Jagetia and Aruna [25], found that administration of AZT to Hela cells before exposure to different doses of γ-radiation resulted in a significant elevation in the yield of MN. This was in contrast to Copaceanu et al. [26] who suggested radioprotection of AZT in Hela cell lines exposed to irradiation in-vitro. Several publications demonstrated that AZT and 3TC alter cell cycle kinetics and increase the proportion of cells in S phase [21,22]. Radiosensitivity of cells differs according to cell type and phase of the cell cycle the cells are in at the time of irradiation [37]. In both studies of Jagetia and Aruna [25] and Copaceanu et al. [26] irradiations were performed on rapidly-dividing Hela cells lines, while in the current study, lymphocytes were irradiated in the G0 phase of the cell cycle.

Following from the MN data on HCWs taking ART, we could not support that either AZT or 3TC have a radiosensitising or radioprotective effect on lymphocytes of healthy individuals. The fact that the increased chromosomal radiosensitivity, seen in HIV positive individuals on ART was not observed in the lymphocytes of HCWs on ART could be due to the following reasons: 1) The duration of the ART was different (more than 6 months for HIV positive patients versus 3-4 weeks in HCWs). 2) It is the ART and the HIV virus in combination with the radiation that led to increased MN yields in HIV-infected patients on ART. 3) It is different ART compounds or the combinations (d4T, TDF and EFV versus AZT) exerting different effects in the cells in response to ionizing radiation.

The results of this study suggest that HIV and certain recent ART (TDF+3TC+EFV) given to HIV positive individuals can lead to enhanced lymphocyte chromosomal radiosensitivity and therefore may impose higher risks for radiation-induced normal tissue complications in these patients. Further investigation of the different compounds of ART in combination with ionizing radiation is warranted, as HIV positive cancer patients undergoing radiotherapy and HIV positive radiation workers may potentially benefit from

having their ART regimens altered according to the radiomodulatory effects of the various drugs.

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8 APPENDIX B: Ethical clearance certificates, informed consents and questionnaires

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

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Olivia Herd

CLEARANCE CERTIFICATE	**M110248**
PROJECT	Analysis of In Vitro Chromosomal Radiosensitivity and Underlying Mechanisms of DNA Repair in South African Women with Breast Cancer (Under approval M091023 Dr Ans Baeyens)
INVESTIGATORS	Ms Olivia Herd.
DEPARTMENT	Department of Radiobiology-Medical Physics
DATE CONSIDERED	Ad hoc
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 18/03/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Ans Baeyens

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...

M110230

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

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Olivia Herd/ Dr Ans Baeyens

CLEARANCE CERTIFICATE	**M110230**
PROJECT	Radiosensitivity in South African Cervical Cancer Patients.
INVESTIGATORS	Ms Olivia Herd/ Dr Ans Baeyens.
DEPARTMENT	Department of Radiation Sciences
DATE CONSIDERED	25/02/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 01/04/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Ans Baeyens

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...

Study title: Analysis of in vitro chromosomal radiosensitivity and underlying mechanisms of DNA repair in South African women with breast cancer
Investigators: Dr A Baeyens, O Herd, X Muller
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 8872 Olivia Herd: 082 778 4929
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

INFORMED CONSENT FORM

Good Day,

We are Dr. A. Baeyens and students Olivia and Xanthene from the Radiobiology research unit of the Department of Radiation Sciences, WITS medical School. We are part of a collaborative study between WITS University and iThemba LABS to investigate the radiosensitivity of breast cancer patients. Information on individual radiosensitivity helps to monitor the radiotherapy treatments.

We invite you to consider participating in a research study. Your participation in this study is entirely voluntary. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.

Should you decide not to participate in the study or if you agree and then change your mind, there will be no implications for you and the best treatment available for you will still be given.

If you agree to participate, we kindly ask you to donate 20ml of blood once. This is not a lot of blood; it is only 4 teaspoons and will not harm you. We will use your blood sample to test the sensitivity to radiation. We also kindly ask you if we can use a part of your tumour tissue that will be removed by the surgeon during your breast operation. This part of tumour tissue we want to use to investigate if there is a link between the radiosensitivity seen in blood and in the tumour. Both your blood sample and tissue sample will be used to unravel the underlying mechanism of radiosensitivity. We also ask permission to view your medical files if we need to obtain any further medical information that may be relevant to our study.

There is no direct benefit to you. But your participation in this study will contribute to the development of greater knowledge of radiosensitivity and may help to ameliorate the radiotherapy treatments of breast cancer patients.

The research is completely confidential, which means that your name will not be recorded on any of our laboratory information. The consent forms will be locked away and only accessible by the researchers. We will require some personal details from you (your age, language, monthly income, do you have children, see questionnaire attached) and we also want to know if you are a smoker or if you have any other major illness, as this can have an influence on our tests.

You are free to ask any questions about this study and discuss any worries you may have with the research staff.

Thank you very much for your time

Regards,

Dr A. Baeyens, Olivia Herd and Xanthene Muller

Study title: Analysis of in vitro chromosomal radiosensitivity and underlying mechanisms of DNA repair in South African women with breast cancer
Investigators: Dr A Baeyens, O Herd, X Muller
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 8872 Olivia Herd: 082 778 4929
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

Study participant number: Date of Birth:

Contact no:

INFORMED CONSENT:

I hereby confirm that I have been informed about the nature, conduct, benefits of the study on radiosensitivity of breast cancer patients

I have also received, read and understood the above written information regarding this study

I have no further questions and declare myself prepared to participate in the study.

PARTICIPANT:

Name (Print):

Signature and date:

STUDY STAFF CONDUCTING CONSENT DISCUSSION:

Name (Print):

Signature and date:

WITNESS (IF APPLICABLE):

Name (Print):

Signature and date:

Study title: Analysis of in vitro chromosomal radiosensitivity and underlying mechanisms of DNA repair in South African women with breast cancer
Investigators: Dr A Baeyens, O Herd, X Muller
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 8872 Olivia Herd: 082 778 4929
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

RADIOSENSITIVITY STUDY ON BREAST CANCER PATIENTS

QUESTIONNAIRE FOR PARTICIPANTS

Age: .....

Home language:.....

Ethnic group:.....

Place of Birth:.....

What is your monthly income?
- None
- less than R500
- between R500 and R1000
- between R1000 and R2000
- between R2000 and R5000
- more than R5000
- unknown

Do you have children?how many?.....

What is your highest grade completed?
- Primary school
- High school
- Tertiary school

Have you ever smoked?/ do you currently smoke?.....

Do you have any other major illness?.....

Do you know your HIV status?.....

Will you disclose your status to me?are you positive or negative?.....

Do you still have monthly bleed (period)?.....

Participant questionnaire

Participant initials:

Study participant number:

Study title: Analysis of radiosensitivity in South African cervical cancer patients
Investigators: Dr A Baeyens, O Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 8872 Olivia Herd: 082 778 4929
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

INFORMED CONSENT FORM (patients)

Good Day,

We are Dr. A. Baeyens and Olivia Herd from the Radiobiology research unit, Department of Radiation Sciences and NRF- iThemba LABS based at Charlotte Maxeke Johannesburg Academic Hospital, Orange block, 4th floor. We are part of a collaborative study between WITS University and NRF - iThemba LABS to investigate the radiosensitivity of cervical cancer patients. Information on individual radiosensitivity helps to monitor the radiotherapy treatments.

We invite you to consider participating in a research study. Your participation in this study is entirely voluntary. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.

Should you decide not to participate in the study or if you agree and then change your mind, there will be no implications for you and the best treatment available for you will still be given.

If you agree to participate, we kindly ask you to donate 5ml of blood once. This is not a lot of blood; it is only 1 teaspoon and will not harm you. We will use your blood sample to test the sensitivity to radiation. We also kindly ask you if we can use a part of your cervical tissue (exfoliated cells) and tumour (biopsy) that will be taken while the doctor is taking samples for routine tests. This may cause you added discomfort. These samples will be used to investigate if there is a link between the radiosensitivity seen in blood and in cervical cells. Both your blood sample and tissue samples will be used to unravel the underlying mechanism of radiosensitivity. We also ask permission to view your medical files if we need to obtain any further medical information that may be relevant to our study.

There is no direct benefit to you. But your participation in this study will contribute to the development of greater knowledge of radiosensitivity and may help to further develop the radiotherapy treatments of cervical cancer patients.

The research is completely confidential, which means that your name will not be recorded on any of our laboratory information. The consent forms will be locked away and only accessible by the researchers. We will require some personal details from you (your age, language, monthly income, see questionnaire attached) and we also want to know if you are a smoker or if you have any other major illness, as this can have an influence on our tests.

You are free to ask any questions about this study and discuss any worries you may have with the research staff.

Thank you very much for your time.

Regards,

Dr A. Baeyens and Olivia Herd

Study title: Analysis of radiosensitivity in South African cervical cancer patients
Investigators: Dr A Baeyens, O Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 8872 Olivia Herd: 082 778 4929
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

INFORMED CONSENT FORM (controls)

Good Day,

We are Dr. A. Baeyens and Olivia Herd from the Radiobiology research unit, Department of Radiation Sciences and iThemba LABS based at Charlotte Maxeke Johannesburg Academic Hospital, Orange block, 4th floor. We are part of a collaborative study between WITS University and NRF - iThemba LABS to investigate the radiosensitivity of cervical cancer patients. Information on individual radiosensitivity helps to monitor the radiotherapy treatments.

We need healthy individuals as a control group for our study. Therefore we invite you to consider participating in a research study. Your participation in this study is entirely voluntary. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.

If you agree to participate, we kindly ask you to donate 5ml of blood once. This is not a lot of blood; it is only 1 teaspoon and will not harm you. We also kindly ask for some left-over cells from your cervical smear that is being done by the doctor for routine tests. This will not give you extra discomfort. We will use these samples to test the sensitivity to radiation. We also ask permission to view your medical files if we need to obtain any further medical information that may be relevant to our study.

There is no direct benefit for you. But your participation in this study will contribute to the development of greater knowledge of radiosensitivity and help to further develop the radiotherapy treatments of cervical cancer patients.

The research is completely confidential, which means that your name will not be recorded on any of our laboratory information. The consent forms will be locked away and only accessible by the researchers. We will require some personal details from you (your age, language, monthly income, see questionnaire attached) and we also want to know if you are a smoker or if you have any other major illness, as this can have an influence on our tests.

You are free to ask any questions about this study and discuss any worries you may have with the research staff.

Thank you very much for your time

Regards,

Dr A. Baeyens and Olivia Herd

Study title: Analysis of radiosensitivity in South African cervical cancer patients
Investigators: Dr A Baeyens, O Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 8872 Olivia Herd: 082 778 4929
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

Study participant number: Date of Birth:

Contact no:

INFORMED CONSENT:

I hereby confirm that I have been informed about the nature, conduct, benefits of the study on radiosensitivity of cervical cancer patients

I have also received, read and understood the above written information regarding this study

I have no further questions and declare myself prepared to participate in the study.

PARTICIPANT:

Name (Print):

Signature and date:

STUDY STAFF CONDUCTING CONSENT DISCUSSION:

Name (Print):

Signature and date:

WITNESS (IF APPLICABLE):

Name (Print):

Signature and date:

Study title: Analysis of radiosensitivity in South African cervical cancer patients
Investigators: Dr A Baeyens, O Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 8872 Olivia Herd: 082 778 4929
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

ANALYSIS OF RADIOSENSITIVITY ON CERVICAL CANCER PATIENTS

QUESTIONNAIRE FOR PARTICIPANTS

Age: .....

Home language:.....

Race:.....

Place of Birth:.....

What is your monthly income?
- None
- less than R500
- between R500 and R1000
- between R1000 and R2000
- between R2000 and R5000
- more than R5000
- unknown

What is your highest grade completed?
- Primary school
- High school
- Tertiary school

Have you ever smoked?/ do you currently smoke?.....

Do you have any other major illness?.....

Do you know your HIV status?.....

Will you disclose your status to me? are you positive or negative?.....

Participant questionnaire

Participant initials:

Study participant number:

9 APPENDIX C: Detailed protocols

9.1 MN assay on lymphocytes

Heparinised blood (0.5 ml) was added to 4.5 ml complete medium (Appendix D) pre-warmed to 37°C and gassed (5%CO_2/95% air). Culture flasks were placed in a phantom-water tank at room temperature (RT) and irradiated with 6 MV X-rays. The distance from the culture flasks to the radiation source was 100 cm at an angle of 90 degrees. The field size at the depth of the sample was 10X10 cm. Once irradiated, lymphocytes were stimulated into division by adding 100 µl stock solution phytohaemagglutinin (Sigma-Aldrich) (Appendix D). After 23 hrs in culture 20 µl stock solution of cytochalasin B (Sigma-Aldrich) (Appendix D) was added to block cytokinesis. 70 hrs post-stimulation, cells were harvested by adding a cold (4°C) hypotonic shock of 7 ml 0.075M KCl (Merck) (Appendix D), followed by fixation in 4:1:5 methanol:acetic acid:Ringer solution (Merck) (Appendix D) at 4°C overnight. Cells were subsequently fixed 3 X in methanol:acetic acid (4:1). Cell suspensions were dropped onto coded slides and slides were mounted with vectashield containing DAPI (Vector Laboratories) before being scanned automatically with the Metafer 4 platform.

9.2 Lymphocyte extraction for γ-H2AX assay

The blood was left to stand in EDTA tubes for 30 mins at RT. Blood was diluted (1:1) with RPMI 1640 (BioWhittaker) and slowly poured at a 45 degree angle onto Histopaque-1077 (Sigma-Aldrich), a solution of polysucrose and sodium diatrizoate that creates a density gradient upon centrifugation and separates white blood cells from red blood cells and plasma. Tubes were centrifuged (slow start/stop) at 1500 rpm for 15 mins to set up the

density gradient. The lymphocyte layer was transferred to a clean tube and washed 3 X with 5 ml complete medium (Appendix D). Cells were counted with trypan blue (Sigma-Aldrich) staining and a haemocytometer. 0.8 X 10^6 cells were planted in 2 ml complete medium.

9.3 γ-H2AX immunostaining

After overnight fixation in 0.5% PFA (Appendix D), slides were washed in 1 X Phosphate Buffer Saline (PBS) (Appendix D) for 5 mins at RT. Cells were covered with ice-cold 0.2% Triton X-100 (Sigma-Aldrich) (Appendix D) at RT in a humidity chamber for 10 mins to increase permeability of membranes. Thereafter, slides were washed 3 X in PBS buffer with 1% Bovine Serum Albumin (BSA) (Roche Diagnostics, Basel, Switzerland) (Appendix D) at RT for 10 mins. Cells were incubated for 1 hr in a humidity chamber at RT with primary mouse-anti-H2AX (1/500 in 1% BSA PBS) (Biolegend, San Diego, USA). This was followed by washing 3 X in 1% BSA PBS at RT for 10 mins. Cells were incubated for 1 hr at RT with secondary antibody, RAM-TRITC (1/1000 in 1% BSA PBS) (Dako, Glostrup, Germany) followed by washing 3 X in PBS at RT for 10 mins. Cells were counterstained with DAPI stock solution (Sigma-Aldrich) (Appendix D) dissolved in Fluoromount mounting medium (1/500) (Sigma-Aldrich).

9.4 Immunohistochemistry to characterize cell cultures

5-µm sections were cut from FFPE tissues with a microtome. Sections were deparaffinised with Toluene (Merck) and rehydrated in ethanol (Merck). Deparaffinised tissue sections were boiled in 1mM citrate acid buffer (Appendix D) for 2 X 5 mins in a microwave oven. Citric acid enables antibodies to reach antigens more easily by breaking down formaldehyde groups in FFPE. 3% Hydrogen Peroxide (Merck) (Appendix D) was added to tissue sections

for 10 mins at RT to remove excess peroxidise that occurs naturally in tissue. The sections were incubated with blocking serum for 30 mins at RT. Blocking serum (Appendix D) contains 5% normal rabbit serum (stops non-specific binding of rabbit antibody) (Dako); 1% BSA (loosely binds to antigens to stop non-specific binding); and 0.2% Tween (increases membrane permeability) (Merck). Sections were incubated for 2 hrs at RT with primary Anti-pan Cytokeratin Antibody AE1+AE3 (1/200 in dilution buffer- Appendix D). Sections were washed with PBS (Appendix D) for 2 X 5 mins, followed by a 30 mins incubation at RT with biotinylated rabbit anti-mouse secondary antibody (1/200 in dilution buffer) (Dako). The final incubation was for 30 mins at RT with horseradish streptavidin labeled with peroxidase (1/200 in dilution buffer) (Dako), followed by treatment with enzyme substrate 3'3 Diaminobenzidine tetrahydrochloride (Sigma-Aldrich) which reacts with peroxidase and forms a visible colour stain. Negative control sections were treated in the same way except that they were incubated with dilution buffer instead of primary antibody. The sections were counterstained with Haematoxyline Von Mayer (Merck) and viewed under a light microscope.

9.5 DNA extractions for HPV typing

9.5.1 Fresh tissue

DNA was extracted from fresh tissue biopsies using a QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands), according to manufacturer instructions. Approximately 25 mg tissue was minced and placed in a 1.5 ml microcentrifuge tube with 180 µl tissue lysis Buffer ATL. Specimens were incubated with 20 µl proteinase K (included in kit) in Buffer ATL at 56°C for 1-3 hrs until tissue lysis. Post tissue lysis, 200 µl cell lysis Buffer AL was added to tubes and specimens were incubated at 70°C for 10 mins. 200 µl 100% ethanol (Merck) was added to

the tubes to precipitate DNA, followed by vortexing. The mixture was added to a QIAamp Mini spin column, centrifuged for 1 min at 8000 rpm. Columns were washed by adding 500 µl Buffer AW1 followed by centrifugation for 1 min at 8000 rpm. For a second wash, 500 µl Buffer AW2 was added to the spin column followed by centrifugation for 1 min at 8000 rpm. Columns were spun for another 1 min at 8000 rpm to remove any residual buffer. To elute DNA, the spin column was placed into a clean microcentrifuge tube and 200 µl elution buffer AE was added. After a 5 mins incubation at RT (optimal time for increased DNA yield), tubes were centrifuged for 1 min at 8000 rpm.

9.5.2 FFPE

For FFPE, DNA was extracted using the phenol-chloroform method. Sections were warmed at 60°C for 2 hrs to soften the paraffin. Sections were washed with xylene (Merck) by adding 1 ml xylene, placing on a plate shaker for 5 mins, spinning for 3 mins at 12000 rpm and removing supernatant. This was repeated 3-5 X until all paraffin was dissolved and removed. The pellet was washed with 1 ml 100% ethanol, spun for 2 mins at 12000 rpm and the supernatant removed. Pellets were incubated overnight in 1 ml sodium thiocyanate (Merck) at 40°C to break crosslinks formed by formaldehyde. The following day, sodium thiocynate was removed and pellets were incubated for 2 hrs at 56°C in 400 µl tissue lysis buffer (Appendix D) with 40 µl proteinase K (10 mg/ml) (Roche Diagnostics) (Appendix D). After the lysis step, samples were incubated for 10 mins at 80°C to inactivate proteinase K. Equal amounts of phenol (Sigma Aldrich) and chloroform (Sigma Aldrich) (450 µl each) were added to samples which were vortexed and spun for 3 mins at 12000 rpm. The supernatant was transferred to a new tube and equal amounts of phenol and chloroform were added (200 µl each) followed by vortexing and spinning for 3 mins at 12000 rpm. The supernatant was

transferred to a clean tube and 400 µl chloroform was added followed by vortexing and spinning for 3 mins at 12000 rpm. The supernatant, containing DNA, was transferred to a clean tube and DNA was precipitated by adding 2.5X ice cold 100% ethanol, 3M sodium acetate (1/10 volume supernatant) (Appendix D) and placing at -70°C overnight. DNA was pelleted by spinning for 30 mins at 12000 rpm (at 4°C) and washed by adding 500 µl ice cold 70% ethanol, followed by spinning for 10 mins at 12000 rpm (4°C). The ethanol was poured off, the pellet dried on the benchtop for 15-30 mins and DNA was dissolved in 50 µl 1XTE buffer (Appendix D).

9.6 Agarose gel electrophoresis for HPV typing

PCR products for HPV genotyping were separated by 2% agarose gel. 1 g Agarose powder (Merck) was dissolved in 50 ml 1X TBE buffer (Appendix D). The gel was left to set at RT in a gel-cast after adding 1 µl of GelRed (10,000X stock solution) (Biotium). PCR products (5 µl) mixed with 1 µl 1X loading dye (6X stock solution diluted 1:6 in dH_2O) (Thermo Scientific) were loaded into well and run at 80-100 Volts for 40 mins. A molecular marker of 0.1 to 1.5 kb (in 0.1kb intervals) (Thermo Scientific) was run concurrently to samples.

10 APPENDIX D: Recipes

Acridine orange stock solution (1 mg/ml)	0.1 g	Acridine orange powder
	100 ml	dH_20
Acridine orange working solution (10 µg/ml)	0.4 ml	Acridine stock solution
	40 ml	Acridine orange buffer
Agarose gel 2% (50 ml)	1 g	Agarose powder made to 50 ml with 1X TBE microwave, add 1 µl GelRed and into cast to set
Blocking Serum	0.1 g	Bovine serum albumin
	0.5 ml	Rabbit serum (normal)
	200 µl	Tween 10 %
	10 ml	1X PBS
Citric acid buffer (1 mM) (1 L)	0.2 g	Citric acid made up to 1 L with dH_2O, pH 6.0
Collagenase alone (1 mg/ml)	1 mg	Collagenase
	1 ml	dH_20
Collagenase/Dispase stock solution (100 mg/ml)	100 mg	Collagenase/Dispase lyophilizate
	1 ml	dH_20
Collagenase/Dispase working solution (1 mg/ml) (1 ml)	10 µl	working solution (100 mg/ml)
	990 µl	1X PBS
Collection Medium (100 ml)	500 µl	Gentamycin (50 µg/ml)
	2.5 ml	Penicillin/Streptomycin (250 U/ml and 0.25 mg/ml)
	97 ml	DMEM/F12
Complete medium (28.75 ml)	3.75 ml	Foetal bovine serum
	25 ml	RPMI 1640 with 50 U/ml penicillin and 50 mg/ml streptomycin
Culture-initiation medium (100 ml)	1 ml	L-Glutamine (2mM)
	1 ml	Penicillin/Streptomycin (100 U/ml and 0.1 mg/ml)

	10 ml	Foetal bovine Serum
	90 ml	DMEM/F12
Cytochalasin B (stock solution 1.5 mg/ml)	5 mg	Cytochalasin B
	3.3 ml	Dimethyl sulphoxide (DMSO)
DAPI stock solution (0.1 mg/ml)	0.1 mg	DAPI powder
	1 ml	dH_20
Dilution buffer for IHC	1 ml	Blocking serum
	9 ml	1X PBS
EDTA 0.5M	146.1 g	EDTA disodium salt made up to 1 L with dH_2O, pH 8
Erythrocyte lysis buffer (500 ml)	0.019 g	EDTA (0.5M)
	0.5 g	$KHCO_3$
	4.1 g	NH_4Cl
		Made up to 500 ml with dH_20
Hydrogen Peroxide (10 ml) 3%	1 ml	30% Hydrogen peroxide
	9 ml	1X PBS
KCl 0.075M	5.6 g	KCl made up to 1 L with dH_2O
Phosphate Buffer Saline 1X (PBS) (1 L)	7.2 g	NaCl
	0.42 g	KH_2PO_4
	1.78 g	Na_2HPO_4
		made up to 1 L with dH_2O, pH 7.2
PBS 1% BSA (100 ml)	1 g	BSA
	100 ml	1X PBS
Paraformaldehyde 8% (PFA)	80 g	Paraformaldehyde powder Dissolved at 40°C with drops of 5M NaOH until dissolved, pH7.4 made up to 1 L with dH_2O
Paraformaldehyde 3%	25 ml	8% PFA
	41 ml	1X PBS

Paraformaldehyde 0.5%	10 ml	3% PFA
	50 ml	1X PBS
Phytohaemagglutinin (stock solution 1 mg/ml)	25 mg	Phytohaemagglutinin
	25 ml	Sabax injection H_2O
Poly-L-Lysine solution 0.1w/v (10 ml) + coating:	1 ml	Poly-L-Lysine
	9 ml	dH2O
		Added to wells and incubated at 37°C for 1 hr
		Wells washed 5X and dried in incubator overnight
Proteinase K (10 mg/ml) (1 ml)	10 mg	proteinase K
	1 ml	dH_2O
Ringer solution (1 L)	0.24 g	$CaCl_2$
	0.42 g	KCl
	9 g	NaCl
		made up to 1 L with dH_2O
TBE buffer 5X (1 L)	20 ml	0.5M EDTA
	24.5 g	Boric acid
	54 g	Tris Base
		made up to 1 L with dH_2O
TE buffer 1X (100 ml)	0.02 ml	0.5M EDTA
	0.012 g	Tris base
	100 ml	dH_2O
Tissue lysis buffer (250 ml)	0.5 ml	0.5M EDTA
	25 ml	Tris-HCl
	1.25 ml	Tween (100%)
Triton-100 0.2% (1 ml)	2 µl	Triton-100
	998 µl	1X PBS
Tween 10% (100 ml)	10 ml	Tween
	90 ml	dH_2O

11 APPENDIX E: Chemicals and consumables

3'3 Diaminobenzidine tetrahydrochloride	Sigma-Aldrich, St Louis, MO, USA
4,6-diamidino-2-phenylindole (DAPI powder)	Sigma-Aldrich, St Louis, MO, USA
70 µm Falcon mesh strainer	BD Biosciences, New Jersey, USA
6X loading dye	Thermo Scientific, Massachusetts, USA
6-well CellBind® (Corning) plates	Corning Incorporated Life Sciences,
24 well plates	Grenier Bio-one, Monroe, North
Acetic Acid	Merck, Darmstadt, Germany
Acridine orange	Sigma-Aldrich, St Louis, MO, USA
Agarose	Merck, Darmstadt, Germany
Anti-pan Cytokeratin Antibody AE1+AE3	Abcam, Cambridge, England, United
Anti-phospho-histone H2AX anti-mouse	Biolegend, San Diego, USA
Biotinylated rabbit anti-mouse secondary	Dako, Glostrup, Denmark
Bovine Serum Albumin (BSA)	Roche Diagnostics, Basel, Switzerland
Chloroform	Sigma-Aldrich, St Louis, MO, USA
Citrate acid buffer	Merck, Darmstadt, Germany
Collagenase	Sigma-Aldrich, St Louis, MO, USA
Collagenase/Dispase	Roche Diagnostics, Basel, Switzerland
Culture flasks 25cm^2	Grenier Bio-one, Monroe, North
Cytochalasin B	Sigma-Aldrich, St Louis, MO, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St Louis, MO, USA
Distilled H_2O	Merck, Darmstadt, Germany
DMEM/F12	BioWhittaker, Walkersville, USA
Ethanol	Merck, Darmstadt, Germany
Ethylene-diamine-tetra-acetate (EDTA)	Merck, Darmstadt, Germany
Fluoromount mounting medium	Sigma-Aldrich, St Louis, MO, USA
Foetal Bovine Serum (FBS)	Gibco-Invitrogen, New York, USA
GelRed	Biotium, California, USA
Gentamycin	BioWhittaker, Walkersville, USA

Haematoxyline Von Mayer	Merck, Darmstadt, Germany
Histopaque-1077	Sigma-Aldrich, St Louis, MO, USA
Horseradish Streptavidin Peroxidase	Dako, Glostrup, Denmark
Hydrogen Chloride (HCl) 1M	Merck, Darmstadt, Germany
Hydrogen Peroxide	Merck, Darmstadt, Germany
Keratinocyte Growth Medium (KGM)	Lonza, Basel, Switzerland
L-Glutamine	Lonza, Basel, Switzerland
Liberase Research Grade Purified Enzyme Blend	Roche Diagnostics, Basel, Switzerland
Methanol	Merck, Darmstadt, Germany
Molecular marker	Thermo Scientific, Massachusetts, USA
Paraformaldehyde	Merck, Darmstadt, Germany
Penicillin/Streptomycin	Gibco-Invitrogen, New York, USA
Phenol	Sigma-Aldrich, St Louis, MO, USA
Poly-L-Lysine	Sigma-Aldrich, St Louis, MO, USA
Poly-L-Lysine-coated slides	Thermo Scientific, Massachusetts, USA
Potassium Chloride (KCl)	Merck, Darmstadt, Germany
Potassium Hydrogen phosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Proteinase K	Roche Diagnostics, Basel, Switzerland
Phytohaemagglutinin	Sigma-Aldrich, St Louis, MO, USA
Rabbit Serum (Normal)	Dako, Glostrup, Denmark
RAM-TRITC antibody	Dako, Glostrup, Denmark
Roswell Park Memorial Institute (RPMI) 1640	BioWhittaker, Walkersville, USA
Sabax injection H_2O	LeBasi Pharmaceuticals, Potchefstroom,
Singlequots	Lonza, Basel, Switzerland
Sodium Acetate	Merck, Darmstadt, Germany
Sodium Chloride (NaCl)	Merck, Darmstadt, Germany
Sodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt, Germany
Sodium Thiocyanate (NaSCN)	Merck, Darmstadt, Germany
Toluene	Merck, Darmstadt, Germany
Tris base	Sigma-Aldrich, St Louis, MO, USA

Triton X-100	Sigma-Aldrich, St Louis, MO, USA
Trypan blue	Sigma-Aldrich, St Louis, MO, USA
Tween	Merck, Darmstadt, Germany
Vectashield with DAPI (4,6-diamidino-2-	Vector Laboratories, Burlingame, CA,
Xylene	Merck, Darmstadt, Germany

12 APPENDIX F: Equipment

Axio Imager M1 microscope	Carl Zeiss, Gottingen, Germany
Centrifuge 5418	Eppendorf, Hamburg, Germany
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Cytospin 4	Thermo Scientific, Massachusetts, USA
Drybath heating block	MRC, Holon, Israel
Fume hood	Erlab, Massachusetts, USA
Gel Documentation System Minibis	DNR Bio-Imaging Systems Ltd, Jerusalem, Israel
Haemocytometer	Marienfeld-Superior, Lauda-Königshofen, Germany
Incubator Direct Heat CO_2	Thermo Scientific, Massachusetts, USA
Laminar Flow Class II BSC	Esco, Oregon, USA
Light Microscope Prima Vert	Carl Zeiss, Gottingen, Germany
Medical Linear Accelerator	Siemens Healthcare, Erlanger, Germany
MyCycler Thermal Cycler	Bio-Rad, California, USA
Nanodrop 2000 spectrophotometer	Thermo Scientific, Massachusetts, USA
Orbital shaker	MRC, Holon, Israel
pH meter	Crison Instruments, Barcelona, Spain
Powersupply Enduro 300V	Labnet, New Jersey, USA
QIAamp DNA Mini Kit	Qiagen, Venlo, Limburg, Netherlands
Qiagen Multiplex PCR kit	Qiagen, Venlo, Limburg, Netherlands
Scale	Adam Equipment, Milton Keynes, UK
Waterbath	Polyscience, Pennsylvania, USA

13 APPENDIX G: HPV Genotyping Primers

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Primer	Sequence
PPX6/F	GCTAAAGGTCCTGTTTCGAGGCGGCTA
PPX6/R	GGCAGCGACCCTTCCACGTACAAT
PPX11/F	GCGTGTTTTGCAGGAATGCACTGAC
PPX11/R	TGCGTCTTGTTTGTCCACCTTGTCC
PPX16U/F	TCCTGCAGGTACCAATGGGGAAGAGG
PPX16U/R	TGCCATACCCGCTGTCTTCGCTTT
PPX18/F	AACAGTCCATTAGGGGAGCGGCTGGA
PPX18/R	TGCCGCCATGTTCGCCATTTG
PPX31/F	GCGGTCCAAACGCTCTACAAAACGCACT
PPX31/R	GCAGGGGCACCAACATCAACAATTCCA
PPX33/F	ACACAGAGGCAGCCCGGGCATTGTTT
PPX33/R	CACGGGTTTGCAGCACGATCAACA
PPX35/F	CCATAACATCGGTGGACGGTGGACAGG
PPX35/R	CCATTACATCCCGTCCCCTCCCCTTCA
PPX39/F	CCGACGGAGTGTCCCTGGACCATCTTA
PPX39/R	CCAGCGTTTTTGGTTCCCTTACCCCGTA
PPX45/F	TGTTGGACATCACACCTACCGTGGA
PPX45/R	TCCGTACCTGACCCAGAAGATGCAA
PPX51/F	CAACTAGCAACGGCGATGGACTG
PPX51/R	CTGCTTCGCGGGCTGACTAGAA
PPX52/F	GGTGTTGGTGCTGGTGCTTTTGCTA
PPX52/R	CAGTTACAGGGGGACGAATGGTGGA
PPX56/F	TGTTGTTTTTCCGCCATTTTGTACATGCAACC
PPX56/R	TGGCCTACATAGTGTATTCTGCAAGCCAAAAC
PPX58/F	GGTAGTACCCCACCGTCTGAGG
PPX58/R	AGACGTGACATTGCCACTGTCA
PPX59/F	CCGAGCAAGACACCTAAGACAGCAACG
PPX59/R	TCGGAGTCGGAGTCAGGTAATTGCT
PPX66/F	GCGGGCGGCTCCTACCTCTTCCTCTTC
PPX66/R	CCACCTAACCTGACACACACTGCCCAAGG
PPXIS/F	TTATCCCGAGTCCCCCAGGCCTTTCT
PPXIS/R	TGGCTTGGCCCCAACTTCCATCA