CHROMOSOMAL RADIOSENSITIVITY AND INSTABILITY IN TRIPLE NEGATIVE AND/OR YOUNG BREAST CANCER AND FANCONI ANAEMIA PATIENTS IN SOUTH AFRICA

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, and the Faculty of Medicine and Health Sciences, Ghent University, Belgium in fulfilment of the requirements for the joint degree of Doctor of Philosophy / Doctor in Health Sciences

Johannesburg, 2018

DECLARATION

I, Flavia Zita Francies, declare that this thesis is my own work. It is being submitted for a joint degree of Doctor of Philosophy / Doctor of Health Sciences in the University of the Witwatersrand, Johannesburg, South Africa and Ghent University, Belgium. It has not been submitted before for any degree or examination at this or any other university.

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Flavia Zita Francies

08 day of February 2018

For my Steph

~ The air beneath my wings ~

PUBLICATION ARISING FROM THIS THESIS

Francies, F. Z., Wainstein, T., De Leeneer, K., Cairns, A., Murdoch, M., Nietz, S., Cubasch, H., Poppe, B., Van Maerken, T., Crombez, B., Coene, I., Kerr, R., Slabbert, J. P., Vral, A., Krause, A., Baeyens, A. & Claes, K. B. M. 2015. *BRCA1*, *BRCA2*, *PALB2* mutations and *CHEK2* c.1100delC in difference South African ethnic groups diagnosed with premenopausal and/or triple negative breast cancer. *BMC*, 15: 912. doi: 10.1186/s12885-015-1913-6 (Chapter 6).

Author contributions: FZF, TW carried out the molecular work, analysed the data and helped draft the manuscript

KDL, BC, IC carried out the molecular work and analysis of data

AC, MM, SN, HC, BP, TVM provided samples for this study

RK, JPS, AV, AK revising the manuscript

AB, KBMC design of the study and drafting the manuscript

Francies, F. Z., Herd, O., Muller, X., Cairns, A., Murdoch, M., Nietz, S., Slabbert, J. P. & Baeyens, A. 2015. Chromosomal radiosensitivity of lymphocytes in South African breast cancer patients of different ethnicity: An indirect measure of cancer susceptibility. *South African Medical Journal*, 105 (8), 675-8. doi:10.7196/SAMJnew.8266 (Chapter 3).

Author contributions: FZF, OH carried out laboratory work, analyse data and helped draft the manuscript

XM assisted in laboratory work

AC, MM, SN provided samples for this study

JPS revising the manuscript

AB design of the study and revising the manuscript

Francies, F. Z., Herd, O., Cairns, A., Neitz, S., Murdoch, M., Slabbert, K., Claes, K., Vral, A., Baeyens, A. 2017. Chromosomal radiosensitivity of triple negative breast cancer patients. In preparation (Chapter 5).

Author contributions: FZF carried out the laboratory work, analysed the data and drafting the manuscript

OH assisted with the laboratory work

AC, SN, MM provided samples for the study

JPS revising the manuscript

KC, AV, AB design of the study and drafting the manuscript

AB design of the study, drafting the manuscript and analysing data

Francies, F. Z., Wainwright, R., Poole, J., De Leeneer, K., Coene, I., Wieme, G., Antoine-Poirel, H., Brichard, B., Vermeulen, S., Vral, A., Slabbert, J., Claes, K. B. M., Baeyens, A. 2017. Diagnosis of Fanconi Anaemia by ionising radiation- or mitomycin-induced micronuclei. *DNA repair*, 61, 17-24. doi: 10.1016/j.dnarep.2017.11.001 (Chapter 7).

Author contributions: FZF carried out the laboratory work, analysed the data and drafting the manuscript

RW, JP, HA, BB provided samples for the study

KDL, IC, GW assisted with molecular work

SV assisted with data analysis

JPS revising the manuscript

AV, KBMC, AB design of the study and drafting the manuscript

Baeyens, A., Herd, O., **Francies, F. Z.,** Cairns, A., Katzman, G., Murdoch, M., Padiachy, D., Morford, M., Vral, A., Slabbert, J. P. 2015. The influence of blood storage time and general anaesthesia on chromosomal radiosensitivity. Mutagenesis, 31, 181-186. doi:10.1093/mutage/gev072 (Chapter 4).

Author contributions: AB design of the study, data analysis and drafting the manuscript

OH, FZF laboratory work and revising the manuscript

AC, GK, MM, DP, MM provided samples for the study

AV, JPS revising the manuscript

CONFERENCE PRESENTATIONS

Oral presentations

1. **Francies, F. Z.,** Wainwright, R., Poole, J., Slabbert, J. P., Baeyens, A. Chromosomal radiosensitivity and genomic instability of Fanconi Anaemia patients in South Africa. *54th National Congress of the South African Association of Physicists in Medicine and Biology (SAAPMB)*, Cape Town, South Africa, September 2016.

2. **Francies, F. Z.,** Herd, O., Cairns, A., Slabbert, J. P., Baeyens, A. Chromosomal radiosensitivity of lymphocytes of BRCA mutation carriers. *53rd National Congress of the South African Association of Physicists in Medicine and Biology (SAAPMB)*, Bloemfontein, South Africa, September 2015.

Poster presentations

1. **Francies, F. Z.,** Wainwright, R., Poole, J., Slabbert, J. P., Baeyens, A. Micronucleus assay for Fanconi Anaemia diagnosis with ionising radiation and mitomycin C. *43rd Annual Meeting of the European Radiation Research Society (ERRS)*, Essen, Germany, September, 2017.

2. **Francies, F. Z.,** Wainwright, R., Poole, J., Slabbert, J. P., Baeyens, A. Chromosomal radiosensitivity and genomic instability of Fanconi Anaemia patients in South Africa. *18th National Congress of South African Society of Clinical & Radiation Oncology (SASCRO) and South African Society of Medical Oncology (SASMO)*, Johannesburg, South Africa, August, 2017.

3. **Francies, F. Z.,** Wainwright, R., Poole, J., Slabbert, J. P., Baeyens, A. Chromosomal radiosensitivity and genomic instability of Fanconi Anaemia patients in South Africa. *European Society for Radiotherapy and Oncology (ESTRO 36)*, Vienna, Austria, May, 2017.

4. **Francies, F. Z.,** Wainwright, R., Poole, J., Slabbert, J. P., Baeyens, A. Chromosomal radiosensitivity and genomic instability of Fanconi Anaemia

patients in South Africa. *School of Clinical Medicine Research day*, University of the Witwatersrand, Johannesburg, South Africa, September, 2015.

5. **Francies, F. Z.,** Herd, O., Cairns, A., Slabbert, J. P., Baeyens, A. Chromosomal radiosensitivity of lymphocytes of BRCA mutation carriers. *17th National Congress of SA Society of Clinical & Radiation Oncology (SASCRO) and SA Society of Medical Oncology (SASMO)*, Cape Town, South Africa, August 2015.

ABSTRACT

Introduction: Breast cancer is the leading cancer in women in South Africa (SA). Triple negative breast cancer (TNBC) is clinically characterised by the lack of expression of estrogen, progesterone and HER2/NEU receptors. These breast cancers occur frequently in young African women and are associated with aggressive disease progression, poor prognosis and *BRCA1* mutations. TN patients with operable tumours may undergo surgery under general anaesthetics. Treatment of TNBC poses a clinical challenge as these tumours are unresponsive to hormonal or HER2 targeted therapy. Defects in *BRCA1* and other DNA repair genes contribute to chromosomal instability and radiosensitivity and cause irregularities in the cell cycle checkpoints in the S/G2 phase.

Studies have shown the overlap of breast cancer susceptibility genes and Fanconi Anaemia (FA) genes. FA is an autosomal recessive disorder defined by cellular hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC) and defects in DNA repair genes. FA patients are known to be radiosensitive and have defects with DNA repair. These patients are at high risk to develop leukaemia and solid tumours that may require radiotherapy. Diagnosis of FA patients often includes detecting chromosomal aberrations induced by a cross-linking agent. Molecular tests are also conducted to identify mutations in FA genes. It has previously been shown that FA patients undergoing radiotherapy display increased clinical radiosensitivity. Evidence suggests that FA patients are chromosomally radiosensitive to ionising radiation (IR).

Chromosomal radiosensitivity can be evaluated using the cytokinesis-block micronucleus (CBMN) assay in different phases of the cell cycle. Micronuclei (MNi) serve as biomarkers for radiation-induced DNA damage repair and defects in DNA repair mechanisms can be reflected in chromosomal radiosensitivity. A number of factors could influence the MNi yield such as storage time and temperature, and cytotoxic agents such as anaesthetics. As radiotherapy is considered a principle treatment in the management of TNBC, it is important to investigate in vitro chromosomal radiosensitivity of South African TN breast cancer patients. Chromosomal instability and radiosensitivity of FA patients has previously not been investigated in SA. The overall aim of this study was to investigate chromosomal instability and radiosensitivity of lymphocytes in South African breast cancer patients, FA patients and parents compared to healthy individuals using the G0 and S/G2 CBMN assay. The effect of age, ethnicity and mutations in breast cancer susceptibility genes was also investigated. Furthermore, storage time and effect of anaesthetics on MNi yield was investigated.

Methods: For the G0 MN assay, heparinised blood in culture medium was irradiated at 0Gy (Baseline), 2 and 4 Gy followed by the immediate stimulation of lymphocytes using phytohaemagglutinin (PHA). Cytochalasin B was added 23 hours later to inhibit cell division. The S/G2 MN assay is a modified version of the G0 MN assay. In this assay, the cultures are first stimulated with PHA and irradiated 72 hours post stimulation. Eight hours post irradiation cells were fixed. The Mitomycin C (MMC) MN assay is similar to the G0 MN assay except the DNA damage is induced using MMC.

Results: Chromosomal instability is significantly elevated in TNBC, young and older breast cancer patients. Radiation-induced MN values in the G0 MN assay are significantly enhanced in a total unselected group of breast cancer patients compared to healthy individuals. However, when subdividing the breast cancer patients in a TNBC group, the enhanced radiation-induced MNi are not observed. We cannot demonstrate a correlation between the age of the patients and chromosomal radiosensitivity but an effect of ethnicity is noted in our breast cancer population. In the S/G2 MN assay, TNBC patients continued to exhibit a decreased chromosomal radiosensitivity. We also demonstrated that increased storage time can influence MNi yields in patients and controls; anaesthetics influenced spontaneous MNi yields.

The FA patients in our study demonstrate higher MNi when compared to parents and controls indicating chromosomal instability and chromosomal radiosensitivity in the G0 as well as in the S/G2 phase of the cell cycle. This is not seen in the FA heterozygotes. With the MMC assay, the detection of significantly higher MN is noted in as well the FA patients as well as the FA carriers.

Conclusions: Chromosomal instability and radiosensitivity of breast cancer and FA patients are notably higher when compared to healthy individuals. The association of BRCA mutations in TN and young patients highlight the importance of radiosensitivity information in the understudied SA population. FA carriers can be at risk for breast cancer with mutations associated with breast cancer susceptibility genes. As a functional assay, the MMC MN assay will be useful in the identification of FA carriers who may be at risk of breast cancer. Data on radiosensitivity of patients with defects in DNA repair genes could provide important information for radiotherapy management of cancer.

ACKNOWLEDGEMENTS

"We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained." ~ Marie Curie

Thank you, Marie Curie for inspiring me to continue my work in radiation biology even when the road was, at times, long and hard. Finally, I see the light at the end of this very long tunnel. Thank you for shattering stereotypes and paving the way for women in science and academia. A very big thank you!

Firstly, I would like to express my sincere gratitude to Ans, my supervisor and mentor. Everything I know about radiobiology today, I owe it most to you. Thank you for always believing in my capabilities and strengths (even when self-doubt creeped in). Thank you for the continuous support from my Honours year to Ph.D., for all the opportunities you've presented to me, for your guidance and love throughout the years.

Anne and Kathleen – an immense thank you to both of you for providing me the opportunity to join your research team in Ghent and access to your labs. As a result, my knowledge in diverse scientific aspects has deepened. Thank you for your valuable comments and suggestions during this research. I was also blessed to meet some amazing people during my time spent in your labs. I hope that our paths will cross again.

I am grateful to Prof. Slabbert and iThemba LABS for always being supportive of my research and providing funding at bleak times. To all my funders: NRF, NECSA, CANSA, UGent (BOF Grant) and VLIR. A heartfelt thank you. Without these funding's, this research would never have seen the light of day.

Prof. Vangu, Prof. Van Der Merwe and the Medical Physics team at CMJAH, your support and assistance are highly appreciated. To Dr. Nietz, Dr. Cairns, Dr. Murdoch, Dr. Wainwright, Dr. Poole and all the nurses in the Paediatric oncology ward at CMJAH, your assistance with the study is highly appreciated. I would like to thank Suretha and Noelene for their genetic counselling assistance with the study.

All my colleagues, past and present: Olivia, watching you complete your Ph.D. was a glimmer of hope for me. Kim, Ilse, Brecht, Liselotte, Xenia, Nathalie, Annelot, the entire staff

of MRB1 and B3: thank you for imparting your technical skills and knowledge to me. All your encouragement, support and friendship are valued. I'm grateful for the time we've had and appreciate the conversations and messages to check if I had my head above water during the course of my Ph.D.

A very special gratitude goes out to all the participants of this research, particularly the patients. You have run this race and continue to do so against cancer. I sincerely hope that this work will add to the knowledge and understanding to fight this battle.

To all my family and friends. My parents and sisters, Jen and Roq, Sonika, Jeroen, Gamze, Greet, Xan, Andrea, Mish and Carrie. All your support and encouragement kept me going.

Steph, my eternal cheerleader, I am immensely grateful for you. Thank you for supporting me spiritually and providing emotional support. You've never ceased to believe in me, always pushed me to do my best and accepted nothing less than excellence from me. This Ph.D. is yours as much as it is mine. You are the reason I was brave enough to face it head-on.

Lastly, thank you Jesus! All of this was possible because You spoke it. I am because of grace.

~ Where God guides, He provides ~

Isaiah 58:11

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer Staging
AT	Ataxia telangiectasia syndrome
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	ATR interacting protein
BACH1	BRCA1-associated C-terminal helicase 1
BARD1	BRCA1 associated RING domain 1
BER	Base excision repair
BN	Binucleated cells
B-NHEJ	Backup-NHEJ
bp	Base pairs
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BRCT	BRCA1 C-terminal
BRIP1	BRCA1-interacting protein C-terminal helicase 1
CBMN	Cytokinesis-block micronucleus
CDC27	Cell division cycle protein 27 homolog
CHEK2	Checkpoint kinase 2
CSA	Cockayne syndrome group A
CSB	Cockayne syndrome group B
CSC	Cancer stem cells
Cyto B	Cytochalasin B
DEB	Diepoxybutane
DCIS	Ductal carcinoma *in situ*
DDR	DNA damage response
DNA–PK	DNA–dependent protein kinase
DNA	Deoxyribonucleic acid
DSB	Double-strand breaks
E. coli	Escherichia coli

E3	Enzyme 3
ER	Estrogen receptor
ERCC1/4	Excision repair cross-complementing protein 1/4
FA	Fanconi Anaemia
FANC	Fanconi anaemia complementation groups
FANCA	Fanconi anaemia complementation group A
FANCB	Fanconi anaemia complementation group B
FANCC	Fanconi anaemia complementation group C
FANCD1	Fanconi anaemia complementation group D1
FANCD2	Fanconi anaemia complementation group D2
FANCE	Fanconi anaemia complementation group E
FANCF	Fanconi anaemia complementation group F
FANCG	Fanconi anaemia complementation group G
FANCI	Fanconi anaemia complementation group I
FANCJ	Fanconi anaemia complementation group J
FANCL	Fanconi anaemia complementation group L
FANCM	Fanconi anaemia complementation group M
FANCN	Fanconi anaemia complementation group N
FANCO	Fanconi anaemia complementation group O
FANCP	Fanconi anaemia complementation group P
FANCQ	Fanconi anaemia complementation group Q
FANCR	Fanconi anaemia complementation group R
FANCS	Fanconi anaemia complementation group S
FANCT	Fanconi anaemia complementation group T
FANCU	Fanconi anaemia complementation group U
FANCV	Fanconi anaemia complementation group V
FANCW	Fanconi anaemia complementation group W
FISH	Fluorescence *in situ* hybridisation
GG-NER	Global-genome NER
H2A/B/3/4	Histone 2A/B/3/4
H2AX	Histone subtype H2A isoform X
HER2	Human epidermal growth factor receptor 2

HIV	Human immunodeficiency virus
HR	Homologous recombination
HSC	Haematopoietic stem cells
HuR	Human antigen R
IARC	International Agency for Research on Cancer
ID2	FANCI and FANCD2 complex
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IR	Ionising radiation
IRP	Individual radiosensitivity parameter
Ku-DNA-PKcs	Ku DNA-dependent protein kinase
LCIS	Lobular carcinoma *in situ*
LET	Linear energy transfer
MAD2L2	Mitotic arrest deficient 2 like 2
Mhp1	MAP-homologous protein 1
MLH	mutL homolog
MLPA	Multiplex Ligation-dependent Probe Amplification
MMC	Mitomycin C
MMEJ	Microhomology-mediated end joining
MMR	Mismatched repair
MNi	Micronuclei
MRE11	Meiotic recombination 11 homolog
MRE11	MRE11 homolog
MRI	Magnetic resonance imaging
MRN	Mre11-Rad50-Nbs1 complex
MSH	MutS homolog
NBS	Nijmegen breakage syndrome
NBN/NBS1	Nibrin
NCR	National Cancer Registry
NER	Nucleotide excision repair

NHEJ	Non-homologous end joining
B-HEJ	Backup-non-homologous end joining
PALB2	Partner and localiser of BRCA2
PBL	Peripheral blood lymphocytes
PHA	Phytohaemagglutinin
PR	Progesterone receptor
PTEN	Phosphatase and Tensin homolog
PTT	Protein truncation test
RAD50	RAD50 homolog
RAD51	RAD51 recombinase
RAD51C	RAD51 paralog C
RAD51D	RAD51 paralog D
RAD52	RAD52 Homolog
RFWD3	Ring finger and WD repeat domain 3
RING-finger domain	Really interesting new gene-finger domain
RNA	Ribonucleic acid
SLX4	SLX4 structure-specific endonuclease subunit
SNP	Single nucleotide polymorphisms
SSA	Single strand annealing
SSCP/HA	Single strand conformation polymorphism/heteroduplex analysis
TC-NER	Transcription-coupled NER
TN	Triple negative
TNBC	Triple negative breast cancer
TNM	Tumour-Nodal-Metastases
TP53	Tumour protein p53
UBE2T	Ubiquitin conjugating enzyme E2 T
XLF	XRCC4-like factor
XP	Xeroderma pigmentosum

XRCC2	X-ray repair cross-complementing 2
XRCC4	X-ray repair cross-complementing 4
XRCC9	X-ray repair cross-complementing 9
β	Beta
γ	Gamma

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 7

LIST OF TABLES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 6

CHAPTER 7

CHAPTER 1
Introduction

1. Breast cancer

Breast cancer is a heterogeneous and multifactorial disease. It can be attributed to various alterations in the genome, including amplification or deletions of genes, insertions, translocation and chromosomal aberrations. Such genetic alterations contribute to the inactivation of tumour suppressor genes and amplification of oncogenic genes. When assembled in a single breast cell, an accumulation of a large number of individual genetic mutations disrupts the control system to the extent that the cell functions autonomously in an erratic and irregular manner. Such corrupted cells may start forming colonies of abnormal cells that may accumulate other aberrant mutations to eventually initiate cancer (Stephens et al., 2009).

1.1 Incidence and mortality of breast cancer

On a global scale, breast cancer is the most common cancer amongst women. In 2012, the International Agency for Research on Cancer (IARC) estimated newly diagnosed breast cancer cases to be 1.67 million (Figure 1) and the number of deaths reported were 522,000 (Globocan, 2012).

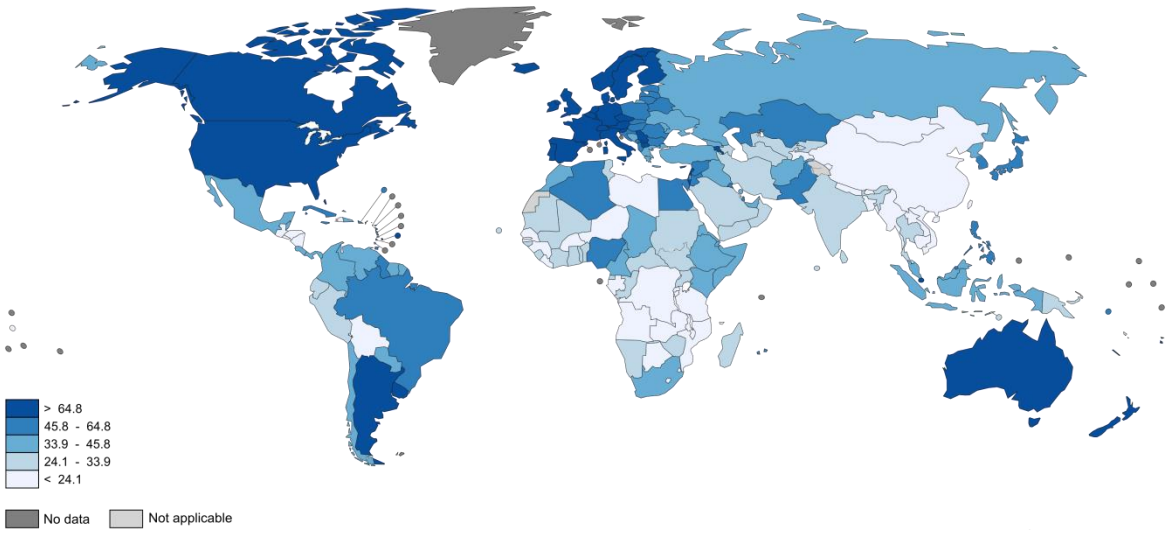

Figure 1: Worldwide breast cancer incidence in women. The estimated age-standardised breast cancer incidence rate per 100, 000 worldwide as reported by the IARC in 2012 (Globocan, 2012).

Although the incidence of breast cancer remains high in developed nations, the incidence of breast cancer in underdeveloped regions is increasing and has a slightly higher mortality rate than developed regions (Tfayli et al., 2010). The incidence rate is increasing owing to urbanisation and change in lifestyle. South African women are highly likely to present with late stage disease as compared to only 5% of American women that present with late stage disease (Kuo et al., 2011). Lack of awareness, limited access to diagnostic centres in rural areas for early detection, and lower standards of healthcare facilities attributed to this trend leading to higher mortality rates in less developed nations (Vorobiof et al., 2001).

In South Africa (SA), breast cancer is the leading cancer in women and the second cause of death (Globocan, 2012). The lifetime risk of breast cancer for women in SA has been estimated to 1 in 28 by the National Cancer registry with 1/51 in black women, 1/11 in white women, 1/19 in Asian women and 1/20 in coloured women (NCR, 2013).

1.2 Breast cancer risk factors

1.2.1. Environmental and lifestyle risk

Multiple factors influence risk of developing breast cancer risk such as lifestyle changes that include change in diet, obesity, increased smoking and hormonal factors. Reproductive factors such as early menarche, late menopause, delayed and decreased parity, and absence of breast feeding are also associated with increased risk (Porter, 2009). These reproductive factors are correlated with exposure to estrogen. Long term exposure of estrogen has been shown to elevate risk; however, decreasing exposure to estrogen has a protective function against breast cancer. For instance, absence of early full-term pregnancy is associated with increased breast cancer risk (Britt et al., 2007, Martin and Weber, 2000).

Young women diagnosed with Hodgkin's lymphoma are often treated with chest radiotherapy and have increased incidence of breast cancer. Breast cancer risk and dose of radiation used are directly proportional. The observed risk primarily prevails in younger women when compared to older women. Consequently, radiotherapy is a factor that plays a role in breast cancer risk (Hill et al., 2005, Travis et al., 2005, van Leeuwen et al., 2003).

The association of breast density with breast cancer risk is evident in literature (Titus-Ernstoff et al., 2006, Boyd et al., 2011, Yaghjyan et al., 2015, Byrne et al., 1995). The risk increases by 4-6 fold in women with dense breast tissue which is often noted in young women (McCormack and dos Santos Silva, 2006). The features of breast tissue can be assessed by mammography. Apart from breast density, obesity is linked with increased breast cancer risk. An alarming 20% of breast cancers result from obesity and lifestyle factors that affect body mass index. However, obesity-related cancers are more prevalent in postmenopausal women (De Pergola and Silvestris, 2013).

Gender is a key factor that influences breast cancer risk. Women are at higher risk of breast cancer than men. Young and older women are both highly likely to be diagnosed with breast cancer. Men are often older at diagnosis with larger and late stage tumours. The occurrence of breast cancer in men is prevalent in the African population (Greif et al., 2012). Only about 1% of all breast cancers in Europe are men. Hereditary causes are the primary reason of male breast cancer (section below) (Fentiman et al., 2006).

1.2.2. Hereditary breast cancer and family history

The occurrence of breast cancer can be either sporadic or hereditary/familial. About 10% of all breast cancers are hereditary and the risk is considerably higher with the presence of mutations in *BRCA1* and/or *BRCA2* (Breast Cancer gene 1 and 2) (Cuzick, 2003), or genetic variations in other DNA repair genes (Goldgar et al., 2011, Casadei et al., 2011, Antoniou et al., 2014, Desrichard et al., 2011). Compared to sporadic breast cancers, hereditary breast cancer also has a low age at onset of disease (Margolin and Lindblom, 2006). Additionally, family history accounts for 15-20% of all breast cancers (Lynch et al., 2008). A positive family history is an important breast cancer risk factor that is shown to increase risk 2 fold with a first or second degree relative affected by the disease. Having two first degree relatives affected, triples the risk of breast cancer (Cuzick, 2003). Accurate information on comprehensive family history is important in individual risk assessment.

1.2.3. Germline mutations in breast cancer susceptibility genes

Germline mutations in DNA repair genes can be heritable and account for 15-20% of familial breast cancer cases (West et al., 2003, Lynch et al., 2008). Genetic predispositions to these genes are associated with enhanced risk of developing breast cancer. These susceptibility genes are divided into high-risk and moderate to low-risk susceptibility genes. Compared to the general population, the risk associated with high-risk genes is four times more; the risk is doubled from the general population with moderate-risk genes and less than twice with low-risk susceptibility genes. *BRCA1* and *BRCA2* are the most described high-risk genes, whereas moderate-risk susceptibility genes include *ATM*, *CHEK2* and *PALB2* (Hollestelle et al., 2010). Single nucleotide polymorphisms have been detected that confer low-risk of breast cancer (Beggs and Hodgson, 2009). Table 1 highlights some important high risk and moderate risk genes associated with breast cancer. Many of these genes are involved in the same pathway for repair of DNA double strand breaks (DSB) through homologous recombination (HR).

In addition, several genome-wide association studies revealed a large number of SNPs, each individually associated with a small increased risk to breast cancer, but a combination of these SNPs may have an additive effect. Large studies conducted by breast cancer consortiums show breast cancer susceptibility SNPs and environmental factors that modify breast cancer risk. Smoking, alcohol consumption, age at menarche and first full term pregnancy were all identified with significant evidence linking it to increased breast cancer risk (Barrdahl et al., 2017). However, breast cancer genetic susceptibility variants are not always associated with increased risk. Certain SNPs also play a protective role against breast cancer (Kirchhoff et al., 2012). Evaluating the function of SNPs in risk assessment of breast cancers is, therefore, essential. Studies are emerging to evaluate if "polygenic risk scores" could have an added value to predict risks for breast and/or ovarian cancer (Li et al., 2017, Kuchenbaecker et al., 2017, Jervis et al., 2014).

Table 1: Breast cancer susceptibility genes showing penetrance and lifetime risk.

Gene	Penetrance	Lifetime risk *	Function	Reference
BRCA1	High	40-70%	Genomic stability and DNA DSB repair	(Takaoka and Miki, 2017, Winter et al., 2016)
BRCA2	High	20-57%	Genomic stability and DNA DSB repair	(Foulkes and Sugano, 2016, Winter et al., 2016)
TP53	High	56-90%	Genomic stability	(Yeo et al., 2016, Dumay et al., 2013)
PTEN	High	50-80%	Genomic stability	(Hopkins et al., 2014, McCabe et al., 2016)
CHEK2	Moderate	25-37%	DNA DSB repair	(Adank et al., 2011, Apostolou and Papasotiriou, 2017)
PALB2	Moderate	20-40%	DNA DSB repair	(Foo et al., 2017)
ATM	Moderate	15-20%	DNA DSB repair	(Goldgar et al., 2011)
BARD1	Moderate	Differs	DNA DSB repair	(Gass et al., 2016, Zhao et al., 2017)

* Modified (Apostolou and Fostira, 2013, van Marcke et al., 2016)

1.2.4. Role of *BRCA1* and *BRCA2*

Germline *BRCA1* and *BRCA2* mutations account for 20-25% of all familial breast cancers and may account for up to 5% of all breast cancers. *BRCA1* and *BRCA2* are tumour suppressor genes playing a significant role in DNA DSB. BRCA1/2 proteins are important in DNA repair pathways and reduced expression or inactivating mutations in these genes can lead to impaired DNA repair. The integrity of the DNA is maintained by BRCA's interaction with recombination proteins that belong to the family of Rad proteins. Defects in *BRCA1/2* contribute to genetic instability and irregularities in the cell cycle checkpoints leading to an increased probability of tumourigenesis (Khanna and Jackson, 2001).

BRCA1 gene is located on chromosome 17 and encodes 1863 amino acids (Figure 2) (Welcsh and King, 2001). The structure of BRCA1 includes 3 main domains of interest; i) the RING-finger domain – accountable for E3 ubiquitin ligase activity, ii) exon 11-13 – region known to contain binding domains for other proteins and iii) the BRCA1-terminal (BRCT) – domain for binding ATM-phosphorylated proteins (Figure 4). In addition to DNA damage response, BRCA1 plays a central role in cell cycle checkpoint activation and transcriptional regulation (Clark et al., 2012).

Chromosome 13 shelters the *BRCA2* gene which encodes 3418 amino acids. In contrast to BRCA1, the N-terminal domain of BRCA2 is a transcriptional activation site proceeded by a series of repeat sequences termed BRC. These sequence motifs are highly conserved and act as a medium for interaction between BRCA2 and RAD51. The nuclear localisation site (NLS) located in the C-terminal contains a DNA-binding site (Shamoo, 2003). The BRCA2 and RAD51 interaction is pivotal in DNA recombination and regulation of DNA repair of single-strand breaks and DSB (Pellegrini and Venkitaraman, 2004).

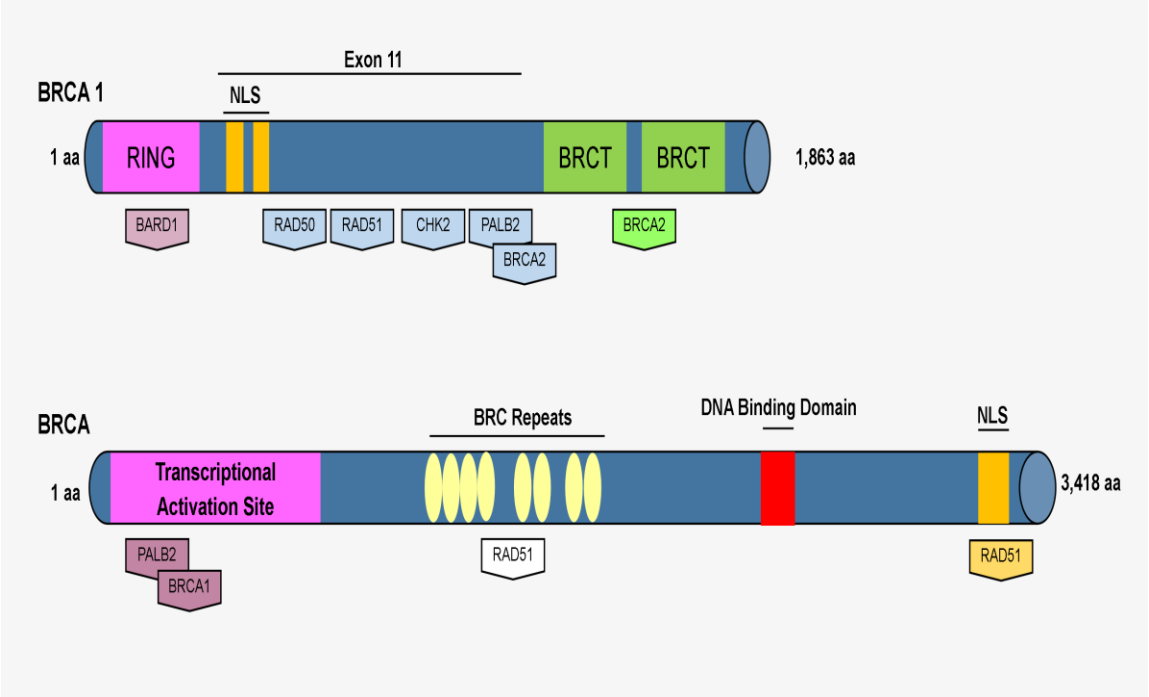

Figure 2: Illustrative structure of BRCA1 and BRCA2. BRCA1 consists of 3 main function domains and encodes 1863 amino acids. BRCA2 consists of 8 BRC repeats important for binding DNA repair proteins. Image adapted from (Venkitaraman, 2001, Orr and Savage, 2015).

1.3. Breast cancer in South Africa

Similar to other developing countries, SA has a higher incidence of early-onset breast cancer. Compared to other developing nations, the age at diagnosis in African women is relatively low (Walker et al., 2004, McCormack et al., 2013). Although breast cancer is predominantly observed in the white population, increasing numbers of young black South African women are frequently diagnosed with the disease. Young African women usually present with advance disease, poor prognosis and disproportionately high mortality rates (Dickens et al., 2014a, Basro and Apffelstaedt, 2010, Matatiele and Van den Heever, 2008). Arguably, an emerging factor linked with increase of breast cancer in young women is parity. Although conferring a protective role in breast cancer, parity is related to increased risk in young African women (Palmer et al., 2003). While the growing incidence of breast cancer in young South African women could also be due to a younger population structure, it is likely that these women are carriers of unique mutations associated with breast cancer. This can further be attributed to genetic predispositions

since *BRCA1/2* mutations are often observed in premenopausal breast cancer (Young et al., 2009). Therefore, carrying a deleterious mutation increases the risk of developing early-onset breast cancer.

1.4. Screening and detection

Regular screening is essential in early detection of benign or malignant breast tumours. Patients with early diagnosis can be provided with appropriate treatment. Screenings include self- and clinical examinations, mammography and ultrasounds frequently for younger women or women with dense breasts, magnetic resonance imaging (MRI) and breast biopsy. Mammography is the mainstream method for breast cancer detection. It functions by exposing breast tissue composition attenuating x-rays to distinguish fat and epithelial tissue (Boyd et al., 2010). Although, mammography has potential benefits in detecting breast cancer, women with dense breast are at a loss as cancerous tissue can be missed, this trend is often observed in young women (Corsetti et al., 2008). Following the detection of an abnormal breast image using mammography, a breast biopsy is conducted for efficient diagnosis prior to any form of treatment (Ely and Vioral, 2007).

Even though MRI is not recommended routinely, it is broadly utilised for screening high risk women; for instance, carriers of germline mutations in high risk breast cancer gene and those with a family history. MRI offers high sensitivity even in women with dense breasts. However, there is lack of evidence that benefits of MRI may precede that of a mammogram (Morrow et al., 2011, Kriege et al., 2004). Due to the higher incidence of breast cancer in young African women, mammography screening in SA is routinely recommended for women 40 years and above (Synman, 2010).

1.5. Classification of breast cancer

Breast cancer is categorised into several histopathological subtypes and staging systems by the American Joint Committee on Cancer staging (AJCC) based on the histopathology type, grade and stage of tumour, and expressions of proteins and genes.

1.5.1. Histopathology

The histopathology is classified into two main categories depending on whether the malignant cells originate in the milk ducts or the lobules (Figure 3). The presence of abnormal cells confined to the lining of the milk ducts is classified as ductal carcinoma *in situ* (DCIS). Similarly, lobular carcinoma *in situ* (LCIS) occurs when abnormal cells are observed in the lobules of breast ducts. LCIS is usually not categorised as malignant, however, it increases the risk of malignancy. The disease is further categorised into invasive or non-invasive (Herbst, 2011). The most common and rapid growing breast cancer which accounts for 80% of all invasive breast cancer is the infiltrating or invasive ductal carcinoma (IDC); the invasive lobular carcinoma accounts for about 10% (Ely and Vioral, 2007).

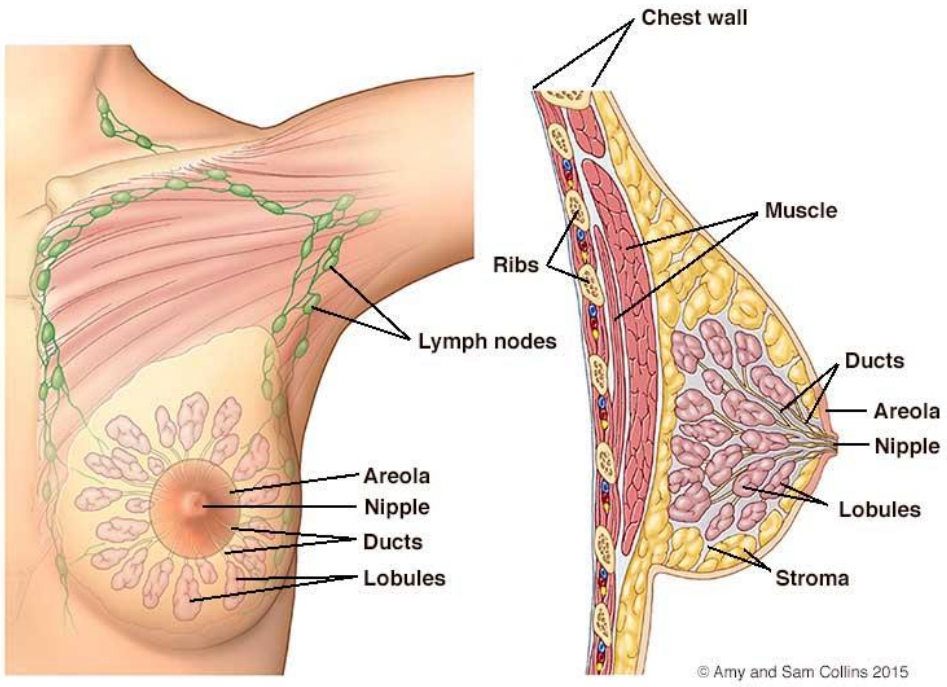

Figure 3: Anatomy of the breast - indicating the ducts and lobules (Komen, 2016).

A second staging system introduced is known as the Tumour-Nodal-Metastases (TNM) considers various aspects such as: i) size of the tumour, ii) presence of cancer cells in the lymph nodes and iii) distant metastases of the cancer cells. The combination of the histopathology

report, staging and the clinical data often provide an indication of the prognosis and is used as guideline in determining the treatment options (Singletary, 2002).

1.5.2. Hormone receptors for breast cancer

In addition to the pathological features and staging, gene expression profiling has deciphered molecular subtypes of breast cancers based on the expression or lack of expression of hormonal receptors. Estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2) are the biomarkers that have prognostic value and are beneficial for treatment evaluation (See section 1.7 on therapy). The presence or absence of the receptors classifies the tumour as hormone receptor-positive or receptor-negative respectively.

Estrogen plays a key role in facilitating the development and progression of breast cancer. The estrogen hormone attaches to the ER receptor and mediates its function of cellular proliferation and differentiation of breast tissue. However, this function is not confined to normal cells only. Cancerous breast cells will also proliferate by estrogen (Russo and Russo, 2006). ER expression is linked with genomic instability by generating oxidative damage to DNA and inducing double strand breaks that can result in breast cancer. Rapidly proliferating cells do not allow sufficient time for DNA damage repair leading to accumulation of DNA damaged cells. ER also interacts with various DNA repair proteins and alters the DNA damage response (Caldon, 2014). In the absence of strictly regulated DNA repair, cell proliferation driven by ER signalling may lead to mutations that may lead to breast cancer (Matta et al., 2016). ER positive breast cancers contribute to about 75% of all breast cancers and are more predominant in postmenopausal women.

The progesterone hormone activates the PR and functions in cell proliferation. Excess PR contributes to the development of breast cancer. PR expression is regulated by ER and gains anti-oncogenic functions in ER-positive tumours. Also, PR is a predictive factor for the response of hormonal therapies in ER-positive cancers (Mohammed et al., 2015, Allred, 2010).

HER2 gene is a proto-oncogene that functions in and regulates cell proliferation and differentiation. Overexpression of HER2 induces malignancy by stimulating the formation of tumours. HER2 is overexpressed in roughly about 20% of breast cancers. In SA, 26% of breast cancer patients are HER2+ (Vanderpuye et al., 2017a). In comparison to HER2- breast cancers, HER2+ phenotypes are thought to be clinically unique and present with poor prognosis (Burstein, 2005).

By molecular classifications, IDC and ILC histological subtypes can further be categorised as 4 phenotypes namely luminal A (ER+/PR+/HER2-), luminal B (ER+/PR+/HER2+), HER2 overexpressing (ER-/PR-/HER2+) and triple negative breast cancer (ER-/PR-/HER2-) (TNBC) (Inic et al., 2014). Luminal cancers are generally associated with good prognosis and the most prominently occurring subtype is the luminal A (Stead et al., 2009, Anders and Carey, 2008). Luminal B cancers are known to have lower expression of ER than luminal A subtypes and generally higher graded tumours (Tamimi et al., 2008).

1.6. Triple negative breast cancer

Tumours lacking the expression of hormone receptors, termed as triple negative (TN), account for 10-20% of breast cancers worldwide (Bauer et al., 2007, Carey et al., 2010, Reis-Filho and Tutt, 2008, Foulkes et al., 2010, Vona-Davis et al., 2008, Alcantara et al., 2017). The prevalence of TNBC in SA ranges between 14-26% (Prodehl, 2016, McCormack et al., 2013, Dickens et al., 2014a, Dickens et al., 2014b), although other African nations have relatively higher prevalence of TNBC compared to SA and the western counterparts (Vanderpuye et al., 2017b). TNBCs are associated with aggressive tumour progression, poor prognosis, higher rate of recurrence and mortality. The frequent occurrence of TNBC is strongly correlated with young or premenopausal African women. TNBC poses a clinical challenge as these tumours are hard to treat with hormonal and/or targeted therapy (See section on therapy below) (Foulkes et al., 2010).

Germline mutations in *BRCA* are associated with the TN phenotype (Dietze et al., 2015), with about 75% *BRCA1* mutation-related breast cancers present with the TN subtype (Bayraktar et al., 2011, Rakha and Chan, 2011). The combination of presenting a *BRCA* mutation and TN

phenotype is frequently observed in early-onset of the disease (Young et al., 2009, Robertson et al., 2012).

1.7. Therapy for breast cancer

Based on the staging, local and systemic treatment options are offered to patients. Local treatments directly affect the tumour only and systemic treatments are generally used to treat the spread of cancer anywhere in the body. Different treatment modalities available for breast cancer are surgery, chemotherapy, radiotherapy, hormonal and targeted therapy (Ely and Vioral, 2007).

Surgery is conducted for inoperable tumours as well as for primary tumours to assist with the staging of the cancer. The two main types of surgery are i) breast conserving surgery such as a lumpectomy where only the tumour is removed and is recommended for early stage cancers and ii) mastectomy involves the removal of the entire breast. For women who are at high risk and have advanced cancers, a double mastectomy is generally recommended (Ely and Vioral, 2007).

Chemotherapy is a mainstream therapy for treating breast cancer. It is administered intravenously in a combination of 2 or 3 drugs and circulates in the bloodstream also reaching cancerous cells that may have spread from the vicinity of the breast tissue (Ely and Vioral, 2007). Despite successful outcome, administering chemotherapy is often accompanied with serious and/or permanent side effects. Older patients receiving chemotherapy are at risk of cardiac toxicity and secondary cancers such as acute myeloid leukaemia. The side effects in the younger patient population are more and comprise fluctuations in menstrual cycle, infertility and premature menopause. Due to its harsh effects, chemotherapy is often administered in cycles allowing the body to recover (Azim et al., 2011).

Residual malignant cells following surgery are frequently treated with radiotherapy. Radiotherapy is the therapeutic use of high-energy beams to eradicate cancer cells which is achieved by external beam therapy or internal beam using a radioactive source. As with chemotherapy, radiotherapy is also associated with important side effects such as edema, fatigue, skin reaction mimicking sun burns (Ely and Vioral, 2007) and ischemic heart disease resulting

from exposure of radiation to the chest area (Darby et al., 2013). To control progression and local-regional recurrence, it is utilized as a principle treatment for TNBC (Dragun et al., 2011) and as a mainstream treatment in the management of most malignancies.

Hormone positive breast cancers are fuelled by estrogen in the blood and are treated systemically with hormone therapy that block the function of estrogen. Target hormonal treatments such as tamoxifen and aromatase inhibitors are available for these cancers and are known to have good prognosis. Tamoxifen is the most commonly used hormonal drug that mediates its effect by blocking the function of estrogen entirely, although it is effective in 5-10% of ER-negative tumours (Manna and Holz, 2016). It is widely used in treating pre- and postmenopausal ER positive breast cancers (Hurvitz and Pietras, 2008). In contrast to tamoxifen, aromatase inhibitors are a class of drugs used to endogenously lower estrogen levels in postmenopausal women. Commonly noted side effects with hormonal therapy are fatigue, hot flashes, bone thinning and seldom blood clots (Ely and Vioral, 2007).

Cancer cells, at times, react to certain proteins that allow them to rapidly multiply. One such protein correlated to breast cancer is HER2. Targeted therapy permits drugs such as Herceptin, which is an anti-HER2 monoclonal antibody, to regulate cell growth. In HER2 overexpressing cancers, Herceptin binds to HER2 to inhibit the growth of cancer cells. In comparison to other treatments, the side effects of targeted therapy are relatively mild such as fever, nausea, vomiting and headaches. Certain targeted drugs are administered in conjunction with chemotherapy (Ely and Vioral, 2007).

Benefits of hormonal or targeted therapy are absent in TNBC patients. Treatment modalities available at present are certain chemotherapy regimens, radiation therapy and surgery. However, the response rate of TNBC to chemotherapy is often associated with poor prognosis (Yagata et al., 2011, Wang, 2011).

2. Fanconi Anaemia

2.1. Incidence of Fanconi anaemia

Fanconi anaemia (FA) is an autosomal recessive trait and cancer-prone chromosomal instability disorder. The incidence of FA in Europe and United States was documented to be approximately 3 in a million. Certain populations have founder mutations and the incidence rate varies (Tischkowitz and Hodgson, 2003). In SA, with mixed ethnicity in the population, prevalence of FA ranges between 1/22 000 for the white Afrikaners to 1/40 000 in the black South Africans (Tipping et al., 2001, Feben et al., 2014a, Rosendorff et al., 1987). The occurrence of FA within the black population is primarily due to the presence of the homozygous *FANCG* founder mutation c.637_643delTACCGCC in 80% of this population (Feben et al., 2014b, Feben et al., 2015).

2.2. Clinical defects

FA is a familial form of aplastic anaemia. The clinical manifestations of FA include congenital and developmental abnormalities, and haematological defects. FA patients are at high risk to develop acute myelogenous leukaemia due to impaired bone marrow function and the incidence of solid tumours are also increased (Alter, 2014). South African black FA patients initially present with severe aplastic anaemia and cytopenia. Other symptoms of the disease include epistaxis and patients are significantly underweight compared to the general population (Feben et al., 2015, Feben et al., 2014c). In the absence of molecular diagnosis, FA is treated as aplastic anaemia which could lead to bone marrow failure. In this instance, the only known curative treatment modality is haematopoietic stem cell (HSC) transplantation. However, graft rejections occur at a high rate and are related to poor survival in patients receiving HSC from related or unrelated donors. Full body radiation is used as myeloablative conditioning to minimise donor rejections. Prior to conditioning with radiation, it is vital to know the molecular diagnosis of aplastic anaemia patients to rule out FA as these patients are highly sensitive to radiation and would pose a challenge with conditioning regimes (Dalle, 2008, MacMillan et al., 2000, Yabe et al., 2006).

Molecularly, FA cells are hypersensitive to DNA cross-linking agents such as mitomycin C (MMC) and diepoxybutane (DEB) which are utilised to quantify chromosomal aberrations diagnostically. Sensitivity to chemotherapy and radiotherapy in FA patients has previously been documented. For patients with cancers associated with haematology, it is recommended that FA diagnostics be conducted before initiating chemotherapy as FA patients are highly sensitive to cytotoxic chemotherapy (Goldsby et al., 1999, Alter, 2002a).

2.3. Molecular pathology

Twenty-two FA patients have been found to carry biallelic pathogenic variants in any of 20 genes, *FANCA* (MIM #607139), *FANCC* (MIM #613899), *FANCD1*/*BRCA2* (MIM #600185), *FANCD2* (MIM #613984), *FANCE* (MIM #613976), *FANCF* (MIM #613897), *FANCG*/*XRCC9* (MIM #602956), *FANCI* (MIM #611360), *FANCJ*/*BRIP1* (MIM #605882), *FANCL* (MIM #608111), *FANCM* (MIM #609644), *FANCN* (MIM #610832)/*PALB2* (MIM #610355), *FANCO* (MIM #613390)/*RAD51C* (MIM #602774), *FANCP* (MIM #613951)/*SLX4* (MIM #613278), *FANCQ* (MIM #615272)/*ERCC4* (MIM #133520)/*XPF* (MIM #278760), *FANCS*/*BRCA1* (MIM #113705), *FANCT* (MIM #616435)/*UBE2T* (MIM #610538), *FANCU* (MIM #617247)/*XRCC2* (MIM #600375), *FANCV* (MIM #617243)/*MAD2L2* (MIM #604094)/*REV7*, and *FANCW*/*RFWD3* (MIM #614151). Variants in *FANCB* (MIM #300515), an X-linked gene and an autosomal dominant variant in *FANCR* (MIM #617244)/*RAD51* (MIM #179617), can also cause the disease, resulting in a total of 22 FA genes reported thus far (Table 2) (Mamrak et al., 2017, Kottemann and Smogorzewska, 2013, Rickman et al., 2015, Wang and Smogorzewska, 2015, Knies et al., 2017). Each of these are involved in DNA interstrand crosslink damage recognition and repair (Dong et al., 2015, Park et al., 2016). Homozygous or compound heterozygous mutations in these complementation groups will result in the FA-phenotype. Amongst these documented FA genes, the most frequently occurring mutations are observed in *FANCA*, *FANCC* and *FANCG* (Brooks et al., 2012). Literature has shown an overlap of the FA and breast cancer genes. For instance, *BRCA2* (*FANCD1*) (Alter et al., 2007) and *BRCA1* (*FANCS*) and *PALB2* (*FANCN*) (Tischkowitz and Xia, 2010, Sawyer et al., 2015) are shown to be associated with a highly increased risk for breast cancer in patients with heterozygous germline mutations in these genes, while biallelic inactivation leads to FA.

Table 2: Overview of the 22 FA genes identified

Complementation Groups	Gene Name / Alternative Name	Chromosomal Location	Estimated Frequency
FA-A	*FANCA*	16q24.3	60-70%
FA-B	*FANCB*	Xp22.31	2%
FA-C	*FANCC*	9p22.3	14%
FA-D1	*FANCD1 / BRCA2*	13q12.3	3%
FA-D2	*FANCD2*	3p25.3	3%
FA-E	*FANCE*	6p21.3	3%
FA-F	*FANCF*	11p15	2%
FA-G	*FANCG*	9p13	10%
FA-I	*FANCI*	15p26.1	1%
FA-J	*FANCJ / BRIP1*	17q22	2%
FA-L	*FANCL*	2p16.1	0.2%
FA-M	*FANCM*	14q21.3	0.2%
FA-N	*FANCN / PALB2*	16p21	0.7%
FA-O	*FANCO / RAD51C*	17q25.1	0.2%
FA-P	*FANCP / SLX4*	16p13.3	0.2%
FA-Q	*FANCQ / ERCC4*	16p13.12	Rare
FA-R	*FANCR / RAD51*	15q15.1	Rare
FA-S	*FANCS / BRCA1*	17q21.31	Rare
FA-T	*FANCT / UBE2T*	1q32.1	Rare
FA-U	*FANCU / XRCC2*	7q36.1	Rare
FA-V	*FANCV / REV7*	1p36.22	Rare
FA-W	*FANCW / RFWD3*	16q23.1	Rare

Modified from (Rosselli, 2016, Wu, 2013)

2.3.1. FA in Ashkenazi Jews

The most prevalent biallelic mutation in FA is observed in *FANCA* and it was the second FANC gene to be cloned in 1996. Expression of *FANCA* ubiquitously conducted in lower amounts. Pathogenic mutations associated with this gene occur as point mutations, base pair changes and often as large deletions. Patients harbouring *FANCA* mutations are commonly compound heterozygous. A founder mutation in this gene has been described in the Afrikaner population (Tischkowitz and Hodgson, 2003).

2.3.2. FA in the Afrikaner population

In 1992, *FANCC* was the first FA gene to be cloned by complementation method. Approximately 14% of FA cases are due to pathogenic mutations in *FANCC* and are most frequently observed in the Ashkenazi Jews as a founder effect. The same founder mutation has been reported in the Japanese population with slightly mild phenotype (Tischkowitz and Hodgson, 2003). However, *FANCC* mutations are not exclusive to these populations alone (de Vries et al., 2012). *FANCC* functions in promoting different forms of DNA repair (Niedzwiedz et al., 2004) and signalling of apoptosis (Taniguchi and D'Andrea, 2006).

2.3.3. FA in black South Africans

The *XRCC9* (X-ray repair cross-complementing protein 9) and *FANCG* are identical genes functioning in chromosomal instability repair. In roughly 82% of the black FA patients in Southern Africa, the presence of a founder mutation has been reported (Dong et al., 2015). In response to DNA damage, FANCG co-localises to RAD51 and interacts with BRCA1 to initiate repair (Hussain et al., 2003).

2.4. Fanconi anaemia pathways

The FA/BRCA repair pathway is pivotal in maintaining genomic instability since the proteins encoded from *FANC* genes are actively involved in DNA repair (Figure 4).

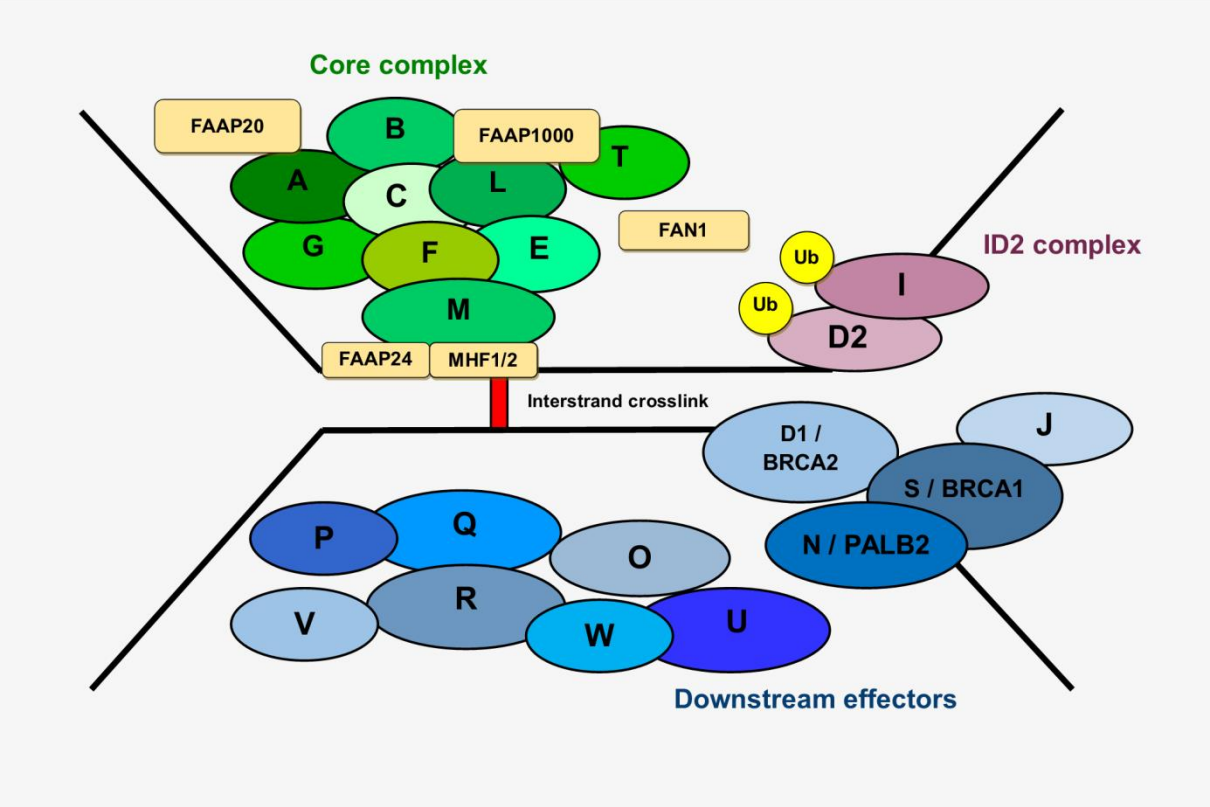

Figure 4: The Fanconi anaemia pathway. Illustration of the Fanconi anaemia pathway displaying the i) core complex (green) ii) ID2 complex (purple) and iii) downstream effectors (blue). Image modified (Haitjema et al., 2013).

The FANC proteins are subgrouped into 3 main categories known as (i) the core complex (ii) ID2 complex and (iii) the downstream effectors. In response to damage caused by DNA cross-linking agents or ionising radiation (IR), the activated core complex formed by 9 FA proteins (FANC-A, B, C, E, F, G, M, L and T) activates the ID2 complex, comprised of FANCD2/FANCI protein, by mono-ubiquitination and phosphorylation. The mono-ubiquitination of ID2 is catalysed by FANCU (also known as UBET2). The ID2 complex plays a critical role in the pathway by translocating to the damage sites which triggers the recruitment of downstream effectors (FANC-D1, J, N, O, P, Q, R, S, U, V and W) in the S/G2 phase. The damage is subsequently repaired by homologous recombination. Following the repair, the ID2 complex undergoes deubiquitination and the FA/BRCA pathway is regulated. Exogenous or endogenous damage causes an increase in the number of FA cells in the G2 phase as a result of S phase checkpoint inefficiency (Sala-Trepat et al., 2000).

3. Radiation and biological effects of radiation

3.1. Ionising radiation

The electromagnetic spectrum illustrates the various types of known radiation (Figure 5). Radiation is energy beams that travel through a vacuum or matter causing excitation or ionisation when energy is absorbed. IR is a type of electromagnetic wave that releases electrons in a process known as ionisation as it interacts with matter. The two forms of ionising radiation are i) electromagnetic waves that include X-rays and γ-rays, and ii) particles which are neutrons, beta and alpha particles (Joiner and van der Kogel, 2009).

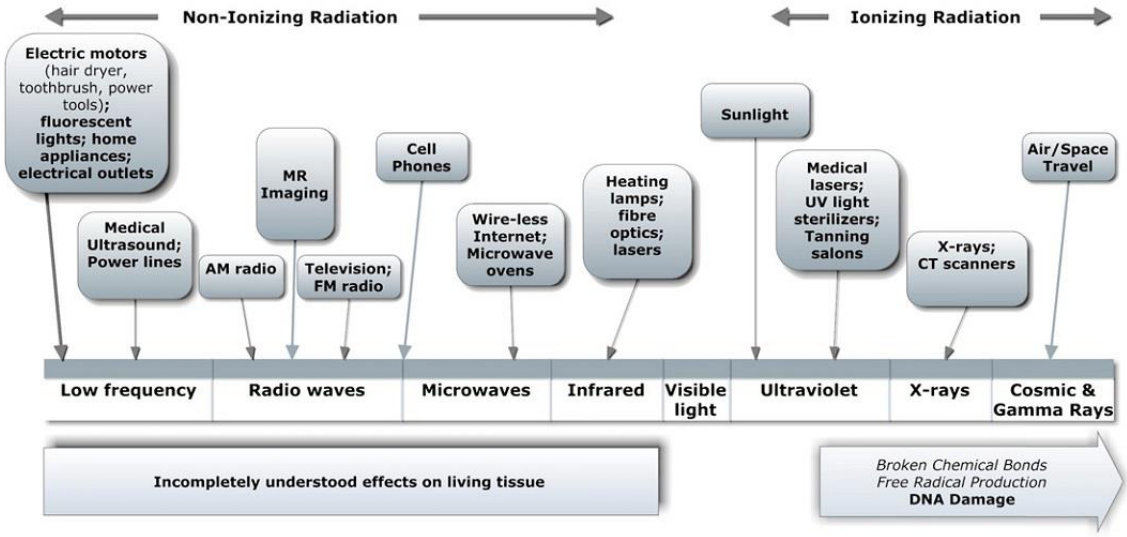

Figure 5: The electromagnetic spectrum. The different ranges of ionising and non-ionising radiation (Genius and Lipp, 2012).

Linear energy transfer (LET) describes the amount of energy transferred by IR per unit distance as it travels the path. When ionisation events are widely spaced, it is termed as low-LET and high-LET radiation deposit greater energy in a small distance. X-rays and γ-rays are low-LET radiation and high-LET radiation includes alpha particles and neutrons. The biological effects of IR depend on its LET (Joiner and van der Kogel, 2009).

3.2. Biological effects of ionising radiation

Increased toxicity and decreased cell survival are consequences of high- and low-LET radiation. Exposure to either forms of LET radiation can cause substantial DNA damage (Baeyens, 2005, Niemantsverdriet et al., 2012). DNA damage triggered by IR can occur either by direct or indirect mode of action (Figure 6). In direct action, DNA is ionised by the deposition of radiation energy introducing chemical alterations. Direct action is predominantly caused by high-LET radiation sources. The indirect action ionises water molecules to produces reactive species that damage target molecules. Indirect action primarily occurs with water to form highly reactive free radicals that damage DNA (Hall, 2012, Baeyens, 2005).

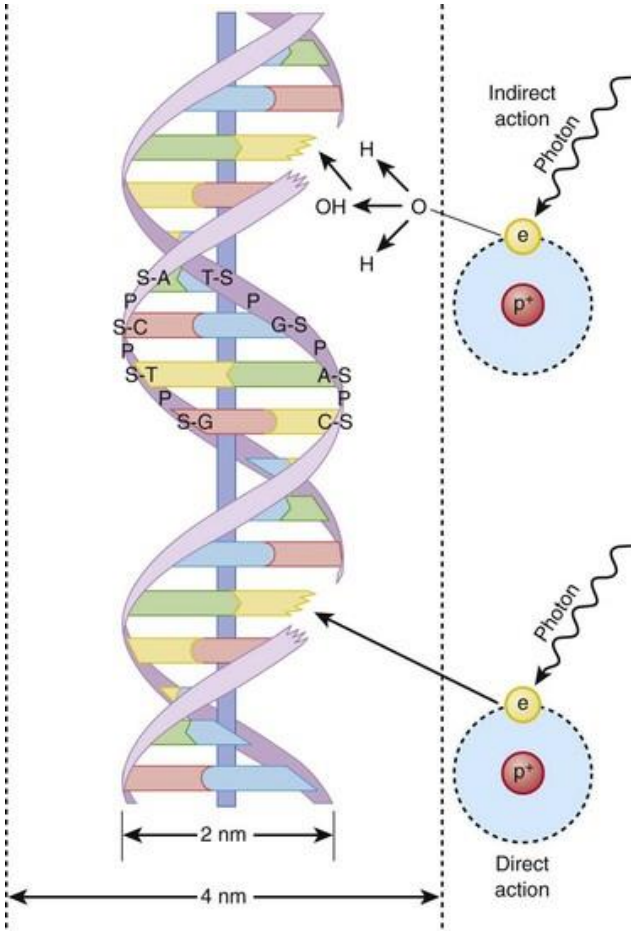

Figure 6: The direct and indirect ionisation mechanism of radiation. In the direct mode, radiation energy is deposited directly on the DNA and in indirect mode, radiation energy is absorbed by the surrounding medium to form reactive species (Frush and Slovis, 2015).

DNA contains genetic material essential for cell survival and damage to DNA is the most significant biological effect. The different types of damage inflicted are i) base damage, ii) single-strand break, iii) DSB, iv) crosslinks and v) bulky lesions (Figure 7). DSB are considered the most lethal form of radiation-induced damage to the DNA. Exposure to IR has chromosomal damaging effects which are the most important effects of radiation inducing various types of DNA damage (Jackson and Bartek, 2009).

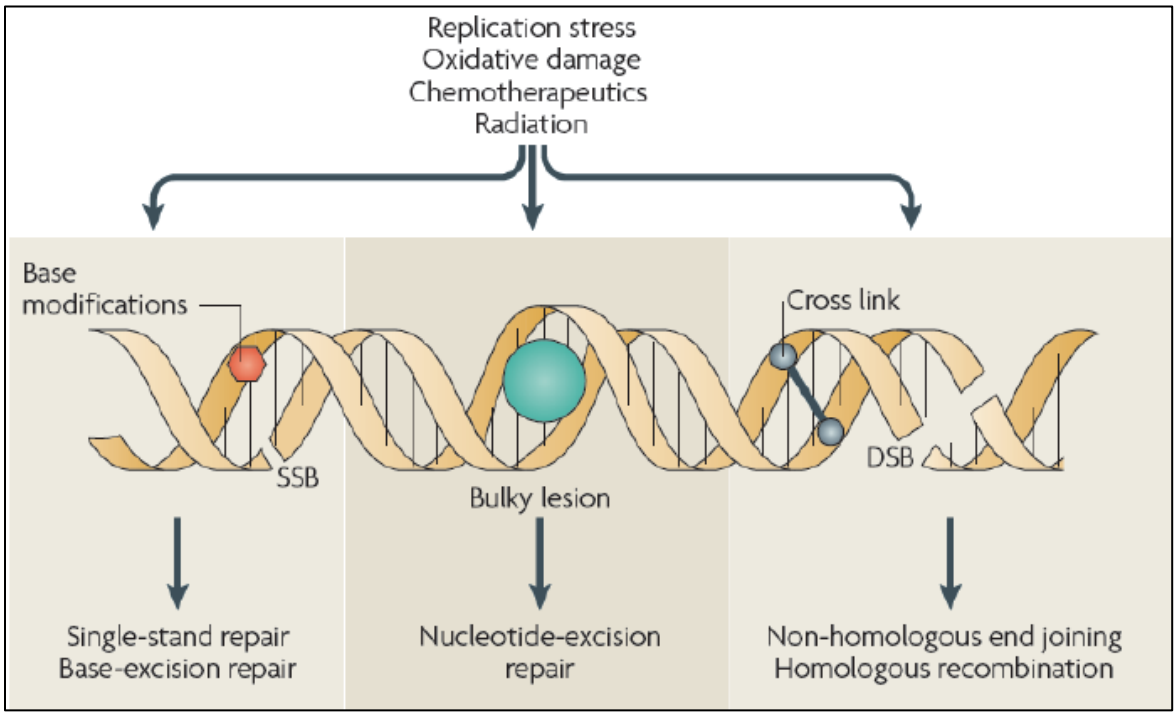

Figure 7: Types of DNA damages. The different types of DNA damage resulting from endogenous or exogenous agents (Arjunan et al., 2015).

DNA damage response (DDR) is a network of pathways that initially sense DNA damage using sensor proteins such as MRN-ATM, Ku-DNA-PKcs and ATRIP-ATR. These sensor proteins then signal DNA damage and activate three effector pathways to determine the outcome of damaged DNA. The 3 effectors pathways are DNA repair, cell cycle checkpoints and apoptosis. Cell cycle checkpoints are activated depending on the integrity on the DNA which leads to cell cycle delay. There are 3 main checkpoints of every stage of the cell cycle: G1/S (Gap 1 phase/Synthesis) checkpoint, intra-S checkpoint and the G2/M (Gap 2 phase/Mitosis) checkpoint. To prevent the introduction of mutations due to misrepair, damaged DNA is repaired during this delay in the cell cycle. However, if the induced damage is intense and cannot be

repaired, the cell undergoes apoptosis (Jackson and Bartek, 2009, Joiner and van der Kogel, 2009).

3.3. DNA repair mechanisms

The cell employs various DNA damage repair pathways namely nucleotide excision repair (NER), base excision repair (BER), mismatched repair (MMR), HR and non-homologous end joining (NHEJ). DSB are predominantly repaired by HR and NHEJ pathways in different phases of the cell cycle.

NER: Xeroderma pigmentosum (XP) is the most documented disorder linked with defective NER pathway. This pathway repairs damage through global-genome NER (GG-NER) or transcription-coupled NER (TC-NER). DNA damage is recognised by specialised proteins that have high affinity for distorted DNA. In GG-NER, DNA damage-binding protein and the XP complementation group C recognises the defect; damage recognition in the TC-NER is facilitated by Cockayne syndrome group A (CSA) and Cockayne syndrome group B (CSB) proteins. DNA duplex is then unwound and large portion of about 27 nucleotides is excised by XP complementation group G and XP complementation group F-ERCC1 endonucleases. Gapped DNA is processed by DNA polymerase using the complementary strand and ligated by DNA ligase (Hakem, 2008).

BER: The BER pathway recognises base damages and repairs through short-patch BER for single bases and long-patch BER for up to 13 bases. To initiate the repair process, DNA glycosylase enzyme removes the damaged base, DNA polymerase-β incorporates a new nucleotide and repair is completed by DNA ligase activity. An impaired BER pathway is associated with an autosomal recessive disorder known as MUTYH-associated polyposis. Biallelic mutations in MUTYH, an important gene in BER, significantly increases the risk of colorectal cancers (Hakem, 2008).

MMR: The MMR pathway is responsible for rectifying insertions, deletions and substitutional errors incurred during DNA synthesis. MSH and MLH family of proteins are important in this repair pathway. The MSH binds to the incorrectly incorporated nucleotides and information is

communicated to replicate machinery by the MLH protein. This is followed by gap-filling by the polymerase and final ligation (Jackson and Bartek, 2009). Hereditary non-polyposis colorectal cancer results from a compromised MMR pathway (Hakem, 2008).

HR: HR, known as the error-free repair, primarily repairs DNA DSB in the S and the G2 phase where a homologous DNA strand participates in the repair (Figure 8). The HR pathway is processed in 3 main steps: i) end-processing ii) the Holliday junction and iii) strand resolution. ATM/ATR mobilises repair factors to the ends of the damaged DNA resulting in the recruitment of the MRN complex - MRE11-RAD50-NBS1 - and the BRCA1/2 proteins. RAD51 protein interacts with BRCA1/2 during the initiation of strand invasion. The nuclease complex, Rad50, facilitates the resection and DNA polymerase extends the damaged strand using the complementary homologous template. The resulting interwound strands, known as the Holliday junction, are resolved into either crossover or non-crossover DNA molecules. Ligation of the two strands occurs by the action of DNA ligase I activity. Other than DSB repair, HR is also associated with interstrand crosslinks. It is implicated in breast cancer and FA (Jackson and Bartek, 2009).

SSA: Another homology-directed repair mechanism is single strand annealing (SSA). Proteins involved in SSA are RAD52, ERCC1 and Rad1/Rad10. Subsequent to end resection, DSB are repaired when sequence repeats are available. The annealing of homology strands form flaps that are clipped away and cause loss of sequence. This way SSA introduces error during repair (Kass and Jasin, 2010).

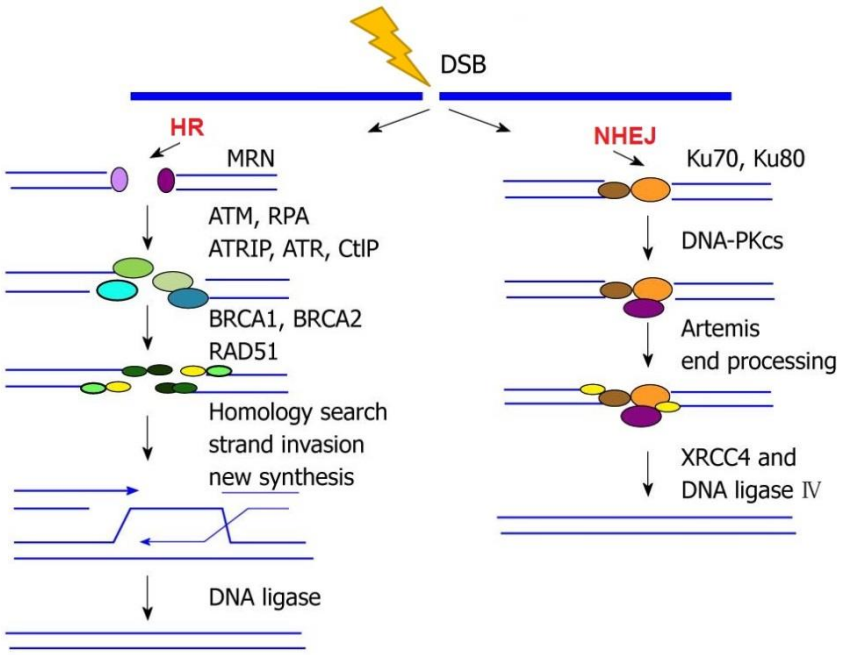

Figure 8: DNA double strand repair pathways. Homologous recombination (left) uses homologous strand to repair damage and non-homologous end joining (right) repair DNA double strand breaks using non-homologous strands (Peng and Lin, 2011).

NHEJ: NHEJ repairs DNA damage using a non-homologous template, therefore it is the preferred repair pathway in phases where there is no homologous template available and it is error-prone (Figure 8). In NHEJ repair pathway, the end recognition is achieved by the Ku70/80 heterodimer that activates the DNA-dependent protein kinase (DNA-PK) catalytic subunit. The binding of the DNA-PK and the nuclease Artemis to the DNA ends results in phosphorylation that cleaves the DNA hairpins. The XRCC4, XLF and the ligase IV are then recruited to the site to ligate the damaged ends. The MRN complex is also capable of endonuclease activity and may play a role in end processing (Helleday et al., 2007). There is evidence that defective NHEJ is associated with immunodeficiency, thymic lymphomas and hereditary autosomal LigIV syndrome. Defects in this pathway are linked with chromosomal instability and radiosensitivity (Hakem, 2008).

MMEJ: An alternate form of NHEJ known as microhomology-mediated end joining (MMEJ) uses a homologous template of 1-16 base pairs (bp) that aligns the damaged ends. This repair process occurs in the early S phase to the G2 phase when a micro homologous template is made

available. Like NHEJ, MMEJ is error-prone since deletions are introduced by the removal of bp to allow the annealing of the homologous template (Sfeir and Symington, 2015).

B-NHEJ: When the repair pathways are all compromised, the cell activates a backup or alternate pathway termed as the B-NHEJ. B-NHEJ is activated when components of the classical NHEJ have defects and cannot be utilised. Despite slower repair kinetics and more prone to error, majority of breaks are repaired. B-NHEJ is less efficient than classical NHEJ. B-NHEJ is advantageous as it functions throughout the cell cycle when needed with increased functionality observed in the G2 phase (Dueva and Iliakis, 2013, Mladenov and Iliakis, 2011).

4. Biomarkers for chromosomal radiosensitivity

Various assays can predict chromosomal radiosensitivity and monitor exposure of IR by evaluating DNA damage. The dicentric assay is the golden standard for biological dosimetry. The cytokinesis-block micronucleus (CBMN) assay is widely used for chromosomal radiosensitivity studies.

4.1. Dicentric assay

Dicentrics are chromosomes with two centromeres that result from misrepair of DNA damage by IR exposure (Figure 9). The number of dicentric chromosomes is directly proportional to the dose of IR. The assay is easily reproducible with low levels of background, sensitive for low doses and quantifies the exposure making it the golden standard. Previously, the assay was known to be laborious and analysing chromosome aberrations is strenuous, however, the assay has recently been automated (Agrawala et al., 2010).

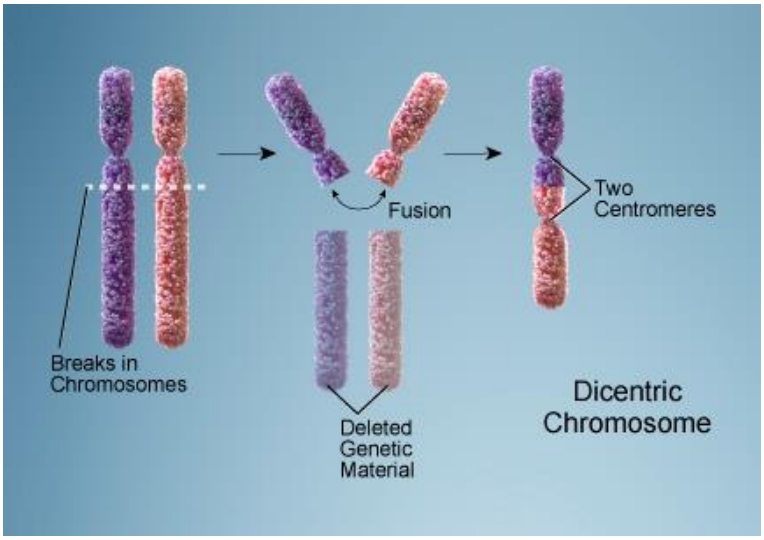

Figure 9: Dicentric chromosome. Abnormal chromosomes with two centromeres resulting from misrepair or abnormal fusion (Medicine, 2017).

4.2. Cytokinesis-block micronucleus assay

Chromosomal radiosensitivity is evaluated by analysing micronuclei (MNi) in cells (Figure 10). MNi are small fragments of DNA that lag behind following a complete nuclear division and serve as biomarkers for radiation induced DNA damage and radiosensitivity. MN contain either whole chromosomes that did not travel to opposite ends of the poles during division or acentric fragments where the centromere is absent (Fenech, 2000). Factors such as HIV, smoking and age influence the formation of MN (Ban et al., 1993, Hallberg et al., 1997, Baeyens et al., 2010).

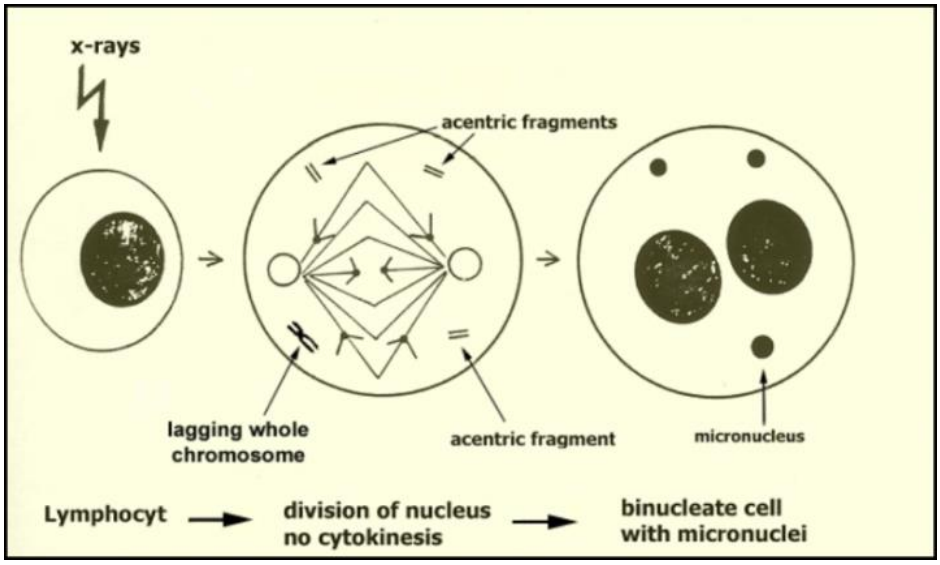

Figure 10: The cytokinesis-block micronucleus assay. Binucleate cells result from lymphocytes that complete one nuclear division after exposure and micronuclei are biomarkers for radiation-induced damage (Baeyens, 2005).

The CBMN assay, commonly also known as the MN assay, can be conducted on peripheral blood lymphocytes (PBL) to evaluate chromosomal radiosensitivity in the G0 (Gap 0) phase. PBLs are easy to obtain by venepuncture and used extensively to detect and analyse radiation-induced damage. For radiosensitivity testing, cells are exposed to IR, lymphocytes are stimulated to cell growth and cytoplasmic division is blocked by the addition of Cytochalasin B (Cyto B). This allows distinguishing of the binucleate cells (BN), cells that have been divided once after exposure, and MN. The BN cells are scored using the automated microscopic system, Metafer (MetaSystems). This is a high throughput scanning system that is capable of rapid scanning of cells at high magnification. This has improved the CBMN assay by decreasing the total scoring time and increasing reproducibility. With the use of fluorescent *in situ* hydridisation (FISH) utilising pan-centromeric probes, the CBMN assay can be made sensitive to differentiating between spontaneous MN and radiation-induced chromosome fragments. This FISH-based MN assay can also be used in biological dosimetry to detect low dose DNA damages.

A modified version of the CBMN assay allows the detection of chromosomal radiosensitivity in the S/G2 phase of the cell cycle. The distinguishable difference in the S/G2 MN assay are i) the lymphocytes cultures are first stimulated to growth, ii) cultures are only irradiated 72 hours after

incubation, iii) cytokinesis is blocked immediately after irradiation and iv) cells are harvested 8 hours post irradiation. This allows the detection of DNA damage prompted by IR in the S/G2 phase. The G/M checkpoint efficiency can also be evaluated by addition of a radiosensitiser such as caffeine. Caffeine abrogates the G2/M checkpoint and permits the progression of damaged cells into mitosis (Pantelias and Terzoudi, 2011). Additionally, the assay is valuable in identifying *BRCA* mutation carriers and could be implemented in a routine diagnostic setting.

5. Biomarkers for DNA damage repair

5.1. γ-H2AX foci assay

A nucleosome complex consists of histone octamers that wrap the DNA. These histone octamers consist of separate histone proteins named H2A, H2B, H3 and H4. H2AX, a variant of H2A protein family, is phosphorylated by ATM/ATR in response to DSB. The phosphorylated H2AX is known as γ-H2AX. The formation of γ-H2AX foci increases as IR exposure increases but disappears as a result of DNA repair. Labelled antibodies to the H2AX foci represent DNA damage and can be visualised microscopically to quantify DNA damage (Figure 11). Therefore the assay is a sensitive biological dosimetry assay (Kuo and Yang, 2008).

With the development in research and imaging, an automated platform to analyse γ-H2AX foci is available through the MetaSystems. Clusters of foci images are captured by the detection of the fluorescent signals from the antibodies. γ-H2AX foci assay can also be performed with a drop of blood (Heylmann and Kaina, 2016).

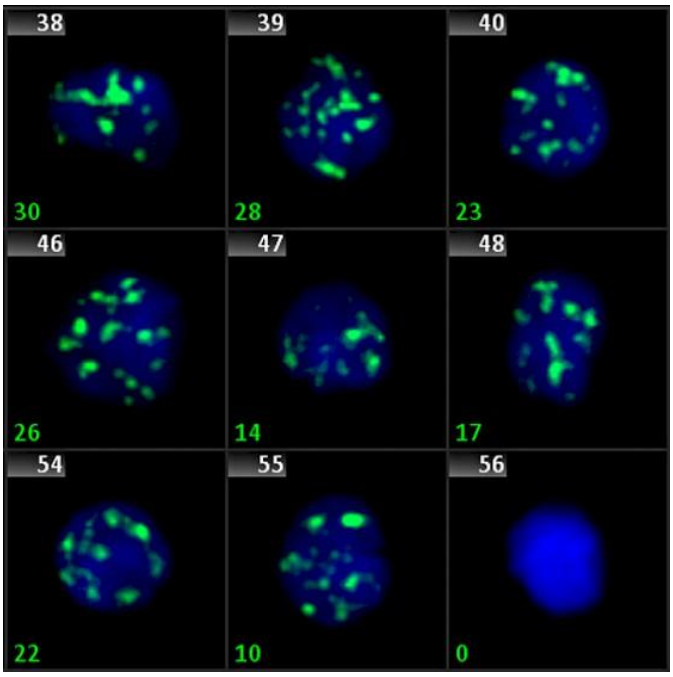

Figure 11: Lymphocytes with γ-H2AX foci. γ-H2AX foci clusters are indicated by green spots. The absence of green spots indicates foci formation (bottom right corner) (Metasystems, 2017).

5.2. RAD51 foci assay

RAD51 is an important protein in HR repair. By interacting with both BRCA1/2, RAD51 is recruited to the damage sites to facilitate in DNA repair by exchanging strands between DNA. In the absence of RAD51 proteins, radiation treatment to mice embryonic cells has caused cell death, highlighting the importance of RAD51 in DNA DSB repair. Exposing cells to IR causes damage and activates RAD51 to accumulate at the damage site. This process can be visualised microscopically following immunofluorescent staining techniques. This allows the quantification of RAD51 foci and examines defects in the HR repair pathway (Figure 12). Defective HR pathway will exhibit decreased accumulation of RAD51 foci. During efficient HR repair, the accumulation of RAD51 foci is larger (Yuan et al., 1999, Shah et al., 2014).

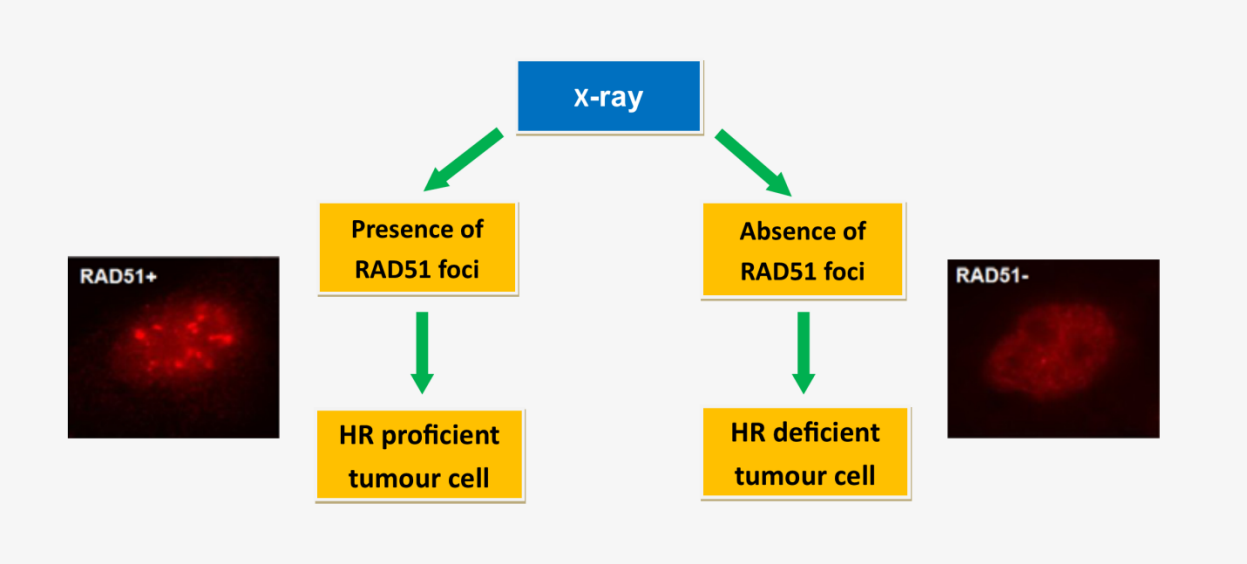

Figure 12: RAD51 foci assay. Following irradiation of cells, the red spots (left block) indicates presence of RAD51 foci. Right block shows no accumulation of RAD51 foci (Vrieling, 2016).

Radiation-induced RAD51 foci formation is suppressed in BRCA2-mutated cells. BRCA1-mutated cells did not show radiation-induced RAD51 foci repression. These results suggest the importance of BRCA2 in RAD51 foci formation (Yuan et al., 1999).

6. Clinical and chromosomal radiosensitivity

Sensitivity of cells to the effects of radiation is known as radiosensitivity. This may result in chromosomal aberrations leading to chromosomal instability. Therefore DNA damage response is highly crucial and extremely critical in maintaining genomic stability (Khanna and Jackson, 2001). Radiosensitivity of cells is dependent on the phase of the cell cycle (Pawlik and Keyomarsi, 2004) and it is also dependent on cell type. Radiosensitivity of different cell types is dependent on factors such as dividing status, cell differentiation and mitotic activity (Washington and Leaver, 2015).

Some patients undergoing radiotherapy display increased clinical radiosensitivity by showing adverse normal tissues side-effects. The first indication for a possible inherited basis for such clinical radiosensitivity came from patients with rare genetics syndromes such as Ataxia Telangiectasia (AT) and Nijmegen breakage syndrome (NBS) (Jeggo and Lavin, 2009). These

patients were shown to display not only clinical, but also *in vitro* chromosomal radiosensitivity (Huo et al., 1994). Patients with these syndromes have germline mutations in genes involved in DNA damage repair. Patients with AT and NBS also display predisposition to many cancers. Their increased chromosomal radiosensitivity led to studies that showed an enhanced *in vitro* chromosomal radiosensitivity in patients with different cancers, such as head and neck, colorectal, prostate, breast and lung cancer (Parshad et al., 1983, Riches et al., 2001, Baeyens et al., 2002a).

6.1. Chromosomal radiosensitivity in breast cancer

European studies have indicated elevated chromosomal radiosensitivity in familial and sporadic breast cancer. Radiation-induced MNi in the G0 cells and chromatid breaks in the G2-phase cells were observed in increased frequencies in breast cancer populations (Baeyens et al., 2002b, Riches et al., 2001, Terzoudi et al., 2000). European breast cancer patients' exhibit elevated sensitivity to chromosomal damage induced by IR (Baria et al., 2001, Poggioli et al., 2010, Ryabchenko et al., 2012, Auer et al., 2014, Varga et al., 2007). Outside of Europe, *in vitro* chromosomal radiosensitivity of breast cancer patients in other populations has also been investigated by evaluating damage in the G2 phase. The non-Hispanic white and Mexican American patients' exhibit enhanced chromosomal radiosensitivity. The results in African American population have been conflicting (Wang et al., 2012, Natarajan et al., 2006). The differences observed in chromosomal radiosensitivity of breast cancer patients with distinct ancestral backgrounds could mean that ethnicity is a potential factor associated with the effects of radiation.

Increased chromosomal radiosensitivity can be correlated to various factors including age and defects in DNA repair proteins such as *BRCA1*/*2* mutations (Auer et al., 2014, Becker et al., 2012). Defects in BRCA contribute to impaired DNA repair and make the cells susceptible to the damaging effects of IR. The association between increased chromosomal radiosensitivity and *BRCA* mutations as a confounding factor was a contradictory subject (Ernestos et al., 2010, Baeyens et al., 2004). The *BRCA* genes are key role players in maintaining genomic stability by interacting with various proteins involved in DNA damage repair. Efficient repair, particularly in

the G2 phase, following exposure to IR ensures optimal DNA damage repair and, additionally, BRCA functions in checkpoint efficiency. The importance of BRCA in chromosomal radiosensitivity has been confirmed in *BRCA1*/*2* mutation carries in the absence of breast cancer compared to non-*BRCA1*/*2* carriers (Baert et al., 2016, Baert et al., 2017, Barwell et al., 2007). Various factors effecting chromosomal radiosensitivity in breast cancer can be studied *in vitro* by mimicking biological responses to IR in breast cancer cell lines (Sprung et al., 2005, Zhu et al., 2015).

Normal tissue effects in breast cancer patients undergoing radiotherapy have been documented. By altering the radiation dose, the normal cellular side effects can be limited. Efficient doses of IR are, however, required to kill malignant cells. Chromosomally radiosensitive breast cancer patients may react severely to IR and display increased normal tissue side effects. By assessing individual chromosomal radiosensitivity of breast cancer patients, the radiotherapy treatments can be tailored to suit individual patients (Huber et al., 2011, Barber et al., 2000). Chromosomally radiosensitive patients are also at high risk in developing secondary cancers. It was reported that 8% of all cancers are radiation-induced secondary malignancies (Habash et al., 2017). Assessing genetic predisposition, particularly in the DNA repair proteins, in these patients is crucial. Acute side effects of radiotherapy are associated with genetic alterations (Andreassen and Alsner, 2009). Therefore, chromosomal radiosensitivity studies are extremely important for patients that require radiotherapy. Despite the breast cancer burden in South Africa, chromosomal radiosensitivity has never been evaluated before.

6.2. Chromosomal radiosensitivity in Fanconi anaemia

FA patients undergoing radiotherapy display increased clinical radiosensitivity by exhibiting adverse normal tissues side-effects (Birkeland et al., 2011, Alter, 2002b). Evidence suggests that FA patients are chromosomally radiosensitive to IR (Higurashi and Conen, 1973, Heddle et al., 1978, Higurashi and Conen, 1971). FA patients exhibit significantly higher DNA damage indicating chromosomal instability (Camelo et al., 2008). Previous studies have shown that FA patients exhibit sensitivity to the damaging effects of IR. Despite available evidence, literature data on chromosomal radiosensitivity of FA patients is very limited.

The FA/BRCA pathway is involved in DNA DSB repair by HR and in the processing of DNA damage induced by MMC. MMC sensitivity in FA patients is widely described (Cohen et al., 1982, Cervenka and Hirsch, 1983, Mozdarani et al., 2011, Talmoudi et al., 2013). In the presence of hematopoietic somatic mosaicism, the diagnosis of FA patients with MMC can be a challenge (Pinto et al., 2009). There is an urgent need for an efficient diagnostic test for FA patients who have deficiencies in FA genes that important for DNA repair.

CHAPTER 2
Research aims and objectives

AIM

The general aim of this study was to evaluate the chromosomal instability and radiosensitivity of South African breast cancer, with focus on the triple negative patients, and FA patients. Patients harbouring mutations in DNA repair genes are expected to be chromosomally instable. TNBC patients frequently have defects in DNA repair pathways. Radiosensitivity information is particularly important in South Africa as radiotherapy is often the only treatment possible for late diagnosed inoperable breast cancers. FA is also characterised by DNA repair defects and associated with chromosomal instability. Chromosomal instability and radiosensitivity in these population groups was investigated using the G0 and S/G2 MN assay. The link between radiosensitivity and DNA repair genes in these population groups was also explored by genetic screening.

OBJECTIVES

The study will be divided as follows:

Primary objectives:

1. Investigate chromosomal instability and radiosensitivity of a selected cohort of triple negative and luminal breast cancer patients in South Africa compared to healthy controls using the G0 and S/G2 MN assay. Collect clinical and social data of patients to investigate if there is an influence of clinical parameters on chromosomal radiosensitivity. Investigate the influence of age and ethnicity on chromosomal radiosensitivity.

2. Investigate chromosomal instability and radiosensitivity of FA patients and parents using the G0 and S/G2 MN assay. Analyse correlations between *FANC* mutations in FA patients with results of the MN assays.

3. Analyse the suitability and sensitivity of the G0 and S/G2 MN assay for the identification of radiosensitive breast cancer and FA patients in South Africa.

4. Optimise a novel chromosomal breakage test using MMC for diagnosis of FA patients. Compare MMC-induced DNA damage in FA patients, parents and controls using the

optimised MMC MN assay. Analyse correlations between *FANC* mutations and results of MMC MN assay.

Secondary objectives:

1. Investigate the mechanisms underlying chromosomal radiosensitivity of breast cancer patients in South Africa by scrutinising mutations in DNA repair genes (*BRCA1*, *BRCA2*, *PALB2*, *CHEK2*) using next generation sequencing (NGS) complemented with multiplex ligation-dependent probe amplification (MLPA).

2. Analyse correlation between the mutation analysis results of breast cancer patients with the MN assays results.

3. Optimise a *FANC* gene panel for mutation analysis for 20 *FANC* genes using NGS complemented with MLPA.

CHAPTER 3

Chromosomal radiosensitivity of lymphocytes in South African breast cancer patients of different ethnicity: An indirect measure of cancer susceptibility

Chromosomal radiosensitivity of lymphocytes in South African breast cancer patients of different ethnicity: An indirect measure of cancer susceptibility

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Background. Breast cancer is the leading cancer among South African (SA) women. SA has citizens from diverse ethnic groups, and the lifetime risk of breast cancer differs according to ethnicity. Candidate genes for increased breast cancer risk are those involved in DNA damage repair pathways, and mutations in these genes are characterised by increased chromosomal radiosensitivity. Several European studies have shown that breast cancer patients are more sensitive to ionising radiation than healthy individuals.
Objectives. To investigate the *in vitro* chromosomal radiosensitivity of SA women with breast cancer and the possible influence of ethnicity and clinical parameters on chromosomal radiosensitivity.
Methods. Chromosomal radiosensitivity was analysed with the micronucleus assay using lymphocytes of breast cancer patients and healthy individuals of different ethnic groups. Lymphocytes were irradiated *in vitro* with 2 Gy or 4 Gy, and micronuclei (MN) were scored 70 hours after irradiation. These MN frequencies were correlated with the ethnicity and clinical parameters of the breast cancer patients.
Results. MN values were higher in breast cancer patients than in healthy controls. This was noted for black and white breast cancer patients at the different radiation doses. No correlations could be demonstrated between MN values and clinical parameters of the breast cancer, except that MN values were significantly higher in oestrogen receptor (ER)-positive breast cancers.
Conclusion. SA breast cancer patients have elevated chromosomal radiosensitivity compared with healthy controls. ER positivity also influences chromosomal radiosensitivity.

S Afr Med J 2015;105(8):675-678. DOI:10.7196/SAMJnew.8266

According to South Africa (SA)'s most recent cancer registry, breast cancer is the leading cancer among SA women, with a lifetime risk of 1 in 34.[1] SA is a country with citizens of diverse ethnicity: black African (80.2%), white Caucasian (8.5%), mixed/coloured (8.9%) and Indian/Asian (2.4%).[2] The lifetime risk of breast cancer differs according to ethnicity: 1/52 in black women, 1/22 in coloured women, 1/19 in Indian women and 1/18 in white women.[1] While the incidence is lowest among black women, it is rising as a result of increased life expectancy and urbanisation, which leads to lifestyle changes that elevate exposure to known risk factors for breast cancer such as dietary changes, decreased exercise, delayed and decreased parity, and reduction in breastfeeding.[3] Although there is a lower incidence of breast cancer in SA in comparison with developed countries, the mortality rate of existing breast cancer patients is higher owing to limited access to diagnostic centres, particularly in rural areas, lack of awareness, low standards of healthcare facilities and limited screening.[4]

Familial breast cancer caused by mutations in high-penetrance genes such as *BRCA 1* and *BRCA 2* accounts for only 5% of all breast cancers. The majority of breast cancers are sporadic and are due to mutations in a number of low-penetrance genes. Candidate genes for breast cancer risk include those involved in DNA damage repair pathways. Mutations in genes regulating these pathways are characterised by increased chromosomal radiosensitivity.[5] Measurement of chromosomal radiosensitivity has been used as an indirect measure of cancer susceptibility. The association between chromosomal radiosensitivity and cancer risk is supported by the following facts: cancer-prone disorders such as ataxia telangiectasia present with high chromosomal radiosensitivity; elevated chromosomal radiosensitivity is an indicator of defects in DNA repair that could lead to the chromosomal instability often observed in cancer; and chromosomal radiosensitivity is linked with early events in carcinogenesis.[6]

Several studies (reviewed in Cardinale *et al.*[7]) on European, Asian and American populations have shown breast cancer patients to have elevated chromosomal radiosensitivity compared with healthy individuals. Studies on the chromosomal radiosensitivity of breast cancer patients have never been performed in SA.

Chromosomal radiosensitivity can be measured using the cytokinesis-block micronucleus assay. 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. MN can contain whole chromosomes (mis-segregated during mitosis) or acentric fragments, which are usually the result of misrepaired or unrepaired DNA double-strand breaks. MN are counted in cells that have undergone a single division; however, cytokinesis is blocked by adding cytochalasin B, which results in binucleated (BN) cells. This assay is well established, robust and can be performed on lymphocytes, which are easily obtained through venepuncture. The automation of MN scoring with the Metafer 4 platform (MetaSystems, Germany) has minimised the variability of the assay and rendered it rapid and less subjective.

Objective

To measure the chromosomal radiosensitivity of SA breast cancer patients in a case-control study design with the micronucleus assay and the Metafer 4 scoring system. Recent studies have shown how tumour characteristics of breast cancer can differ among different ethnic groups.[8,9] The differences between the ethnic groups point to differences in the underlying biology of the disease and led to the idea of comparing chromosomal radiosensitivity in different ethnic groups. We also assessed whether there was an influence of clinical parameters on chromosomal radiosensitivity.

Materials and methods

Subjects

Blood samples were collected from 68 breast cancer patients (mean age (standard deviation (SD)) 52 (12)) recruited from Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), a public hospital in Johannesburg, Gauteng Province, South Africa, and Donald Gordon Medical Centre, a private hospital in Johannesburg. We included 30 black breast cancer patients (mean age 47 (12) years), 25 white breast cancer patients (mean age 59 (11) years), 7 Indian breast cancer patients (mean age 45 (8) years) and 6 coloured breast cancer patients (mean age 49 (8) years). Exclusion criteria included prior chemo- and/or radiotherapy. Clinical and biographical information on the patients was obtained through questionnaires and hospital files. All patients were categorised by race (black, white, Indian, coloured) based on patients' self-reported data from the questionnaires. Most of the breast cancer patients (80.0%) had invasive ductal carcinomas, of which 31.1% were stage 0 - I, 53.3% stage II and 15.6% stage III. Overall 74.1% were oestrogen receptor (ER)-positive, 63.8% were progesterone receptor (PG)-positive and 78.6% were human epidermal growth factor receptor 2 (HER2)-negative. No participant was HIV-positive.

Blood samples from 70 healthy controls (mean age (SD) 35 (12) years), including 20 black women (mean age 36 (15) years), 35 white women (mean age 36 (10) years), 8 Indian women (mean age 32 (6) years) and 7 coloured women (mean age 31 (13) years), were also collected. The healthy donors were staff members and students from CMJAH, where the study was undertaken. All donors signed informed consent. Ethical approval for the study was obtained through the Human Research Ethics Committee, University of the Witwatersrand, Johannesburg (M110248).

Irradiations and micronucleus assay

The protocol for the micronucleus assay described by Herd *et al.*[10] was used in this study. In brief, 0.5 mL of heparinised blood was added to 4.5 mL of RPMI 1640 (Bio-Whittaker, USA) supplemented with 13% fetal bovine serum (Gibco-Invitrogen, USA), and antibiotics (50 U/mL penicillin and 50 mg/mL streptomycin; Gibco-Invitrogen, USA). The medium was pre-warmed to 37°C and gassed (5% CO_2/95% air). Culture flasks with blood and medium were irradiated with doses of 2 Gy or 4 Gy of X-rays using a 6 MV photon beam from a medical linear accelerator (Siemens Healthcare, Germany). A 0 Gy dose was used as a sham-irradiated control. For each dose point, two cultures were set up. Immediately after irradiation the lymphocytes were stimulated with 100 µL phytohaemagglutinin (stock solution 1 mg/mL; Sigma-Aldrich, USA), and 23 hours later 20 µL cytochalasin B (stock solution of 1.5 mg/mL; Sigma-Aldrich) was added to block cytokinesis. Cells were harvested at 70 hours after stimulation using a cold (4°C) hypotonic shock with 7 mL 0.075M KCl (Merck, Germany). This was followed by fixation in methanol:acetic acid:Ringer (0.9% NaCl) solution (4:1:5) (Merck). Fixed cell suspensions were dropped on coded slides and stored at 4°C. Slides were mounted with Vectashield containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories, USA) before being scanned automatically with the Metafer 4 system. The classifier and scoring method was based on Herd *et al.*[10] Each dose point was scored by at least two scorers. All results were normalised to an MN frequency in 1 000 BN

Table 1. Spontaneous and radiation-induced MN values in breast cancer patients and healthy controls, according to ethnicity

Group	Patients	Controls	*p*-values
Black			
n	30	20	
MN/1 000 BN cells, mean (SD)			
0 Gy	14 (8)*	10 (4)	0.0087
2 Gy	179 (30)*	159 (31)	0.0273
4 Gy	498 (91)*	449 (66)	0.0324
White			
n	25	35	
MN/1 000 BN cells, mean (SD)			
0 Gy	16 (7)*	11 (4)	0.0083
2 Gy	172 (24)*	158 (23)	0.0253
4 Gy	507 (79)*	443 (35)	0.0006
Coloured			
n	6	7	
MN/1 000 BN cells, mean (SD)			
0 Gy	12 (9)	12 (7)	0.6043
2 Gy	193 (49)	169 (20)	0.4697
4 Gy	487 (103)	444 (51)	0.5281
Indian			
n	7	8	
MN/1 000 BN cells, mean (SD)			
0 Gy	11 (6)	11 (5)	0.8427
2 Gy	172 (28)	177 (34)	0.8000
4 Gy	467 (91)	470 (74)	0.9305
All groups			
n	68	70	
MN/1 000 BN cells, mean (SD)			
0 Gy	14 (7)*	11 (5)	0.0012
2 Gy	177 (30)*	161 (27)	0.0014
4 Gy	497 (86)*	448 (52)	0.0001

*Significantly different from controls.

cells. Radiation-induced MN values were obtained by subtracting baseline (0 Gy dose) values from those obtained in irradiated samples.

Statistical analysis was performed with Graphpad Prism 6. Differences between means of MN yields of patients and controls in black and white populations were tested for significance with the unpaired Student's *t*-test. For comparison of differences in MN values in the Indian and coloured subgroups, and between ER receptor subgroups, the Mann-Whitney *U*-test was applied. This test is used for small sample sizes. To analyse the correlations between age, clinical parameters and MN values, we used Pearson's correlation coefficient. The confidence level of the statistical tests was 95%, and statistical significance was set at $p<0.05$.

Results

The results obtained with the micronucleus assay on the samples of 68 breast cancer patients and 70 healthy individuals are presented in Table 1. The mean spontaneous MN yields of all the breast cancer patients were significantly higher than those of the healthy controls ($p<0.005$). The spontaneous MN yields were significantly correlated with the age of the healthy individuals, but this correlation could not be observed in the breast cancer patient group ($p<0.005$). To investigate whether ethnicity had an influence on chromosomal radiosensitivity, we split the breast cancer patients and the healthy controls into four subgroups (black, white, coloured and Indian). When the patients and controls were grouped according to their ethnicity, the significantly higher number of spontaneous MN was only seen in the black and white patients compared with the healthy individuals of the same ethnicity.

The radiation-induced MN yield was calculated by subtracting the spontaneous yield from the yield in the irradiated cells. For the whole group of breast cancer patients, the mean MN yields were significantly higher than in the whole group of healthy individuals for both 2 Gy and 4 Gy irradiations. Grouping the samples according to their ethnicity revealed significantly higher radiation-induced MN values in the black and white breast cancer patients for both 2 Gy and 4 Gy. This could not be observed in the coloured and Indian subgroups (Table 1). Histograms of radiation-induced MN after 4 Gy for the four ethnic subgroups are presented in Fig. 1. The MN distribution after 2 Gy showed similar patterns for the four subgroups (data not shown). Although no significant differences

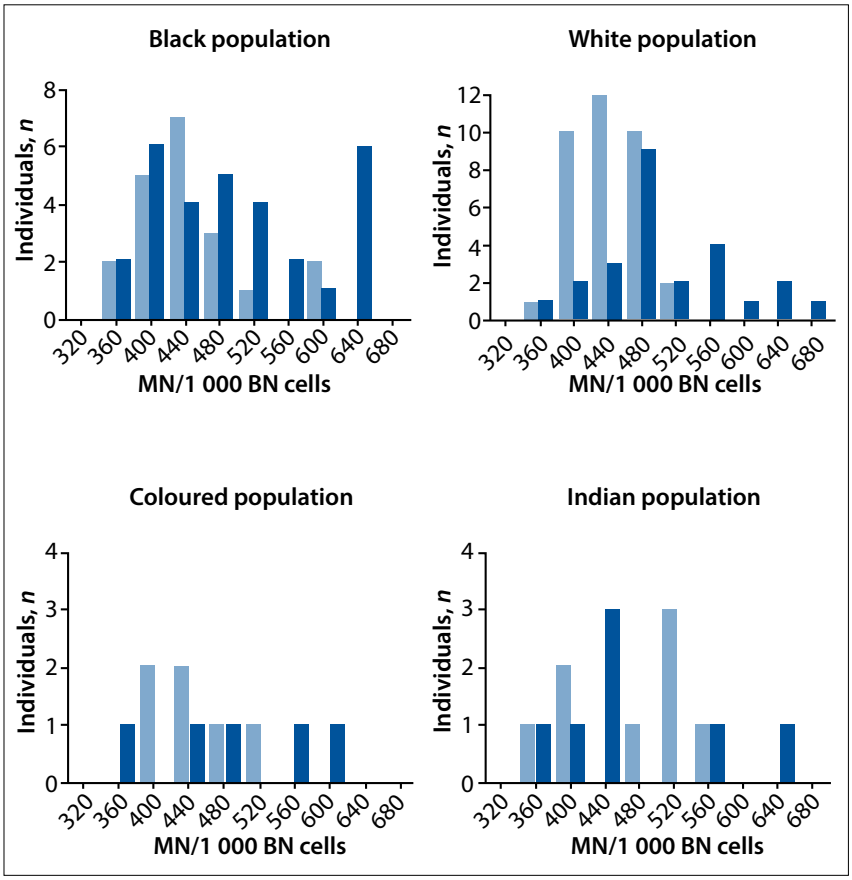

Fig. 1. Distribution of MN yields after 4 Gy of breast cancer patients and healthy individuals. (Dark blue bars = breast cancer patients; light blue bars = healthy individuals.)

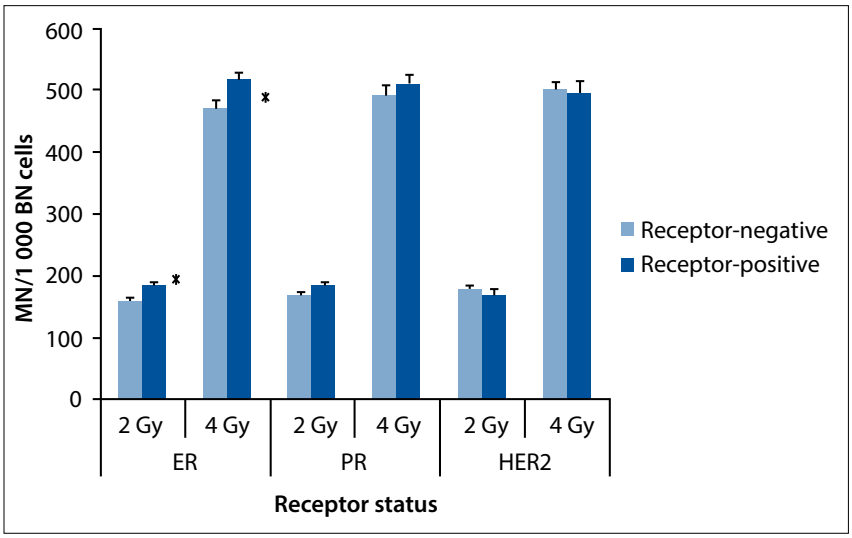

*Fig. 2. Radiation-induced MN values of breast cancer patients grouped according to receptor status. (*Significantly different from the receptor-negative group.)*

in mean MN values could be seen in the coloured group, there was a shift of values towards the higher range. This shift was not noted in the Indian population.

All the breast cancer patients were also split into groups according to clinical parameters, and MN values in these groups were compared. No significant correlation could be found between clinical parameters (tumour histological type, size and staging) and MN yields of the breast cancer patients. There was an effect of ER positivity on the MN yields (Fig. 2). Breast cancers positive for ER receptors had significantly higher radiation-induced MN values than ER-negative breast cancers for both 2 Gy and 4 Gy (ER-positive

185 MN/1 000 BN cells (2 Gy), 518 MN/1 000 BN cells (4 Gy); ER-negative 159 MN/1 000 BN cells (2 Gy), 468 MN/1 000 BN cells (4 Gy)) (p=0.0031 and p=0.044, respectively).

Discussion

This study investigated whether SA breast cancer patients are more sensitive than healthy individuals to DNA damage caused by ionising radiation. Since differences in breast tumour characteristics are noted between ethnic populations, we also evaluated the possible differences of chromosomal radiosensitivity in the four SA ethnic groups.

Higher spontaneous mean MN frequencies, which were seen in breast cancer patients in this study, have been linked with higher levels of genetic instability. These significantly higher mean MN values occurred mainly in the white and black patients, who had significantly higher MN values than controls of the same ethnicity. The higher ages of the cancer patients, which is a limitation in our study, could have played a role in the elevated spontaneous MN values; Thierens *et al.*[11] have suggested an increase of 0.58 MN/year. The higher levels of spontaneous MN in the breast cancer patients could also suggest higher chromosomal instability, which is associated with an increased risk of cancer.

The higher chromosomal radiosensitivity observed in the whole group of SA breast cancer patients and in the white patients in this study is in agreement with several international studies. It is interesting that the significantly higher MN values were also seen in black breast cancer patients, who have never been studied for chromosomal radiosensitivity with the micronucleus assay. This trend is in contrast with a study performed by Wang *et al.*,[12] who looked at chromatid breaks in young breast cancer patients and noted significantly higher chromatid breaks in white American breast cancer patients than white controls, but not in African-American breast cancer patients compared with black controls.

The MN values of the coloured patients and controls were not significantly different, although higher MN values were observed in the patients. However, the small sample size of this subgroup limits conclusions. The other small subgroups were the Indian patients and controls, between whom no differences in mean MN values were found.

Black African breast cancer patients are known to have more aggressive tumour phenotypes than white women, and a higher prevalence of triple-negative and premenopausal breast cancers.[8,9] These differences were not reflected in differences in chromosomal radiosensitivity of white and black breast cancer patients in our study. We found no correlation between the MN values and most of the clinical parameters investigated, which is in agreement with Baeyens *et al.*[13] There was an effect of ER status on MN values, with ER-positive women having significantly higher MN values. A similar trend was observed in the study of Riches *et al.*,[14] where patients with increased G2 radiosensitivity had a higher proportion of ER-positive tumours. The underlying reason for the higher radiation-induced MN in this type of breast cancer is unknown, but it could be based on interactions between the double-strand break repair kinase DNA-PK and ERs.[15] The link between ERs and MN could suggest a prognostic value of the micronucleus assay for ER-positive breast cancers. Black and coloured women with breast cancer have a higher incidence of ER-negative cancers,[8,9] which could have led to lower MN values in these groups. However, we did not observe this in our study. Enlarging the sample sizes and subgrouping the cancer patients into ethnic groups and into ER-positive and negative patients could provide greater insight.

Conclusion

Our results showed that SA breast cancer patients have elevated chromosomal radiosensitivity compared with healthy controls. The presence of ER positivity also influenced this radiosensitivity. More rigorous extended studies on the different ethnic groups are needed to validate our findings and to unravel the underlying mechanisms.

Acknowledgments. This research was supported by a grant of the South African Medical Research Council, by a 'VLIR Own Initiative Programme' between Belgium and SA (ZEIN2011PR387), and by a grant under the Nuclear Technologies in Medicine and the Biosciences Inititiative (NTeMBI), a national technology platform developed and managed by the SA Nuclear Energy Corporation and supported by the Department of Science and Technology, SA. The authors thank all the patients and healthy individuals who participated in this study.

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Accepted 1 April 2015.

CHAPTER 4

The influence of blood storage time and general anaesthesia on chromosomal radiosensitivity assessment

Mutagenesis, 2015, 00, 1–6
doi:10.1093/mutage/gev072
Original Manuscript
Advance Access Publication Date: xx xxxx xxxx

OXFORD

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The influence of blood storage time and general anaesthesia on chromosomal radiosensitivity assessment

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Received 15 April 2015; Revised 7 August 2015; Accepted 11 September 2015.

Abstract

The micronucleus assay (MN assay) is a well-established assay in genetic toxicology, biomonitoring of mutagen-exposed populations and chromosomal radiosensitivity testing. To evaluate the effect of storage time on the chromosomal radiosensitivity assessment in lymphocytes, micronuclei (MN) yields in blood samples received and processed on the same day were compared with MN yields obtained when blood cultures were set up 24 and 48 h after blood sampling. Furthermore, the influence of general anaesthesia on MN and binucleated cells (BN) yields in the MN assay was considered. Blood samples of 10 healthy donors were irradiated and blood cultures were set up during the same day of blood sampling or with a delay of 24 or 48 h. The MN assay was also performed on two blood samples from 60 women undergoing breast surgery. The first blood sample was taken before general anaesthesia and the second sample, 2 h after anaesthesia induction. Fifty percent of the blood samples were transported to the cytogenetics lab within 2 h while the other 50% reached the lab after 24 h. The results of this study show a decrease in BN and an increase in MN yields with increasing storage time before irradiation and setting up of the MN assay for both healthy controls and patients. The administration of general anaesthesia in patients resulted in lower BN yields, higher spontaneous MN yields but no differences in radiation-induced MN yields. In conclusion, this study indicates that the time between blood sampling and the *in vitro* irradiation of the samples for the MN assay influences the MN yields. Delays of more than 24 h should be avoided. To assess chromosomal radiosensitivity in patients, blood samples should be taken before induction of general anaesthesia as anaesthesia can have an impact on the reliability of the MN results.

Introduction

The micronucleus assay (MN assay), is a well-established assay to assess chromosomal damage in the field of genetic toxicology, human biomonitoring of mutagen-exposed populations and chromosomal radiosensitivity testing. Over the years, many case–control radiosensitivity studies performed on cancer patients have shown that MN can also be used as biomarker for cancer predisposition. Cancer patients may have defects in repair of DNA damage and this can be reflected in enhanced chromosomal radiosensitivity (1–3). To assess chromosomal radiosensitivity with the MN assay, lymphocytes of patients are irradiated *in vitro* and stimulated into division. Micronuclei (MN), which represent small nuclei that form in the cytoplasm when chromosomes or chromosome fragments are not

incorporated into the daughter nuclei subsequent to cell division, are scored in binucleated cells (BN), which can be accumulated by blocking cytokinesis with cytochalasin-B. MN can contain: whole chromosomes (missegregated during mitosis), acentric fragments resulting from misrepaired or unrepaired DNA double-strand breaks or other complex chromosomal fragments (4). Automation of the MN scoring with the Metafer platform (Metasystems) has further improved this test by allowing it to become especially rapid and reproducible (5,6). The automated Metafer system scans the slide for BN and subsequently counts the number of MN in these cells based on a number of pre-programmed settings or classifiers (7). The cells are then displayed in a gallery, where they can be manually checked and corrected for false positives and negatives. A factor influencing the reliability and sensitivity of the automated MN scoring is the yield of BN per sample.

The blood samples, on which the MN assay will be performed, often need to be transported to cytogenetic laboratories. This transit time can vary from 1 h to more than 100 h (8) and the conditions (temperature changes) during this transit time can influence the outcome of the MN assay (9,10). We noticed at the start of our radiosensitivity study on breast cancer patients that the time between blood sampling and the initiation of the MN assay could vary between 2 and 24 h, due to time delays and other logistics involved in the different theatre procedures.

Besides blood storage time, another confounding factor in chromosomal radiosensitivity studies is the administration of general anaesthesia. Often blood samples of cancer patients are collected when the patients undergo curative procedures and are under general anaesthesia. Although the mutagenic/genotoxic effects of anaesthesia have been widely studied in lymphocytes of patients and personnel, the effect of anaesthetic agents on *in vitro* chromosomal radiosensitivity assessment using the MN assay has not been investigated (11,12).

In the present study, we investigated the effect of blood storage time on *in vitro* chromosomal radiosensitivity, by comparing the spontaneous and in *vitro* radiation-induced MN yields, in blood samples of 10 healthy individuals processed for radiosensitivity assessment on the same day and 24 or 48 h after blood sampling. Additionally, the effect of blood storage time on *in vitro* chromosomal radiosensitivity was also analysed in a group of patients undergoing breast surgery. In this patient group, we also investigated the influence of general anaesthesia on spontaneous and *in vitro* radiation-induced MN.

Materials and methods

Study population and blood sampling

Ten milliliters of heparinised peripheral blood was collected from 10 healthy women (mean age: 29 years; staff members and students from the University of Witwatersrand). These volunteers had no known previous exposure to cytotoxic or genotoxic agents. The blood samples were stored at room temperature in the cytogenetic lab and the MN assay was started up immediately (within 2 h) after venepuncture (Day 0); within 24 h after venepuncture (Day 1) and 48 h after venepuncture (Day 2).

Concurrently, 60 female patients were recruited from Wits University Donald Gordon Medical Centre, a private hospital in Johannesburg where they were undergoing breast surgery for cancer treatment or elective procedures. None of the patients had received chemotherapy or radiotherapy prior to sample collection. Both the effect of blood storage time and anaesthesia on MN yields was assessed in these patients. To assess the effect of general anaesthesia on MN results, heparinised venous blood (10 ml) was drawn from patients at two time points: before the induction of general anaesthesia and just before completion of surgery, ~120 min after the anaesthesia was administered. To assess the effect of blood storage time, we performed the MN assay on blood samples of patients taken before and after anaesthesia, and that were transported to the cytogenetics laboratory within 2 h of surgery (group A—Day 0) or that were stored at room temperature in the theatre and transported to the cytogenetics lab after 24 h (group B—Day 1). There were 30 patients (mean age: 46 years; age range: 27–65 years) in group A and 30 patients (mean age: 45 years; age range: 21–87 years) in group B. All blood donors signed an informed consent and the study was approved by the Human Research Ethics Committee, University of Witwatersrand, Johannesburg, South Africa (M10372).

Anaesthesia procedure of patients going for breast operation

The anaesthetic procedure included the following steps: all patients were fully monitored during surgery with standard American Society of Anaesthesiology (ASA) monitoring consisting of three lead electrocardiogram, peripheral oxygen saturation (SpO2), non-invasive arterial pressure (systolic and diastolic), end-tidal C02 (PETCO2) and end-tidal Desflurane (DSF). Patients were given the choice of midazolam oral premedication (15 mg) or none. All patients were given Cefazolin (1 g) prior to induction. All patients were induced with Propofol (2 mg/kg, iv), in addition to Fentanyl (2 ug/kg, iv) and maintained with DSF at a minimum alveolar concentration of at least 1.0 and no greater than 1.3. All patients received neuromuscular blockade with rocuronium bromide (0.9 mg/kg, iv). The lungs were mechanically ventilated using volume control mode with tidal volumes of 8 ml/kg and inspired oxygen concentration (FiO_2) of between 40 and 50% in air at 0.5–1 l/min. Respiratory rate was titrated to maintain PETCO2 concentration at 34–40 mmHg. Balanced analgesia with Morphine (titrated up to 10 mg, IV), Paracetamol (1g, IV) and Parecoxib (40 mg, IV) was given to all patients. All patients received Granisetron (1 mg, IV) and Decadron (8 mg, IV) as antiemetics. All patients had reversal of their neuromuscular blockade with neostigmine (2.5 mg, IV) and Glycopyrulate (0.4 mg, IV).

Automated-MN assay

Cultures for the MN assay were initiated according to a standard protocol in our laboratory. Briefly, 0.5 ml of blood was diluted in 4.5 ml of complete culture medium [RPMI (Roswell Park Memorial Institute) 1640 (BioWhittaker, Walkersville, MD, USA) supplemented with 13% foetal bovine serum (Gibco-Invitrogen, New York, NY, USA) and 50 U/ml penicillin and 50 mg/ml streptomycin (Gibco-Invitrogen). The blood cultures were irradiated with 2 and 4 Gy 6 MV X-rays at a dose rate of ~1.33 Gy/min. A 0 Gy dose was used as a sham-irradiated control. All cultures were set up in duplicate. After irradiation, 100 µl phytohaemagglutinin (PHA) (stock solution 1 mg/ml) (Sigma-Aldrich, St Louis, MO, USA) was added as mitogen and 23 h later 20 µl cytochalasin B (stock solution of 1.5 mg/ml, Sigma-Aldrich) was added to block cytokinesis. Cells were harvested 70 h after stimulation using a cold (4°C) hypotonic shock with 7 ml 0.075 M KCl (Merck, Darmstadt, Germany), followed by fixation in methanol:acetic acid:Ringer (0.9% NaCl) solution (4:1:5) (Merck) at 4°C (6). A day later, cells were then fixed another three times with methanol:acetic acid (4:1) (Merck) and stored overnight at 4°C. To standardise the slide preparation, all cells were concentrated in 250 µl fixative and 40 µl of cell suspensions were dropped on clean slides

the following day. Mounting was done with vectashield containing DAPI (4,6-diamidino-2-ohenylindole; Vector Laboratories, Brussels, Belgium). Slides were coded and scanned with the MSearch software module of the Metafer 4 scanning system (MetaSystems), using a Zeiss Imager.Z2 microsope (Zeiss). A maximum of 1000 BN per slide was scanned. The classifier and scoring method was based on Herd *et al.* (13). Briefly, Msearch software module of Metasystems identifies BNs and displays them in an image gallery with a MN count per cell. All automated scorings (BN and MN) were visually checked and validated by two scorers to correct for false positive and false negative MN and to reject unsuitable cells. The BN yields represented in this article are the total number of true BN cells scanned on two slides. The MN results were normalised to a MN yield in 1000 BN.

Statistical analysis

Statistical analysis was performed with the software Graphpad Prism 6. The comparison of mean MN yields and BN yields between the two patient groups was done with the unpaired student *t*-test, while the comparison of the blood samples before and after anaesthesia were compared with the paired student *t*-test. The mean MN yields and BN yields of the healthy controls at the three time points were compared with the Wilcoxon test. The level of statistical significance was set at $P < 0.05$.

Results

Effect of blood storage time on MN and BN yields in healthy individuals

To study the effect of blood storage time on the MN results, the MN assay was performed in blood samples of healthy individuals with different storage times before initiating the MN assay. The delayed initiation of the MN assay had no effect on the spontaneous MN values. However, the radiation-induced MN yields increased significantly as the time between blood sampling and irradiations and initiation of the MN assay increased ($P = 0.0137$ and $P = 0.0020$ for 2 and 4 Gy, respectively at Day 1; $P = 0.0039$ and $P = 0.0020$ for 2 and 4 Gy, respectively at Day 2) (Figure 1; Table 1). The BN yields decreased significantly after 24 h (Day 1) for the 4 Gy dose ($P = 0.0195$) and after 48 h (Day 2) for both doses ($P = 0.0098$ and $P = 0.002$ for 2 and 4 Gy, respectively).

Effect of blood storage time on MN and BN yields in patients undergoing breast operations

The effect of blood storage time on the MN results was also analysed in a group of patients undergoing breast operations. For patients in group A, the blood samples were processed for MN analysis within 2 h after venepuncture while for patients belonging to group B the blood samples were processed 1 day after blood sampling. The MN yields of both groups are presented in Figure 2. The spontaneous MN yields between both groups were not significantly different. On the other hand, the radiation-induced MN yields for both the 2 and 4 Gy doses were significantly higher in group B ($P = 0.0027$ and $P = 0.0030$ for 2 and 4 Gy, respectively). The differences noted in MN yields between the two patient groups, were not reflected in the BN yields, although the BN yields decreased as the dose increased (Table 2).

Effect of anaesthetics on the MN yields in patients undergoing breast operations

Table 3 shows mean MN yields and BN yields obtained with the MN assay performed on blood samples of breast surgery patients

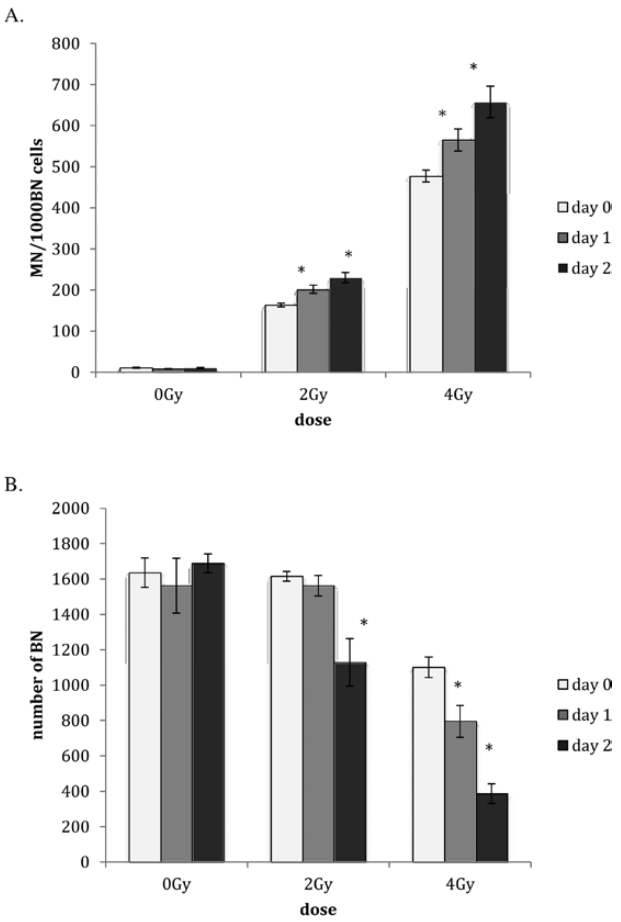

Figure 1. Mean MN yields per 1000 BN cells (**A**) and BN yields (**B**) of 10 healthy individuals with MN assay initiated immediately after venepuncture (Day 0); within 24 h after venepuncture (Day 1) and 48 h after venepuncture (Day 2). Error bars = SEM; *=Significantly different from Day 0.

before they received anaesthetic agents and on blood samples of the same patients after 2 h of general anaesthesia. The spontaneous MN yields were significantly increased after administration of anaesthesia ($P = 0.0017$). No significant differences were observed between the mean radiation-induced MN yields before or after anaesthesia, but the coefficients of variation were higher in the 'after anaesthesia' samples. The mean yields of BN were significantly lower in the samples from the 'after anaesthesia' group for all radiation doses ($P < 0.01$).

Discussion

The effect of delays between blood sampling time and set up of the MN assay (*in vitro* irradiation and culture initiation) was investigated in the frame of chromosomal radiosensitivity assessment with the MN assay. The effect on both spontaneous and *in vitro* radiation-induced MN was investigated. Several studies have shown influences on the MN yields of storage temperature, storage time and delayed mitogenic stimulation after the blood has been exposed to radiation *in vivo* (14). A reduction of MN yields is often observed in samples cultured more than 24 h post-irradiation (14), although others have seen no influence of storage time on the MN yields of exposed lymphocytes (9).

Regarding spontaneous MN yields, the influence of blood storage temperatures has been studied before, with storage temperatures ranging from −196°C (liquid nitrogen) up to 37°C. The effect of

Table 1. Mean BN yields and MN yields per 1000 BN cells for 10 healthy individuals with MN assay initiated immediately after venepuncture (Day 0); within 24 h after venepuncture (Day 1) and 48 h after venepuncture (Day 2)

		Day 0		Day 1		Day 2	
		BN	MN	BN	MN	BN	MN
0 Gy	Mean	1636.0	10.7	1563.0	7.8	1689.0	10.0
	SEM	82.7	1.2	155.5	1.4	52.2	1.6
	CV (%)	16.0	35.3	31.5	58.5	9.8	51.0
	Range (min–max)	1017–1847	6–17	593–1839	3–18	1355–1861	4–18
	P value versus day 0			0.5566	0.07	0.5566	0.9121
2 Gy	Mean	1615.0	163.4	1562.0	201.9	1129.0	230.1
	SEM	27.9	4.8	58.2	9.9	134.8	12.5
	CV (%)	5.5	9.3	11.8	15.5	37.7	17.1
	Range (min–max)	1490–1728	144–197	1158–1747	166–269	642–1790	163–292
	P value versus Day 0			0.6953	0.0137*	0.0195*	0.002*
4 Gy	Mean	1101.0	477.4	795.5	565.1	386.1	657.5
	SEM	58.5	14.8	90.3	27.0	55.7	38.3
	CV (%)	16.8	9.8	35.9	15.1	45.7	18.4
	Range (min–max)	794–1401	384–544	450–1293	441–749	223–785	460–860
	P value versus Day 0			0.0098*	0.0039*	0.002*	0.002*

*Significantly different from Day 0.

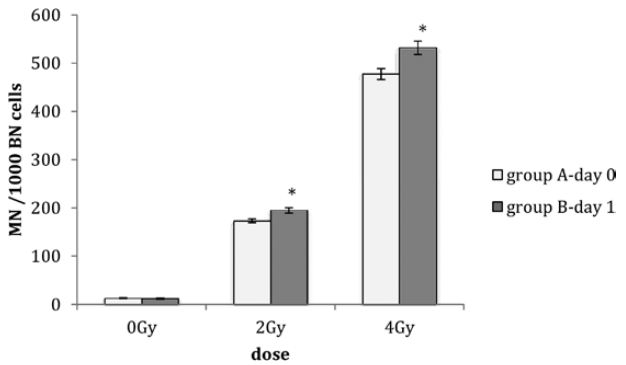

Figure 2. Mean MN yields per 1000 BN for 60 patients under going breast surgery. Group A (n = 30): blood samples received and MN cultures set up within 24 h after venepuncture (Day 0). Group B (n = 30): blood samples received and MN cultures set up 1 day after blood sampling (Day 1). Error bars = SEM. *Significantly different from group A.

Table 2. Mean MN yields per 1000 BN for 60 patients under going breast surgery

		Group A—Day 0	Group B—Day 1
		MN	MN
0 Gy	Mean	12.7	12.1
	SEM	1.1	1.2
	CV (%)	48.7	56.0
	Range (min–max)	3–29	3–39
	P value versus group A		0.7072
2 Gy	Mean	173.1	194.9
	SEM	4.2	5.6
	CV (%)	13.1	15.7
	Range (min–max)	128–211	141–250
	P value versus group A		0.0027*
4 Gy	Mean	477.4	532.0
	SEM	11.3	13.6
	CV (%)	13.0	13.6
	Range (min–max)	370–652	413–694
	P value versus group A		0.003*

Group A (n = 30): blood samples received and MN cultures set up within 24 h after venepuncture (Day 0); group B (n = 30): blood samples received and MN cultures set up 1 day after blood sampling (Day 1).
*Significantly different from group A.

storage time has also been previously studied with storage times ranging from 4 days to more than 2 years. All these studies reported contradictory results (9,15–18). In our present study, we did not find significantly higher spontaneous MN yields after 24 and 48 h of blood storage at room temperature. Comparably, Belloni *et al.* (18) observed no enhanced apoptosis and no enhanced chromosomal radiosensitivity in whole blood cultures stored for 24 or 48 h. The spontaneous MN yields were correlated with the age of the donors, which is widely described in literature (7,19).

The reason for the higher radiation-induced MN yields observed in blood samples stored at room temperature for longer than 24 h before initiation of the MN assay are still unclear. A possible explanation could be that physiological cellular processes are impaired when the blood is stored at room temperature (20) and that DNA repair processes are not optimally functioning at the moment the cells are exposed to ionising radiation. Another hypothesis for the higher MN yields after longer blood storage times could be due to the fact that blood cells, during storage at room temperature, will release stress factors such as cytokines (21) and oxidative radicals that may affect the DNA before the cells are exposed to *in vitro* irradiation. A third explanation could be that during blood storage, cells become hypoxic and acute hypoxia can increase the level of oxidative DNA damage and affect the chromosomal instability (22). The result of this oxidative stress may be undetectable in non-irradiated samples but may result in an additive effect when combined with exposure to ionising radiation.

The decrease in BN yields observed after higher doses of radiation is in agreement with other literature data and is due to the fact that higher doses will result in more highly damaged cells, which will not divide anymore and will go into apoptosis upon exposure (23). This will result in lower BN counts using the automated Metafer platform. If the highly

Table 3. Mean MN yields per 1000 BN and BN yields for 60 patients that underwent breast surgery

		Before Anaesthesia		After Anaesthesia	
		BN	MN	BN	MN
0 Gy	Mean	1781.0	12.4	1667.0	14.9
	SD	118.7	6.5	312.0	7.9
	CV (%)	6.7	52.0	18.7	53.2
	Range (min–max)	1058–1951	3–39	553–1916	3–42
	P values			0.0098*	0.0017*
2 Gy	Mean	1623.0	184.0	1340.0	178.8
	SD	229.5	28.9	465.3	32.8
	CV (%)	14.1	15.7	34.7	18.3
	Range (min–max)	871–1891	128–250	314–1888	127–262
	P values			<0.0001*	0.2228
4 Gy	Mean	1133.0	503.7	829.9	494.5
	SD	402.5	72.0	464.3	94.2
	CV (%)	35.5	14.3	56.0	19.0
	Range (min–max)	290–1805	370–694	156–1782	351–733
	P values			<0.0001*	0.6219

*Significantly different from 'before anaesthesia' group with paired *t*-test.

damaged cells in the samples that were stored more than 24 h before starting the MN assay undergo apoptosis, we can assume that, depending on blood storage time, we are looking with the MN assay at another pool of surviving lymphocytes that are still able to divide but with a lower DNA integrity or lower DNA repair capacity. Although Belloni *et al.* (18) did not detect higher levels of apoptosis in blood samples stored at 20°C for 48 h, Carloni *et al.* (24) has shown that adding PHA to lymphocyte cultures prevents the cells from undergoing apoptosis. Whether the DNA integrity or repair capacity has decreased during the delays in sample processing remains to be investigated.

In the second part of the study, the influence of general anaesthesia on the MN assay was investigated. For this part, we compared the MN yields of blood samples from patients before and after induction of general anaesthesia to study the effect of anaesthetic agents on the spontaneous and radiation-induced MN. Several studies have shown that anaesthetic agents have genotoxic, mutagenic and hematologic effects and can also have an influence on DNA damage/repair processes and apoptosis. In some studies, it was shown that the MN yields are increased in operating theatre personnel occupationally exposed to different anaesthetic gases (12,25,26). However, Wiesner *et al.* (11) only observed increased MN yields in anaesthetists exposed to high levels of anaesthetics. Anaesthetic agents have been demonstrated to induce oxidative stress by increasing the concentrations of reactive oxygen species, which could cause DNA damage (27–29). Later studies showed contradictory results (30,31). The conflicting results on the genotoxicity of general anaesthetics in operating personnel or patients going for surgery could be due to different combinations and concentrations of anaesthetic agents used in the different studies. In our study, we detected a significant increase in the spontaneous MN yields in blood samples of patients that received general anaesthesia before blood sampling. According to Karabiyik *et al.* (32) anaesthetic agent-induced DNA damage would be completely repaired by the cells by the fifth postoperative day. The observed increase in the spontaneous MN yields in blood samples of patients that received general anaesthesia could also be a result of oxidative stress caused by surgical trauma (33,34).

Unexpectedly, we could not observe differences in mean radiation-induced MN yields between the samples before and after anaesthesia, although higher variations in MN yields were seen in the samples after anaesthesia. The DNA damage induced by irradiating cells with 2 and 4 Gy X-rays possibly dominates the effect of the oxidative stress induced by the anaesthetic agents, which is only reflected in the higher spontaneous MN in non-irradiated samples. As general anaesthesia can also have aneugenic besides clastogenic characteristics (26), the use of a pancentromere probe in combination with the MN assay on these samples may clarify the origin of the observed MN. The effect of anaesthesia on BN yields (significantly lower BN yields after anaesthesia) can be a limiting factor influencing the reliability of the assay. It has been reported in the literature that apoptosis is enhanced during the early post-operative period in lymphocytes isolated from patients undergoing surgery under general anaesthesia (33). Although we do not see an effect of general anaesthesia on radiation-induced MN, exposure to general anaesthesia in combination with ionising radiation should be further investigated, as this can be important for the recently developed intraoperative radiotherapy treatments for several cancers. Our results suggest that blood samples after administration of general anaesthesia could be used to assess chromosomal radiosensitivity of individuals but the influence on lowering BN could be a limiting factor.

In conclusion, the results of this study indicate that blood storage time has an influence on the radiation-induced MN yields and by this can be a confounding factor when assessing the *in vitro* chromosomal radiosensitivity in patients by mean of the MN assay. Fresh blood samples from cancer patients should also preferably be taken before administration of general anaesthesia, as the anaesthetics can have an impact on the reliability of the MN results.

Funding

This research was supported by a grant of the South African Medical Research Council, by a 'VLIR Own Initiative Programme' between Belgium and South Africa (ZEIN2011PR387).

Acknowledgements

The authors would like to thank all the patients and healthy individuals who participated in this study.
Conflict of interest statement: None declared.

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CHAPTER 5

Chromosomal radiosensitivity of triple negative breast cancer patients

Chromosomal Radiosensitivity of Triple Negative Breast Cancer Patients

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INTRODUCTION

Breast cancer is the most common cancer in South Africa (SA) and the second overall cause of mortality. As estimated by the national cancer registry, the lifetime risk in SA is 1 in 26 (NCR, 2013). Environmental factors, lifestyle changes, reproductive and hormonal changes all contribute to breast cancer risk (Porter, 2009, Li et al., 2016). The risk is considerably greater with family history; women with a first degree relative with breast cancer have a 3.3-fold higher risk. Familial breast cancer is primarily linked with early-onset of the disease (Skol et al., 2016). With a young population structure, SA has a high incidence of breast cancer in young women exhibiting adverse tumour pathology. Additionally, genetic predisposition can contribute to the rise of early-onset breast cancer incidence (McCormack et al., 2013, Basro and Apffelstaedt, 2010). Germline mutations in DNA repair genes such as *BRCA1* and *BRCA2* also confer to high breast cancer risk accounting for 15-20% of familial breast cancer and about 5% of all breast cancers (Nathanson et al., 2001).

Breast cancer is subtyped based on the presence of receptors on the tumour. The presence of estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2) have prognostic values and are essential in patient management. Breast cancers expressing receptors are known as luminal subtypes. Luminal A is ER+/PR+/HER2- whereas luminal B is ER+/PR+/HER2+. When HER2 is highly expressed, the tumour is

classified as HER2+ (Inic et al., 2014). Triple negative breast cancer (TNBC) is defined by the absence of ER, PR and HER2 and accounts for 10-20% of all breast cancers worldwide (Foulkes et al., 2010, Reis-Filho and Tutt, 2008, Alcantara et al., 2017) and ranges between 14-26% in SA (Dickens et al., 2014, McCormack et al., 2013). Aggressive tumour progression, higher grade tumours and metastases are features of TNBC that contribute to higher mortality rates. Population studies show the frequent occurrence of TNBC in young African women compared to Caucasian women (Sturtz et al., 2014). Women with TNBC are more likely to have mutated BRCA genes (Yeh et al., 2017, Dietze et al., 2015, Bowen et al., 2008). About 75% of *BRCA1* mutation-related breast cancers present with the triple negative (TN) subtype (Bayraktar et al., 2011, Rakha and Chan, 2011) and *BRCA1* mutations largely being identified in TN patients with family history; the possibility of germline mutations is elevated in young TN patients (Robertson et al., 2012). The available treatment modalities for TN are chemotherapy regimens, radiotherapy and surgery. Targeted hormonal therapy was considered insignificant in treating TN tumours until recently. TN patients with androgen receptors, frequently expressed in breast cancer tumours, showed improved outcome following tamoxifen treatment (Hilborn et al., 2016) and increased chemosensitivity (Wang et al., 2017). To control progression and local-regional recurrence, radiotherapy is also utilised as the principle treatment for TNBC (Dragun et al., 2011).

The most detrimental biological effect of radiation is the production of double strand breaks (DSB) of DNA. The two main DNA repair pathways that function in DSB repair are homologous recombination (HR) and non-homologous end-joining (NHEJ). A third repair mechanism, the alternate-end-joining (Alt-EJ), has also been described in the repair of DSB (Ceccaldi et al., 2016, Iliakis et al., 2015). DNA repair genes, such as BRCA, are allied with chromosomal stability and have critical functions in repair pathways. TN tumours with BRCA mutations can be, therefore, deficient in the HR DNA repair pathway (Sharma, 2016). The availability of a homologous template of DNA for repair determines the pathway to be utilised. Hence, HR exclusively repairs damage in the late S and G2 phase of the cell cycle. NHEJ utilises a non-homologous template for repair and therefore is available for repair throughout the cell cycle. Similar to the NHEJ, alt-EJ pathway operates throughout all phases of the cell cycle and acts as a backup pathway when NHEJ and HR are absent or defective. Due to the error-prone

mechanism of alt-EJ, the pathway generally gives rise to translocations, mutations and alterations leading to chromosomal instability (Iliakis et al., 2015). As part of the response to DNA damage repair, at every stage of the cell cycle, checkpoints are activated to prevent replication of damaged DNA by allowing adequate time for repair. A number of proteins, including BRCA, are involved in the activation of these checkpoints (Fernet et al., 2010). *BRCA1/2* genes are essential in regulation of the G2/M checkpoint and HR repair (Prakash et al., 2015). The G2/M checkpoints can be abrogated in the presence of caffeine and allows the progression of cells to mitosis (Jiang et al., 2000). By exploiting this trait of caffeine, the efficiency of the checkpoints and repair pathways can be evaluated. Upon exposure to ionising radiation (IR), the checkpoints are also activated as response to damaged DNA. Therefore, defects in checkpoints or its activating proteins are associated with radiosensitivity (Slonina et al., 2016).

Deficiencies in DNA repair genes are linked with predisposition to cancer and elevate chromosomal radiosensitivity in breast cancer patients (Khanna and Jackson, 2001, Terzoudi et al., 2000). TNBC patients, particularly, have reduced expression of DNA repair genes and associated with deleterious BRCA mutations. Breast cancer patients have been shown to exhibit chromosomal radiosensitivity in the G0 and S/G2 phase where DSB are primarily repaired by NHEJ and HR respectively (Poggioli et al., 2010, Baeyens et al., 2005, Scott et al., 1999, Baeyens et al., 2002). Chromosomal radiosensitivity can be evaluated using the micronucleus (MN) assay (Fenech, 2000). The assay is extensively used in human biomonitoring subsequent to mutagen exposure and to analyse radiation-induced damage (Vral et al., 2011). Defects in DNA repair genes and age are factors that commonly influence the frequency of micronuclei (MNi) (Fenech and Bonassi, 2011).

In the present study, we assessed the *in vitro* chromosomal radiosensitivity of TNBC compared to luminal breast cancer patients and healthy individuals by performing the MN assay in the G0 and S/G2 phases of the cell cycle. The influence of age in this group of patients and the influence of positive mutations in breast cancer susceptibility genes such as *BRCA* on chromosomal radiosensitivity was also assessed.

METHODS AND MATERIALS

Sample collection: Patients were recruited from Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and WITS Donald Gordon Medical Centre. Blood samples were collected from 83 breast cancer patients. Previous neo-adjuvant chemotherapy and radiotherapy was the exclusion criteria of the study. Patients were categorised as young/old TN and young/old luminal breast cancer patients (Figure 1). All patients less than 50 years of age were categorised as young patients. All patients 50 years or above were categorised as older patients. Ninety healthy individuals with no personal history of breast cancer, who were students and staff members from CMJAH, were also enrolled in the study as the control population. A signed consent was obtained from all patients and controls. The study was approved by the Human Research Ethics Committee, University of the Witwatersrand, Johannesburg (M110248).

The mean age of the older patients was 62 years; the mean age of all the young patients was 41 years. TN patients had a mean age of 50 years and mean age of luminal patients were 53 years. The patients with positive mutations in breast cancer susceptibility genes had a mean age of 49 years.

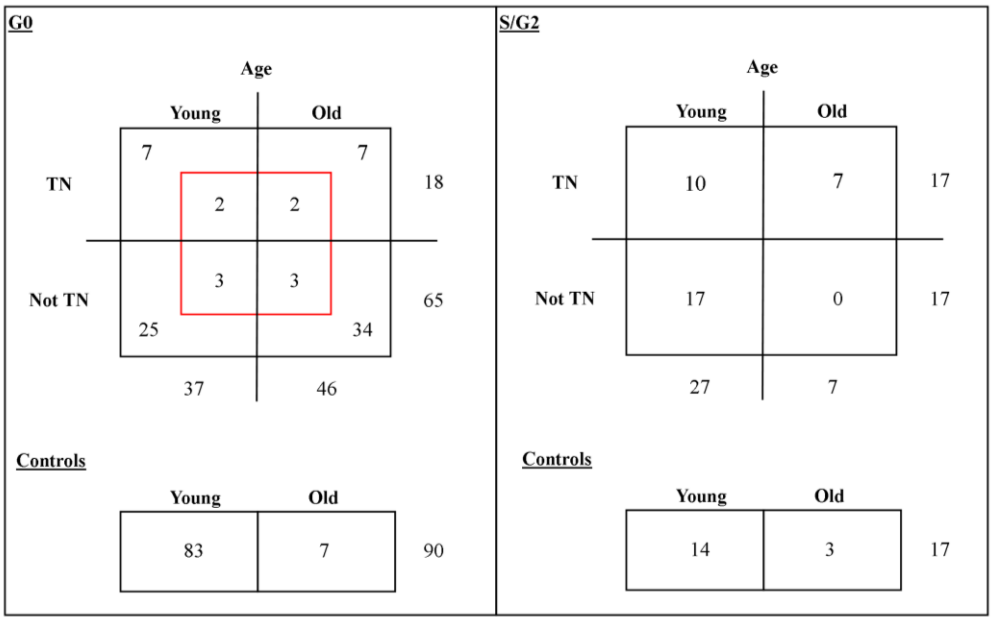

Figure 1: Overview of patient subgroups included in the study for the G0 and S/G2 MN assays. The red inner box indicates patients with positive mutations in breast cancer susceptibility genes.

Mutation analysis: All TNBC and patients ≤50 years were screened for *BRCA1*, *BRCA2*, *PALB2* and *CHEK2* c.1100delC allele mutations. Mutation analysis was conducted using the MiSeq platform and large genomic arrangements were identified using MLPA (Francies et al., 2015b).

G0 MN assay: As previously described, the G0 MN assay was initiated with 0.5ml heparin blood samples supplemented with 4.5 ml of pre-warmed culture medium (Baeyens et al., 2016). The culture medium comprised of RPMI-1640 with added L-glutamine (Bio-Whittaker, USA), antibiotics (10 000 U/ml penicillin and 10 000 µg/ml streptomycin; Gibco-Invitrogen, USA) and additional 13% foetal bovine serum (FBS; Gibco-Invitrogen, USA). Following the initial incubation at 37°C in 5% CO_2, the cells were irradiated using a linear accelerator (Siemens Healthcare, Germany) to doses of 2 and 4 Gy X-rays. A 0 Gy dose was used as a control to detect spontaneously occurring MNi. Immediately after irradiation, the lymphocytes were stimulated to divide with phytohaemagglutinin (PHA; 100 µl, stock solution 1 mg/ml; Sigma-Aldrich, USA) and further incubated for 23hrs. Cytokinesis block was then induced by addition of 20 µl cytochalasin B (Cyto B; stock solution 1.5 mg/ml; Sigma-Aldrich, USA). Cell were then harvested 70 hrs post stimulation using an ice cold hypotonic shock of 7 ml of KCL (0.075 M; Merck, Germany) and washed in a methanol: acetic acid: ringer solution in a ratio of 4:1:5 (Merck, Germany). After an overnight storage at 4°C, the cells were washed an additional 3 times with a methanol: acetic acid solution (4:1; Merck, Germany). The slides were prepared by dropping a cell suspension of 40 µl onto methanol-cleaned slides and counter stained with DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories). All slides were scanned on the automated platform, Metafer MNScore Software (MetaSystems), and scoring of MNi was performed on duplicate slides for each condition.

S/G2 MN assay: A modified version of the G0-MN assay was used to detect chromosomal radiosensitivity in the S/G2 phase of the cell cycle (Baert et al., 2017). Heparinised blood samples were supplemented and cultured in the same manner as the G0 MN assay described above. Prior to irradiation, the assay was initiated with the addition of 100 µl PHA (100 µl, stock solution 1 mg/ml; Sigma-Aldrich, USA) to stimulate the lymphocytes into division and then incubated at 37°C in 5% CO_2 for 62 hrs. The cultures were then exposed to X-rays of 2 and 4 Gy using a linear accelerator (Siemens Healthcare, Germany). Here, a 0 Gy control was also set up

to detect spontaneous MNi in the S/G2 phase. Instantaneously after irradiation, cytokinesis block is induced by addition of 20 µL Cyto B (stock solution 1.5 mg/mL; Sigma-Aldrich, USA). To half of the irradiated cultures, 200 µl caffeine (stock solution 100mM; Sigma-Aldrich, USA) was added. Caffeine abrogates the G2/M checkpoint and permits the progression of damaged cells into mitosis. The efficiency of the G2/M checkpoint can be evaluated with addition of caffeine. The harvesting of cells, staining and scoring was accomplished as described above in the G0 MN assay.

Radiosensitivity indicator (RIND) score: To evaluate individual radiosensitivity, a radiosensitivity score is computed. As a threshold value to determine radiosensitivity, the mean MN yield and standard deviation (SD) of the healthy population was utilised. For mild radiosensitivity, a score of 1 was assigned when individual MN yield is higher than the mean MN yield of healthy controls +1SD. Individual MN yields higher than the mean MN yield +2SD of healthy controls were assigned a score of 2 indicating severe radiosensitivity. Any individual MN value lower than the mean MN yield +1SD of healthy controls was scored as 0 (Baert et al., 2017).

To compute a G2/M checkpoint efficiency ratio, the MN values with addition of caffeine was divided with the MN values without caffeine (MNCaf+/MNCaf-). Radiosensitivity would be indicated by a low checkpoint ratio. The G2/M checkpoint efficiency ratio was converted in a similar manner as the radiosensitivity score. For a score of 1, the individual values were lower than the mean checkpoint ratio –1SD of healthy controls. Whereas for a score of 2, the values were lower than the mean –2SD of healthy controls. If the individual values were greater than the mean –1SD of healthy controls, the score was 0 (Baert et al., 2016).

The RIND score was utilised as a comprehensive scoring method to assess radiosensitivity in individuals. For the G0 MN assay, a final RIND score was the sum of the RIND score for the 2 Gy and for the 4 Gy doses. This final G0 RIND score could vary from 0 to 4. For the G2 MN assay, we also included the checkpoint efficiency ratio in the calculation of the final RIND score. The final RIND score here could vary between 0 and 8. The final RIND score was used to categorise our patients into levels of radiosensitivity (Baert et al., 2016).

Statistical Analysis: The statistical significance of the study was analysed using GraphPad Prism 7 software. For the MN score comparison between each group and the control group, the non-parametric Mann-Whitney test was utilized. The significance level was set at <0.05. Statistical measure for the difference in RIND score for the G0 and S/G2 test between the groups were analysed using the Fisher's exact test.

RESULTS

G0 Micronucleus assay:

Chromosomal instability is stipulated by MNi frequency occurring spontaneously (0 Gy) which was evaluated in all breast cancer samples. The spontaneous MN values in the G0 MN assay of all breast cancer patients are significantly higher when compared to healthy individuals (p=0.0008) (Figure 2).

The effect of age on chromosomal instability and radiosensitivity was investigated between the all young and all older patients. Chromosomal instability indicated by enhanced MN values were significantly different for all the young patients (p=0.01) and older patients compared to the controls (Figure 2). However, the significant difference were higher in the older patients (p=0.0044). Compared to the controls, chromosomal radiosensitivity was elevated in all breast cancer patients (2 Gy: p=0.0018; 4 Gy: p=0.0084), young and old patients (2 Gy: p=0.0014; 4 Gy: p=0.0481) with enhanced MN values following radiation (Figure 3). Although, when comparing MN values for the 2 Gy dose, no significant differences were observed (p=0.0778) in the young patients. The correlation between MN values in the young and old patients were assessed and no significant differences were observed for spontaneous and irradiated MN values. No correlation between age and chromosomal radiosensitivity was demonstrated in our cohort.

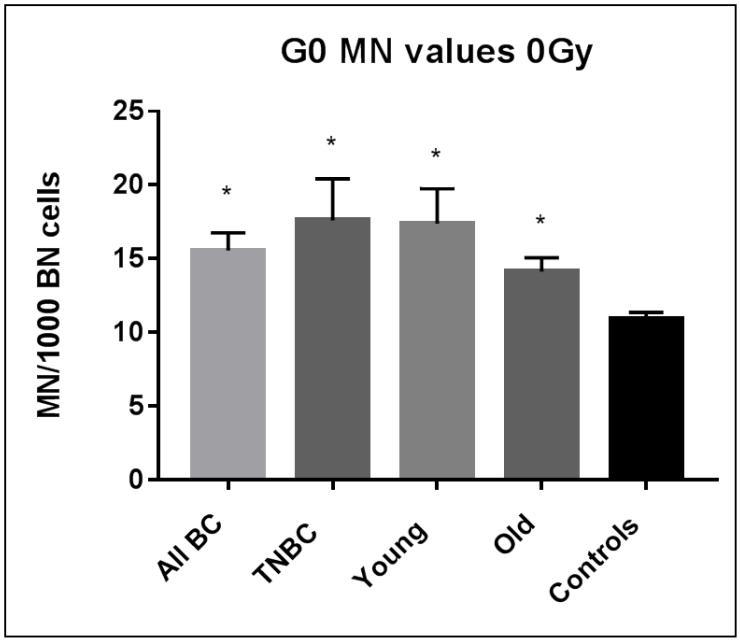

Figure 2: Spontaneous MNi frequency of all breast cancer, triple negative, young and older breast cancer patients compared to controls G0 MN assay. *Statistically significant from controls ($p<0.05$).

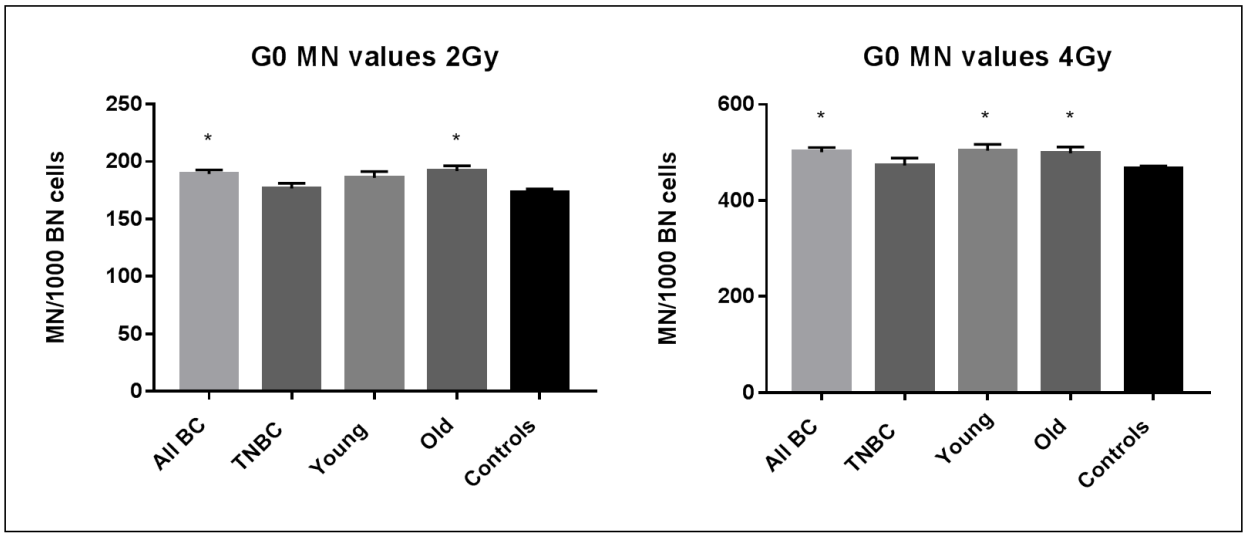

Figure 3: 2 and 4Gy irradiated MN frequencies for triple negative, young and older patients in the G0 MN assay. *Statistically significant from controls ($p<0.05$)

Since there was no correlation between age and chromosomal radiosensitivity, the patients were all group as TN or luminal breast cancer patients to investigate the effect of TN phenotype on

chromosomal radiosensitivity. The TNBC (p=0.0031) and luminal patients (p=0.0066) exhibit chromosomal instability with significantly higher spontaneous MN values.

Similar to the spontaneous MN values, when comparing all breast cancer patients, the MNi frequency was significantly different from the controls for both the 2 and 4 Gy irradiation. Although, after irradiation, the MN values of TNBC patients were not significantly different when compared to controls (2 Gy: p=0.6049; 4 Gy: p=0.9984). The opposite was observed for luminal patients (2 Gy: p=0.005; 4Gy: p=0.0016) for both IR doses (Figure 4). Chromosomal instability and radiosensitivity was also assessed in the 10 breast cancer patients who were positive for mutations in breast cancer susceptibility genes. No significant differences were observed in these patients in the spontaneous and irradiated MN values when compared to the other breast cancer patients.

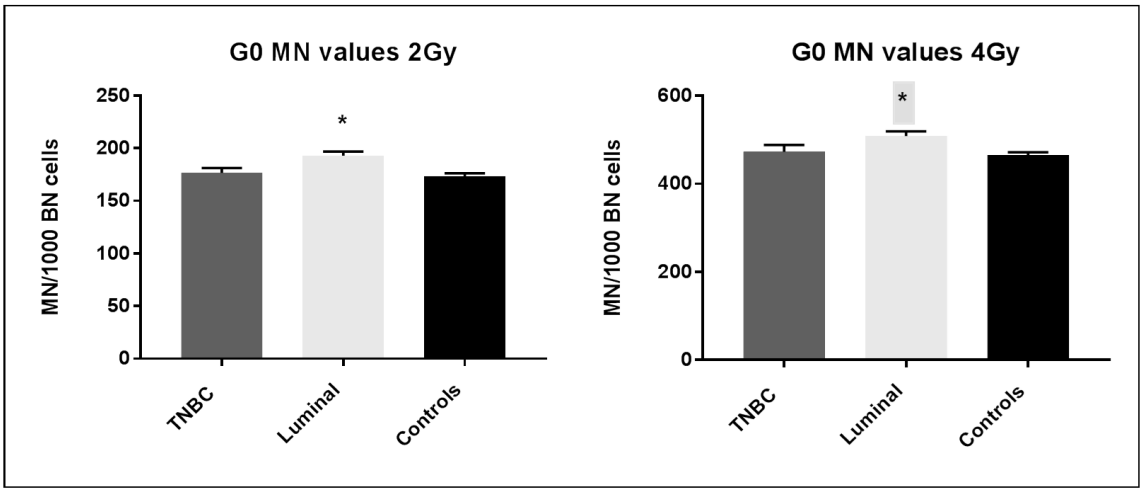

Figure 4: 2 and 4Gy irradiated MN frequencies for triple negative and luminal patients compared to controls in the G0 MN assay. *Statistically significant from controls (p<0.05)

The RIND score was computed to assess individual radiosensitivity. A RIND score above 2 was considered as radiosensitive, with a score of 0 and 1 being radioresistant. Using this threshold for the G0 assay, 88% of individuals in the control group were not radiosensitive. Only 11% of TN patients exhibited a radiosensitive phenotype compared to 32% of luminal patients with enhanced chromosomal radiosensitivity. The remaining 89% of TN patients were not radiosensitive compared to 68% of luminal breast cancer patients. When comparing chromosomal radiosensitivity in patients who are either TN or luminal, there is a shift in

distribution with higher levels of chromosomal radiosensitivity in the luminal patients (Figure 5). The correlation between MN values in the TN and luminal patients were assessed and no significant differences were observed for irradiated MN values.

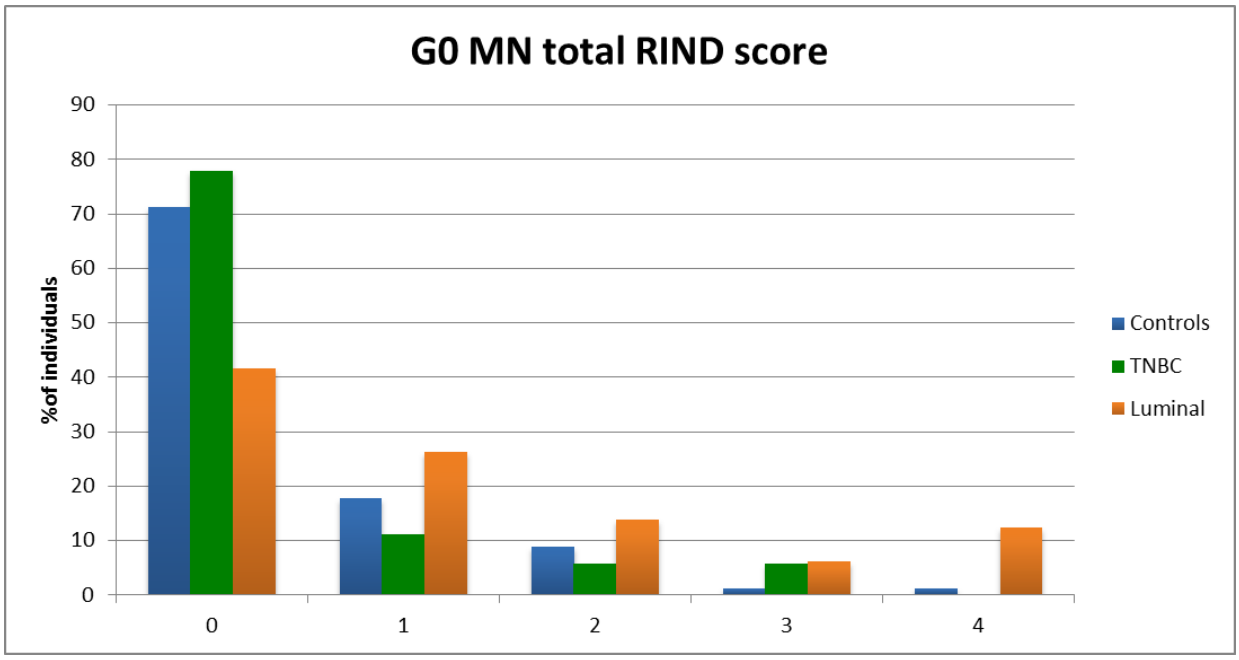

Figure 5: G0 RIND scores to assess individual radiosensitivity in triple negative and luminal patients compared to controls.

S/G2 Micronucleus assay:

In vitro chromosomal radiosensitivity focusing in the S/G2 phase of the cell cycle was evaluated in 17 TN and 17 luminal breast cancer patients and compared to 17 controls (Figure 1). When comparing spontaneous MNi frequencies of patients with controls, 24% all breast cancer patients (8/34), 12% of all TN patients (2/17) display chromosomal instability as compared to 35% (6/17) luminal patients. By assigning RIND scores, we evaluated the chromosomal radiosensitivity. In the TN patients, 76% did not exhibit a radiosensitive phenotype; whereas 47% of luminal patients were radiosensitive. Similar to the G0 results, the distribution of luminal breast cancer patients show a shift towards chromosomal radiosensitivity (Figure 6).

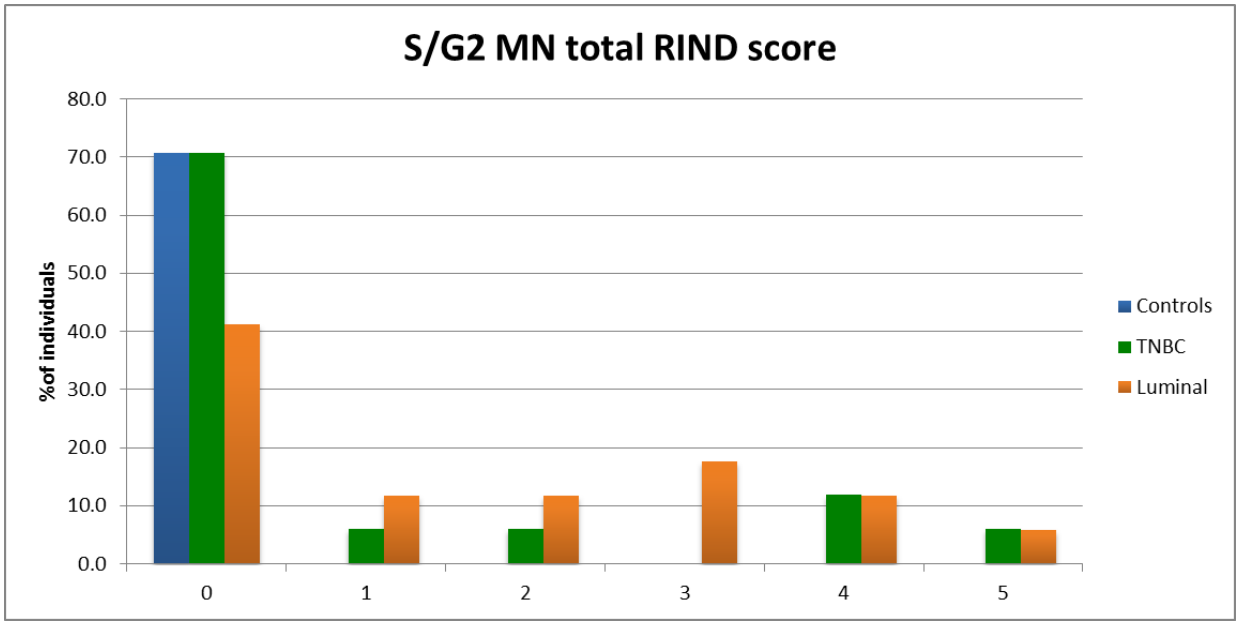

Figure 6: S/G2 RIND scores to assess individual radiosensitivity in triple negative and luminal patients compared to controls.

DISCUSSION

The *in vitro* chromosomal radiosensitivity of TN breast cancer patients in South Africa was evaluated in this study and compared to luminal breast cancer patients and healthy individuals. As TNBC are often young patients, we investigated if age has an influence on the chromosomal radiosensitivity in this population. Since BRCA mutations are also often observed in TNBC populations, the effect of mutations in BRCA and other breast cancer susceptibility genes on radiosensitivity was also analysed. DNA damage was induced by IR during two different phases of the cell cycle: G0 and S/G2 phase. DNA repair pathways in these phases differ and defects in these pathways can be reflected as chromosomal radiosensitivity.

The mean spontaneous MNi frequencies in the G0 MN assay are significantly increased in the breast cancer patients indicating chromosomal instability. This result is in agreement with our previous study where we showed increased chromosomal instability in an unselected South African breast cancer population of all ages (Francies et al., 2015a). Various factors influence spontaneously occurring MNi such as age, environmental and genetic factors (Orta and Gunebakan, 2012, Jones et al., 2011). When comparing the different subgroups of breast cancer

patients in our study, we were able to demonstrate significantly increased mean spontaneous MN values in both the TN (mean age = 50) and luminal breast cancer patients (mean age = 53) compared to controls. This could be influenced by the different genetic background of the patients. In older patients, age-associated decline in DNA repair leads to accumulation of DNA damage, possibly contributing to the elevated spontaneous MN values (Thierens et al., 2000). Other factors that could influence chromosomal instability in older patients are age-related oxidative damage and aneuploidy (Luzhna et al., 2013).

For IR induced damage, MN values were significantly higher, for both the 2 and 4Gy, in the whole breast cancer group as expected from our previous findings (Francies et al., 2015a). Interestingly, when sub grouping patients in age groups, there were no significant differences in MNi between young and older patients. TNBC patients showed lower yield in MN values as compared to the luminal patients. Recent studies have brought to light the "radioresistant" phenotype in TNBC cell lines compared to non-TNBC cell lines. The TN tumours characteristics could contribute to the "radioresistant" phenotype observed in TN patients or TN cell lines. One such factor is the microRNAs (miRNAs) that function in post-transcriptional regulation of gene expression. miRNAs are implemented in the progression of tumours, metastases and response to therapy in breast cancer (Kurozumi et al., 2017). A subset of microRNA's has been implicated in regulating the cellular response of TN cells to IR. A study by Ren et al., (2015) verified the overexpression of miRNA 27a in TNBC cell lines and its regulation in radiosensitivity and cell proliferation (Ren et al., 2015). Another study showed that miRNA-129-5p is expressed in lower levels in TN cell lines compared to luminal cell lines. Upon exposure to irradiation, survival fractions were elevated when miRNA-129-5p was knocked down in luminal cell lines and in TN cells lines, survival fractions were decreased when miRNA-129-5p was overexpression (Luo et al., 2015).

In the second part of the study, individual chromosomal instability and radiosensitivity was analysed in the S/G2 phase. Chromosomal instability was more predominant in luminal breast cancer patients (35%) than in TNBC patients (12%) when compared to controls. A study of Djuzenova et al. using the S/G2 MN assay on peripheral blood mononuclear cells showed that an unselected breast cancer population did not exhibit increased spontaneous MN values compared

to the controls. Enhanced spontaneous MN values were only observed in breast cancer patients that previously had radiotherapy (Djuzenova et al., 2006). Contrary to their results, we were able to demonstrate G2 chromosomal radiosensitivity in luminal patients.

For the evaluation of the 2 Gy - and 4 Gy - induced DNA damage in S/G2 cell phase, the RIND scoring system was introduced to evaluated individual radiosensitivity. It was previously suggested that patients with high RIND scores could reflect defects in repair and G2/M arrest capacity (Baert et al., 2016). The checkpoint efficiency is indicated by a ratio of MNi with caffeine divided by MNi without caffeine (MNCaf+/MNCaf-). By combining radiation induced MN values and checkpoint efficiency ratio, RIND score distribution of luminal breast cancer patients showed a shift towards a radiosensitive phenotype, while the TNBC showed radiosensitivity levels were comparable with the healthy individuals. Patients exhibiting radiosensitivity could have defects in HR repair and G2/M checkpoint control and expressing low levels of DNA repair genes. This defect may be reflected as chromosomal radiosensitivity in the S/G2 phase.

A radioresistant phenotype was exhibited in 76% of TN patients. A recent study showed upregulation of RAD51 in TNBC tumours when compared to other breast cancer. RAD51 is a primary component of the HR pathway. TN cells with enhanced HR repair exhibit radioresistance to IR (Gasparini et al., 2014). The overexpression of RAD51 leads to enhanced HR capacity which could lead to a "radioresistant" phenotype of TN lymphocytes.

In our previous study, we showed that ER positive breast cancer patients exhibited increased chromosomal radiosensitivity (Francies et al., 2015a). It can be hypothesised that in the absence of ER receptors in TN cells, the DNA damaging effects of estrogen cannot be mediated and could contribute to a "radioresistant" phenotype. Chen et al. (2017) showed elevated radiation-induced DSB breaks in TN cell lines transfected with ERα and delay in repair compared to wild type TN cell line. In the ER-transfected TN cells, the radiation-induced G2/M arrest was enhanced and a time-dependent decrease in S phase was observed compared to wild type cells. The HR pathway is enhanced in the S phase for efficient DSB repair. A shorter S phase implies less time for DNA repair (Chen et al., 2017). These results suggest that the absence of ER in TN

cells permits improved DNA repair and decreased G2/M arrest. Furthermore, cell cycle and DNA repair genes are overexpressed in TNBC cells. Therefore, it can be hypothesised that the observed chromosomal radioresistance could be attributed to increased expression of DNA damage response genes (Engebraaten et al., 2013).

A second hypothesis of the observed radioresistance in TN cells could be the overexpression of β-catenin upon exposure to IR. Irregularities of the Wnt/β-catenin pathway are essential for stem cell maintenance (Yin et al., 2016). Following IR, cancer stem cells (CSC) in breast cancers are reinforced and have enhanced Wnt/β-catenin pathway. CSC are associated with radioresistance. CSC in TNBC cells could be maintained by this pathway (Krause et al., 2017, Pohl et al., 2017).

As a third hypothesis, TN radioresistance could also be attributed to HuR, a mRNA binding protein that is endogenously overexpressed in TN cells. Resistance to therapy and poor prognosis of TNBC was linked to HuR. A study by Mehta et al. (2016) showed significant increase in radiation-induced γ-H2AX foci in HuR knockdown TN cells. The scrambled control, however, had a higher cell survival rate. When HuR is knocked down, DNA repair genes involved in HR were suppressed (Mehta et al., 2016). The overexpression of HuR, therefore, may also maintain DNA repair genes in HR for improved repair.

As previously reported in the literature, 17% (3/18) of all our TN patients were positive for germline *BRCA1/2* mutations (Peshkin et al., 2010, Gonzalez-Angulo et al., 2011) and a slightly higher proportion (2/9; 22%) of young patients that have TNBC (Young et al., 2009). This could be due to the unique genetic makeup of the under-studied African population.

Studies have shown that defects in *BRCA1/2* can be reflected as enhanced chromosomal radiosensitivity (Ernestos et al., 2010, Baert et al., 2016, Baert et al., 2017, Barwell et al., 2007). BRCA1/2, and other important proteins, plays a central role in the G2/M checkpoint control and HR repair. Breast cancer patients with BRCA mutations were shown to have significantly higher chromatid breaks indicating chromosomal radiosensitivity (Ernestos et al., 2010). Also, Baert et al., (2016 & 2017) confirmed enhanced G2 chromosomal radiosensitivity of *BRCA1* and *BRCA2* mutation carriers without breast cancer (Baert et al., 2016, Baert et al., 2017). These results

highlight the significant role of *BRCA1/2* in efficient DNA repair and cell cycle checkpoint control to eliminate chromosomal aberrations. We were not able to demonstrate significant differences in BRCA mutation carriers.

The observations of our study describe chromosomal radiosensitivity in TNBC and luminal breast cancer patients. Radiosensitivity data in TN patients is primarily important as they lack effective treatment options. We were able to demonstrate a radioresistant phenotype of lymphocytes in TN patients. Our results suggest that the healthy lymphocytes in TN patients are equipped to handle higher doses of IR, therefore, treatment plans could be altered with adequate doses for TNBC patients. However, TNBC patient's positive for *BRCA1/2* mutations should be treated with caution and further validations of chromosomal radiosensitivity in the S/G2 phase in larger cohorts is necessary.

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CHAPTER 6

BRCA1, *BRCA2* and *PALB2* mutations and *CHEK2* c.1100delC in different South African ethnic groups diagnosed with premenopausal and/or triple negative breast cancer

Francies et al. BMC Cancer (2015) 15:912
DOI 10.1186/s12885-015-1913-6

BMC Cancer

RESEARCH ARTICLE Open Access

BRCA1, *BRCA2* and *PALB2* mutations and *CHEK2* c.1100delC in different South African ethnic groups diagnosed with premenopausal and/or triple negative breast cancer

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Abstract

Background: Current knowledge of the aetiology of hereditary breast cancer in the four main South African population groups (black, coloured, Indian and white) is limited. Risk assessments in the black, coloured and Indian population groups are challenging because of restricted information regarding the underlying genetic contributions to inherited breast cancer in these populations. We focused this study on premenopausal patients (diagnosed with breast cancer before the age of 50; $n = 78$) and triple negative breast cancer (TNBC) patients ($n = 30$) from the four South African ethnic groups. The aim of this study was to determine the frequency and spectrum of germline mutations in *BRCA1*, *BRCA2* and *PALB2* and to evaluate the presence of the *CHEK2* c.1100delC allele in these patients.

Methods: In total, 108 South African breast cancer patients underwent mutation screening using a Next-Generation Sequencing (NGS) approach in combination with Multiplex Ligation-dependent Probe Amplification (MLPA) to detect large rearrangements in *BRCA1* and *BRCA2*.

Results: In 13 (12 %) patients a deleterious mutation in *BRCA1/2* was detected, three of which were novel mutations in black patients. None of the study participants was found to have an unequivocal pathogenic mutation in *PALB2*. Two (white) patients tested positive for the *CHEK2* c.1100delC mutation, however, one of these also carried a deleterious *BRCA2* mutation. Additionally, six variants of unknown clinical significance were identified (4 in *BRCA2*, 2 in *PALB2*), all in black patients. Within the group of TNBC patients, a higher mutation frequency was obtained (23.3 %; 7/30) than in the group of patients diagnosed before the age of 50 (7.7 %; 6/78).

Conclusion: This study highlights the importance of evaluating germline mutations in major breast cancer genes in all of the South African population groups. This NGS study shows that mutation analysis is warranted in South African patients with triple negative and/or in premenopausal breast cancer.

Keywords: Triple negative breast cancer, Premenopausal breast cancer, *BRCA* mutations, South Africa

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Background

Breast cancer is the most common cancer amongst South African women with a lifetime risk of 1 in 32 [1]. South Africa is a country consisting of citizens from diverse ethnic groups. These include: black/African (79.8 %), white/Caucasian (8.7 %), mixed ancestry/coloured (9.0 %) and Indian/Asian (2.5 %) (Statistics South Africa, 2013) [2]. According to the most recent report from the National Cancer Registry of South Africa, 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 (National Cancer registry, NHLS, 2006) [1].

Breast cancer has a strong heritable component, with approximately 15–20 % of cases exhibiting a family history of the disease [3, 4]. Mutations in genes such as *BRCA1* and *BRCA2* lead to autosomal dominant inherited cancer susceptibility and confer a high lifetime risk of breast cancer, as well as ovarian and other cancers. Recently it was suggested that the risk to develop breast cancer for *PALB2* mutation carriers is as high as the risk borne by *BRCA2* mutation carriers [5]. Identification of mutations in these genes through clinical genetic testing enables patients to undergo screening and prevention strategies, some of which provide reduced morbidity. In addition, the c.1100delC mutation in *CHEK2* has been identified as a susceptibility allele with incomplete penetrance and is associated with moderate lifetime risks of breast cancer. Data on the prevalence and spectrum of mutations in these genes are widely available for individuals of European descent. However, data for cohorts with African ancestry are scarce [6].

A few South African studies on mutations in *BRCA1*, *BRCA2* and *PALB2* are available [7–10].Three South African population groups exist in which the presence of *BRCA1/2* founder mutations occur; these are the Ashkenazi Jewish population [11], the Afrikaans population [7] and the black Xhosa population [10]. Other family-specific mutations have also been identified, as is typical of populations elsewhere. Table 1 shows data from studies done in South Africa to date. These studies have been performed mostly in white breast cancer patient cohorts. Furthermore, African populations are known to exhibit greater genomic diversity when compared to white populations, and genetic findings in one population cannot necessarily be extrapolated to another [12]. Consequently, there is a need to establish the aetiology of inherited breast cancer in this population. The epidemiology of breast cancer in South African black populations exhibits a number of unique trends when compared to other population groups worldwide. The difference in underlying genetic architecture, family structure, limited financial and human resources, limited community knowledge of breast cancer, limited information on family history and historical difficulty accessing health care, makes it more complex to perform risk assessments in these populations [13]. Overall, the cancer incidence in sub-Saharan Africa is lower as compared to developed countries but there is evidence to suggest changes in the disease burden as the impact of communicable diseases is mitigated [14]. South African women tend to be diagnosed with breast cancer at younger ages [15–17]. However, the diagnosis only occurs at advanced stage due to the lack of awareness, access to diagnostic centres available and limited screening. Hence, the inclusion criterion for a "young" breast cancer or premenopausal (PM) breast cancer patient was set at 50 years (See Additional file 1: Table S1). While this could be due to a younger population structure, it is possible that these younger women carry unique mutations in certain genes. Breast cancer in young women is correlated with aggressive tumour progression, lack of expression of receptors and poor prognosis [18]. Furthermore, it is often attributed to a genetic predisposition with germline mutations in the *BRCA1/2* genes [19–22]. Younger women of African descent are known to be in the high-risk group with decreased survival rates [23].

Another factor that is generally considered as an indicator of genetic susceptibility to breast cancer is the so-called "triple negative" histological phenotype. Approximately 15 % of breast cancers lack the expression of estrogen receptors, progesterone receptors and HER2/NEU receptors and are known as triple negative breast cancer (TNBC) [24]. This type of breast cancer is associated with an aggressive disease progression, higher histological grade, poor prognosis, high rate of recurrence and decreased survival rates. The frequent occurrence of TNBC is strongly correlated with younger patients of African descent and increased incidence has been noted among black South African breast cancer patients [16, 17, 25]. The strong association between TNBC and mutations in the *BRCA1* gene, seen in European and American populations [26, 27], has not been investigated in a South African cohort.

This study aimed to evaluate the contribution of germline *BRCA1*, *BRCA2* and *PALB2* mutations and the *CHEK2* c.1100delC allele to breast cancer in a high-risk South African cohort. Individuals included in the study were of different ethnicities (with a majority from the understudied black population) and had been diagnosed with premenopausal breast cancer (less than 50 years) or exhibited the "triple negative" histological phenotype. We chose to analyse *BRCA1*, *BRCA2* and *PALB2* as associated risks are well established and clinically relevant. In addition, the prevalence of *CHEK2* c.1100delC was evaluated in this cohort and compared with the prevalence in individuals of

Table 1 Literature overview on *BRCA1* and *BRCA2* mutations detected in a South African population

Study (Reference)	Ethnic group	Gene	Mutation detected	Patients/families tested	Frequency (%)	Detection method
Yawitch & Van Rensburg 2000 [51]	Black	*BRCA1*	N/A	0/206	0	PTT and SSCP/HA; limited to regions with Afrikaner founder mutations
Reeves et al., 2004 [7]	White/Ashkenazi Jewish	*BRCA1*	c.68_69delAG	4/18	4.4	PTT and SSCP/HA
	White	*BRCA1*	c.329dupA	1/18	1.1	
	White	*BRCA1*	c.1008dupA	1/18	1.1	
	White	*BRCA1*	c.1352C > A; p.S451*	1/18	1.1	
	White/Afrikaner	*BRCA1*	c.1374delC	2/18	2.2	
	White/Afrikaner	*BRCA1*	c.2641G > T; p.E881*	5/18	5.6	
	Indian	*BRCA1*	c.4957insC	1/18	1.1	
	White/Ashkenazi Jewish	*BRCA1*	c.5266dupC	3/18	3.3	
Schlebusch et al., 2010 [52]	White/Afrikaner, Ashkenazi Jewish, Black, Indian	*BRCA1*	N/A	26/129	20.2	PTT and SSCP/HA and MLPA
		BRCA2	N/A	43/129	33.3	
Sluiter et al., 2011 [9]	White/Afrikaner	*BRCA1 + BRCA2*	N/A	0/36		MLPA
	White/Ashkenazi Jewish	*BRCA1*	Ex23-24del	1/30	3.3	
		BRCA2	N/A	0/30		
Van der Merwe et al., 2012 [10]	Coloured	*BRCA1*	c. 1504_1508delTTAAA	1/105	1.0	PTT and SSCP/HA
		BRCA1	c. 2641G > T;p. E881*	1/105	1.0	
		BRCA2	c. 2826_2829delAATT	1/105	1.0	
		BRCA2	c. 5771_5774delTTCA	4/105	3.8	
		BRCA2	c. 6448dupTA	1/105	1.0	
		BRCA2	c. 7934delG	1/105	1.0	
	Black	*BRCA2*	c. 5771_5774delTTCA	4/16	25.0	
Schoeman et al., 2013 [13]	White, Mixed Ancestry, Black	*BRCA1*	c. 2641G > T; p. E881*	7/302	2.3	SSCP/HA
		BRCA1	c. 68_69delAG	2/302	0.7	
		BRCA1	c. 1374delC	2/302	0.7	
		BRCA1	c. 5266dupC	1/302	0.3	
		BRCA2	c. 7934delG	17/302	5.6	
		BRCA2	c. 5771_5774delTTCA	7/302	2.3	
		BRCA1	N/A	4/302	1.3	PTT
		BRCA2	N/A	5/302	1.7	
		BRCA1	N/A	2/302	0.7	Sequencing
		BRCA2	N/A	2/302	0.7	Sequencing
		BRCA1	N/A	18/302	6.0	

PTT protein truncation test, *SSCP/HA* PCR-single strand conformation polymorphism/heteroduplex analysis, *N/A* mutations were not described; * indicates the presence of a premature stop codon (cfr. nomenclature HGVS (Human Genome Variation Society))

European ancestry. We applied a cost efficient next generation sequencing (NGS) approach for analysis of the complete coding regions of *BRCA1*, *BRCA2* and *PALB2* [28]. Furthermore, large rearrangements have been reported in both *BRCA1* and *BRCA2* in several populations which may be missed by sequencing. We therefore complemented the sequencing approach with multiplex ligation-dependent probe amplification (MLPA), for these two genes.

Methods

Patients

EDTA blood samples of 108 breast cancer patients were collected from breast clinics in two state hospitals and a private hospital in Johannesburg - Charlotte Maxeke Johannesburg Academic Hospital, Chris Hani Baragwanath Academic Hospital and Wits Donald Gordon Medical Centre respectively. Patients were selected if their tumour was triple-negative (TN), and/or their breast cancer diagnosis was premenopausal. All patients were categorized as black, white, Indian or coloured based on patients' self-reported data from questionnaires. The cohort consisted of 85 black patients (78.7 %), 16 white patients (14.8 %), 5 Indians (4.6 %) and 2 coloureds (1.9 %). Table 2 presents the overview of the distribution of ethnicity in the cohort. All patients signed informed consent. Pathology data were obtained from the hospital files. Genetic counselling was offered to the patients, prior to obtaining their consent.

The study was approved by the Human Research Ethics Committee (Medical), University of the Witwatersrand (No. M091023; M110922; M130450).

DNA extraction

Genomic DNA was extracted from 4 - 6 ml of peripheral blood using a modified version of the standard salting out method [29].

Target enrichment, library preparation and sequencing

BRCA1, *BRCA2* and *PALB2* analysis was successfully conducted on 108 samples using Illumina's Miseq desktop sequencer. Target enrichment was achieved by high throughput PCR. Primers were designed for the complete coding region including splice site regions of *BRCA1* (31 amplicons), *BRCA2* (42 amplicons) and *PALB2* (19 amplicons) using Primer XL (www.pxlence.com). PCR conditions according to the protocol described by De Leeneer et al. were utilised [28].

Library preparation was performed using a modified version of the Nextera XT (Illumina) protocol. Sequencing was conducted on the MiSeq v2 instrument (Illumina Inc.) according to manufacturer's instructions. The approach is described in detail by De Leeneer et al. [28].

Table 2 Overview of distribution of ethnicity in our South African cohort

		Black (%)	White (%)	Indian (%)	Coloured (%)
Dx < 50 *n* = 92	TNBC	7 (7.6)	4 (4.3)	2 (2.2)	1 (1.1)
	Not TNBC	70 (76.1)	5 (5.4)	2 (2.2)	1 (1.1)
Dx > 50 *n* = 16	TNBC	8 (50.0)	7 (43.8)	1 (6.3)	0
Total *n* = 108		85 (78.7)	16 (14.8)	5 (4.6)	2 (1.9)

Dx: Age at diagnosis

Sanger sequencing

All genetic variants and pathogenic mutations identified via NGS were confirmed with Sanger sequencing. For confirmation by Sanger sequencing, an independent PCR amplification step was performed. In addition, the presence of all deleterious mutations was confirmed on an independently extracted DNA sample. All fragments with a coverage of <28× were also analysed by Sanger sequencing. For an overview of the number of amplicons that required Sanger sequencing, refer to Additional file 2: Table S2.

Nucleotide positions and protein translation correspond to reference sequence and Genbank account number NM_007294.3; NP_009225.1 for *BRCA1*, NM_000059.3; NP_000050.2 for *BRCA2*, NM_024675.3; NP_078951.2 for *PALB2* and NM_007194.3 for *CHEK2* c.1100delC. Nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide 1.

MLPA

Large genomic rearrangements and/or gene dosage alterations in both the *BRCA1* and *BRCA2* genes were screened for in 108 patient samples using MLPA. *BRCA1* MLPA analysis was performed using the SALSA MLPA P002 probemix (version C2-1113) (MRC-Holland) and *BRCA2*/*CHEK2* MLPA using the SALSA MLPA P045 probemix (version B3-1113) (MRC-Holland). MLPA setup was performed according to the manufacturer's protocol. Fragment detection and sizing was conducted using capillary gel electrophoresis on the ABI 3730XL genetic analyser (Applied Biosciences). All fragments positive for the *CHEK2* mutation (c.1100delC) in the MLPA analysis were confirmed with Sanger sequencing.

The screening was performed in a research setting. We used the infrastructure and the protocols supplied by a molecular diagnostic laboratory with an ISO15189 accreditation.

Data analysis

Mapping of sequencing data was performed with CLC bio Genomics Workbench v6 software (CLC bio Inc.). Various in-house scripts were used for sequence analysis [28]. The Sanger sequencing data were analysed using SeqPilot v4.1.2 build 512 and SeqSpace v2.5.0. MLPA data were analysed using Coffalyser (MRC-Holland).

Variants of unknown significance (VUS) were evaluated using *in silico* mutation interpretation software – Alamut. We used the computational algorithms of SIFT, AlignGVGD, Polyphen and Mutation Taster for missense varaints and the splice site prediction programs SpliceSiteFinder, MaxEntScan, NNSPLICE, GeneSplicer and Human Splicing Finder for intronic, silent and missense variants. Based on these predictions and in

combination with a study of the literature and published minor allele frequencies, variants were classified in five classes. Unfortunately, due to limited availability of data, Bayesian likelihood analyses could not be performed to calculate the degree of likelihood of pathogenicity. Therefore, we applied the following rules:

- Variants with a MAF (minor allele frequency) of > 0.01 were classified as class 1 (data not shown)
- Variants were classified as class 2 if all prediction programs provided neutral scores (data not shown)
- Variants with two or more programs with deleterious predictions were allocated to class 3 (Table 5)
- All truncating and unequivocal splice site variants were considered as deleterious, in addition to missense variants in the RING domain of BRCA1 (class 4–5) (Table 3)

Statistical analysis

Mutation frequency was calculated with 95 % confidence intervals. The Fisher's exact test was used to compare mutation frequencies in the different groups of patients. Statistical analysis was performed with Graphpad Prism software.

Results

In the total study population ($n = 108$), 15 heterozygous pathogenic mutations in 14 patients were identified (12.9 %; 95 % CI = 7.3–20.8 %): six in *BRCA1,* seven in *BRCA2*; two patients were found to carry *CHEK2* c.1100delC of which one patient also harboured a deleterious *BRCA2* mutation. All mutations were identified by sequencing on Miseq, except a large deletion in *BRCA1* and the *CHEK2* c.1100delC mutation which were detected by MLPA. No unequivocal deleterious mutations were identified in the *PALB2* gene (Table 3).

The distribution of *BRCA1/2* mutations among the different subgroups (TNBC and/or PM) and based on ethnicity is presented in Table 4. A significantly higher mutation detection ratio was obtained within the group of TNBC patients (7/30; 23.3 %; 95 % CI = 9.9–42.3 %) compared to the premenopausal breast cancer group without TNBC (6/78; 7.7 %; 95 % CI = 2.9–16.0 %) ($p = 0.0432$). Not surprisingly, the highest mutation detection ratio was obtained within the subgroup of TNBC patients diagnosed before the age of 50 (5/14; 35.7 %; 95 % CI = 12.7–64.9 %).

The *BRCA2* c.7934delG Afrikaner founder mutation was identified in 2 (white) patients, one with TNBC and one diagnosed with premenopausal breast cancer. In the black patient population, two previously unreported mutations were identified in *BRCA1* (c.1155G > A and c.1953_1954insA) and one in *BRCA2* (c.582G > A) (see Table 3). Six (6/85; 7.1 %; 95 % CI = 2.6–14.7 %) pathogenic *BRCA1/2* mutations were observed in the black population group and five (5/16; 31.3 %; 95 % CI = 11.0–58.7 %) in the white population group. Two mutations were identified in the Indian group (2/5; 40 %; 95 % CI = 5.3–85.3 %) and no mutations were identified either in

Table 3 *BRCA1, BRCA2* and *CHEK2* germline pathogenic mutations identified in triple negative and premenopausal breast cancer patients using NGS and MLPA

Patient no.	Ethnicity	Category	Gene	Exon	Nucleotide change	Amino acid change	Mutation effect	Reference
1	White	TNBC/PM	*BRCA1*	4	c.181 T > G	p.Cys61Gly	Missense	[53]
2	Black	TNBC/PM	*BRCA1*	4	c.212G > A	p.Arg71Lys	Missense	[54]
3	Indian	TNBC/PM	*BRCA1*	10	c.3593 T > A	p.Leu1198*	Nonsense	[55]
4	Black	PM	*BRCA1*	10	c.1155G > A	p.Trp385*	Nonsense	Novel
5	Black	PM	*BRCA1*	10	c.1953_1954insA	p.Lys652fs	Frameshift	Novel
6	White	TNBC	*BRCA1*[a]	1–2	-	-	Deletion	[30]
7	Black	PM	*BRCA2*	7	c.582G > A	p.Trp194*	Nonsense	Novel
8	Black	TNBC	*BRCA2*	11	c.5771_5774delTTCA	p.Ile1924fs	Frameshift	[10]
9	White	PM	*BRCA2*	11	c.5213_5216delCTTA	p.Thr1738fs	Frameshift	[56]
			CHEK2[a]	11	c.1100delC	p.Thr367fs	Frameshift	[39]
10	White	TNBC	*BRCA2*	17	c.7934delG	p.Arg2645fs	Frameshift	[10]
11	White	PM	*BRCA2*	17	c.7934delG	p.Arg2645fs	Frameshift	[10]
12	Indian	TNBC/PM	*BRCA2*	21	c.8754 + 1G > A	Non-coding	Splice site	[57]
13	Black	PM	*BRCA2*	23	c.9097_9098insA	p.Thr3033fs	Frameshift	[53]
14	White	PM	*CHEK2*[a]	11	c.1100delC	p.Thr367fs	Frameshift	[39]

PM Premenopausal
[a]MLPA results
*indicates the presence of a premature stop codon (cfr. nomenclature HGVS (Human Genome Variation Society))

Table 4 *BRCA1* and *BRCA2* germline pathogenic mutations identified using NGS and MLPA in a South African cohort divided according to premenopausal diagnosis, triple negative status and ethnicity

Total *n* = 108	Dx < 50 *n* = 92 (85.2 %)				Dx > 50 *n* = 16 (14.8 %)		Total no. of mutations per ethnic group
	TNBC *n* = 14 (13.0 %)		Not TNBC *n* = 78 (72.2 %)		TNBC		
Black *n* = 85 (78.7 %)	*n* = 7		*n* = 70		*n* = 8		6 (7.1 %)
Mutations	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2	
	c.212G > A	-	c.1155G > A	c.582G > A	-	c.5771_5774delTTCA	
	-	-	c.1953_1954insA	c.9097_9098insA	-	-	
White *n* = 16 (14.8 %)	*n* = 4		*n* = 5		*n* = 7		5 (31.3 %)
Mutations	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2	
	c.181 T > G	c.7934delG	-	c.7934delG	Exon 1a-2 del	-	
	-	-	-	c.5213_5216delCTTA	-	-	
Indian *n* = 5 (4.6 %)	*n* = 2		*n* = 2		*n* = 1		2 (40.0 %)
Mutations	BRCA1	BRCA2	BRCA1	BRCA2	BRCA1	BRCA2	
	c.3593 T > A	c.8754 + 1G > A	-	-	-	-	
Coloured *n* = 2 (1.9 %)	*n* = 1		*n* = 1		0		0
Mutations	-		-		-		
Total mutations per subgroup	5 (35.7 %)		6 (7.7 %)		2 (12.5 %)		

BRCA1 or *BRCA2* in the two coloured individuals studied.

To detect large genomic rearrangements in *BRCA1* and *BRCA2*, 108 samples were analysed using MLPA. A white TNBC patient was found to be heterozygous for a *BRCA1* exon 1a-2 deletion. Several deletions including these exons but with different breakpoints have previously been described (for an overview of deletions affecting these exons: [30]). As the number of large rearrangements reported in *PALB2* is extremely small [31], MLPA for *PALB2* was not conducted in this cohort.

The *CHEK2* mutation (c.1100delC) was observed in 2/108 (1.9 %) patients. Both of these patients were white, premenopausal patients. One of these patients was also positive for a deleterious *BRCA2* mutation.

In addition to pathogenic mutations, several VUS were identified: 1 in *BRCA1*, 3 in *BRCA2* and 2 in *PALB2*. In Table 5 we provide an overview of the variants which were classified as class 3 based on *in silico* prediction programs. Three of the four *in silico* prediction programs used classified the *BRCA2* variant c.9875C > T and c.7712A > G as "probably damaging". The *BRCA2* variant c.9875C > T was

Table 5 *In silico* predictions obtained for variants of unknown significance in the South African cohort

					In silico prediction programs					
Ethnicity	Variant	Gene	Amino acid change	Occurrence	Classification	Align GVGD[a]	SIFT	Mutation Taster	PolyPhen	Refs
Black	c.1843_1845delTCT	*BRCA1*	p.Ser615del	1	3	-	-	-	-	[58–60]
Black	c.4798_4800delAAT	*BRCA2*	p.Asn1600del	1	3	-	-	-	-	[61]
Black	c.7712A > G	*BRCA2*	p.Glu2571Gly	1	3	C0	Deleterious	Disease causing	Probably damaging	[62]
Black	c.9875C > T	*BRCA2*	p.Pro3292Leu	2	3	C0	Affect protein function	Disease causing	Probably damaging	[63]
Black	c.118A > G	*PALB2*	p.Arg40Gly	1	3	C0	Affect protein function	Polymorphism	Probably damaging	Novel
Black	c.2845 T > C	*PALB2*	p.Cys949Arg	1	3	C0	Affect protein function	Disease causing	Probably damaging	Novel

[a]Spectrum of prediction classes (C0, C15, C25, C35, C45, C55, C65) with C0 less likely to be deleterious and C65 most likely

identified in two black patients. Two of the four prediction programs consulted classified the *PALB2* variants c.118A > G and c.2845 T > C as "probably damaging".

Discussion

The current study is the first study performing mutation analyses in *BRCA1, BRCA2* and *PALB2* and determining the frequency of *CHEK2* c.1100delC in triple negative and/or premenopausal breast cancer patients in South Africa through both next generation sequencing and large rearrangement testing. In total we detected 13 *BRCA1/2* mutations in our study cohort of 108 patients (12 %; 95 % CI = 6.6–19.7 %), thus reinforcing the important contribution of germline *BRCA1* and *BRCA2* mutations to inherited breast cancer in this mixed South Africa cohort. Two patients harboured a *CHEK2* c.1100delC mutation, one of them in combination with a deleterious *BRCA2* mutation. Previous studies done on South African breast cancer populations reported *BRCA1/2* mutation frequenciess of 1 to 25 % [7–10] (for an overview: see Table 1). The prevalence of mutations in *BRCA1/2* genes in these South African studies varies by inclusion criteria, ethnicity and mutation screening techniques used. None of these studies looked specifically at TNBC or premenopausal patients.

The mutation frequency was higher in the subgroup of TNBC than in the premenopausal breast cancer patients: 23.3 % (7/30) of TNBC patients harbour a pathogenic mutation in either *BRCA1* or *BRCA2,* compared to 12.0 % (11/92) of all premenopausal breast cancer patients.

Various studies have shown the frequency of *BRCA1* mutations to be higher than *BRCA2* in patients exhibiting the triple negative phenotype [27, 32, 33]. In our study 13.3 % (4/30) of TNBC patients had a pathogenic mutation in *BRCA1* compared to 10 % (3/30) in *BRCA2.*

In our premenopausal cohort, the prevalence of *BRCA1* mutations were similar (5/92; 5.4 %) to *BRCA2* mutations (6/92; 6.5 %). *BRCA2* mutations are in general less frequent than *BRCA1* in younger white women with breast cancer [19]. A relatively high number of *BRCA2* mutations compared to *BRCA1* has been reported in other studies of young black populations [34–36] and is contradictory to the scenario in Western populations. This could be due to the unique genetic background of African patients.

In the black population, the overall frequency of mutations identified was 7.1 % as compared to 31.3 % in the white population. Due to the presence of the *BRCA2* c.7934delG Afrikaner founder mutation, *BRCA2* is the most important contributor in the white population in our study cohort, while *BRCA1* and *BRCA2* mutations were observed in equal numbers in the black patients studied. We identified neither the Ashkenazi Jewish nor the Xhosa mutations in our study groups. Our patient cohort was recruited in the region of Johannesburg and is characterized by diverse population structure/ethnic backgrounds. Therefore we did not anticipate finding a large number of founder mutations.

The *CHEK2* c.1100delC allele contributes to a moderate increased breast cancer risk. The frequency is estimated to be only 1 % in familial breast cancer and 0.5 % in early onset breast cancer [37, 38]. In the Dutch population the prevalence in the general population is 1.1 %, 2.5 % in unselected breast cancer cases, and up to 4.9 % in familial breast cancer cases [39]. Within our South African cohort we identified this allele in two white patients (2/16 = 12.5 %), but in none of the patients from other ethnicities (0/92). White Afrikaner South Africans mainly descend from Dutch immigrants which could explain the higher percentage of *CHEK2* c.1100delC in this cohort.

Previous studies that aimed to clarify the prevalence of *BRCA1/2* mutations in black populations from other parts of Africa and African Americans have indicated similar rates [6, 22, 27, 36, 40]; although it is difficult to compare them since eligibility criteria for study participation varies extensively. Churpek et al. [40] reported a pick-up rate of 26 % (47/180) for pathogenic mutations in a group of black patients with early onset disease (age of diagnosis <45) and 25 % pick-up rate (26/103) for pathogenic mutations in triple negative black patients. Here we report *BRCA1/2* mutation frequency of 14 % (1/7) in the premenopausal triple negative black subgroup. Our overall mutation detection rate of *BRCA1/2* mutations in the black premenopausal breast cancer patients was 6.5 % (5/77). This is similar to the mutation rate reported in a study by Pal et al. [22] in young black African American breast cancer patients (9 %; 13/144). Although the prevalences are similar among the studies on West African, African American breast cancers and our study, we identified 3 novel mutations in the South African black patients. Furthermore, historical evidence has shown that African Americans descend from West African ancestry and so it is not surprising that there are some differences between these two and the South African black population, who have some distinct genetic differences at the population level [12, 41].

Large genomic rearrangements in *BRCA*, detected with MLPA, were only observed in 0.9 % (1/108) of our cohort. No large rearrangements were identified in the black South African breast cancer patients. Generally, low frequencies for large rearrangements have been reported in black patients, e.g. Pal et al., [22], detected 2 rearrangements in 144 young African-American women with breast cancer (1.4 %), both of which were in *BRCA1*. Zhang et al., [42] reported one *BRCA1* exon deletion (0.3 %) in a cohort of 352 Nigerian breast cancer

patients. In another South African study on 52 unrelated families of European ancestry, only 1 large deletion was detected in *BRCA1* [9]. The lack of detection in *BRCA2* led the authors to suggest that large rearrangements in *BRCA2* might not play a role in inherited breast cancer in South African patients [9]. However, to draw final conclusions on the presence of large rearrangements in both white and black South African breast cancer patients, a larger patient population should be extensively studied.

Gene sequencing techniques also resulted in the identification of several VUS. Based on *in silico* predictions, we assigned a class (class 1– 3) to each VUS for clinical interpretation [43]. VUS with a probability of increased pathogenicity are assigned a higher class. A number of studies have presented models and performed functional assays for the classification of VUS in *BRCA1/2* [43–46]. We detected six VUS in the 85 black patients of our cohort and none in the 16 white patients. Also other studies suggested that the frequency of VUS is higher in patients of African descent, for instance Nanda et al. [47].

A previous study conducted in a South African cohort revealed a pathogenic *PALB2* mutation in 2 % of early onset white breast cancer patients [8]. Our cohort consisted of a small number of white patients and no unequivocal deleterious mutations in *PALB2* were identified. However two missense variants with suggestive *in silico* predictions were identified (Table 5) that warrant further functional analyses. Until recently, the pathogenic effect of *PALB2* missense variants has not been firmly proven. For some missense variants in the WD40 domain (from amino acids 853–1186) [48] altered patterns of direct binding to the RAD51C, RAD51 and BRCA2 h proteins in biochemical assays have been shown [49]. We identified a missense variant in the WD40 domain (c.2845 T > C; p.Cys949Arg). In order to elucidate the pathogenicity of missense variants in *PALB2*, additional (functional, segregation) analyses are required.

We focused on identifying mutations in *BRCA1*, *BRCA2* and *PALB2* and the *CHEK2* c.1100delC mutation, as the risks for the development of breast and associated cancers with these genes have been determined by analysing large study populations. The search for the remaining genetic contribution towards breast and ovarian cancer has been carried out extensively, with numerous other genes being identified. However, at this time, the contribution and associated risks of mutations in most of these genes is not yet well established. As the prevalence of mutations in each of these genes is much lower than germline *BRCA1/2* mutations in the large cohorts (white American) of patients investigated up until now [50], international collaborations in populations of different ethnicities will be required to gain insight into the exact risks associated with mutations in these genes.

Conclusion

This study is the first to evaluate the use of NGS technology as a diagnostic testing platform for inherited breast cancer in a South African cohort. The results presented herein are particularly relevant for inherited cancer testing in the black population of South Africa, a previously under-researched group. The NGS approach applied [28] is a cost and time effective approach; it shows great promise for *BRCA1/2* screening in developing countries like South Africa. The advent of NGS allows the costs of mutation analysis to fall dramatically, which should allow testing to become more widely available, especially in countries with limited healthcare resources, like South Africa. This will create opportunities to improve patient treatment and challenges for breast cancer multidisciplinary teams. The finding of a germline deleterious mutation could alter treatment decisions; for instance, women with germline mutations might opt for more radical surgery or may consider prophylactic surgery to the contralateral breast or ovaries.

Our results have highlighted the contribution of *BRCA1/2* germline mutations in South African breast cancer patients with triple negative breast tumours and/or premenopausal breast cancer of different ethnicities.

Additional files

Additional file 1: Table S1. Overview of grading and staging of breast cancer on diagnosis (DOC 30 kb)

Additional file 2: Table S2. Overview of sequencing coverage per run (DOC 29 kb)

Abbreviations

MAF: Minor allele frequency; MLPA: Multiplex ligation-dependent probe amplification; NGS: Next-generation sequencing; PM: Premenopausal; TN: Triple negative; TNBC: Triple negative breast cancer; VUS: Variants of unknown significance.

Competing interests

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

Authors' contributions

FZF, TW carried out the molecular work, analysed data and helped draft the manuscript. KDL, BC, IC carried out the molecular work and analysis of data. AC, MM, SN, HC, BP, TVM provided samples for this study. RK, JPS, AV, AK revising the manuscript. AB, KBMC design of the study and drafting the manuscript. All authors have read and approved the manuscript.

Acknowledgements

This research was supported by a 'VLIR Own Initiative Programme' between Belgium and South Africa (ZEIN2011PR387), a grant of the South African Medical Research Council, a grant from CANSA (Cancer Association of South Africa) and partially funded by Stichting tegen Kanker (project 2012–198). The authors wish to thank Mrs. Marianne Gomes for providing genetic counselling to a number of patients in the study. Additionally the lab staffs at the Center for Medical Genetics, Ghent University Hospital, Belgium and

the Division of Human Genetics, NHLS, and School of Pathology, University of the Witwatersrand, Johannesburg are acknowledged for their assistance. We are also grateful to Dr. Sarah Rayne for helping in collecting samples. We would like to thank all the patients who participated in this study.

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Received: 24 March 2015 Accepted: 5 November 2015
Published online: 17 November 2015

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CHAPTER 7

Diagnosis of Fanconi Anaemia by ionising radiation- or mitomycin C-induced micronuclei

DNA Repair 61 (2018) 17–24

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Diagnosis of Fanconi Anaemia by ionising radiation- or mitomycin C-induced micronuclei

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ARTICLE INFO

Keywords:
Fanconi Anaemia
DNA repair
Chromosomal radiosensitivity
Genomic instability
Radiosensitivity

ABSTRACT

Fanconi Anaemia (FA) is an autosomal recessive disorder characterised by defects in DNA repair, associated with chromosomal instability and cellular hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC). The FA repair pathway involves complex DNA repair mechanisms crucial for genomic stability. Deficiencies in DNA repair genes give rise to chromosomal radiosensitivity. FA patients have shown increased clinical radiosensitivity by exhibiting adverse normal tissue side-effects. The study aimed to investigate chromosomal radiosensitivity of homozygous and heterozygous carriers of FA mutations using three micronucleus (MN) assays. The G0 and S/G2 MN assays are cytogenetic assays to evaluate DNA damage induced by ionising radiation in different phases of the cell cycle. The MMC MN assay detects DNA damage induced by a crosslinking agent in the G0 phase. Patients with a clinical diagnosis of FA and their parents were screened for the complete coding region of 20 FA genes. Blood samples of all FA patients and parents were exposed to ionising radiation of 2 and 4 Gy. Chromosomal radiosensitivity was evaluated in the G0 and S/G2 phase. Most of our patients were homozygous for the founder mutation *FANCG* c.637_643delTACCGCC; p.(Tyr213Lysfs*6) while one patient was compound heterozygous for *FANCG* c.637_643delTACCGCC and *FANCG* c.1379G > A, p.(Gly460Asp), a novel missense mutation. Another patient was compound heterozygous for two deleterious *FANCA* mutations. In FA patients, the G0- and S/G2-MN assays show significantly increased chromosomal radiosensitivity and genomic instability. Moreover, chromosomal damage was significantly elevated in MMC treated FA cells. We also observed an increase in chromosomal radiosensitivity and genomic instability in the parents using 3 assays. The effect was significant using the MMC MN assay. The MMC MN assay is advantageous as it is less labour intense, time effective and has potential as a reliable alternative method for detecting FA patients from parents and controls.

1. Introduction

Fanconi Anaemia (FA) is primarily an autosomal recessive disorder that is clinically characterized by congenital malformations, progressive development of hypoplastic anaemia and cancer predisposition that often results in haematological malignancies such as acute

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https://doi.org/10.1016/j.dnarep.2017.11.001
Received 28 June 2017; Received in revised form 25 August 2017; Accepted 3 November 2017
Available online 08 November 2017
1568-7864/

myelogenous leukaemia (AML) or myelodysplasia (MDS) as well as various solid tumours, especially head and neck squamous carcinoma. Recent determination of the carrier frequency gave an estimate of more than 1/200 [1], with an expected prevalence at birth of at least 1/160 000. In certain populations, the carrier frequency is much higher, due to founder mutations. In South Africa, with mixed ethnicity in the population, the prevalence of FA ranges between 1/22 000 for the white Afrikaners to 1/40 000 in the black South Africans [2,3].

Twenty-one different FANC genes have been reported in literature *FANCA*, *FANCB*, *FANCC*, *FANCD1* (also known as *BRCA2*), *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ* (also known as *BRIP1* or *BACH1*), *FANCL*, *FANCM*, *FANCN* (also known as *PALB2*), *FANCO* (also known as *RAD51C*), *FANCP* (also known as *SLX4*), *FANCQ* (also known as *XPF* or *ERCC4*), *FANCR* (also known as *RAD51*), *FANCS* (also known as *BRCA1*), *FANCT* (also known as *UBE2T*), *FANCU* (also known as *XRCC2*) [4,5], and very recently *REV7* (also known as *MAD2L2* or *FANCV*) was identified as a novel FA gene [6]. FA is most often inherited in an autosomal recessive manner, however, also autosomal dominant (*FANCR/RAD51*) and X-linked forms (*FANCB*) have been reported [7]. Next generation sequencing (NGS) allows efficient identification of causal mutations in this genetically heterogeneous disease. Deleterious mutations in *FANCA*, *FANCC* and *FANCG* are identified in 80–90% of the FA cases [8].

All currently known FA genes encode for a cluster of proteins responsible for repair of stalled DNA replication forks by unhooking DNA interstrand cross-links (ICL) and promoting homologous recombination (HR). The FA/BRCA repair pathway is pivotal in maintaining genomic stability. The FANC genes are sub grouped into 3 main categories known as (i) the core complex (ii) ID2 complex and (iii) the downstream effectors. In response to damage caused by DNA crosslinking agents or ionising radiation (IR), the activated core complex formed by 8 FA genes (A, B, C, E, F, G, M, and L) activates the ID2 complex, comprised of *FANCD2/FANCI* gene, by mono-ubiquitination and phosphorylation. The mono-ubiquitination of ID2 is catalysed by the E2 ubiquitin-conjugating enzyme *FANCT/UBE2T* [9]. The ID2 complex plays a critical role in the pathway by translocating to the damage sites which triggers the recruitment of downstream effectors (D1, J, N, O, P, Q, R, S and U) in the S/G2 phase. The damage is subsequently repaired by HR. Following the repair, the ID2 complex undergoes deubiquitination and the core complex genes are unassembled [10,4,11]. The latest identified FA gene, *REV7/FANCV*, a subunit of DNA polymerase ζ involved in translesion DNA synthesis (TLS) seems to act as a necessary downstream effector of the FA-BRCA pathway, most likely functioning in the TLS step of ICL repair [12].

In FA patients' cancer treatment by chemotherapy or radiotherapy is complicated because of the possibility of side effects due to the underlying defect in DNA damage response. Sensitivity to chemotherapy and radiotherapy in FA patients has previously been documented [13,14]. Radiosensitivity is the susceptibility of cells to the DNA damaging effects of IR. Exposure to IR causes a variety of DNA damage of which the double strand breaks are the most important [15]. Double strand breaks (dsb) are repaired predominantly by two main DNA repair pathways: i) HR which requires a homologous DNA strand for repair and ii) non-homologous end-joining (NHEJ). The cell is selective on which DNA repair pathway to use depending on the phase of the cell cycle. In the S/G2 phase where a homologous strand is present, the HR repair pathway is preferred over NHEJ. The NHEJ repairs double strand breaks is predominant in the remaining phases of the cell cycle [16].

Some older studies suggested that FA patients, similar to patients with ataxia telangiectasia or Nijmegen breakage syndrome, are characterised by an increased *in vitro* chromosomal radiosensitivity [17,18], while others report the contrary [19]. However, literature on chromosomal radiosensitivity of FA patients is very limited.

It is well established that FA cells are hypersensitive to DNA crosslinking agents such as mitomycin C (MMC) and diepoxybutane (DEB) [20]. Chromosome fragility is pathognomonic in the diagnosis: FA is classically diagnosed by treating lymphocytes of patients with MMC or DEB and subsequent quantification of all types of chromosomal breakages and radial forms [21]. The major challenge with this assay is the low quality slides and the need of highly experienced personnel to analyse chromosomal breakages; furthermore, it is labour intense and time consuming. We propose here a more robust assay to identify FA patients by detecting micronuclei (MNi) and evaluate if this can be used to distinguish heterozygous carriers from non-carriers. The cytokinesis-block micronucleus assay (CBMN) in human lymphocytes is a well-established assay for measuring chromosomal aberrations after *in vivo* or *in vitro* exposure to genotoxic agents. Here, we propose a novel protocol where MMC is applied to induce MN. Furthermore, we evaluated two MN protocols using ionising radiation as genotoxic agent: the G0 MN assay, a well-known cytogenetic assay where cells are irradiated in the G0 phase of the cell cycle [22,23], and a S/G2 MN assay, in which the damage is induced in the S/G2 phase [24,25]. In the S/G2 MN assay, cells were also subjected to caffeine treatment. The addition of caffeine to cells abrogates the G2/M cell cycle checkpoint. The abrogation of the checkpoint permits the progression of damaged cells into mitosis [26]. In this way the efficiency of the repair pathway activated when cells are irradiated in the S/G2 phase can be evaluated.

2. Methods and material

2.1. Study population

Thirteen patients with clinical manifestation of FA and their parents were recruited from the Paediatric Oncology department at Charlotte Maxeke Johannesburg Academic hospital and Chris Hani Baragwanath hospital in South Africa. In addition, one patient and parent was recruited from Cliniques Universitaires Saint-Luc, Belgium. In total, fourteen FA patients and 14 parents were enrolled in the study and heparin blood samples were collected. Blood samples from 14 healthy donors were also collected from student and staff members from the university and hospital. The mean age of the patients is 11 years. The youngest patient enrolled in the study was 5 years and the oldest being 17 years. The mean age of the parents enrolled in the study was 40 years and healthy individuals had a mean age of 30 years. Informed consents were obtained from all donors. Ethics for the study was approved by the Wits Human Research Ethics Committee (Medical) (clearance number M141031).

2.2. Mutation analysis

2.2.1. DNA extraction

Genomic DNA was extracted from peripheral blood using the automated Tecan Freedom EVO®-HSM Workstation (Promega). DNA extraction for 14 patients with clinical manifestations of FA and 14 parent samples was carried out as per manufacturer's instructions.

2.2.2. Target enrichment, library preparation and sequencing

Singleplex PCR reactions were performed for the coding region of all 20 known FA genes. Primers for the coding regions and splice site regions of all the genes were designed using PrimerXL (www.pxlence.com). The PCR conditions and the modified version of the Nextera XT (Illumina) library preparation protocol utilised in this study were conducted as previously described [27]. Subsequently sequencing was performed on the Miseq instrument (Illumina).

2.2.3. Sanger sequencing

All pathogenic mutations and variants classified as likely pathogenic were confirmed by Sanger sequencing on the ABI3730XL (ThermoFisher) instrument in the patients. Carriership of a heterozygous mutation was also confirmed in DNA of the parents available.

2.2.4. MLPA

In order to detect large exon spanning deletions/duplications, MLPA was conducted using the commercially available MLPA kits (MRC-Holland) for some FA genes (MLPA P031-*FANCA* mix 1 and P032-*FANCA* mix 2 (version: B2-0116), P057-*FANCD2-PALB2* (version: B2-0415) and P260-*PALB2-RAD50-RAD51C-RAD51D* (version: B1-1114)). Fragment separation is achieved by capillary electrophoresis using the ABI model 3730XL sequencer (ThermoFisher).

2.2.5. Data analysis

Sequencing data obtained from the Miseq run was mapped using CLC Bio Genomics Workbench v7 software (CLC bio Inc.). The sequencing analysis was also conducted using other in-house scripts as previously described [27]. Sanger sequencing data were analysed using the SeqPilot software v4.1.2 build 512. Coffalyer.NET (MRC-Holland) software was used for MLPA data analysis.

2.3. The cytokinesis-block micronucleus (CBMN) assay to assess radiation induced DNA damage

2.3.1. G0 CBMN assay

To quantify the chromosomal radiosensitivity of FA patients and heterozygous carriers, the MN assay was performed as previously described. Heparinised blood (0.5 ml) was added to 4.5 ml of pre-warmed RPMI-1640 supplemented with L-glutamine (Bio-Whittaker, USA), 13% of foetal bovine serum (FBS; Gibco-Invitrogen, USA) and antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin; Gibco-Invitrogen, USA). The cells were irradiated in the G0 phase of the cell cycle with doses of 2 and 4 Gy 6 MV X-rays at a dose rate of 1.33 Gy/min using a linear accelerator (Siemens Healthcare, Germany). A sham irradiated control (0 Gy) culture was also set up to detect spontaneously occurring MNi. After irradiation, cell division was stimulated in lymphocytes by the addition 100 µl of phytohaemagglutanin (PHA − stock solution 1 mg/ml; Sigma-Aldrich, USA), and cytokinesis was blocked after 23hr by adding 20 µl cytochalasin B (Cyto B − stock solution 1.5 mg/ml; Sigma-Aldrich, USA). Seventy hours post PHA-stimulation, cells were harvested using a cold hypotonic shock of 7 ml of KCL (0.075 M; Merck, Germany) and fixed in a methanol: acetic acid: ringer solution (4:1:5) (Merck, Germany). After overnight storage at 4 °C, the cells were further fixed 3 times by using a methanol: acetic acid solution (4:1). A suspension of cells (40 µl) was dropped onto slides and stained with acridine orange stain (10 µg/ml) (Sigma-Aldrich, USA). Duplicate slides were made of each sample, coded and 500 BN cells per slide were scored using the Zeiss Axioskop fluorescent microscope (Carl Zeiss, Gottingen, Germany). To obtain radiation-induced MN values, the spontaneous MN were deducted from the MN values of irradiated samples.

2.3.2. S/G2 CBMN assay

Compared to the G0 MN assay, this assay is modified to analyse chromosomal radiosensitivity when cells are irradiated in the S/G2 phase of the cell cycle as previously described [25]. In short, heparinised blood (0.5 ml) in culture medium was set up and lymphocyte stimulation was immediately achieved by addition of 100 µl PHA (stock solution 1 mg/ml; Sigma-Aldrich, USA). Following stimulation, the cultures were incubated at 37 °C and 5% CO_2. The lymphocytes were irradiated after 72 h to doses of 2 and 4 Gy. To detect spontaneous MNi in the S/G2 phase of the cell cycle, a control (not irradiated) culture was also started. Cytokinesis was immediately inhibited after irradiation by the addition of 20 µl Cyto B (stock solution 1.5 mg/ml; Sigma-Aldrich, USA) and 200 µl caffeine (stock solution 100 mM; Sigma-Aldrich, USA) was added to a part of the cultures. The cells were harvested 8 h post irradiation and fixed as described above. Duplicate slides were stained, coded and 500 BN per slide were scored. Also here, radiation-induced MN values were obtained by deduction of spontaneous MN from the MN values of irradiated samples.

2.4. Cytokinesis-block micronucleus assay to assess MMC induced DNA damage

Mitomycin C Micronucleus assay: This assay was optimized to analyse the extent of chromosomal breakage induced by MMC in FA patients and parents (heterozygous carriers). Cultures were initiated by the addition of 0.5 ml heparinized blood in 4.5 ml culture medium. To optimize this technique, we used 2 different concentrations of MMC that can induce detectable damage. Concentrations of 0.02 µg/ml or 0.1 µg/ml MMC (stock solution 0.5 mg/ml; Sigma-Aldrich, USA) were added and lymphocytes were immediately stimulated with PHA (Sigma-Aldrich, USA). The cultures were incubated at 37 °C and supplemented with 5% CO_2. Similar to the G0 MN assay, 23 h later the cytokinesis was blocked by the addition of Cyto B (Sigma-Aldrich, USA). Cells were harvested 70 h post stimulation. The staining and scoring was conducted as described above. MMC-induced MN values were obtained by deducting spontaneous MN from the MN values in the samples treated with MMC.

2.5. Nuclear division index

The nuclear division index (NDI) is a measure of cell proliferation where poor nuclear division is indicated by a low NDI value and a higher NDI value suggests good proliferative capacity of the cells. The NDI was calculated for each of the assays by evaluating the number of mononucleate (N1), binucleate (N2), trinucleate (N3) and polynucleate (N4) cells. A total (Ntotal) of 500 cells per slide was scored. The formula used to calculate NDI is: NDI = (N1 + 2N2 + 3N3 + 4N4)/Ntotal.

2.6. Statistical analysis

GraphPad Prism 7 was used to analyse the statistical significance of the study. The comparison of the MN scores between the groups was conducted using the non-parametric Mann-Whitney. The significance level was set at < 0.05.

3. Results

3.1. Germline mutations

As expected in the 13 black South African patients the large majority (12/13) was found to be homozygous for the *FANCG* founder mutation c.637_643delTACCGCC; p. (Tyr213Lysfs*6). One patient was heterozygous for the *FANCG* c.637_643delTACCGCC mutation, and a novel heterozygous *FANCG* c.1379G > A, p. (Gly460Asp) missense variant, which has not been previously described. This variant is not reported in large databases like ExAC or gnomAD. By this substitution a highly conserved amino acid (Gly460) is changed into an amino acid (Asp) with different physicochemical properties (Grantham distance: 94 [0–215]). Prediction programs like SIFT, Polyphen and MutationTaster all support a deleterious effect for this variant. The clinical symptoms manifested in this patient included microcephaly, hypopigmentation, short stature, flattened thenar eminence and the bone marrow aspirate showed aplasia strongly supporting a FA diagnosis. No deleterious mutation was identified in the other FA genes screened.

The patient recruited in Belgium, of Cameroonian origin, was found to be compound heterozygote for two *FANCA* mutations: c.987_990delTCAC, p. (His330Alafs*4) and a large deletion spanning exons 22–28 (c.1901-?_2778 + ?del).

In all 14 parents, from whom DNA was available, we confirmed heterozygous carriership of the relevant *FANCG* or *FANCA* mutation.

3.2. G0 Micronucleus assay

Table 1 shows the data for the 3 endpoints scored in the G0 MN assay. The spontaneously occurring mean MNi values (non-irradiated

Table 1
G0 MN assay: Comparison of mean ± SD spontaneous (0 Gy) and radiation-induced (at 2 and 4 Gy) MNi values in 1000 binucleated cells of FA patients, parents and healthy controls.

	0 Gy	2 Gy	4 Gy
[a]Patients	27.93 ± 9.94*	356.6 ± 70.67**	915.0 ± 160.5**
[a]Parents	23.21 ± 9.80	286.5 ± 78.26	776.2 ± 185.3
[a]Controls	18.29 ± 8.73	255.4 ± 58.48	706.6 ± 179.6

[a] Mean and SD of 14 patients, 14 parents and 14 controls.
* Significantly different from controls ($p < 0.05$).
** Significantly different from parents and controls ($p < 0.05$).

cells) of the FA patients are significantly higher compared to the control group ($p = 0.01$; see Supplementary tables for p values) indicating genomic instability in the FA patients. The mean MNi values observed in BN cells irradiated with 2 Gy and 4 Gy are significantly higher in the patient group when compared to the control group (2 Gy: $p = 0.0003$; 4 Gy: $p = 0.0025$); however, MN frequencies between patients and controls overlap at an individual level. Significant differences in radiation-induced MNi are also observed between FA homozygotes and FA heterozygous carriers (2 Gy: $p = 0.02119$; 4 Gy: $p = 0.0444$). Although the MN yields were higher in the heterozygous carriers compared to controls, no significant differences in either the spontaneous or irradiated MNi values were demonstrated. In the 2 and 4 Gy irradiated samples respectively, a 1.5 and 1.3 fold increase of MNi was observed in the FA patients compared to controls. The parents showed a 1.1 fold increase both by 2 and 4 Gy radiations. The mean NDI values of the G0 cultures were lower in patients when compared to parents and controls. This difference was significant in irradiated cultures ($p < 0.05$).

3.3. S/G2 Micronucleus assay

Table 2 represents the response to DNA damage induction in the S/G2 phase of the cell cycle, the MNi values of cells irradiated in the S/G2 phase, in the presence of caffeine were compared between the 3 groups. In this phase of the cell cycle, spontaneously occurring MNi values in the FA patients and interestingly also in the heterozygous carriers were significantly higher compared to the control group. The differences were more pronounced than in the G0 phase. FA patients showed significant higher radiation-induced MN values than the heterozygous carriers and controls. Also, when adding caffeine we noticed significant differences in MNi between the FA patients and the control group after 2 Gy and 4 Gy. Similar to the G0 MN assay results, significant differences were observed at a group level. When comparing the FA heterozygotes with the control group significant differences were seen after irradiation with 4 Gy and addition of caffeine. In response to IR and caffeine, proliferative capacity of FA cells in the G2 phase was lower when compared to the controls ($p < 0.05$). For radiation-induced samples without caffeine, FA patients had a 1.9 and 2.1 fold increase in MNi values for the 2 and 4 Gy respectively, as compared to the controls. The fold increase in FA heterozygote carriers was 1.2 for both the 2 and 4 Gy radiation-induced samples. MNi values of radiation-induced samples in the presence of caffeine for both FA patients and parents were similar to the fold increase as those without caffeine.

Table 3
MMC MN assay: Comparison of mean ± SD spontaneous (0 μg/ml) and MMC-induced (at 0.02 and 0.1 μg/ml) MNi values in 1000 binucleated cells of FA patients, parents and healthy controls.

	0 μg/ml	0.02 μg/ml	0.1 μg/ml
[a]Patients.	30.77 ± 10.87**	466.30 ± 218.40**	[b]979.00 ± 348.10**
[a]Parents	21.42 ± 8.88	42.45 ± 21.54	[b]256.80 ± 90.09*
[a]Controls	17.69 ± 8.21	31.54 ± 17.76	101.10 ± 34.45

[a] Mean and SD of 13 FA patients, 12 parents and 13 controls.
[b] Mean and SD of 6 FA patients and 9 parents.
* Significantly different from controls ($p < 0.05$).
** Significantly different from parents and controls ($p < 0.05$).

3.4. Mitomycin C micronucleus assay

In order to quantify the sensitivity to DNA crosslinking agents, cells were subjected to MMC. This assay detects MMC-induced damage during the G0 phase of the cell cycle. The results of this assay are shown in Table 3. Spontaneous MNi values of patients obtained in the sham treated cultures set up together with the radiation and MMC treated cultures are similar as expected (Table 1 and Table 3). Both concentrations of MMC induced significantly higher MNi in the FA patients compared to the FA heterozygotes and control group. The higher concentration of MMC even resulted in significant differences in MNi between the FA heterozygotes and the controls ($p < 0.0001$). On average, a 9.7 fold increase of MNi was observed in the FA homozygotes compared to the control group and a 2.5 fold increase of MNi in the parents versus the control group was seen after treatment with the higher concentration of MMC (0.1 μg/ml).

Fig. 1 demonstrates the individual fold increase in FA patients and parents versus the mean of the control population for 0.02 μg/ml and 0.1 μg/ml MMC concentrations; also illustrating similar results in *FANCG* and *FANCA* patients. The horizontal lines represent one standard deviation (1SD) and two standard deviations (2SD) of the mean. As presented in Fig. 1, the fold increase can be utilised to identify FA patients successfully with MMC treatment of 0.02 μg/ml as all patients have a fold increase above the 2SD. A better discrimination between parents and controls at the individual level was demonstrated when 0.1 μg/ml MMC concentration was used and parents show a fold increase above 1SD. However, with the higher dose of 0.1 μg/ml MMC, more experimental failures were observed in the patient group. FA patients also showed significantly lower NDI values following MMC treatment ($p < 0.05$); the NDI values were further decreased when FA cells were treated with larger concentrations of MMC ($p < 0.05$).

4. Discussion

In this study we applied different MN assays in patients diagnosed with Fanconi Anaemia and their parents. We compared the results of these two groups with a healthy control group. The genotypes in all patients were determined and showed biallelic inactivation of *FANCG* in the South African patients or *FANCA* in a patient of Cameroonian descent. Carriership testing confirmed heterozygosity for a deleterious *FANCG/FANCA* mutation in the parents. Our study corroborates the

Table 2
G2 MN assay: Comparison of mean ± SD of spontaneous (0 Gy) and radiation-induced (at 2 and 4 Gy) MNi values in 1000 binucleated cells of FA patients, parents and healthy controls.

	0 Gy	2 Gy	4 Gy	2 Gy C+	4 Gy C+
[a]Patients	27.14 ± 9.16**	91.00 ± 40.67**	198.80 ± 73.18**	262.80 ± 121.10**	494.60 ± 257.30*
[a]Parents	18.36 ± 8.57*	57.21 ± 19.54	117.80 ± 31.58	147.40 ± 66.12	346.80 ± 144.70*
[a]Controls	11.50 ± 6.01	48.36 ± 14.51	95.43 ± 32.42	112.20 ± 40.67	248.20 ± 89.61

[a] Mean and SD of 14 patients, 14 parents and 14 controls.
* Significantly different from controls ($p < 0.05$).
** Significantly different from parents and controls ($p < 0.05$).

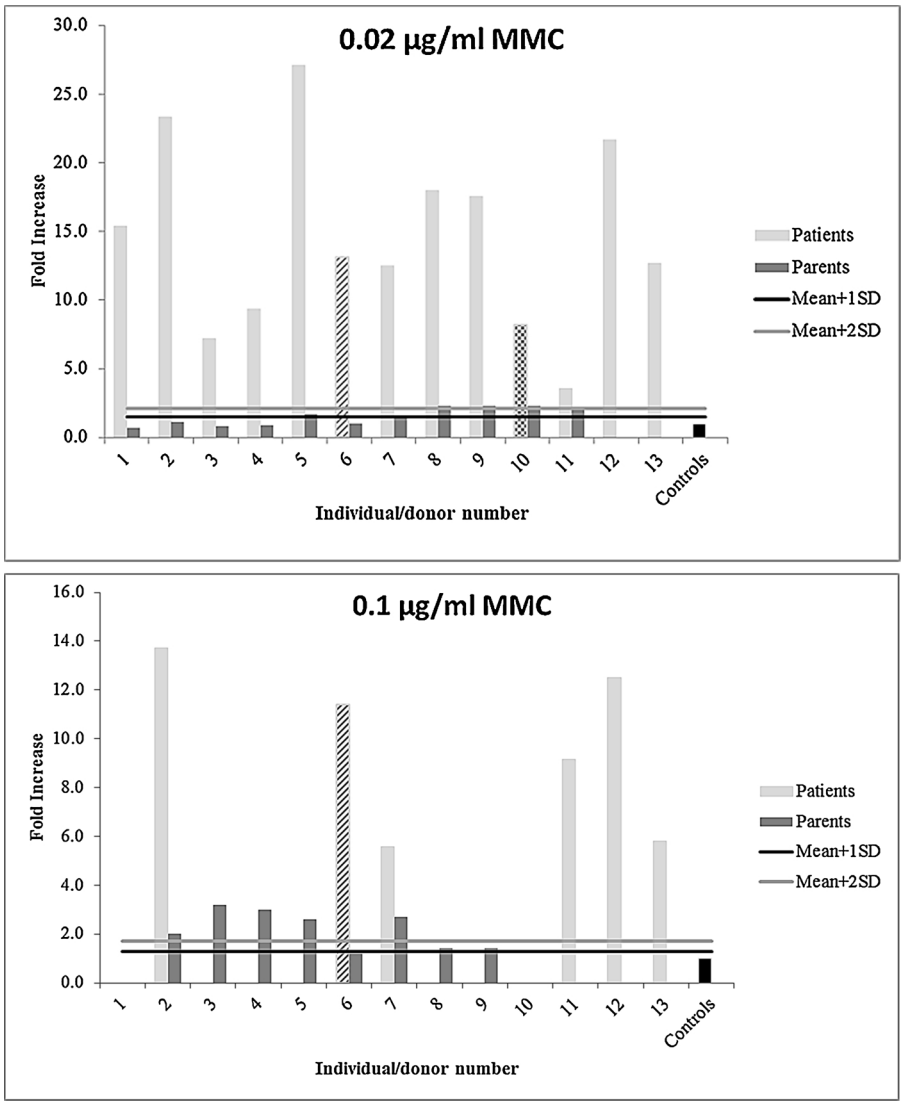

Fig. 1. Fold increase of MNi following MMC treatment. The individual fold increase of FA patients and parents versus mean of controls for concentrations of 0.02 µg/ml and 0.1 µg/ml MMC is presented as bars. The horizontal lines represent one standard deviation (1SD) and two standard deviations (2SD) of the mean of controls. Bar with diagonal lines (donor 6): *FANCG* compound heterozygote patient; bar with checked blocks (donor 10): *FANCA* compound heterozygote patient. All other patients are homozygous for *FANCG*.

high prevalence of the *FANCG* c.637_643delTACCGCC founder mutation in black FA patients [2,28,29]. However, a portion of black FA individuals present with a heterozygous mutation [30]; this requires additional screening of the *FANCG* gene in first instance and other FA genes, if no second deleterious mutation in *FANCG* can be identified. We report here a novel *FANCG* missense variant c.1379G > A, p.(Gly460Asp) which is likely pathogenic, in a black South African patient heterozygous for *FANCG* c.637_643delTACCGCC. The patient had a clear clinical diagnosis of FA and the applied assays showed similar aberrant values like in individuals homozygous for *FANCG* c.637_643delTACCGCC, indicative for a deleterious effect of this variant. Carriership testing in the mother confirmed heterozygosity for the *FANCG* c.637_643delTACCGCC mutation and absence of c.1379G > A, p.(Gly460Asp); DNA of the father was not available. This observation supports the hypothesis that both variants are most likely *in trans* in the child. Our study confirms that besides the founder mutation *FANCG* c.637_643delTACCGCC a second common *FANCG* mutation is unlikely in the black South African population [31].

We evaluated three MN assays to identify FA patients, as these may be more robust than the classically applied chromosome-breakage analysis on Giemsa-stained metaphases of lymphocytes/fibroblasts after addition of MMC or DEB. We induced MNi by addition of MMC in G0 cells or by irradiation of cells in G0 or S/G2 phase of the cell cycle. NHEJ is the preferentially used DNA repair pathway for dsb in the G0 phase while in S/G2 phase HR plays an important role. The FA/BRCA pathway is involved in DNA dsb repair by HR and in the processing of DNA damage induced by MMC.

In first instance, we evaluated spontaneously occurring MNi in two different phases of the cell cycle. The FA patients' exhibit, on average, significantly higher spontaneous MNi frequencies in the G0 phase compared to controls and heterozygous parents. This confirms the report of Camelo et al. and suggests genomic instability in FA patients [32]. With the S/G2 MN assay we also observed on average significantly more spontaneous MNi in both patients and heterozygous parents compared to controls. This could be attributed to homozygous or heterozygous defects in a FA gene, involved in the HR pathway which is preferentially used during the S/G2 phase of the cell cycle. However, due to the heterogeneity in spontaneous MNi it was not possible to consistently demonstrate higher spontaneous MNi values in each individual FA patient/parent compared to controls.

Subsequently, we scored MNi in irradiated lymphocytes of FA patients, FA parents and controls. Our results show significantly higher mean MNi frequencies in irradiated lymphocytes of patients in both phases of the cell cycle when compared to heterozygotes and controls. Loss-of-function in the FA genes causes disruptions in cell cycle checkpoints which could explain the enhanced sensitivity in irradiated FA cells. Increased radiosensitivity in FA cells was also observed in older and much smaller studies performed on an average of 4 FA

patients [18,33,17]. (missing a reference here; reference is mentioned in query notes).

Overall, the mean differences are larger in S/G2 MNi frequencies than the mean differences in G0 MNi frequencies between FA patients, FA heterozygous carriers and controls. A previous study has also shown a similar trend in chromosomal radiosensitivity in the G2 phase [34]. This could be due to the impaired repair pathways and checkpoints and accumulation of unrepaired DNA damage in the G2 phase in FA cells. Upon exposure to exogenous or endogenous damage, the number of FA cells in the G2 phase increases and is attributed to the S-phase checkpoint inefficiency [35]. This is reflected in our observation by the lower NDI values in FA patients when compared to controls that is also in agreement with previous studies describing poorer proliferative capacity and a prolonged G2 phase in FA lymphocytes [19]. In a normal cell cycle, the cells with unrepaired damage are blocked by G2/M checkpoint in order to repair the damage prior to division. In G2, the damaged cells are repaired by HR [36]. This characteristic is absent in the FA cells as they are thought to override the G2/M checkpoint and a significant quantity of unrepaired DNA is carried through to mitosis. Therefore FA cells have accumulated a greater number of damaged DNA and are highly prone to cancer [37].

The understanding of the FA pathway is still evolving and new components continue to be identified. The FA core complex is normally formed during S/G2 phases and cells preferentially use HR during this phase of the cell cycle. However, recent studies described cells derived from patients with FA mutations in the FA core complex that were not severely defective in HR repair of dsb and FA core complex-deficient cells that had impaired HR pathway and thereby favoured NHEJ [38,39]. These controversial findings evoke further investigation of the connections between the FA pathway and the DNA repair pathways.

The ability of caffeine to induce DNA damage and G2/M cell cycle checkpoint abrogation was used in combination with the S/G2 MN assay in our study. The higher number of MNi in all three groups after treatment of the cells with IR and caffeine confirmed this effect of caffeine on the induction of MNi. The results obtained with the S/G2 MN assay in combination with caffeine also showed higher MN values in the FA homozygotes compared to heterozygotes and controls, supporting the study of [40] where it was stated that cells deficient in *FANCD2* developed higher levels of γ-H2AX foci when exposed to caffeine and where was suggested that patients with germline or somatic *FANCD2* mutations may be hypersensitive to cytotoxicity of coffee [40]. Although our FA patients are carriers of *FANCG/FANCA* mutations, we also noticed elevated radiation-induced MNi values. Inactivation of *FANCG* attributes to a functional collapse of the FA core complex and reduces DNA damage repair capacity. The MN values in heterozygous carriers are higher but not significantly different from the values obtained in the control group after 2 Gy irradiation in combination with caffeine. However, after a 4 Gy dose of IR and caffeine we found significantly higher levels of residual DNA damage suggesting that less damage is repaired after exposure to caffeine and IR even when a functional allele of the FA gene is available.

As a group, FA patients showed significantly elevated radiosensitivity with the G0 and S/G2 MN assays compared to parents and controls. However, the assays are not suitable as a biomarker for detecting individual FA patients. Since the risk of cancer is elevated in FA patients, radiosensitivity information in this patient group is relevant and may be taken into consideration prior to start treatment using IR.

The MMC MN assay is very promising as an individual biomarker (Table 3 and Fig. 1). Even though cells were treated with low MMC concentrations, a clear distinction in MNi frequencies between FA homozygotes, FA heterozygotes and controls was observed. However, only a low number of binucleated cells were detectable in cells of some FA patients treated with the highest 0.1 μg/ml concentration of MMC. This is attributed to the toxic nature of MMC that induces unrepairable interstrand- crosslinks which lowers cell proliferation of FA cells as reflected in the NDI values. Despite the poor cell proliferation, we were able to produce conclusive results to identify FA patients. The test is more robust when applying only 0.02 μg/ml and is optimal for distinguishing individual FA patients from individual controls and parents. Evaluating metaphases in chromosomal-breakage analysis of MMC or DEB requires a trained eye and can be labour intense. In comparison, scoring MNi using the MMC MN assay is amenable to automation, it is relatively straightforward and time efficient. Therefore, we think that our MMC MN assay has the potential for a reliable alternative for the classic chromosome breakage assay.

The majority of our FA patients are homozygous for the *FANCG* founder mutation. Our cohort also includes two patients that are compound heterozygotes; one patient with two mutations in *FANCG* and the other with two mutations in *FANCA*. The phenotype observed in the *FANCG* homozygous patients are also demonstrated in the *FANCG* and *FANCA* compound heterozygous patients. The observation suggests that the phenotype is valid for both the FANC genes that form part of the FA core complex. Although, further validations in larger patient and parent groups with different FANC genotypes are required prior to implementation as a standard test in clinical setting.

Interestingly, the application of higher MMC concentrations in our MMC MN assay revealed significant differences between the group of FA parents and the control group. This distinction could not be made with a MMC-based chromosome breakage assay [41]. Similarly, identifying FA parents from controls using the DEB test has shown to be unsuccessful with chromosomal breaks of parents overlapping with controls [42,43].

Identification of heterozygous mutation carriers may be clinically relevant in the context of breast cancer prevention as heterozygous mutations in several FA genes were shown to be associated with an increased risk for breast cancer [44–49]. The best known breast cancer gene, *BRCA1 (=FANCS)* has been shown to interact directly with *FANCD2* and *FANCA* in response to DNA damage [50]. Similar to our study, the damaging effects of IR and MMC were evaluated in lymphocytes from heterozygous *BRCA1/FANCS* and *BRCA2/FANCD1* mutation carriers (breast cancer patients and healthy carriers). In response to IR, the heterozygous carriers of both genes did not show elevated MNi values, but upon exposure to MMC enhanced chromosomal sensitivity was detected in lymphocytes of individuals with heterozygous *BRCA2* mutations [51]. It is clear that larger studies are warranted to validate our findings but the MMC MN assay may be promising as an individual biomarker for functional deficiencies in at least some of the FA genes.

5. Conclusions

In conclusion, this study compared 3 different MN assays to characterize FA patients with biallelic inactivated *FANCA/FANCG* alleles and to compare the results with heterozygous carriers of *FANCA* and *FANCG* mutations and controls. Chromosomal radiosensitivity of the FA patients was demonstrated with the G0 and S/G2 MN assay. The MN assay utilising IR showed on average an increased number of MNi in FA patients and heterozygous carriers compared to controls, but cannot be used to identify individual patients. Using the MMC MN assay, we were able to distinguish FA patients from heterozygous carriers and controls. Interestingly, with this assay, higher MNi scores were obtained in the heterozygous parents compared to controls. Further studies are warranted to evaluate the sensitivity and specificity of this test in FA patients and parents with other genotypes/mutated FANC genes. The big advantage of the MMC MN assay is that is less labour intense, more time effective and less subjective compared to the classic chromosomal breakage assay. Future research can indicate if this assay can differentiate FANC heterozygote genotypes from healthy controls and can be used as a functional assay identifying individuals with a heterozygous defect in genes related to the FA/BRCA pathway.

Conflict of interest statement

The authors declare there are no conflicts of interest.

Acknowledgements

This work was supported by a CANSA (Cancer Association of South Africa) grant, a grant under the Nuclear Technologies in Medicine and the Biosciences Initiative (NTeMBI – project ref. NTeMBI2016), a national technology platform developed and managed by the SA Nuclear Energy Corporation and supported by the Department of Science and Technology of South Africa and the BOF-DOS (Special Research Fund) scholarship (Scholarship code: 01W04115), Ghent University. The authors wish to thank Suretha Erasmus and Noelene Kinsley for providing genetic counselling to the patients and parents in the study. Additionally the lab staffs at the Center for Medical Genetics, Ghent University Hospital, Belgium are acknowledged for their assistance. We would like to thank all the patients, parents and volunteers who participated in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dnarep.2017.11.001.

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CHAPTER 8
General discussion

1. Breast cancer

Breast cancer is often treated with radiotherapy. As radiotherapy is a very useful tool to kill cancer cells, it needs to be cautiously used to spare surrounding normal tissue. Although an effective treatment, about 5% of patients treated with radiotherapy suffer from normal tissue side effects (Sprung et al., 2005). Several parameters will influence the normal tissue tolerance of breast cancer patients (Barnett et al., 2009, Mukesh et al., 2012). Normal tissue toxicity is presented as clinical manifestations with early and late reactions. Chromosomal radiosensitivity has been proposed as a predictor for normal tissue tolerance (Habash et al., 2017, Vandevoorde et al., 2016).

Chromosomal radiosensitivity has previously also been shown to be a marker for breast cancer predisposition. Literature data for chromosomal radiosensitivity in breast cancer patients has been explored in various populations (Scott et al., 1999, Baria et al., 2001, Ernestos et al., 2010, Terzoudi et al., 2000, Poggioli et al., 2010, Burrill et al., 2000, Baeyens et al., 2002, Baeyens et al., 2005). Despite the breast cancer burden, chromosomal radiosensitivity has previously not been studied in South Africa. The clinicopathological presentations of South African breast cancer patients differ from their European counterparts. South African breast cancer patients present with aggressive premenopausal tumours, whereas postmenopausal breast cancer is prevalent in the European population; the underlying genetic mechanisms in the two populations may also differ (Vanderpuye et al., 2017, Abdulrahman and Rahman, 2012, Huo et al., 2017). A large systematic review of breast cancer in sub-Saharan Africa highlighted the tumour characteristics of women in this region. Black African women were shown to be 10-15 years younger in age at diagnosis compared to women in developing countries (Jedy-Agba et al., 2016). In this thesis, we investigated the chromosomal instability and radiosensitivity in South African breast cancer patients. Young African women are affected by aggressive subtypes of breast cancer (Gabriel and Domchek, 2010, Bowen et al., 2008). A number of studies have described the association of TNBC in young African women (Anders et al., 2009, Carvalho et al., 2010, Assi et al., 2013). Genetic screening threshold for breast cancer patients recommended are 50 years. Robertson et al. recommended *BRCA1* screening for TNBC patients below the age of 50 (Robertson et al., 2012). Additionally, patients below 51 are considered premenopausal and treated accordingly. Consequently, assessing the chromosomal instability and radiosensitivity of TN patients are essential. The effect of age on chromosomal radiosensitivity was

investigated between all young and older patients. Patients who were ≤50 years were classified as young and patients >50 years were consider as older patients.

1.1. Chromosomal instability in breast cancer patients

Owing to population diversity in South Africa, we first assessed the influence of ethnicity on chromosomal instability and radiosensitivity using the G0 MN assay (Chapter 3). Using this assay we analysed the spontaneously occurring spontaneously occurring MNi that are indicative of chromosomal instability. Several papers have described environmental and genetics factors contributing to the increase in spontaneous MNi frequencies (Jones et al., 2011, Ramsey et al., 1995, Bolognesi et al., 1997, Orta and Gunebakan, 2012). In chapter 3, all breast cancer patients were subgrouped by ethnicity: i) black, ii) white, iii) Indian and iv) coloured (mixed race) patients. In our unselected breast cancer cohort, patients manifest chromosomal instability compared to healthy individuals. When subgrouping the patients based on ethnicity, higher spontaneous MN values were observed in the white (mean age = 59 years) and black (mean age = 47 years) patients suggesting that ethnicity has an effect on chromosomal instability. When chromosomal instability was assessed in lymphocytes in Caucasian and African American breast cancer patients, the MN values were significantly higher in Caucasian women and significantly lower in African American women (Aboalela et al., 2015). Our results demonstrate that black South African patients also exhibit enhanced chromosomal instability as measured by the G0 MN assay; these results promote the notion that chromosomal instability may vary based on ethnicity. The age of the patients could have contributed to enhanced spontaneous MN values.

Similar to the general breast cancer population (Chapter 3), chromosomal instability with increased spontaneous MN values was depicted between young and old patients (Chapter 5); the enhanced chromosomal instability with the differences being higher in the older patients. This could be attributed to the increase of 0.58MN/year as suggested by Thierens et al. (2000) (Thierens et al., 2000). Young breast cancer patients have more aggressive tumour features and could possibly have a unique genetic composition that results in increased spontaneous MNi frequency (Basro and Apffelstaedt, 2010). The age-associated decline in DNA repair leads to accumulation of DNA damage, possibly contributing to the elevated spontaneous MN values in the older patient group. Other factors that could influence

chromosomal instability in these patients are age-related oxidative damage and aneuploidy (Luzhna et al., 2013).

Similar to the general breast cancer population (Chapter 3), G0 chromosomal instability with increased spontaneous MN values was depicted in TN lymphocytes. In chapter 5, we compared the chromosomal instability of all TN (n = 17) and luminal patients (n = 10) in the S/G2 phase of the cell cycle. Comparing spontaneous MN values in the S/G2 phase of the 2 subgroups revealed 35% of the luminal patients as chromosomally instable. Only 14% of TN patients had high spontaneous MNi compared to the controls. Given that majority of *BRCA1* carriers develop TNBC, chromosomal instability was also evaluated in all mutation carriers in breast cancer susceptibility genes. No statistical significance was observed in the 10 mutation carriers compared to the controls. In a recent study, BRCA positive populations show elevated chromosomal instability (Baert et al., 2016, Baert et al., 2017). Further studies are warranted to confirm S/G2 chromosomal instability in TNBC patients who are also mutation carriers in breast cancer susceptibility genes, particularly in BRCA.

1.2. Effect of age on chromosomal radiosensitivity

African breast cancer populations are often distinct from other breast cancer populations by the different age distributions (Sighoko et al., 2013, Newman, 2005). The incidence of breast cancer is more prominent in young black populations (Basro and Apffelstaedt, 2010, Shavers et al., 2003); therefore the focus of the next part in this study was the effect of age on chromosomal radiosensitivity by investigating young and older breast cancer patients. Elevated MNi frequency occurring spontaneously is an indicator of chromosomal instability whereas chromosomal radiosensitivity is indicated by a higher frequency of MN values induced by radiation. In chapter 3, G0 lymphocytes from a group of unselected patients (n = 68; mean age = 52) irradiated with 2 and 4 Gy showed increased MN values compared to a group of healthy individuals suggesting chromosomal radiosensitivity in the breast cancer group. These results are in agreement with previous European studies (Baeyens et al., 2002, Baeyens, 2005, Scott et al., 1998). Similar to our study, Baeyens et al. (2002) investigated the G0 chromosomal radiosensitivity in an unselected (Belgian) breast cancer cohort but using γ-rays at high and low dose rate. With a similar sample size as used in our study, they reported significantly increased mean radiation-induced MN values in the patient's population when compared to healthy individuals. The mean age of the breast cancer cohort used in our study

was slightly higher (52±12) than in Baeyens et al. (2002) (45±10) (Baeyens et al., 2002). These results confirm enhanced chromosomal radiosensitivity in the general breast cancer population regardless of age.

Auer et al. (2014) investigated the effect of age on chromosomal radiosensitivity in breast cancer patients with a 2 Gy dose. The technique for the effect of age was a 3 colour FISH to detect metaphase spreads, different from the MN assay used in our study. Their study reported increased chromosomal radiosensitivity in an unselected cohort of breast cancer patients. Interestingly, they described that enhanced chromosomal radiosensitivity was primarily observed in patients aged between 40 and 50 years (Auer et al., 2014). We were able to demonstrate increased chromosomal radiosensitivity in a young and older breast cancer cohort (Chapter 5). Young patients only displayed significantly enhanced levels of MN values for the 4 Gy dose of IR, whereas the increase was significant for the older patients for both 2 and 4 Gy doses compared to the controls. However, no significant correlation between age and chromosomal radiosensitivity in breast cancer patients in our study was demonstrated.

1.3. Effect of ethnicity on chromosomal radiosensitivity

Differences in breast cancer exist between ethnic groups in South Africa (Dickens et al., 2014, Vorobiof et al., 2001). For instance, black women are known to have more TN subtypes than their white counterparts (Awadelkarim et al., 2008) and the incidence of breast cancer in the black population is prominent in younger women (Stark et al., 2010). We, therefore, wanted to investigate the link between chromosomal radiosensitivity and ethnic groups (Chapter 3). As seen in previous international studies, our results are in agreement by demonstrating chromosomal radiosensitivity in the white population (Poggioli et al., 2010, Burrill et al., 2000, Baeyens et al., 2005). Black patients (n = 30) also showed increased chromosomal radiosensitivity for both the 2 and 4 Gy irradiations in the G0 phase. Whereas the coloured patients (n = 6) had higher MN values compared to healthy controls although no significant differences were noted. The increase in MN values was not observed in the Indian cohort. The small group of the Indian and coloured population (n = 6 and n=7) included in our study restricts conclusions. Further validations are necessary in order to draw conclusions in both the coloured and Indian breast cancer patients. The enhanced chromosomal radiosensitivity observed in the black and white patients' group but not in the coloured and

Indian patients' group suggests diverse DNA damage response and repair mechanisms in different ethnic populations. A study has highlighted the role of DNA damage response in African American and Caucasian breast cancer populations (Rodriguez-Gil et al., 2010). Their results show lymphocytes of young African American breast cancer patient's show significantly increased levels γ-H2AX foci after irradiation compared to Caucasian women. γ-H2AX foci denotes the presence of DNA DSB. These results suggest that African American patients are prone to elevated DNA damage (Rodriguez-Gil et al., 2010). Similarly, our study shows increased chromosomal radiosensitivity in the black patients' further highlighting the difference in African patients and other ethnic groups. There is a need for improved understanding of DNA repair in different South African patients, per ethnic group, which could be clinically relevant in managing risk assessment and disease prevention.

1.4. Effect of tumour characteristics and triple negative phenotype on chromosomal radiosensitivity

Due to the tumour heterogeneity, we investigated the potential effects of clinical parameters on chromosomal radiosensitivity (Chapter 3). By subgrouping all patients on tumour receptor expression, histological type, size and staging, radiation-induced MN values were elevated in the ER positive patients. ER is a steroid hormone and a transcription factor that mediates its biological effects through two receptors – ER alpha (ERα) and ER beta (ERβ). ER positive breast cancers are more predominant in postmenopausal women (Hurvitz and Pietras, 2008). Oxidative damage to the DNA induced by estrogen in the ER positive cells is enhanced by exposure to IR. Increase in MNi in ER positive cancers could be a result of pre-existing DNA damage plus the radiation-induced DNA damage (Mobley and Brueggemeier, 2004). There is evidence of chromosomal radiosensitivity of lymphocytes of ER positive patients was also demonstrated in the G2 phase by evaluating chromatid breaks (Riches et al., 2001). As an extension of this study, the effect of ERα and ERβ on breast cancer radiosensitivity will be investigated in our research group.

Breast cancer patients are clinically categorised into different subtypes. One of the classification systems is based on expression of hormonal receptors (ER, PR and HER2). In our first study of chromosomal radiosensitivity on unselected breast cancer group we noted that the ER-positive breast cancer patients had a more radiosensitive phenotype compared to the ER-negative breast cancer patients. The lack of receptors on TN cells may reduce

sensitivity to IR. TNBC occurrence is prominently noted in young African women; therefore chromosomal radiosensitivity data is of utmost importance in the South African context. In chapter 5, we investigated the G0 chromosomal radiosensitivity of breast cancer patients grouped as TN and luminal patients based on the receptor status and age. Chromosomal radiosensitivity data in TNBC patients has recently encouraged great interest and results are indicative for the radioresistance of TN cells (Ren et al., 2015, Chen et al., 2017a, Mehta et al., 2016, Li et al., 2017). Contrary to the observed radioresistant phenotype, there is evidence that TNBC have defects in DNA repair genes (Ribeiro et al., 2013). DNA repair genes are crucial in damage repair and are involved in different repair pathways; the role of different repair pathways was assessed in the study using the G0 and S/G2 MN assay. TNBC patients exhibited radioresistance for exposure to IR. In the TN patients, the potential factors contributing to radioresistance could be the unique biology of the tumours. High mitotic activity is a hallmark of TNBC that can promote radioresistance. Onozato et al. (2017) recently demonstrated the radioresistance in highly proliferating cells after IR treatment (Onozato et al., 2017). Furthermore, cell cycle and DNA repair genes are overexpressed in TNBC. Therefore, it can be hypothesised that the observed chromosomal radioresistance could be attributed to increased expression of DNA repair genes (Engebraaten et al., 2013).

DSB are repaired predominantly by two main DNA repair pathways: HR and NHEJ. The cell is selective on which DNA repair pathway to use depending on the phase of the cell cycle. In the S/G2 phase where a homologous strand is present, the HR repair pathway is preferred over NHEJ. NHEJ repair of DSB is predominant in the remaining phases of the cell cycle. The role of HR in chromosomal radiosensitivity was investigated in the group of breast cancer patients by using an adapted protocol of the G0-MN assay. In this S/G2-MN assay the radiation damage was induced in the S/G2 phase (Claes et al., 2013, Baert et al., 2016). Next, the S/G2 chromosomal radiosensitivity of TN and luminal patients was investigated (Chapter 5). Radiation-induced MN values for 2 and 4 Gy demonstrated elevated chromosomal radiosensitivity in luminal patients only. Interestingly, lower radiation-induced MN yields were observed in TN patients for both doses of IR indicating a “radioresistant” phenotype. The first hypothesis for radioresistance observed in TNBC patients could be the result of the overexpression of β-catenin upon exposure to IR (Yin et al., 2016). Irregularities of the Wnt/β-catenin pathway is essential for stem cell maintenance (Pohl et al., 2017). Cancer stem cells (CSC) in breast cancers with enhanced Wnt/β-catenin pathway are reinforced by exposure to IR. This may contribute to the constant maintenance of cancer stem cells in

TNBC cells. CSC are implicated with chemoresistance, radioresistance and metastases (Bartucci et al., 2015, Krause et al., 2017). Furthermore, they also have increased DNA repair capacity contributing to therapy resistance (Pranatharthi et al., 2016). Radioresistance noticed in TN cells could be a result of enhanced Wnt/β-catenin signalling. Recent studies have identified microRNAs as a factor that can regulate radiosensitivity in TNBC (Ren et al., 2015, Li et al., 2017, Gasparini et al., 2014). Ren et al. (2015) demonstrated the over expression of microRNA-27a in TNBC cell lines compared to normal breast cancer cell lines. MicroRNA-27a was shown to directly target CDC27, a protein related with chromosomal integrity, and downregulate CDC27 rendering the TN cells radioresistant (Ren et al., 2015). Similarly, microRNA-454 was also shown to be highly expressed in TN cells. Recently, Li et al. (2017) studied the role of microRNA-454 in TN cancers and its response to IR. MicroRNA-454 was shown to inhibit apoptosis following the exposure to ionising radiation and improving survival of TNBC cells (Li et al., 2017). The IR-induced cell survival leads to radioresistance. Although, these studies are conducted on cell lines, microRNAs regulate lymphocyte function and development (Lawrie et al., 2008, Belver et al., 2011). Therefore, the decrease in IR-induced MNi frequency in lymphocytes of TNBC patients could be a result of overexpression of microRNAs that regulate radiosensitivity.

Similar to our results, Nosrati et al. (2017) have shown higher MN values in luminal breast cancer patients compared to the control population and a decline in the MNi frequency in TNBC patients (Nosrati et al., 2017). The second hypothesis of radioresistance in TNBC patients could be due to the enhanced DNA repair in the absence of ER as shown by Chen et al. (2017) (Chen et al., 2017b). Using DNA recombinant technology, viral vectors with ERα genes were transfected in TNBC cell lines resulting in expression of ER in TN cells. Thus, the effect of ERα on TNBC radiosensitivity could be assessed. The authors show increased radiation-induced DSB breaks in TN cells transfected with ERα and delay in repair compared to wild type TN cells. Compared to the wild type cells, radiation-induced G2/M arrest was increased and, in a time-dependent manner, the S phase was decreased in the ER-transfected cell. The HR pathway is enhanced in the S phase for efficient DSB repair. A shorter S phase implies less time for DNA repair to occur in S phase. This finding suggests that ER impairs DNA repair by altering the cell cycle (Chen et al., 2017b). Further investigations on the impact of ER on chromosomal radiosensitivity will be investigated in our research group.

In chapter 5, we evaluated checkpoint efficiency using caffeine in all TN (n=17) and luminal (n=17) breast cancer patients. In the presence of DNA damage, checkpoints are activated in order to regulate accurate and effective replication. Caffeine, however, abrogates the checkpoints and permits damaged cells to progress through the cell cycle (Vandevoorde et al., 2016). The RIND score was computed to assess individual radiosensitivity. A RIND score above 2 was considered as radiosensitive. For the radiation-induced MN values for TN and luminal patients, 24% of TN versus 47% luminal patients showed elevated chromosomal radiosensitivity compared to healthy controls. Chromosomal instability and radiosensitivity in the S/G2 phase was previously observed in breast cancer patients (Baria et al., 2001). The mean age of breast cancer patients was 56 years. The chromosomal radiosensitivity in this cohort was quantified by scoring metaphase spreads in lymphocytes. Chromosomal instability in the breast cancer patients group in this study showed no significant increase compared to the controls. Significant differences were, however, observed in radiation-induced chromosomal aberrations (Baria et al., 2001). In the TN patients, 76% of the radioresistant phenotype observed could be due to the dominant effect of the underlying mechanisms seen in TN cells. The direct or indirect impairment of the HR repair pathway, significant in the G2 phase, could have detrimental effects on DNA damage repair leading to increased chromosomal radiosensitivity. A recent study showed upregulation of RAD51 in TNBC tumours when compared to other breast cancer. RAD51 is a primary component of the HR pathway. TN cells with enhanced HR repair exhibit radioresistance to IR (Gasparini et al., 2014). The third hypothesis of radioresistance seen in TNBC patients could be a factor as recently shown by Mehta et al. (2016). They showed the association between an mRNA-binding protein, HuR, and radiosensitivity in 3 different TNBC cell lines. HuR was linked to cancer therapy resistance and therefore resulting in poor prognosis as seen in TN cancers. HuR is endogenous expressed and enhanced expression is described in TN cells. The study showed that knockdown of HuR leads to radiosensitisation by inducing oxidative stress and increased DNA damage. They saw a significant increase in γ-H2AX foci in HuR knockdown TN cells compared to control cells after IR exposure. The scrambled control had a higher cell survival rate. Various DNA repair genes involved in HR were suppressed by the silencing of HuR; thereby indicating silencing HuR leads to radiosensitisation. (Mehta et al., 2016). Since HuR is endogenous expressed and enhanced in TNBC cells, this could be a contributing factor to the observed radioresistant phenotype of TNBC lymphocytes in our study.

Additional to the G0 MN assay, the radioresistant phenotype seen in the TN patients in the S/G2 phase as well may suggest possible differences in cell cycle controls in TN compared to luminal breast cancer patients. The S/G2 MN assay with addition of caffeine was previously conducted on AT families; the AT patients were classified as highly radiosensitive and carriers as radiosensitive (Claes et al., 2013). Another study demonstrated radiosensitivity of lymphocytes of AT carriers only to IR in the G2 phase (Terzoudi et al., 2005). AT patients have mutated ATM; ATM plays a vital role in the G2/M checkpoint and in DSB repair. When ATM is mutated, the DNA damage will override the checkpoint. These results suggest that defects in the G2/M checkpoint will be reflected as chromosomal radiosensitivity as seen in our luminal breast cancer patients cohort.

1.5. Effect of mutations in breast cancer susceptibility genes and chromosomal radiosensitivity

The association of TNBC and *BRCA1* mutations has been widely described (Bayraktar et al., 2011, Gonzalez-Angulo et al., 2011, Lee et al., 2011, Peshkin et al., 2010, Robertson et al., 2012, Young et al., 2009). Defects in breast cancer susceptibility genes or DNA repair genes often make cells vulnerable to effects of IR. TN and/or young South African patients could carry unique defects in DNA repair genes. In chapter 6, we aimed to investigate the underlying mechanism of this chromosomal radiosensitivity by screening breast cancer patients selected for TN status or patients ≤50 years for *BRCA1*, *BRCA2*, *PALB2* and the *CHEK2* allele c.1100delC. In our cohort, we detected a total of 12 *BRCA1/2* mutations (12.8%) and 1 *CHEK2* c.1100delC allele (1.1%). Three novel mutations were identified in black patients; two in *BRCA1* (c.1155G>A; p.Trp385* and c.1953_1954insA; p.Lys652fs) and 1 in *BRCA2* (c.582G>A; p.Trp194*). Previous South African studies reported deleterious *BRCA1* mutations in about 20% of the familial breast/ovarian cancers. At that time, the standard mutation analysis strategy in South Africa was the protein truncation test (PTT) and PCR-single strand conformation polymorphism/heteroduplex analysis (SSCP/HA) (Reeves et al., 2004). As these techniques do not allow the detection of exon-spanning deletions/duplications, it is advised to complement the strategy with MLPA analysis. Reeves et al. applied stringent inclusive criteria for genetic screening compared to our study and screened Afrikaner breast cancer families with different founder mutations than the black South African breast cancer patients. Furthermore, the applied genetic screening techniques are less sensitive than NGS used in our study. Sluiter et al. (2010) screened 52 hereditary

breast/ovarian South African families with MLPA who turned out to be negative after analysis with PCR-based techniques. With this technique, they identified 1 family with a large genomic arrangement in *BRCA1* (Sluiter and van Rensburg, 2010). We also applied MLPA but did not detect a large deletion/duplication in our cohort. This may indicate that these types of mutations are a major contributing factor in the South African population. Screening large South African breast cancer cohorts are necessary in order to identify BRCA mutations unique to this population, especially in young TN patients. Robertson et al. (2012) showed that over 10% of young TN patients are likely to be *BRCA1* mutations carriers (Robertson et al., 2012). In the TN cohort in our study, 17 % of all TN patients (n = 18) and 22% of young patients with TN phenotype (n = 9) are *BRCA1* mutation carriers.TN patients a priori a higher likelihood of being *BRCA1/2* mutation carriers. The prevalence of *BRCA1* mutations in TNBC patients ranges between 9-37% (mean = 35%) and for *BRCA2* the range is 1-12% (mean = 8%) (Rashid et al., 2016, Li et al., 2014, Peshkin et al., 2010). Despite the prevalence of Ashkenazi Jewish and Xhosa founder mutations in the South African population, these founder mutations are absent in our population as neither Ashkenazi nor Xhosa patients were included in our study. Although *BRCA1/2* are the most sequenced genes worldwide, we identified three novel mutations, never described before, in our study cohort of black patients, indicating the need for large screenings of young and/or TN patients in South Africa widely.

Our study did not identify *PALB2* mutations in the selected cohort of TNBC and young breast cancer patients. However, previously studies have reported the presence of pathogenic *PALB2* (2%) mutations in young white South African breast cancer patients (Sluiter et al., 2009). These findings reinforce the need for breast cancer susceptibility screening for South African patients with early onset breast cancer or who are TN. Further studies are recommended to uncover mutations with DNA repair genes that are specific to our population. There is increasing evidence linking breast cancer with DNA repair genes (Chae et al., 2016). South African breast cancer women are an understudied population. Increasing mutation analysis within this population will contribute to the limited aetiology and assist in disease management.

Patients carrying mutations in breast cancer genes in our study were also subject to chromosomal radiosensitivity analysis (Chapter 5). The mean age of patient positive for mutations in DNA repair genes (n = 10) in our study was 49 years and mutations primarily

occurred in white patients; 40% were confirmed TN patients. In the 10 mutation carrier patients analysed for chromosomal radiosensitivity, no statistical significance in MN values were observed when compared to the controls. In other recent studies performed, BRCA positive populations show elevated chromosomal radiosensitivity to IR. In the *BRCA1/2* positive study population studied by Baert et al. (2016), 72% of *BRCA1* and 50% *BRCA2* healthy carriers were reported as chromosomally radiosensitive (Baert et al., 2016) (Baert et al., 2017). Screening TN and young South African breast cancer patients for *BRCA1/2* and other breast cancer susceptibility genes is important as it can improve individual patient treatment by assessing chromosomal radiosensitivity prior to radiation therapy. Mutations in *BRCA1/2* genes compromise DNA repair mechanisms resulting in chromosomal instability and chromosomal radiosensitivity. Larger studies to evaluate the effect of BRCA on chromosomal radiosensitivity in South African patients are further warranted.

1.6. Effect of storage and anaesthetics on MN yields in breast cancer patients

In our study, blood samples of patients are often transported to the labs following curative breast surgeries. Patients are mostly under the influence of general anaesthesia prior to drawing of the blood. A major challenge is the inadequate transportation of blood samples with lack of controlled temperature and delay in sample processing. Both factors, time and general anaesthesia, could affect the MN yields. In chapter 4, we investigated the influence of i) storage time of blood samples and ii) anaesthesia on the chromosomal radiosensitivity. In the first part of the study investigating storage time of blood samples, blood samples from a control population and patient group were collected. No differences in spontaneous MN values were detected in both the controls and patients following the delay in processing time. The radiation-induced MNi frequencies were, however, significantly increased with increasing storage times in patients and controls. Temperature control plays an essential role in blood storage. Blood samples from our study were stored at room temperature prior to conducting the MN assay. Belloni et al. (2010) showed the effect of temperature during blood storage on the yield of chromosomal aberrations using metaphase spreads. Samples prior to irradiation stored at 4 or 20°C with PHA, did not exhibit any chromosomal damage. When irradiated lymphocytes were stored for 24 and 48 hours with no PHA and at 20°C, they observed a significant increase in apoptosis and significant decrease in dicentrics compared with storage with PHA and 4°C (Belloni et al., 2010). In our study, the BN yield depression was noted in the patients when dose increased and after 48 hours of storage, the BN yield

decreased for both doses in the control group. This can be attributed to increased apoptosis due to storage of sample at room temperature for 48 hours as suggested by Belloni et al. (2010) (Belloni et al., 2010). Storage and transportation of blood samples should be cautiously handled by controlling temperature. If processing of samples is delayed, samples can then be stored at 4°C with PHA until 48 hours for reliable results (Belloni et al., 2010).

Breast cancer patient undergoing surgeries are administered general anaesthetics. Anaesthetics are widely known to have genotoxic potential and its effects have been described (Chandrasekhar et al., 2006, Rozgaj and Kasuba, 2000, Lucio et al., 2017, Aldrieny et al., 2013). We assessed the influence of anaesthetics on the number of chromosomal aberrations in blood samples from patients undergoing breast surgeries (Chapter 4). Exposure to anaesthesia contributed to decrease in BN yield. Blood samples collected after administering general anaesthesia exhibit significantly enhanced spontaneous MN values. Our findings are in agreement with a study by Ajauro et al. (2013) where they showed that lymphocytes of operating room personnel exposed to anaesthetic gases exhibited significantly increased MN values when compared to personnel's not exposed (Araujo et al., 2013). For the radiation-induced samples after anaesthetics, MN values were significantly enhanced for samples processed after 24 hours for both doses. The effects of radiation on lymphocytes are considerably more harmful and could override the effect of anaesthetics in patients undergoing breast surgery. Our results suggest that for radiosensitivity studies, blood samples should preferably be collected prior to the administration of general anaesthetics to reflect true radiation-induced MN values in breast cancer patients.

2. Fanconi Anaemia

The next aim of the study was to assess chromosomal radiosensitivity in FA patients using the G0 and S/G2 MN assays (Chapter 7). FA patients are known to be hypersensitive to DNA crosslinking agents and this trait was utilised to detect chromosomal breakage induced by MMC to distinguish homozygous or heterozygous mutation carriers of the FA genes. A large number of genes are associated with FA and new components of the FA pathway are still being identified; we optimised a FANC gene panel for the mutation analysis of the 22 FANC genes known at the start of the study using NGS in combination with MLPA was used to identify large deletions. Furthermore, the correlation between chromosomal radiosensitivity and FA mutations was investigated.

Founder mutations in South Africa are particularly associated with the black South Africans (*FANCG* c.637_643delTACCGCC), Afrikaner (*FANCA* del E12-17; del E11-17; c.3398delA) and Ashkenazi (*FANCC* c.456+4A>T) population groups (Morgan et al., 2005, Wainstein et al., 2013, Feben et al., 2017a). Consequently, molecular screening for FA patients in South Africa is based on ethnicity. However, previous studies have shown that a subset of patients do not adhere to this pattern and might require further testing (Wainstein et al., 2013). The need for a FANC panel, therefore, was crucial in identifying and diagnosing FA patients who are clinically symptomatic and negative for the common mutations. Advances in genetic screening have paved the way for multi-gene panel tests. With the involvement of genetics in a number of diseases, gene panel testing is widely available (Nguyen et al., 2017, Ring et al., 2016, Silva-Smith and Sussman, 2017, van Marcke et al., 2016, Wing et al., 2017). Our gene panel consisted of 20 FANC genes (Dong et al., 2015, Park et al., 2016). We optimised singleplex PCR for all coding exons of the FANC genes. Next, the modified Nextera XT (Illumina) library preparation protocol was applied as previously described (De Leeneer et al., 2015). To identify large genomic rearrangements in the FANC genes, MLPA was performed using the commercially available probemix for FANC genes (MRC-Holland) (Chapter 7). Lastly, all pathogenic mutations and variants identified on the Miseq platform were confirmed using Sanger sequencing. Following the validation, clinical diagnosis of 12 unrelated patients and 2 siblings were molecularly confirmed using this approach. Patients were subjected to both the panel screening and MLPA to detect large amplification or deletions.

All South African patients (n = 13) screened in this study had clear clinical symptoms of FA prior to mutation analysis. The homozygous *FANCG* c.637_643delTACCGCC founder mutation was detected in 92% of the black South African patients. This is slightly higher than a study performed by Morgan et al. (2005) who reported 82% prevalence of the black South African founder mutation (Morgan et al., 2005). In addition, we identified a black patient compound heterozygous for the recurrent FANCG mutation and a novel missense variant (c.1379G>A; p.(Gly460Asp)), likely to be pathogenic. Also, others detected two *FANCG* heterozygous mutations in black South African FA population (Wainstein et al., 2013). Our results confirm the unlikelihood of a second common *FANCG* mutation in the black South African population. A recent study identified two black South African patients compound heterozygous for *FANCD1*/*BRCA2* mutations (Feben et al., 2017b). The deleterious *BRCA2* mutation in allele 1 in both patients was c.5771_5774delTTCA; p.Ile924Argfs*38 which is associated with enhanced breast cancer risk (van der Merwe et al., 2012). We also identified this mutation in our TNBC patient cohort (Chapter 6). The second mutation in allele 2 was c.582G>A (p.Trp194*) which has also been was previously reported in a South African breast cancer cohort (Chen, 2015). All parents are mandatory heterozygous carriers and heterozygosity in FANC genes are shown to be associated with early onset breast cancer, ovarian and pancreatic cancer risk. FA carriers harbouring deleterious mutations in *FANCD1*/*BRCA2* are of higher risk for these cancers. In conjunction with our results, in case of absence of the recurrent *FANCG* mutation, there is a clear need for testing black South African FA patients for deleterious mutations in other FANC genes.

Following the identification of the FANC mutations in all 14 patients and confirmed heterozygosity in parents, the next aim was to investigate the chromosomal instability and chromosomal radiosensitivity in both patient and parent this group. The FA/BRCA pathway plays an integral part in maintaining chromosomal stability and is fundamental in DNA damage response. However, the pathway is compromised if DNA repair genes that control and regulate the pathway carry a defect as seen in FA patients and heterozygous carriers. The defect could be reflected as chromosomal instability which is a frequent trait in FA patients (Palovcak et al., 2017, Cerabona et al., 2014). The chromosomal instability was confirmed by the significantly higher spontaneous MN values in the G0 and S/G2 phases of the cell cycle in our study. Heterozygous carriers also displayed higher spontaneous MN values also probably due to defects in FANC genes.

Chromosomal radiosensitivity information in the FA population is also important. The likelihood of developing solid tumours, leukaemia or other cancers in FA patients is substantially high (Mathew, 2006, Velleuer and Dietrich, 2014). The cumulative risk for various solid tumours assessed was 700-fold increase for acute myeloid leukaemia, 600-fold for head and neck squamous cell carcinoma, 3000-fold for vulvar squamous cell carcinoma and 6000-fold for myelodysplastic syndrome (Alter, 2014). Compared to the general population, FA patients are reported to have a 50-fold increase in risk of solid tumours. (Rosenberg et al., 2003). When evaluating cancer risks in heterozygous carriers, a study by Berwick et al. (Berwick et al., 2007) showed increased incidence of breast cancer in FA carriers. Chromosomal radiosensitivity can be seen as a marker of predisposition to cancer. We show that FA lymphocytes also presented significantly enhanced radiation-induced MN values in the G0 and S/G2 phases of the cell cycle. Chromosomal radiosensitivity of FA cells in the G0 phases has previously been contradictory (Duckworth and Taylor, 1981). However, some literature data still demonstrate that FA cells are significantly more radiosensitive when compared to controls (Higurashi and Conen, 1971, Higurashi and Conen, 1973, Alter, 2002, Heddle et al., 1978). We were able to demonstrate enhanced chromosomal radiosensitivity of FA lymphocytes compared to FA parents and healthy individuals with significant differences in radiation-induced MNi in both the G0 and S/G2 phase. This is in agreement with a study by Bigelow et al. (Bigelow et al., 1979) who also evaluated radiosensitivity using lymphocytes. However, this is a much older study performed on smaller cohorts. The FA/BRCA genes play a fundamental role in DNA repair in the G2 phase. When comparing chromosomal radiosensitivity in the G0 and S/G2 phases, significant differences observed in the S/G2 phase for both doses were more pronounced than in the G0 phase. The enhanced S/G2 chromosomal radiosensitivity could be attributed to defects DNA repair pathway in FA patients (Sala-Trepat et al., 2000) due to mutated *FANCG*. The FANCG forms part of the core complex in the FA/BRCA pathway. Defects in the core complex could render the pathway as inefficient. During DNA repair, FANCG interacts with BRCA1 and BRCA2; FANCG directly binds to the BRC repeats on BRCA2. BRC repeats have the capability of binding to RAD51. The presence of a mutation in *FANCG* deteriorates the binding affinity of RAD51 contributing to the increase of breast cancer susceptibility (Hussain et al., 2003) and enhanced chromosomal radiosensitivity. All FA patients with *FANCG* deleterious mutations (n = 13) could exhibit chromosomal radiosensitivity due to the loss of binding affinity of RAD51 that impairs the FA/BRCA pathway and the HR repair mechanism.

Defective checkpoints are a trait that has previously been observed in FA cells (Sala-Trepat et al., 2000). To evaluate the G2/M checkpoint efficiency in our FA cohort, the checkpoint was abrogated by the addition of caffeine to the irradiated cultures in the S/G2 MN assay. The checkpoint efficiency is indicated by a ratio of MNi with caffeine divided by MNi without caffeine (MNCaf+/MNCaf-). A lower ratio indicated an impaired checkpoint. In older studies, FA patients' cells treated with radiation and caffeine showed elevated chromosomal damage compared to healthy individuals that was quantified by metaphase spreads. Two heterozygous carriers were also assessed in the study and displayed increased chromosomal damage with caffeine treatment alone (Pincheira et al., 1988). The FA parents in our study showed impaired checkpoint only at 4 Gy dose of IR with significantly enhanced MNCaf+ values. It was suggested that the addition of caffeine to irradiated FA cells may inhibit DNA repair process in G2 (Sabatier and Dutrillaux, 1988). The significantly higher MNCaf+ value in FA patients seen in our study may be due to the inhibition of DNA damage repair and abrogation of the G2/M checkpoint. Although no significant differences were observed, the higher MNCaf+ values in the FA parents may be due to presence of a defective FANC allele hindering damage repair and overriding the G2/M checkpoint.

The standard chromosomal breakage analysis is a preferred method to diagnose FA by quantifying MMC-induced chromosomal aberrations in metaphase spreads (Krynyckyi et al., 2005). Due to the variations encountered with this technique and the labour intensity in scoring metaphase spreads, we have proposed an alternative method to investigate MMC-induced MN. MMC induced DNA crosslinks in FA patients' results in higher MN values that are statistically significant when compared to heterozygous carriers and controls. FA patients were clearly distinguishable from FA parents and controls when either a lower and higher concentration of MMC was used. However, due to the toxicity of MMC, the FA patients had low numbers of BN cells when MMC concentration was increased. We show that with the lower concentration of 0.02 µg/ml MMC, we could also differentiate heterozygous carriers from healthy controls. The heterozygous carriers had values in between patients and controls. Older studies conducted on lymphocytes of FA parents using DEB and MMC could not differentiate FA parents from controls. An older study induced chromosomal breakage using DEB and MMC for the identification of FA carriers were also unsuccessful with the overlapping of results between the FA parents and control population (Cervenka and Hirsch, 1983, Deviren et al., 2003, Rosendorff and Bernstein, 1988, Cohen et al., 1982).

The chromosomal breakage analysis requires the quantification of metaphase spreads. Different protocols are utilised across cytogenetics laboratories that may cause variations in results correlated with very low yields of metaphases. Analysing metaphase spreads require extensive experience and results could be inconclusive results in the absence of an experienced analyser (Oostra et al., 2012). The chromosomal breakage analysis is highly labour intensive. The MMC MN assay we've proposed has proven to be less labour intense and quantifying MNi is less subjective. Another advantage of the MMC MN assay is the reduction in cost. It is more economical and a robust technique that allows distinguishing of FA patients, carriers and controls. Since, the MMC MN assay is time effective, less subjective and less labour intensive, it can be utilised in a clinical setting to diagnose FA patients particularly in South Africa. South African FA patients manifest clinically symptoms prior to genetic screening referrals and usually present with late stage disease with severe haematological presentations. Due to the subtlety of the physical defects, some patients clinically not diagnosed which still remains a challenge (Feben et al., 2014). There is an urgent need for a quick and reliable assay for the clinical diagnosis of FA patients in South Africa prior to severe clinical symptoms. With further validations, the MMC MN assay is a promising functional assay to identify individuals with defects in FA genes.

CONCLUSIONS

As the primary aim of this research, the chromosomal instability and chromosomal radiosensitivity of breast cancer patients in South Africa was investigated. Breast cancer in South Africa is the leading cancer amongst women and has a high incidence of early-onset of the disease. Young African women are frequently diagnosed with TNBC. The occurrence of TN is often associated with *BRCA1/2* mutations that play a central role in DNA repair; defects in DNA repair genes can lead to chromosomal instability and chromosomal radiosensitivity. In a selected cohort of breast cancer patients, 12% were positive for *BRCA1/2* mutations; 3 were novel mutations identified in black South African patients. Six variants with unknown clinical significance were also identified in the black population; this highlights the need for further genetic screening in the under studied population, explicitly in young TN patients. Screening a larger population of TN and young patients will permit the identification of deleterious mutations in breast cancer susceptibility genes and contribute to the mutation spectrum in South African breast cancer patients.

Information on breast cancer radiosensitivity is important, particularly in TN breast cancer patients who present with more aggressive tumours, poor prognosis and limited treatment options. TNBC patients are treated with IR and the information on radiosensitivity is very valuable. The observations of the MN assay suggest increased chromosomal instability and chromosomal radiosensitivity in South African breast cancer patients. The increase in spontaneous and radiation-induced MNi frequency was observed in young as in older patients. Although age influences spontaneous MN, an age effect on radiation-induced MN could not been shown. With a mixed ethnicity in South Africa, a correlation between chromosomal radiosensitivity and ethnicity was investigated and revealed differences in radiosensitivity in different ethnic South African groups. Overall, in the different ethnic groups, black and white patients were more chromosomally radiosensitive and the radiosensitivity was more pronounced in estrogen receptor positive patients. In the absence of receptors, TN patients presented with a “radioresistant phenotype”. Our results suggest that the healthy lymphocytes in TN patients are equipped to handle higher doses of IR, therefore, treatment plans could be altered with adequate doses for TNBC patients. Further validations of chromosomal radiosensitivity in larger TN patients population is necessary. Furthermore, larger cohorts are warranted to validate chromosomal instability and chromosomal radiosensitivity in the presence of germline mutations in DNA repair genes such as BRCA.

The secondary aim of the study was to investigate the chromosomal instability and chromosomal radiosensitivity in FA patients and parents compared to healthy individuals. Of all the South African FA patients recruited for the study, 92% were positive the *FANCG* founder mutations and a *FANCA* mutation was identified in a patient with African descent. The radiosensitivity data for the FA patients and parents presented here are, therefore, valid for the *FANCG* and *FANCA* homozygous and heterozygous carriers. Further studies are warranted to test FA homozygotes or compound heterozygotes of other FA genes. As a marker for cancer predisposition, chromosomal radiosensitivity is crucial in cancer prevention and monitoring. Additionally, these patients are at risk to develop secondary cancers as a result of radiotherapy, it is recommended that these patients are treated with caution. In the FA patients, chromosomal instability and chromosomal radiosensitivity was significantly elevated. This was observed in both the G0 and S/G2 phase of the cell cycle. As obligate carriers, the FA parents also exhibited chromosomal instability in G0 and S/G2 phase. Chromosomal radiosensitivity in FA parents was not detected. Using IR, the MN assay proves to be beneficial for detecting FA homozygotes and heterozygotes at a group level but not on an individual level. On the contrary, when we induced DNA damage by MMC instead of IR, and using the MMC MN assay, identification of FA homozygotes and FA heterozygotes from controls was possible. Identification of heterozygous mutation carriers may be clinically relevant in the context of breast cancer prevention as heterozygous mutations in several FA genes were shown to be associated with an increased risk for breast cancer.

The optimised MMC MN assay in our study has shown to be beneficial as it is less labour intense, more time effective and less subjective than the classic MMC chromosomal breakage assay. Since this technique can undeniably differentiate FANC homozygotes and heterozygote genotypes from healthy controls, we propose the MMC MN assay as a potential new functional assay supportive for the identification of individuals with a deficiency in genes related to the FA/BRCA pathway.

FUTURE PERSPECTIVES

This study has highlighted the various factors that affect chromosomal radiosensitivity in breast cancer patients. Future experiments could continue the investigation of chromosomal instability and chromosomal radiosensitivity of breast cancer patients by scrutinising other potential factors such as the effect of ER. ER mediates its biological effects through two receptors – ER alpha (ERα) and ER beta (ERβ). These receptors are co-expressed in breast cancer cells and the ratios of expression vary. The TNBC are known to lack the expression of ER. However, it was recently shown that TN cells lack the expression of ERα but a portion of TNBC express ERβ. The lack of ER expression was suggested in our study as a factor that influences chromosomal radiosensitivity in TN cells. Therefore, further investigations on which ER subtype influences chromosomal radiosensitivity will be assessed. Overexpression and knockdown of the estrogen receptors in several breast cancer types by using recombinant DNA technology are proposed in our future studies.

Our results of the S/G2 MN assay show an inclination of increased chromosomal radiosensitivity in luminal patients but not in TNBC patients. It would be of great interest to further evaluate the chromosomal radiosensitivity in the S/G2 phase of TN and luminal patients by increasing sample numbers. In the future, the effect of ethnicity should also be evaluated with larger cohorts.

FA patients regularly receive blood cell transfusions due to anaemia, thrombocytopenia and low platelet counts. Receiving sufficient blood samples from these patients can often be a challenge. Using fibroblasts from skin biopsies from FA patients to investigate radiation- or MMC- induced DNA damage could overcome the problem of insufficient blood in FA patients and could exclude the interference of recent blood transfusions with the quality of results. Apart from biomarkers tested in our study, future work in FA patients can scrutinise DNA repair proteins. HR repair is crucial in the FA/BRCA pathway. RAD51 is a downstream effector and plays an important role in the pathway. The *FANCG* gene forms part of the core complex and mutations in this gene are frequent in the black FA patients in SA. Using RAD51 foci assay could allow detecting the interaction between the two genes and repair efficiency. RAD51 foci assay can also be used to investigate the repair kinetics in FA patients and parents. Other proteins involved in the FA/BRCA pathway could be investigated with

specific foci assays and could reveal more information on the underlying mechanism of radiosensitivity in the breast cancer and FA patients.

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APPENDIX 1

Supplementary tables for chapter 6

Supplementary Table 1: Overview of grading and staging of breast cancer on diagnosis.

Age	*Average*	45
	Median	42
	Range	25 – 77
Grade	*Grade 1*	7%
	Grade 2	51%
	Grade 3	42%
Stage	*Stage I*	9%
	Stage II	55%
	Stage III	32%
	Stage IV	2%

Supplementary Table 2: Overview of sequencing coverage per run.

Run	**Total no. of amplicons sequenced**	**No. of amplicons with coverage <28x**	**No. of amplicons with coverage <5x**	**Average per run**	**Median per run**	**Range per run**
1	4416	177 (4%)	123 (3%)	313	238	0-7000
2	1248	38 (3%)	25 (2%)	208	185	0-1000
3	2400	23 (1%)	20 (1%)	509	441	0-2500

Supplementary tables for chapter 7

Supplementary Table 1: G0 MN assay p values

Dose	Patients vs Controls	Parents vs Controls	Patients vs Parents
0Gy	0.0100*	0.2397	0.3000
2Gy	0.0003*	0.3459	0.0219*
4Gy	0.0025*	0.2456	0.0444*

*Statistically significant

Supplementary Table 2: S/G2 MN assay p values

Dose	Patients vs Controls	Parents vs Controls	Patients vs Parents
0Gy	<0.0001*	0.0164*	0.0108*
2Gy C-	0.0040*	0.1996	0.0430*
4Gy C-	<0.0001*	0.0962	0.0006*
2Gy C+	<0.0001*	0.1499	0.0051*
4Gy C+	0.0025*	0.0310*	0.1371

*Statistically significant

Supplementary Table 3: MMC MN assay p values

Dose	Patients vs Controls	Parents vs Controls	Patients vs Parents
0 MMC	0.0032*	0.2858	0.0307*
0.02 µg/ml	<0.0001*	0.2013	<0.0001*
0.1 µg/ml	<0.0001*	0.0002*	0.0004*

*Statistically significant

APPENDIX 2

Ethics clearance certificate for breast cancer study

M130450

R14/49 Dr A Baeyens/Ms F Francies

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130450

NAME: (Principal Investigator)	Dr A Baeyens/Ms F Francies
DEPARTMENT:	Dept of Radiation Sciences/Radiobiology CM Johannesburg Academic Hospital
PROJECT TITLE:	Chromosomal Radiosensitivity of Triple Negative and Young Breast Cancer Patients in South Africa
DATE CONSIDERED:	26/04/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Ans Baeyens
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 12/06/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature

Date 20/06/2013

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Ethics clearance certificate for Fanconi anaemia study

R14/49 Ms FZ Francies & Dr A Baeyens

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M141031

NAME: (Principal Investigator)	Ms FZ Francies & Dr A Baeyens
DEPARTMENT:	Radiation Sciences Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Evaluation of 3 Cytogenic Assays as Diagnostic Tools in Genetic Screening of Franconi Anemia Patients
DATE CONSIDERED:	31/10/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Ans Baeyens
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	06/02/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX 3

Informed consent and questionnaire for health individuals

Informed consent and questionnaire for breast cancer patients

Informed consent and questionnaire for Fanconi Anaemia patients/parents

Study title: Chromosomal radiosensitivity of triple negative and/or young breast cancer patients in South Africa
Investigators: Dr Ans Baeyens, Flavia Francies, Olivia Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Olivia Herd: 082 778 49 29
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

INFORMED CONSENT FORM - HEALTHY DONORS

Good Day,

We are Dr A. Baeyens and students Flavia Francies and Olivia Herd from the Radiobiology lab based at WITS medical School. We are part of a collaborative study between WITS University and iThemba LABS to investigate the radiosensitivity of breast cancer patients. Radiosensitivity explains how well the radiation works on your tumour and normal organs; it means an increased susceptibility of cells, tissues or organs to the harmful effects of ionizing radiation. Ionizing radiation is used in radiotherapy to treat cancer. Information on individual radiosensitivity helps to monitor the radiotherapy treatments.

We need healthy individuals, without breast cancer, 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.

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.

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. This study has been approved by the University of Witwatersrand, Human Research Ethics Committee. If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301 or peter.cleaton-jones@wits.ac.za.

Thank you very much for your time

Regards,
Dr A. Baeyens, F. Francies, O. Herd

Study title: Chromosomal radiosensitivity of triple negative and/or young breast cancer patients in South Africa
Investigators: Dr Ans Baeyens, Flavia Francies, Olivia Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Olivia Herd: 082 778 49 29
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: Chromosomal radiosensitivity of triple negative and/or young breast cancer patients in South Africa

Investigators: Dr Ans Baeyens, Flavia Francies, Olivia Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Olivia Herd: 082 778 49 29
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? .....

Are you pre- or postmenopausal? .....

Family history of cancer?

……
…

Participant questionnaire

Participant initials:

Study participant number

Study title: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

INFORMATION SHEET FOR HEALTHY DONORS

Good Day,

We are Dr. A. Baeyens and student Flavia Francies 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 chromosomal instability and radiosensitivity in Fanconi Anaemia (FA) patients. Radiosensitivity is the susceptibility of cells to the harmful effects of ionizing radiation. This may be due to possible defects in DNA repair.

We need healthy individuals, without FA, 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.

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. 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.

There is no direct benefit to you. But your participation in this study will contribute to the development of greater knowledge of radiosensitivity and diagnostics in FA 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. This study has been approved by the University of Witwatersrand, Human Research Ethics Committee. If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301 or peter.cleaton-jones@wits.ac.za.

Thank you very much for your time

Regards,
Dr A. Baeyens, F. Francies

Study title: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

Study participant number: Date of Birth:
Contact no:

INFORMED CONSENT FOR HEALTHY DONORS:

I hereby confirm that I have been informed about the nature, conduct, benefits of the study on radiosensitivity of Fanconi Anaemia 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: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

QUESTIONNAIRE FOR PARTICIPANTS

Age: .....

Home language: .....

Ethnic group: .....

Place of birth: .....

Do you have children? How many? .....

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 initials:

Study participant number:

Study title: Chromosomal radiosensitivity of triple negative and/or young breast cancer patients in South Africa
Investigators: Dr Ans Baeyens, Flavia Francies, Olivia Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Olivia Herd: 082 778 49 29
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

INFORMED CONSENT FORM - PATIENTS

Good Day,

We are Dr A. Baeyens and students Flavia Francies and Olivia 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. Radiosensitivity explains how well the radiation works on your tumour and normal organs; it means an increased susceptibility of cells, tissues or organs to the harmful effects of ionizing radiation. Ionizing radiation is used in radiotherapy to treat cancer. Information on individual radiosensitivity helps to monitor the radiotherapy treatments.

We invite you to consider participating in our 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 will be 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.

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 will only be 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. This study has been approved by the University of Witwatersrand, Human Research Ethics Committee. If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301 or peter.cleaton-jones@wits.ac.za.

Thank you very much for your time
Regards,
Dr. A. Baeyens, F. Francies and O. Herd

Study title: Chromosomal radiosensitivity of triple negative and/or young breast cancer patients in South Africa
Investigators: Dr Ans Baeyens, Flavia Francies, Olivia Herd
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Contact numbers: Dr A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Olivia Herd: 082 778 49 29
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: Chromosomal radiosensitivity of triple negative and/or young breast cancer patients in South Africa
Investigators: Dr Ans Baeyens, Flavia Francies, Olivia Herd
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Olivia Herd: 082 778 49 29
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? .....

Are you pre- or postmenopausal? .....

Family history of cancer?

……

……..

Participant questionnaire

Participant initials:

Study participant number:

Study title: Chromosomal radiosensitivity of triple negative and/or young breast cancer patients in South Africa
Investigators: Dr Ans Baeyens, Flavia Francies, Olivia Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Olivia Herd: 082 778 49 29
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

INFORMED CONSENT FORM (GENETIC COUNSELLING AND MOLECULAR WORK):

Good Day,

We are Dr A. Baeyens and students Flavia Francies and Olivia 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. Radiosensitivity explains how well the radiation works on your tumour and normal organs; it means an increased susceptibility of cells, tissues or organs to the harmful effects of ionizing radiation. Ionizing radiation is used in radiotherapy to treat cancer. Information on individual radiosensitivity helps to monitor the radiotherapy treatments.

We invite you to consider participating in our 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 will be 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. You will also receive genetic counselling which is the process of evaluating family/medical history to explain what genetic factors may play in risk to develop cancer for you and your family. The counselors will give you advice of the consequences of the breast cancer and will also provide assistance in making further medical decisions such as frequent screenings for breast cancer.

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 will only be 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. This study has been approved by the University of Witwatersrand, Human Research Ethics Committee. If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301 or peter.cleaton-jones@wits.ac.za.

Thank you very much for your time.

Regards,
Dr. A. Baeyens, F. Francies and O. Herd

Study title: Chromosomal radiosensitivity of triple negative and/or young breast cancer patients in South Africa
Investigators: Dr Ans Baeyens, Flavia Francies, Olivia Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Olivia Herd: 082 778 49 29
Ethics Committee Chairperson: Prof Cleaton-Jones: 011 717 23 01

Study participant number: Date of Birth:
Contact no:

INFORMED CONSENT (GENETIC COUNSELLING AND MOLECULAR WORK):

I hereby confirm that I have been informed about the nature, conduct, benefits of the molecular 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: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

INFORMATION SHEET FOR FA PATIENT AND PARENTS

Good Day,

We are Dr. A. Baeyens and student Flavia Francies 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 chromosomal instability and radiosensitivity in Fanconi Anemia (FA) patients. Radiosensitivity is the susceptibility of cells to the harmful effects of ionizing radiation. This may be due to possible defects in DNA repair.

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

Should you decide not to grant permission for your child to participate in the study or if you agree and then change your mind, there will be no implications for you or your child. The best treatment available will still be provided for your child. If you agree to grant permission for your child to participate, we kindly ask your child to donate 20ml of blood once. This is not a lot of blood; it is only 4 teaspoons and will not harm your child. We will use your child's blood sample to test the sensitivity to radiation. The blood sample will be used to unravel the underlying mechanism of radiosensitivity in FA patients.

There is no direct benefit to you or your child. But your child's participation in this study will contribute to the development of greater knowledge of radiosensitivity and diagnostics in FA patients. The research is completely confidential, which means that your child's name will not be recorded on any of our laboratory information. The consent forms will be locked away and will only be accessible by the researchers. We also ask permission to view your child's medical files if we need to obtain any further medical information that may be relevant to our study.

You are free to ask any questions about this study and discuss any worries you may have with the research staff. This study has been approved by the University of Witwatersrand, Human Research Ethics Committee. If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301 or peter.cleaton-jones@wits.ac.za.

Thank you very much for your time

Regards,
Dr. A. Baeyens and F. Francies

Study participant number:

ASSENT FORM

We are Dr. A. Baeyens and student Flavia Francies from WITS Medical School. We are doing a study to figure out if some children have Fanconi Anaemia by doing some tests. We are asking you to take part in the research study because you have been visiting the hospital for treatment for Fanconi Anaemia.

- For this study, we will need some blood. You may need a needle poke, so we can test some of your blood. The poke for the blood test can hurt a little.
- We will also look at your hospital file to get some information about your treatment.

This study will not help you or no bad thing will happen to you. But we hope that someday this study will help other children who have Fanconi Anaemia like you do.

- If you want to take part in this study then you can just say "Yes" or "no" if you do not wish to take part.
- You do not have to be in this study if you don't want to. We will not mind if you do not want to participate.
- If you do not want to participate, you will still receive the best treatment and the doctors will continue to take good care of you.
- Your parents said it is ok for you to be in this study. But you can still decide if you want to take part or not.
- You can stop being part of this study at any time.
- You can ask any questions you want to ask. If you think of a question later, you or your parents can contact me.

If you understand everything and you want to be part of this study then write your name below. I will write my name too. This is to show that we both spoke about the study and you want to be part of it.

PARTICIPANT:

Name:

Signature and date:

STUDY STAFFF OBTAINING ASSENT:

Name:

Signature and date:

Study title: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

Contact no:

INFORMED CONSENT FOR FA PATIENTS AND PARENTS:

I hereby confirm that I have been informed about the nature, conduct, benefits of the study on chromosomal instability and radiosensitivity of Fanconi Anaemia patients.

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

I have no further questions and hereby grant permission for my child to participate in the study.

PARENT OR LEGAL GUARDIAN OF 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: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

INFORMATION SHEET FOR PARENTS OF FA PATIENTS

Good Day,

We are Dr. A. Baeyens and student Flavia Francies 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 chromosomal instability and radiosensitivity in Fanconi Anaemia (FA) patients. Radiosensitivity is the susceptibility of cells to the harmful effects of ionizing radiation. This may be due to possible defects in DNA repair.

We need individuals who are asymptomatic carriers of the FA gene. 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.

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. 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. The blood sample will be used to unravel the underlying mechanism of radiosensitivity in asymptomatic carriers.

There is no direct benefit to you. But your participation in this study will contribute to the development of greater knowledge of radiosensitivity and diagnostics in FA 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. This study has been approved by the University of Witwatersrand, Human Research Ethics Committee. If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301 or peter.cleaton-jones@wits.ac.za.

Thank you very much for your time

Regards,
Dr. A. Baeyens, F. Francies

Study title: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

Study participant number: Date of Birth:
Contact no:

INFORMED CONSENT FOR PARENTS OF FA PATIENTS:

I hereby confirm that I have been informed about the nature, conduct, benefits of the study on radiosensitivity of Fanconi Anaemia 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: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

QUESTIONNAIRE FOR PARTICIPANTS

Age: .....

Home language: .....

Ethnic group: .....

Place of birth: .....

Do you have children? How many? .....

Have you ever smoked? Do you currently smoke? .....

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

Do you know your HIV status? Are you prepared to tell us? .....

Participant initials:

Study participant number:

Study title: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies, Olivia Herd
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Olivia Herd: 082 778 49 29
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

GENETIC TESTING PROTOCOL: INFORMATION SHEET FOR FA PATIENTS AND/OR PARENTS (CARRIERS)

Good Day,

We are Dr. A. Baeyens and student Flavia Francies 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 chromosomal instability and radiosensitivity in Fanconi Anaemia (FA) patients. Radiosensitivity is the susceptibility of cells to the harmful effects of ionizing radiation. This may be due to possible defects in DNA repair.

What is the reason for the genetic testing protocol in this research study?
FA is an inherited condition and results from two mutations (faults) in one of the many genes (FA genes) associated with the diagnosis. It is mostly inherited in an autosomal recessive manner, meaning that both parents to a child with FA each carry one mutation in a disease-causing FA gene. There are over 15 FA genes.

The mutation, in a FA gene, affects the structure of chromosomes in people with FA, and therefore also the stability and radiosensitivity. Different types of tests (cytogenetic) are used to identify these unstable chromosomes. These results are used for diagnosis.

The aim of this study is to determine the usefulness of different cytogenetic tests to identify chromosomal instability and radiosensitivity in FA patients, and their carrier parents. The underlying genetic cause for the diagnosis will be required to investigate if there is a link between the specific mutations and the cytogenetic test result. The purpose of the genetic testing protocol is therefore to identify the underlying genetic cause for the FA diagnosis in patients and their carrier parents.

Is participation in the genetic testing voluntary?
Your child has been selected for the genetic testing protocol because he/she has a confirmed clinical diagnosis of FA, and both the underlying disease-causing gene mutations have not been identified. We invite you to consider the participation of your child and yourself in our research study. Your/your child's participation in the genetic testing protocol is entirely voluntary. If you decide that your child can take part in this study, you will be asked to sign the consent form to confirm that you understand the purpose for genetic testing. You will be given a copy to keep.

Should you decide not to grant permission for your child for genetic testing or if you agree and then change your mind, there will be no implications for you or your child. The best treatment available will still be provided for your child.

What is required of your child and/or you for genetic testing?
Your child and/or you will be asked for a blood sample of 10ml. This is not a lot of blood; it is equal to about 2 teaspoons and the test is not harmful. You will not need to fast before being tested. Genetic testing will be performed to identify the presence of the mutations in one of 15 FA genes. The testing will be performed at the Diagnostic Molecular Laboratory, University of Ghent, Belgium.

Prior to testing you will receive genetic counselling. A family history will be drawn up and the counsellor will use this and medical history to discuss the diagnosis of FA in your family, including the genetic cause, symptoms, progression of the disease, inheritance and genetic testing. You will receive an explanation of the types of genetic tests that will be performed, and the associated risks and limitations. As parents, the results from your child's test will be used to identify your underlying mutation. The benefit of testing parents is to assist with interpretation of results. On occasion the test result may be inconclusive.

Will you get the results from the genetic test?
A genetic counsellor will communicate the result from your genetic test to you in a separate session. The counsellors will advise you of the consequences of being a carrier of the FA gene and the impact on future medical decisions and other family members.

How will your child and/or you benefit from the genetic testing protocol?
The benefit of the genetic testing will be that the underlying genetic cause for FA in the family is identified and this can be used to determine the risk of FA for other family members and in future children. This information can be used in the research project to identify links between gene mutations and cytogenetic test results. This research will not benefit you or your child directly, but will contribute to the development of greater knowledge of radiosensitive and diagnostics in FA patients.

Are there any risks associated with the genetic testing protocol?
The risks associated with the genetic test are in the sampling, the testing procedure and interpretation of the result. These will be discussed with you and are detailed in the consent form.

Will you receive payment to participate?
There is no payment for participation.

You are free to ask any questions about this study and genetic testing protocol and discuss any worries you may have with the research staff. This study has been approved by the University of the Witwatersrand, Human Research Ethics Committee. If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human

Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301 or peter.cleaton-jones@wits.ac.za.

Thank you very much for your time.

Regards,
Dr. A. Baeyens, F. Francies

Study title: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

Contact no:

INFORMED CONSENT FOR GENETIC TESTING - FA PATIENTS AND/OR PARENTS (CARRIER)

I hereby confirm that I have been informed about the nature, conduct, benefits of the study on chromosomal instability and radiosensitivity of Fanconi Anaemia patients.

I have also received, read and understood the above written information regarding the genetic testing protocol.

I have been informed and understand the purpose of this genetic test. A genetic counsellor has discussed with me the limitations, benefits and the risks of this testing, and I have had my questions answered. I understand the following:

- The test procedure is specific to the diagnosis of Fanconi Anaemia and cannot determine the complete genetic makeup of an individual.
- The method and risks of the sampling procedure(s) has/have been explained to me.
- The test may reveal previously unrecognized biological relationships, such as non-paternity. DNA tests also may reveal a genetic condition in another family member.
- My/my child's DNA sample may be used to interpret that of a family member.
- The test result may not always be definitive, and rarely are they uninformative or difficult to interpret.
- There is a small potential error for all test results (generally < 1%). In addition, the laboratory may have difficulties analyzing the sample and a second sample may be requested.
- I may withdraw my consent at any time without giving a reason and without this affecting my/my child's future medical care. However, the sample remains the property of the pathology laboratory unless otherwise instructed by me.
- Medical confidentiality will be respected.
- My/my child's result will not be released to anyone except me through my referring doctor and genetic counsellor (if applicable). They will only be released to other medical professionals or other parties with my written consent.
- By giving written informed consent, you are giving permission for the researchers to use the data collected or generated in this study for scientific purposes ONLY and that all data will be treated confidentially. You have the right to have any questions you may have about the research project answered by the project head or any of the researchers involved in the study.
- The DNA sample will not be stored for future use and will be destroyed in accordance with the Wits Research rules

Study title: Evaluation of 3 cytogenetic assays as diagnostic tools in genetic screening of Fanconi Anaemia patients.
Investigators: Dr. Ans Baeyens, Flavia Francies
Institution: WITS University and iThemba LABS
Contact numbers: Dr. A Baeyens: 072 919 88 72 Flavia Francies: 0798786949
Ethics Committee Chairperson: Prof. Cleaton-Jones: 011 717 23 01

I give my consent to have a sample taken for genetic testing on the individual named below for the condition, Fanconi Anaemia:

PARTICIPANT:

Name:

Date of Birth:

IF PARTICIPANT IS A PARENT:

Patient's name:

Relation to patient:

PARENT OR LEGAL GUARDIAN OF PARTICIPANT:

Name (Print):

Signature and date:

STUDY STAFF CONDUCTING CONSENT DISCUSSION:

Name (Print):

Signature and date:

WITNESS (IF APPLICABLE):

Name (Print):

Signature and date: