The Effect of Selected Polymorphisms in the p53 Pathway as Potential Genetic Modifiers of Cancer Risk and Penetrance in Female Afrikaner *BRCA2* Carriers

By

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DECLARATION

I certify that the dissertation hereby submitted by me for the degree M.Med.Sc. at the University of the Free State is my independent effort and had not previously been submitted for a degree at another University/Faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

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B. K. Dajee

Thank you for your love throughout the years.
How else could I become what I've become?
All your plans and hopes and even fears
Now come together in what I have done.
Know that I am grateful for your love.
Your hard work is mirrored now in mine.
On you all my accomplishments must shine.
Underneath my pride, your spirits move.

N. Gordon

This dissertation is dedicated to my parents.

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Abbreviations

3’	3 prime end
5’	5 prime end
72Pro	codon 72 Proline
72Arg	codon 72 Arginine
Arg	arginine (amino acid)
ATM	Ataxia telangiectasia (mutated) gene
BAP1	BRCA-associated protein
BARD1	BRCA1-associated RING domain
BC	Breast cancer
BCLC	Breast Cancer Linkage Consortium
BIC	Breast Cancer Information Core database
bp	base pair
BRC	BRCA2 repeat motif
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
BRCA3	Breast cancer susceptibility gene 3
BRCT	BRCA1 carboxy-terminus
BRIP	BRCA1-interacting protein
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
CHEK2	Checkpoint kinase 2 gene
CI	confidence interval
CIMBA	Consortium of Investigators of Modifiers of *BRCA1* and *BRCA2*
CTD	C-terminus DNA-binding domain
C-terminus	carboxy terminus
CtIP	C-terminal-binding –protein-interacting protein
DBD	DNA binding domain
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleic triphosphates
DSBs	double strand breaks

DSS1	Deleted in split-hand/split-foot 1 region
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
FA	Fanconi anaemia
FANCD1	FA complementation group D1
FANCD2	FA complementation group D2 ,
FANCJ	BRCA1 FA complementation group J
g/l	grams per litre
Gly	Glycine
HR	homologous recombination
HRT	hormone replacement therapy
IARC	International Agency of Research in Cancer
kDa	kilodalton
LFL	Li-Fraumeni Like syndrome
LFS	Li-Fraumeni syndrome
M	molar (moles per liter)
MDM2	mouse double minute 2 gene
mM	millimolar
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NCR	National Cancer Registry
NES	nuclear export signal
ng	nanograms
ng/µl	nanograms per microliter
NLS	nuclear localization sequence
NoLS	nucleolar localization signal
N-terminus	amino terminus
OB	oligonucleotide/oligosaccharide-binding
OC	oral contraceptives
OMIM	Online Mendelian Inheritance in Man
OR	odds ratio
OVC	ovarian cancer
p21	*WAF1* gene product

PALB2	Partner and localizer of *BRCA2* gene
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pmol/µl	pico moles per microliter
PR	progesterone receptor
PRD	proline-rich domain
Pro	proline (amino acid)
PTEN	phosphatase and tension homolog
RAD51	homolog of RecA of *E.* coli
RR	relative risk
SA	South Africa
Ser	serine (amino acid)
SET	sodium chloride EDTA-Tris HCl
SNPs	single nucleotide polymorphisms
SSCP	single-strand conformation polymorphism
STK11	serine/threonine kinase gene
TAD	transactivation domain
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA buffer
TET	tetramerization domain
Tp53	tumour suppressor p53 gene
Tris	2-Amino-2-(hydromethyl)-1,3-propanediol
U	units
UTR	untranslated region
$V.cm^{-1}$	Volts per centimetre
v/v	volume per volume
Val	Valine
w/v	weight per volume
WAF1	wild type p53 activated fragment 1 gene
µg	microgram
µl	microlitre
$\mu l.mg^{-1}$	microgram per millitre

Abstract

Germline mutations in *BRCA2* confer a high risk for the development of breast cancer in the Afrikaner population. A great deal of variability in the development of the disease has been observed among mutation positive family members. Evidence suggested that genes affecting breast cancer risk in the general population could potentially also affect breast cancer risk in *BRCA* mutation carriers. The cell cycle control pathway was selected as a candidate as the functional loss of the tumour suppressor protein p53 is a common feature in diverse human cancers. The ability of this protein to sense cellular damage and halt the progression of the cell cycle or direct the cells to apoptosis is essential in preventing tumourigenesis.

The aim of the study was an attempt to identify potential genetic modifiers of breast cancer risk and penetrance in Afrikaner women carrying the South African founder *BRCA*2 c.8162delG mutation. It involved environmental factors as well as six polymorphisms detected in critical genes of the *Tp53* pathway. The investigated polymorphisms included three variants previously detected in *Tp53* (intron 3, exon 4 and intron 6), a polymorphism present in the promoter of *MDM2* and two SNPs identified in *WAF1* (intron 2 and exon 2).

The epidemiological study failed to identify any specific characteristic associated with an increased or protective breast cancer risk and did not explain the observed residual variation. Of the six polymorphisms studied, only one proved to be statistically significant, namely the 5' splice-site variant in intron 2 of *WAF1.* This polymorphism seemed to explain the variation in penetrance for some of the families, but needs to be confirmed by more extensive studies. A breast cancer recombinant haplotype was compiled using the most informative variants, namely the polymorphism in the *MDM2* promoter, the 5' splice-site variant in intron 2 of *WAF1* and the SNP in exon 4 of *Tp53*, but proved to be uninformative. Association studies including gene to gene and gene to environment interactions could assist researchers in their understanding of the mechanistic basis of the polygenic nature of breast cancer.

Chapter 1

Introduction

Since the discovery of the two hereditary breast cancer genes in the mid 1990s, considerable progress has been made in the characterization of the genetic component of breast cancer (BC) (Struewing *et al.*, 1995; Tonin *et al.*, 1999; Zeegers *et al.*, 2004; Sokolenko *et al.*, 2006). In South Africa, research projects aimed at elucidating the role of *BRCA1* (breast cancer susceptibility gene one) and *BRCA2* (breast cancer susceptibility gene two) within the Caucasian Afrikaner led to the identification of three founder mutations, namely *BRCA1* c.1493delC, BRCA1 p.E881X and *BRCA2* c.8162delG (Reeves *et al.*, 2004; NC van der Merwe, personal communication). Of the three, the *BRCA2* mutation is currently the most common mutation observed within the Caucasian Afrikaner, for it was observed in 42% of all the studied BC families (NC van der Merwe, personal communication). Although these results were met with high expectations, considerable variation in the phenotypic expression of BC was observed among individuals carrying an identical *BRCA2* mutation, for the age at onset varied considerably among these women.

A similar tendency has also been observed internationally amongst BC families (Easton, 1999; Nathanson and Weber, 2001; Antoniou *et al*., 2003; Dapic *et al*., 2005) and these observations led to various questions. The questions focused on other potential genetic models and mechanisms that could explain the remaining familial BC risk. Researchers also hypothesized to what extent a combination of environmental and genetic factors could modify BC risk among *BRCA2* mutation carriers specifically (Tryggvadottir *et al.*, 2003; Haile *et al.*, 2006).

The variation in phenotypic expression could potentially be attributed to genetic and/or environmental factors called “modifiers”. These modifiers are assumed to be the cause of these differences in terms of both the risk of developing BC and the appearance of other associated tumours (Antoniou and Easton, 2006; Levy-Lahad

and Friedman, 2007). The identification of these modifying factors that could influence BC risk is further complicated by the interaction of genetic factors with one another and with the environment.

Genes involved in the various pathways contributing to tumourigenesis have been targeted specifically. These include pathways such as carcinogen metabolising systems, cell apoptosis, DNA repair and hormone metabolism (Rebbeck, 2002). Investigations into these genes led to the discovery of various single nucleotide polymorphisms (SNPs) that, in combination with each other, could act as low to intermediate genetic modifiers of BC risk.

In order to improve the risk assessment in *BRCA2* mutation positive women, a group of SNPs present in genes involved in fundamental pathways were selected. The cell cycle control pathway was selected as a candidate as the functional loss of the tumour suppressor protein p53 is a common feature in diverse human cancers (Lain and Lane, 2003; Joerger and Fersht, 2007). The ability of this protein to sense cellular damage and halt the progression of the cell cycle or direct the cells to apoptosis is essential in preventing tumourigenesis (Levine *et al*., 2006).

The p53 protein regulates the transcription of the mouse double minute 2 gene (*MDM2*) by means of an auto regulatory feedback loop (Lacroix *et al*., 2006; Levine *et al.*, 2006). The interaction between the MDM2 protein and p53 results in the inactivation of the p53 tumour suppressor function. Wild type p53 regulates the G1 checkpoint in response to DNA damage and can induce cell cycle arrest through transcriptional activation of the wild type p53 activated fragment 1 gene (*WAF1*) or by apoptosis (El-Deiry *et al.*, 1993). In tumour cells containing altered forms of p53, p21 (the protein encoded by *WAF1*) levels are greatly reduced or are totally absent, leading to abnormal control of cell-cycle progression (El-Deiry *et al.*, 1993).

Polymorphisms previously identified in these genes regulating the p53 pathway have been investigated and classified as potential modifiers of p53 gene function (Bond *et al*., 2004). Understanding the nature and mechanisms of these modifying effects as well as the biological context in which they occur, will open new

perspectives for approaches aimed at controlling the clinical impact of the tumour suppressor p53 gene (*Tp53*) mutations.

The aims of this study are:

- to screen the Caucasian Afrikaner population for the presence of potential modifying polymorphisms in the *Tp53*, *WAF1* and *MDM2* genes that are involved in the p53 pathway and
- to assess whether any of these polymorphisms potentially modifies the phenotypic expression of BC risk and penetrance in *BRCA2* c.8162delG mutation carriers.

Chapter 2

Literature review

Despite major advances in our understanding of human disease, the mechanisms underlying many common diseases such as heart disease and cancer remain elusive. Cancer is often referred to as a genetic disease since the transition from a normal to cancerous cell involves the acquisition of genetic alterations. These alterations confer a growth advantage to the aberrant cells, ultimately resulting in malignant transformation (Ishikawa *et al*., 2006; Shimada and Nakanishi, 2006). These alterations may be acquired somatically or be present in the germline (Kenemans *et al*., 2004, Lux *et al*., 2006).

Carcinogenesis is a multi-step process characterized by genetic alterations in genes that affect major biological pathways (Kenemans *et al*., 2004). These pathways regulate cell growth and tissue homeostasis involving the cell cycle, apoptosis and differentiation and function as an integrated network. Perturbations in any of these pathways can have profound consequences on others such as deoxyribonucleic acid (DNA) repair, genomic stability, senescence and many more (Kotnis *et al.*, 2005; Ishikawa *et al*., 2006).

2.1 Worldwide incidence of breast cancer

BC is a malignancy affecting the breast of females as well as males. Worldwide it has been observed as the most frequent cancer in females and is the leading cause of death among women (United Nations World Health Organizations, 2007). BC used to be a problem that mostly afflicted affluent Caucasian women in the developed and westernised countries. However, the face of BC is changing. Over the past decade an increase in BC incidence was observed Asia, Africa, the Middle East, Eastern Europe and Latin America (Jones, 1999). The reason for the change is due the extended life span in females from low- and middle income groups which have risen from age 50 in 1965 to 65 in 2005. This increase is due to better

sanitation, improved public health and a greater availability of food. All these factors allow women to age into the normal sporadic BC demographic. As people adapt to western lifestyles, they also tend to increase their BC risk by smoking, eating fatty foods and not exercising enough. It is alleged that by 2020, 70% of all BC cases will occur in developing countries (Jones, 1999).

2.2 Incidence of breast cancer in South Africa

South Africa (SA) is one of the few nations on the African continent that support population-based collection of cancer data via the National Cancer Registry (NCR). According to this registry, BC is the leading cancer for women in SA, with one in 27 women diagnosed during their lifetime. Since this registry relies on information submitted mostly by pathology laboratories for case ascertainment, it is suspected that their statistics may be underestimating the actual cancer incidence (Vorobiof *et al.*, 2001).

The age standardised death rate in 2000 was 10 per 100 000 as stated in the mortality report of the Medical Research Council. The age standardised incidence rate was quoted as 33 per 100 000 in 1999 according to the NCR. They recorded a total of 5 606 (18.6%) and 5 901 (19.4%) BC cases in 1998 and 1999 respectively. In 1998, cancer of the breast was second only to cervical cancer, but changed to the leading cancer in 1999 (Mqoqi *et al.,* 2004). Age specific incidence rates greater than 80 per 100 000 were recorded in women older than 49 years, with an incidence of 161 per 100 000 recorded in women of 75 years and older (Mqoqi *et al.,* 2004).

Breast cancer is extremely common among SA Caucasian females and constitutes on average 20% of all cancer types reported. Of all the BC cases reported in 1998 and 1999, 2 412 and 2 468 were observed in Caucasian women, comprising on average 45% of all cancer reported for that group in those years. BC is also common among females representing the Asian and Mixed Ancestry populations. Vorobiof and co-workers (2001) published lifetime risks for BC as being one in 13 for Caucasians, one in 63 for the Mixed Ancestry and one in 81 for the Black SA

females. The latest findings (Mqoqi *et al.*, 2004) show an increase in risk for certain population groups such as Caucasians (1 in 12), Asians and Mixed Ancestry (1 in 18). Females representing the Black population still have the lowest incidence rates of all (Mqoqi *et al.*, 2004).

When compared to other countries, the BC incidence rate in SA Caucasian women was fourth highest, with an incidence of 76.5 per 100 000 (Fig 2.1) (Parkin *et al.,* 2002). The incidence rates in Black SA women represented the second lowest with an incidence of 18.4 per 100 000. An interesting phenomenon was observed, namely that the incidence of BC of Asian women in SA was nearly double that of women in their country of origin (Fig 2.1).

2.3 Hereditary breast cancer

Hereditary (familial) cancer implies an inherited predisposition to cancer and is characterized by clustering of the disease within families, typical of a dominantly inherited trait. This familial clustering was first documented 100 years ago by the French surgeon Broca in 1866.

The proportion of BC cases that is directly attributable to hereditary factors has been estimated to be only 5-10% (Claus *et al*., 1996), with the highest risks among first degree relatives. Characteristics that indicate the presence of a genetic predisposition to BC include an early age at diagnosis (younger than 35 years), the presence of bilateral female BC and male BC within the family (Lux *et al*., 2006). The expression of the disease depends on genetic and various environmental factors such as gender, age, diet and hormonal exposure.

Proof of dominant BC inheritance was obtained with the location of *BRCA1* (Online Mendelian Inheritance in Man (OMIM 113705)) and *BRCA2* (OMIM 600185*)* on chromosomes 17 and 13 (Miki *et al.*, 1994; Wooster *et al.*, 1995). Soon after their discovery, mutation screening commenced. It revealed that individuals carrying a germline mutation in *BRCA1* or *BRCA2* had a significantly increased lifetime risk of developing BC and/or ovarian cancer (OVC). These genes are associated with

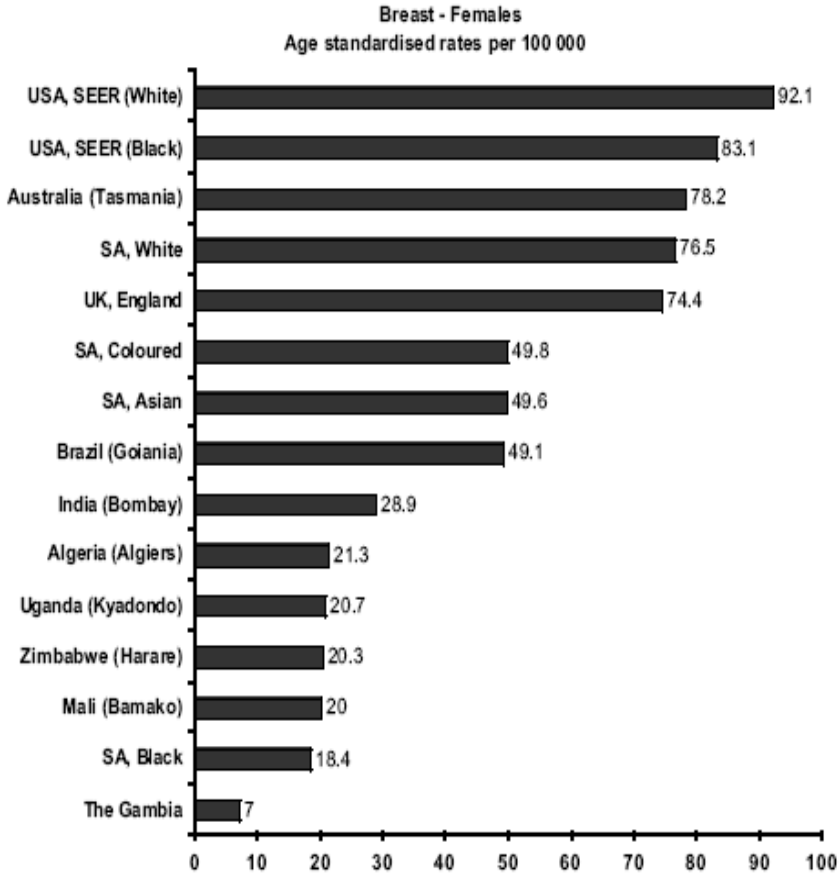


Figure 2.1 International BC rates standardised according to age per 100 000 for various populations (Parkin *et al.,* 2002).

DNA damage at various cell cycle checkpoints, repair functions and apoptosis. Most of the *BRCA* mutations affect the activity of the protein product (Honrado *et al*., 2005), thereby leading to a high-risk predisposition for BC.

Since BC is a feature of several cancer syndromes, various other genes have also been found to be mutated in familial BC. These include Li-Fraumeni syndrome (LFS) (OMIM 151623, germline mutations in *Tp53* - OMIM 191170) (Li and Fraumeni, 1969), Cowden syndrome (OMIM 158350, mutations in the phosphatase and tensin homolog gene *PTEN* - OMIM 601728) (Liaw *et al.*, 1997) and Petz-Jeghers syndrome (OMIM 175200, mutations in the serine/threonine kinase gene *STK11* - OMIM 602216) (Boardman *et al.,* 1998).

There also appears to be an increased risk for BC and OVC when mutations occur in the ataxia-telangiectasia gene (*ATM* - OMIM 208900) (Khanna, 2000), checkpoint kinase 2 gene (*CHEK2* - OMIM 604373) (Lee *et al.*, 2000), Fanconi anaemia (FA - OMIM 227650) genes, Partner and localizer of *BRCA2* (*PALB2* - OMIM 610355) and BRCA1 interacting protein (*BIRP* - OMIM 605882) (Seal *et al.*, 2006). These are all examples of rare intermediate penetrance BC genes conferring a relative risk (RR) of 2-3. Together they account for ~ 2.3% of familial RR for BC.

2.3.1 The *BRCA* genes

Studies based on early onset BC families reported linkage to chromosome 17q12 (Hall *et al.*, 1990). Four years later, *BRCA1* was cloned by Miki and colleagues (1994). Mutations within this gene proved to be linked to numerous families with multiple early onset of BC and OVC. Linkage studies using families with multiple cases of early-onset BC indicated co-segregation of the disease with chromosome 13q markers, which led to the identification of *BRCA2* (Wooster *et al.*, 1995).

2.3.1.1 Breast cancer susceptibility gene 1 (*BRCA1)*

BRCA1 is a large gene spread over 80 Kb of genomic DNA. It comprises 22 coding exons that are transcribed into a 7.8 kb messenger ribonucleic acid (mRNA)

encoding a protein of 1863 amino acids (Miki *et al.*, 1994). The molecular mass of the protein is approximately 200 kDa. The gene construction and sequence bears no homology to any other genes, with the exception of a RING finger motif at the amino terminus (N-terminus) (Fig 2.2 A) (Miki *et al.*, 1994). The BRCA1-associated RING domain protein (BARD1) (Wu *et al*., 1996) is thought to be a critical factor in BRCA1-mediated tumor suppression (Thai *et al*., 1998). The presence of this motif implicates BRCA1 interaction with other proteins to form protein complexes. The protein also has a nuclear localization sequence (NLS) (Thakur *et al*., 1997) and a conserved acidic carboxy terminus (C-terminus) called the *BRCA1* carboxyl terminal (BRCT) domain (Fig 2.2 A) (Bork *et al*., 1997).

The BRCA1 protein is expressed in several tissues including the ovary, testis and mammary gland epithelial cells (Miki *et al*., 1994). The conserved BRCT domain acts as a protein-binding site and is present in a number of other DNA repair, DNA damage-response and cell cycle control proteins (Bork *et al*., 1997). BRCA1-interacting proteins include C-terminal-binding protein-interacting protein (CtIP), BRIP, BRIP/ FA complementation group J (FANCJ) and p300. These factors interact via the BRCT domain, which serves as a multi-purpose protein to protein interaction module (Fig 2.2 A) (Deng and Brodie, 2000).

2.3.1.2 Breast cancer susceptibility gene 2 (*BRCA2*)

BRCA2 is a large gene consisting of 27 exons (Wooster *et al.*, 1995) that encodes a transcript of approximately 12 kb contained within 70 kb of genomic DNA. The protein consists of 3418 amino acids and similar to BRCA1, shows no homology to any other proteins (Wooster *et al.*, 1995; Tavtigian *et al*., 1996).

BRCA2 is expressed in several tissues including the mammary gland, spleen, ovary, lung, testis and thymus and has many unique features (Tavtigian *et al*., 1996). These include eight evolutionary conserved sequences, termed the BRC repeat (Fig 2.2 B) (Bork *et al.*, 1996). Each BRC motif is ~ 70 amino acids in length with a core sequence of 26 amino acids. The function of these motifs is to mediate direct binding of the protein to the RAD51 recombinase (Fig 2.2 B) (Wong *et al.*,

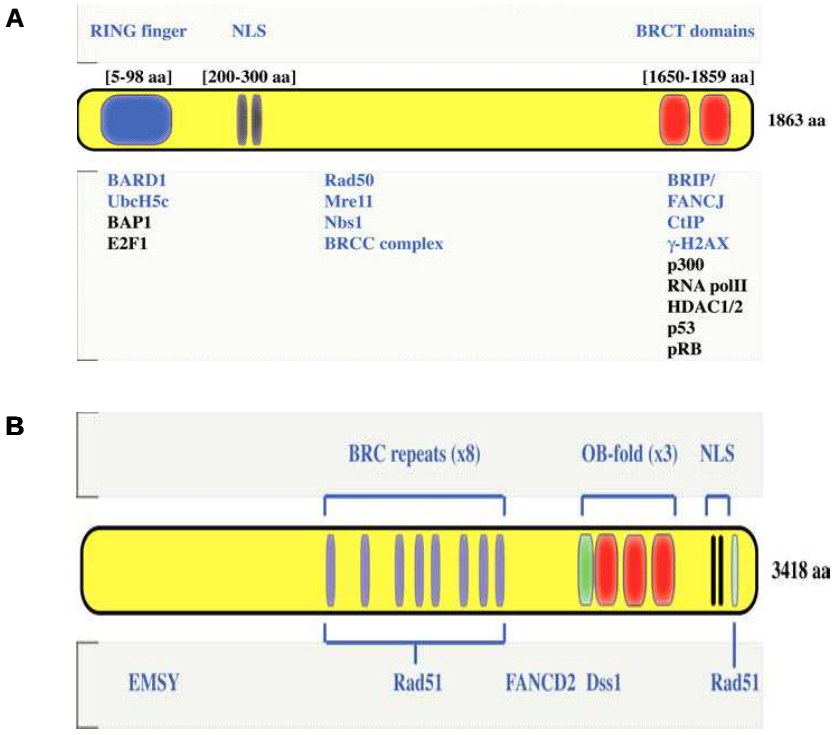


Figure 2.2 Schematic presentations of the BRCA proteins. **A** Diagram depicting the position of the RING, NLS and the BRCT domains of BRCA1. The BRCA1-interacting proteins are shown under the region of BRCA1 that are required for their association. **B** Diagram depicting the position of the eight BRC motifs, three OB folds, the single RAD51-binding site and two NLS regions of BRCA2 C-terminus. The BRCA2-interacting proteins are shown underneath the specific regions involved in the interaction (Boulton, 2006).

1997). The two NLS motifs are situated within the final residues of the C-terminus and are responsible for the nuclear localization and proper function of the BRCA2 protein.

The binding of BRCA2 to Deleted in split-hand/split-foot 1 region (DSS1) is essential for the protein to function during repair (Fig 2.2 B) (Yang *et al.*, 2002). DSS1 is a highly conserved 70 amino acid protein that interacts with the C-terminus DNA/DSS1-binding domain of BRCA2 (Yang *et al.*, 2002). It binds to BRCA2 in an extended conformation interacting with numerous residues within a helical domain containing three oligonucleotide binding folds (OB1, OB2 and OB3) (Fig 2.2 B) (Yang *et al.*, 2002).

DSS1 is important for the maintenance of genome stability and plays a role in BRCA2-dependent recombination. DSS1 is a conserved component of the homologous recombination (HR) pathway that functions with BRCA2 to efficiently target a homolog of RecA of *E.* coli (RAD51) to sites of double strand breaks (DSBs) (Venkitaraman, 2002). FA complementation group D2 (FANCD2), a component of the FA DNA repair pathway, also binds directly to BRCA2 (Hussian *et al.*, 2004). Moreover, biallelic inactivation of BRCA2 results in FA and was therefore assigned as FA complementation group D1 (FANCD1) (Howlett *et al.*, 2002). Evidence supports a role for FANCD2, FANCD1/BRCA2 and the FA pathway in coordinating lesion repair via HR and trans-lesion bypass pathways (Nakanishi *et al.*, 2005).

2.3.1.3 Functions of the BRCA proteins

Although the function of the BRCA proteins are linked with key metabolic processes such as DNA-damage repair, regulation of gene expression, cell cycle control and others (Honrado *et al.*, 2005), their functional roles have not been completely elucidated.

Both proteins localize to the nucleus of dividing cells and work in pathways that are required for the maintenance of chromosome structure (Venkitaraman, 2001).

Here they function as caretakers to suppress genomic instability (Moynahan *et al.,* 2001). Since BRCA2 is also known as FANCD1 protein, it also plays a role in a complex series of nuclear events that promotes DNA cross linking repair (Howlett *et al*., 2002).

The two proteins are involved in multiple tumour types and directly regulate the growth of tumours by inhibiting growth or promoting cell death. According to Knudson's double hit hypothesis, the inactivation of a tumour suppressor gene requires two mutations (usually one germline and one somatic) leading to tumour development (Knudson, 1971). Although mutations in these caretaker genes do not result in tumour formation itself, they cause genetic instability which results in the occurrence of more genomic mutations that eventually leads to tumour formation (Moynahan *et al.,* 2001; Shimada and Nakanishi, 2006). How these proteins exert their tumour suppressor functions is however still not completely understood.

Some of the functions of BRCA1 and BRCA2 will now be further discussed in detail to illustrate where these proteins can play a role in tumour suppression. What remains unclear however is how disruption of the fundamental roles in essential cellular processes can lead to a tissue specific cancer phenotype associated with mutations in these genes.

2.3.1.3.1 DNA damage sensing and repair

The model in Figure 2.3 suggests that a macromolecular complex involving BRCA1, BRCA2, BARD1 and RAD51 repair DSBs by means of HR. According to Cortez *et al.* (1999), the initial step in response to DNA damage involves the sensing of the damaged DNA and sending of a signal to downstream effectors. Protein kinases such as ATM initiate the signal by phosphorylating downstream proteins including BRCA1 (Fig 2.3) (Cortez *et al.*, 1999). The BRCA1-Associated Genome Surveillance Complex of which BRCA1 forms a part acts as a sensor and responds to the damage by participating in various cellular pathways including cell cycle regulation and DNA repair (Wang *et al*., 2000).

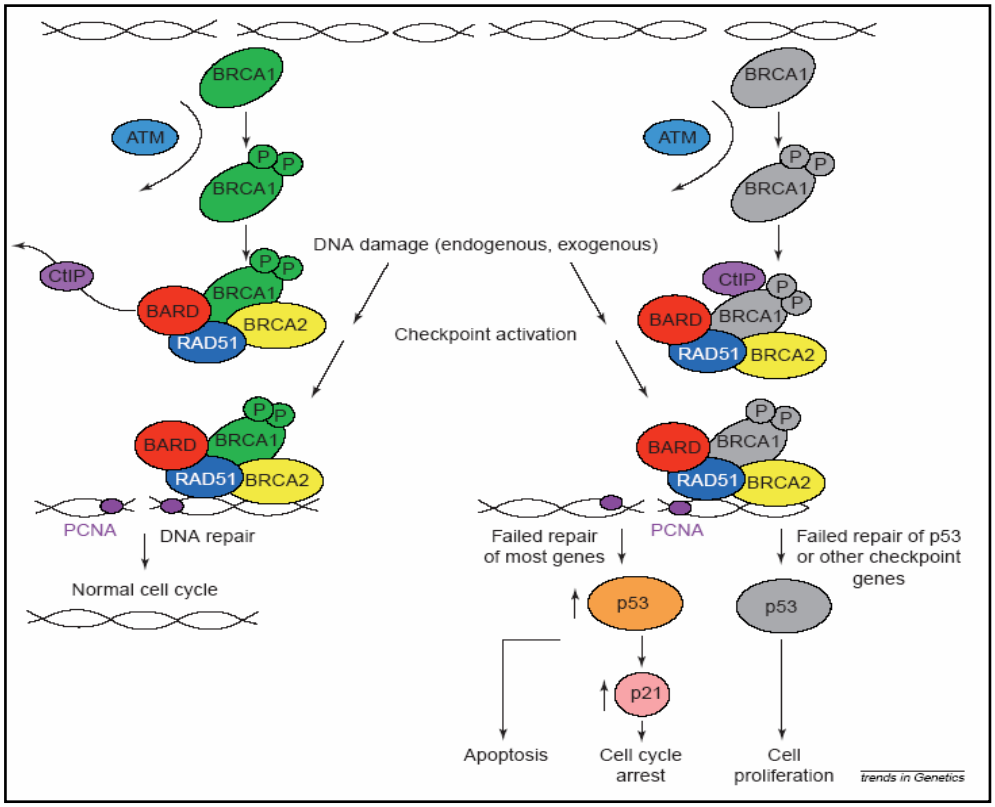


Figure 2.3 Illustration of the macromolecular complexes involved in various physiological responses to DNA damage (Welcsh *et al.*, 2000).

DNA repair commences by means of HR once the sensor has received the signal. In order to repair the break, BRCA1 associates with RAD51 during the S phase of the cell cycle, where after both re-localize to the site of repair (Fig 2.3) (Venkitaraman, 2001). RAD51 coats the single stranded DNA in order to form a nucleoprotein filament. The purpose of this filament is to invade and pair with a homologous DNA duplex in order to initiate strand exchange and to ensure the most accurate repair (Venkitaraman, 2002). BRCA2 mediates the recruitment of RAD51 to DSB sites where it binds to a processed DSB and promotes the nucleation of the RAD51 nucleoprotein filament (Fig 2.3). Once the RAD51 filament has formed, BRCA2 stimulates RAD51-mediated strand exchange and D-loop formation (Boulton, 2006). The main aim of this interaction is for replication arrest to be activated.

Although the involvement of BRCA2 appears to be more direct than that of BRCA1, their functional abrogation leads to gross chromosomal abnormalities presumably due to incorrect repair of DSBs. In the absence of BRCA2, no RAD51 foci will be formed upon DNA damage (Venkitaraman, 2002). BRCA2 therefore dictates RAD51 availability and activity.

2.3.1.3.2 Checkpoint control

In order for DNA damage to be repaired, the cell cycle must be arrested. Genome integrity is maintained by precisely ordering and timing cell cycle events in order to prevent mutations that can disrupt normal growth control (Ishikawa *et al*., 2006). The cell cycle progression can be arrested by cell cycle checkpoints during the G1/S and the G2/M to allow time for repairing DNA damage. The checkpoints monitor DNA status and ensure the completion of the previous phase before advancing to the next phase (Shimada and Nakanishi, 2006).

The dominant checkpoint in response to DNA damage goes through G1. This involves the activation of the ATM-CHEK2-p53/MDM2-p21 pathway (Fig 2.3), which is capable of inducing sustained and sometimes permanent G1 arrest.

Unlike BRCA1, the checkpoint function is preserved in BRCA2-deficient primary cells (Patel *et al*., 1998). Both *Brca1* -/- and *Brca2* -/- mice die during early stages of embryogenesis. Loss of *Tp53* and *WAF1* delays embryogenic lethality for a few days (Patel *et al*., 1998). This indicates that the absence of checkpoint control might be a crucial step in tumourigenesis. Most BRCA1 and BRCA2 null cells undergo apoptosis because of intact checkpoint controls. Cells in which BRCA1 and BRCA2 are disrupted and key checkpoint proteins such as p53 or p21 are inactivated, survive in the presence of genomic instability (Fig 2.3) (Tirkkonen *et al*., 1997; Gretarsdottir *et al*., 1998). Most tumours from women with *BRCA1* and *BRCA2* mutations show loss of the corresponding wild type allele. However some tumours arise in the presence of an intact wild type allele. It has been proposed that a second event in tumourigenesis might involve the inactivation of a checkpoint gene rather than loss of a second *BRCA1* or *BRCA2* allele (Venkitaraman, 2002).

2.3.1.3.3 Transcriptional response

BRCA1 has been implicated in the transcriptional regulation of several genes activated in response to DNA damage by mediating gene-specific transcription control (Jasin, 2002; Venkitaraman, 2002). As a sequence specific DNA-binding transcription factor, the universal tumour suppressor p53 may represent an important link between BRCA1 and gene-specific transcription control (Somasundaram *et al*., 1997). p53 lies at the heart of a cell signalling pathway that is triggered by genotoxic stresses, including DNA damage. Stress-induced p53 initiated cell cycle arrest and/or apoptosis ensures timely repair or elimination of potentially deleterious genetic lesions (Levine *et al.*, 2006). Significantly, *Tp53* and *BRCA1* appear to regulate transcription from an overlapping set of DNA damage inducible target genes, including *WAF1*, thereby linking the biochemical activities of these proteins to a common pathway of tumour suppression (Somasundaram *et al*., 1997; Ouchi *et al*., 1998).

2.3.2 Germline mutations in *BRCA1* and *BRCA2*

The morphological and molecular characteristics of BC arising in *BRCA* mutation

carriers differ from sporadic age-matched controls and non *BRCA1/2* familial BC cases (Thompson and Easton, 2004). Women with a familial predisposition develop the disease at a younger age and are more frequently diagnosed with bilateral disease compared to sporadic cases. Loss of heterozygosity was observed at *BRCA1* and *BRCA2* loci in sporadic BC although the remaining allele almost never mutates (Kenemans *et al*., 2004; Lux *et al*., 2006). This evidence confers that *BRCA*-associated BC is a different entity (Lux *et al*., 2006).

The Breast Cancer Information Core (BIC) database has thus far recorded 1536 distinct germline *BRCA1* and 1885 *BRCA2* mutations. Of these, 878 (57%) and 1140 (60%) have been reported once only. Mutations within *BRCA1/2* appear to be distributed across the entire coding sequences with no obvious mutational hot spots (Thompson and Easton, 2004; Cipollini *et al.*, 2004).

Most disease causing mutations found in BC and/or OVC families truncate the protein product. The most common mutations are small frameshift insertions or deletions, nonsense mutations and splice site mutations. The deletion of complete or partial exons results in the insertion of intronic sequences (Honrado *et al*., 2005).

Mutations in the central part of *BRCA1* are associated with a lower BC risk, whereas mutations in the 3' end have a lower OVC risk (Thompson and Easton, 2002). This genotype-phenotype correlation also exists for *BRCA2*, for the central part of the gene contains an OVC cluster region. Individuals containing these mutations occurring within this region have a higher OVC and lower BC risk (Thompson and Easton, 2001).

The presence of a *BRCA2* disease-causing mutation is also associated with an increased risk for male BC, prostate, pancreas, colon, gall bladder, bile duct and stomach cancers, as well as malignant melanoma (Breast Cancer Linkage Consortium (BCLC), 1999).

2.3.3 Prevalence and founder effects of *BRCA* mutations

The incidence of mutations in high-risk families varies among different populations. Some populations present a wide spectrum of mutations, whereas in other groups specific mutations are common due to the presence of founder effects (Neuhausen, 2000). Founder effects can originate due to geographic or religious isolation and result in a potentially rare mutation frequently occurring. Haplotype analysis of families carrying the same *BRCA* mutation can determine whether these high-frequency alleles are derived from an older or more recent single mutational event or whether they have arisen independently more than once (Ferla *et al*., 2007).

Founder mutations have been identified for various populations within the *BRCA* genes, such as the French Canadian (Tonin *et al.,* 1999; Oros *et al.,* 2006), Icelandic (Roa *et al.,* 1996), Dutch (Hartmann *et al.,* 2004; Zeegers *et al.,* 2004), Japanese (Ikeda *et al.,* 2001; Sekine *et al.,* 2001), African American (Olopade *et al.,* 2003; Pal *et al.,* 2004) and Ashkenazi Jewish populations (Struewing *et al.,* 1995; Roa *et al.,* 1996; Sokolenko *et al.,* 2006).

The three most common *BRCA* mutations are found within the Ashkenazi Jewish population, namely c.185delAG and c.5382insC in *BRCA1* and c.6174delT in *BRCA2*. Although the large majority of c.185delAG carriers are of Ashkenazi Jewish origin, the mutation has also been reported for other Jewish populations indicating an older origin (Struewing *et al.,* 1995). The frequencies of the c.185delAG and c.6174delT mutations have been estimated to be 1 in 100, whereas the frequency of the c.5382insC mutation is estimated to be 1 in 400. In this population, these mutations are present in ~ 30% of BC cases diagnosed before age 40 years (Sokolenko *et al.,* 2006).

A single *BRCA2* mutation, c.999del5, has been identified in the Icelandic population and is present in the majority of multiple BC families. Approximately 1 in every 200 Icelanders are thought to carry this founder mutation, which is a higher frequency than that of all combined mutations in larger, genetically more heterogeneous populations. In this population, the c.999del5 mutation is estimated to account for

24% of BC diagnosed before age 40 years and 38% of male BC cases (Roa *et al.,* 1996).

2.3.3.1. South African founder mutations

The first results regarding the presence and prevalence of *BRCA* mutations in the South African population was published by Reeves and co-workers during 2004. They screened a total of 138 high risk families containing three or more affected individuals. Various *BRCA1* mutations were identified of which approximately half were novel. Together, these mutations accounted for 28% of all the families studied. The results obtained for *BRCA1* are similar to that recorded for populations from Western Europe and America (Reeves *et al*., 2004).

Two recurring *BRCA1* mutations were observed within the Caucasian Afrikaner families, namely c.1493delC and p.E881X. These mutations were novel and unique to the Afrikaner (Reeves *et al*., 2004). Haplotype analysis, together with genealogical and historical data indicated that these mutations originated from single mutational events more than 300 years ago, for all the families shared a common haplotype (Reeves *et al.*, 2004).

Screening of the *BRCA1* negative families revealed the presence of a major founder mutation in *BRCA2*, namely c.8162delG in exon 17 (NC van der Merwe, personal communication). This mutation has only once been reported to the BIC and is therefore considered unique to the Caucasian Afrikaner. The investigators observed a mutation frequency of 42% for this specific mutation in *BRCA2* (NC van der Merwe, personal communication), making it South Africa's most common *BRCA* mutation. Collectively these three mutations accounted for 92% of all the *BRCA* mutation positive Afrikaner families studied thus far (NC van der Merwe, personal communication).

Common mutations and/or marker haplotypes among Afrikaners have been established for several heritable disorders (usually at a high frequency and five to ten times higher than in other populations), including *Porphyria variegata*,

keratolytic winter erythema, hypercholesterolemia, progressive familial heart block, Huntington's chorea, Fanconi anemia, myotonic dystrophy and Gilles de la Tourette syndrome (Simonic *et al*., 1998; Karayiorgou *et al*., 2004).

The Afrikaner population meets several criteria that make it an ideal population for mapping complex traits. This is due to a small number of initial founders that likely allowed a relatively restricted set of mutations to have been introduced into the population. The large current population size allows the identification of sufficient case numbers. Furthermore, a strong infrastructure allows genealogical research as well as collection of good quality clinical data (Karayiorgou *et al*., 2004). Moreover, unusually low allelic diversity at the associated disease loci has been observed. This is expected of genetic drift in a population of this size.

2.3.4 Penetrance of *BRCA1* and *BRCA2*

The risk of developing the disease is commonly measured in terms of the lifetime probability of developing BC or OVC. The average cumulative risk and penetrance for developing BC and OVC in *BRCA1* mutation carriers by age 70 years is 65% (95% CI 44-78%) and 39% (95% CI 18-54%) respectively. The estimates for *BRCA2* mutation carriers are considerable lower, namely 45% for BC (95% CI 31-56%) and 11% for OVC (95% CI 2.4-19%) (Antoniou *et al*., 2003). The values of Antoniou *et al.* (2003) may be an overestimation, due to the fact that affected family members are more inclined to go for testing than non-affected relatives. These estimates still provide a more reliable basis of risk assessment for *BRCA1* and *BRCA2* mutation carriers, for it is based on population studies using cases unselected for a family history.

The lifetime risk for developing BC in women who carry a deleterious *BRCA1* and *BRCA2* mutation is estimated to be as high as 80% or roughly 10 times greater than that of the general population. This number may vary according to the specific mutation present and the country of residence (Antoniou *et al.*, 2003; Narod, 2006).

2.3.5 Breast cancer susceptibility gene 3 (*BRCA3*)

In a study conducted by the BCLC, it was stated that 65% of all the hereditary BC families were linked neither to *BRCA1* nor *BRCA2* (Ford *et al*., 1998). This finding demonstrates that additional BC susceptibility genes remain to be identified.

Several candidate loci have been proposed to harbour novel BC predisposing genes, including the regions 8p12-22 (Kerangueven *et al*., 1995; Seitz *et al*., 1997), 13q21 (Kainu *et al*., 2000) and 2q32.2 (Huusko *et al*., 2004). These three loci have been strongly contested by analysis from data representing independent families (Rahman *et al*., 2000; Thompson *et al*., 2002; Huusko *et al*., 2004).

The search for *BRCA3* has led to some assumptions, namely that the remaining BC families are genetically heterogenous. Secondly, there is no distinct phenotype available to classify these families and thirdly the difficulty involved in trying to identify novel high-to-moderate penetrant genes may be due to the fact that they do not exist (Nathanson and Weber, 2001).

2.4 Low penetrance genes as genetic modifiers

It is speculated that the residual familial risk could be due to low-penetrance alleles, which may act in an additive fashion to increase a woman's risk for BC, the so-called polygenic model (Easton, 1999; Antoniou *et al*., 2003; Dapic *et al*., 2005; Antoniou and Easton, 2006). This could contribute to the inter-individual phenotypic differences observed among *BRCA* mutation carriers. These differences affect not only the occurrence of the disease, but also the characteristics of the tumour and the age at onset (Nathanson and Weber, 2001; Narod, 2006; Levy-Lahad and Friedman, 2007). Shared environmental risk factors that cluster within families could also contribute as much as 10% to some of the remaining unknown familial risk (Jonker *et al*., 2003).

Altered function of these low penetrance genes due to SNPs may affect the gene-environment and gene-gene interactions, thereby increasing the risk of BC

development (Peto, 2002). The selection of cancer susceptibility genes with a low penetrance depends on the knowledge of biochemical and physiological pathways that are known to be involved in the process of carcinogenesis. Potential candidates are represented in a wide variety of pathways, ranging from the detoxification of environmental carcinogens to steroid hormone metabolism and DNA damage repair (Rebbeck, 2002). Due to carcinogen exposure, development of BC may be facilitated by a cumulative effect of mutations or polymorphisms in these genes. Under a polygenic model, each allele confers a small genotypic risk which, when combined additively confer a range of susceptibilities (Kotnis *et al*., 2005).

Identification of these additional low penetrant genes will require different research methods and significant advances in molecular genetic technology, for linkage studies to date have been unable to detect any additional regions of interest (Easton, 1999; Dapic *et al*., 2005; Antoniou and Easton, 2006). Case control association studies have identified some candidate associations but the results have not been reproducible (Dapic *et al*., 2005). Identifying multiple low risk polymorphisms that collectively confer a high risk of BC will be difficult using existing methods, but is expected to be feasible in the future as the methods for typing such polymorphisms becomes faster and less expensive (Easton, 1999).

2.4.1 Consortium of Investigators of Modifiers of *BRCA1* and *BRCA2*

Due to the increasing interest in modifying genes, the Consortium of Investigators of Modifiers of *BRCA1* and *BRCA2* (CIMBA) have been initiated. CIMBA consists of 30 affiliated groups who collect DNA and clinical data from *BRCA1* and *BRCA2* mutation carriers. More than 10 000 *BRCA1* and 5 000 *BRCA2* mutation carriers have already been collected (Chenevix-Trench *et al*., 2007).

CIMBA aims to identify genetic modifiers of BC risk since that will lead to an improved understanding of BC in *BRCA* mutation carriers and that may prove useful for determination of individualized risk (Chenevix-Trench *et al*., 2007). The advantage of CIMBA is the utilization of larger sample populations, as previous

studies have been limited in size and statistical power. They focus on the validation of common SNPs that have been previously associated with risk in smaller studies.

2.5 Potential genetic modifiers involved in the p53 pathway

The p53 pathway is an ideal candidate to investigate for the presence of modifiers since it plays a critical role in cell cycle control, DNA repair and apoptosis during cellular stress situations such as DNA damage or oncogene expression (Fig 2.4) (Levine *et al.*, 2006). Another reason for its selection is the fact that p53 interacts directly with *BRCA1* and *BRCA2* in carrying out the above functions (Jonkers *et al*., 2001; Cheung *et al*., 2004).

p53 is present at low levels under unperturbed conditions but becomes rapidly activated in response to a variety of stimuli including DNA damage. The occurrence of one or more stress signals is associated with the post-translational modification of p53 by upstream protein kinases which leads to a dramatic increase in the half-life of the protein (Appella and Anderson, 2001; Brooks and Gu, 2006). As the p53 protein concentration increases, it becomes an active transcription factor. This is mediated by inactivation of a key negative regulator of p53, namely MDM2 (Fig 2.4) (Fakharzadeh *et al*., 1991). MDM2 is the major p53 protein ubiquitin ligase responsible for inhibiting p53 activity and promoting its degradation (Kubbutat and Vousden, 1997).

Once activated, the p53 transcription factor binds to a specific set of DNA sequences that regulate the transcription rates of p53-responsive genes that at least in part implement the responses to these stress signals (Liu and Kulesz-Martin, 2006). p53 exerts its control on the cell cycle primarily through the G1/S checkpoint and can arrest the cell cycle at G1 by regulating *WAF1* (El-Deiry *et al*., 1993). *WAF1* encodes for the p21 protein that associates with and inhibits the cyclin-dependent kinases (CDKs) (Fig 2.4), the principal enzymes needed for cell cycle progression. As p21 is an important post transcriptional effector in the p53 pathway of cell cycle control it may have prognostic significance in BC. The G 1

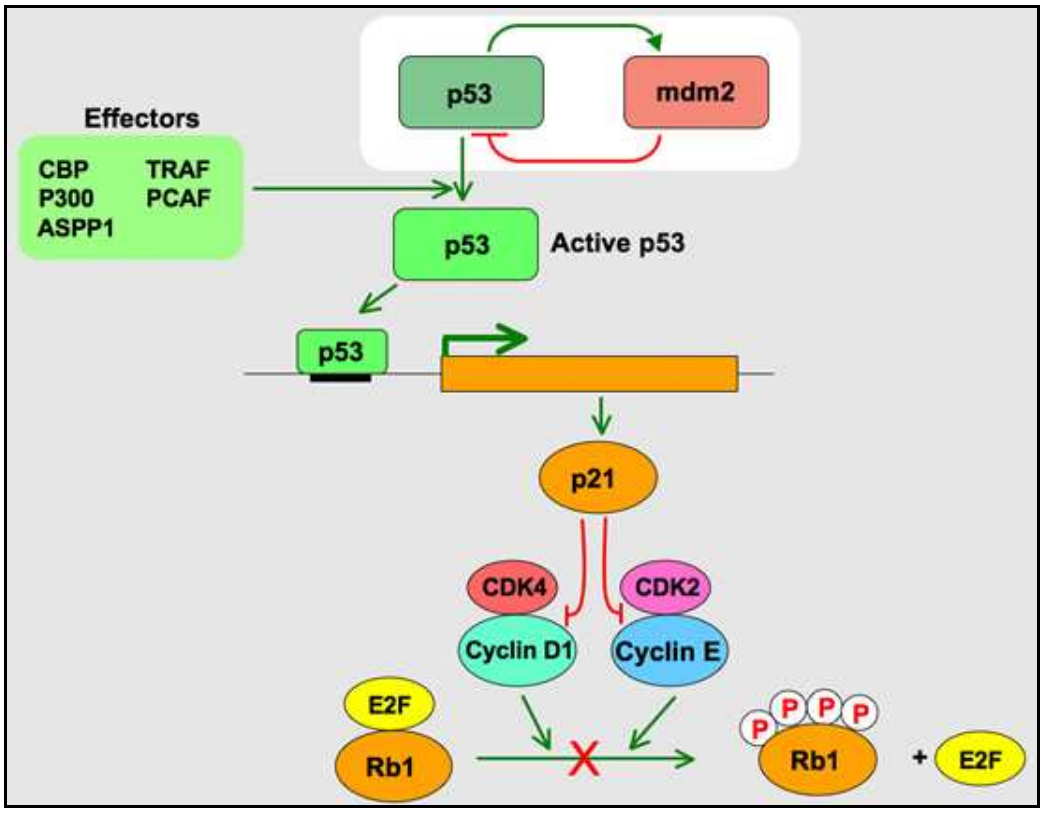


Figure 2.4 The p53 signalling pathway in response to DNA damage (http://p53.free.fr/p53_info/p53_pathways.html).

phase of the cell cycle is important as cell differentiation or death occurs at this stage (Shimada and Nakanishi, 2006). Mutations in G1 checkpoints allow cell proliferation, survival and tumour progression, which result in further DNA replication stress, DSBs, increased genomic instability and selective pressure for accumulating mutations (Moynahan *et al.,* 2001; Ishikawa *et al*., 2006).

The purpose of this signal transduction pathway is to ensure fidelity of the duplication process of the DNA in the cells. Stress, such as DNA damage, increases the error rate for the duplication of DNA. Thus, cell cycle arrest provides the time to repair the DNA before duplication, whereas senescence and apoptosis eliminate clones of cells that would otherwise propagate with a high error rate (Levine *et al.*, 2006; Vousden and Lane, 2007).

The interaction between the gene products of *Tp53*, *MDM2* and *WAF1* affects the anti-oncogenic properties of p53 (Fig 2.4) (Levine *et al*., 2006). As BC is a complex disease in which one genetic variant alone in any of these genes cannot influence disease risk, it is hypothesized that multiple variants along related biological pathways could interact to alter BC risk (Murphy, 2006; Pietsch *et al*., 2006).

2.5.1 Impact of SNPs in the p53 pathway

The age-specific incidence of cancers is dependent upon a minimum of three factors, including (a) the number of rate limiting mutations required for a given cancer, (b) the mutation rate per cell division and (c) the net proliferation rate of the affected cells (Knudson, 2001). Since the efficiency of the p53 pathway can potentially be affected by each of these variables, it can lead to both the initiation and propagation of a cancer (Bond *et al*., 2004). A SNP that has the ability to modulate the efficiency of the p53 pathway could therefore affect the age at which an individual develop cancer.

The p53 signal transduction pathway involves more than 100 proteins (Bond *et al.*, 2005). Many of the genes encoding these proteins have SNPs in their coding or regulatory regions, which could affect the efficiency of the signalling pathway. In

the 82 genes involved in the p53 pathway, already 1335 SNPs have been identified for the non coding (n = 977) and coding regions (n = 358) (Bond *et al*., 2005).

The p53 pathway is crucial for the prevention of tumour formation. Humans harbouring a germline inactivating mutation in one allele of *Tp53* develop tumours early in life and at dramatically high frequencies (Lain and Lane, 2003). Somatic inactivating mutations of *Tp53* are also found in over 50% of human tumours (Lain and Lane, 2003; Joerger and Fersht, 2007). These observations support the importance of the p53 pathway in tumour suppression. Attenuation of the p53 pathway by SNPs in critical nodes of the pathway could affect all three factors, for it has been shown to influence the age-specific incidence by accelerating carcinogenesis in an individual.

2.6 The tumour suppressor protein p53 (*Tp53* OMIM 191170)

Tp53 was initially described as an oncogene due to the protein's ability to bind to the large T-antigen in Simian virus 40-transformed cells (Linzer and Levine, 1979). It was later found that the 'transforming' *Tp53* was in fact a mutated form and that the wild type *Tp53* actually suppressed transformation, which finally categorized p53 as a tumour suppressor protein (Finlay *et al.*, 1989).

The p53 protein is referred to as the guardian of the genome and represents a key regulator in cellular growth control. The protein is dispensable for normal development but is pivotal in the cellular response of cells to extra-cellular damage (Liu and Kulesz-Martin, 2001; Levine *et al*., 2006). This protein is situated at the crossroads of a network of signalling pathways that are essential for cell growth. Regulation of p53 activity is crucial for homeostasis and tumour suppression since overexpression of *Tp53* leads to cell death, whereas under expression results in tumour development (Vousden and Lane, 2007; Iwakuma and Lozano, 2007). p53 functions as a transcription factor that regulates the expression of a large group of responsive genes, initiating feedback loops with the p53 protein and the core gene products (Lacroix *et al*., 2006; Levine *et al.*, 2006). These feedback loops connect the core p53 activities to other signal transduction pathways. When a positive feedback loop is activated, it results in apoptosis and cell death, whereas a

negative feedback loop attenuates the p53 pathway. This results in the integration of a stress signal with other pathways in the cell to regulate cell growth, division or cell death (Lacroix *et al*., 2006).

Other roles of p53 include the regulation of longevity and ageing and glycolytic pathways that might determine the endurance, overall fitness and apoptotic responses during various types of stress (Vousden and Lane, 2007). Evidence of genetic variation in the activity of the p53 pathway in humans gives these proposed roles extra relevance (Murphy, 2006; Pietsch *et al*., 2006).

2.6.1 *Tp53* gene and protein structure

Tp53 is located on chromosome 17p13 and has 11 exons encoding a polypeptide consisting of 393 amino acids with a molecular mass of 53 kDa (Vogelstein and Kinzler, 1994). The protein contains several different functional domains that are involved in processes important to p53 regulation and function (Fig 2.5) (Vogelstein and Kinzler, 1994).

The first 42 amino acids in the N-terminus of p53 contain the acidic transactivation domain (TAD) (Fig 2.5) and the MDM2 protein binding site. The TAD interacts with a number of regulatory proteins such as the negative regulator MDM2 which regulates cellular levels of p53, components of the transcription initiation complex, the acetyltransferases p300 and cAMP response element-binding protein (Thut *et al*., 1995). These proteins act as co-activators and regulate p53 function via acetylation of its C-terminus (Gu *et al*., 1997).

A proline-rich domain comprising amino acids 40–92 follows the TAD domain and is presumably required for the interaction with various proteins involved in the induction of apoptosis (Fig 2.5) (Baptiste *et al*., 2002; Edwards *et al*., 2003). It contains five repeats of a SH3-binding motif (PXXP where P represents proline and X any other amino acid). It also mediates the co-factor binding through interaction with acetyltransferase p300 (Dornan *et al*., 2003).

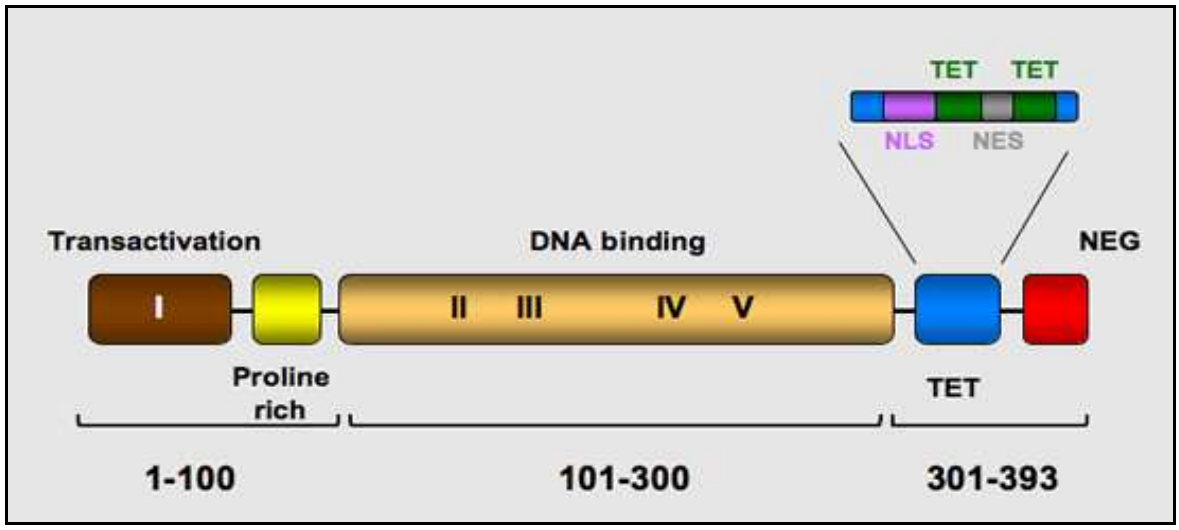


Figure 2.5 Structure and domains of the human p53 protein (http://p53.free.fr/p53_info/p53_Protein.html).

The central domain (residues 101-306) contains the highly conserved DNA binding domain (DBD) (Fig 2.5). This domain is required for its transcriptional properties and for recognizing the *Tp53* DNA consensus elements in its target promoters (Kern *et al*., 1991). The core domain (residues 94-292) binds specifically to doublestranded target DNA in a complex (El-Deiry *et al.,* 1992). The affinity with which p53 binds these elements varies depending on the sequence (Inga *et al*., 2002). The protein generally binds with high affinity to recognition elements of genes involved in cell cycle arrest, whereas all the lower affinity-binding sites are found in genes involved in apoptosis (Liu and Kulesz-Martin, 2001).

The C-terminus of p53 is composed of a linker region, oligomerization or tetramerization (TET) site and a basic C-terminus DNA-binding domain (CTD) (Fig 2.5). The CTD contains a number of phosphorylation, acetylation, sumoylation and ubiquitination sites associated with the regulation of p53 that has been associated with DNA damage recognition (Appella and Anderson, 2001; Brooks and Gu, 2003). While the DBD recognizes specific target sequences, the CTD binds DNA without any sequence specificity (Kim and Deppert, 2006; Liu and Kulesz-Martin, 2006).

The activity of p53 can be controlled through its cellular localization by the regulation of the NLS and nuclear export signal (NES) (Shaulsky *et al.,* 1991), as well as interactions with some of its partner proteins. The protein has a major NLS in its linker region and two other NLS sequences in its C-terminus (Fig 2.5) (Shaulsky *et al.,* 1991). These sequences are recognized by a heterodimeric protein complex that allows nuclear export. The NLS regulates nuclear translocation of p53 from the cytoplasm into the nucleus following DNA damage. The NES lies within the tetramerization domain. It is proposed that when p53 tetramerizes by binding DNA, the NES is covered preventing p53 export from the nucleus (Stommel *et al.,* 1999). Another NES is situated in the p53 N-terminus in the MDM2 binding domain (Zhang and Xiong, 2001).

2.6.2. Mutations in *Tp53*

Mutations in *Tp53* are extremely common and more than 24 000 already documented that are present in ~50% of all human cancers (Lain and Lane, 2003; Joerger and Fersht, 2007). They have been identified in 20-40% of all BC cases (Kalemi *et al.,* 2005; Boersma *et al.,* 2006). A unique feature of *Tp53* compared to other tumour suppressor genes, is its mode of inactivation. Mutations observed in most tumour suppressor genes are inactivated, resulting in the absence of protein synthesis. However, the majority of alterations in *Tp53* are missense mutations that leaves the full-length protein stable and intact (Soussi, 2007).

Eighty percent of all the *Tp53* mutations observed in breast tumours are missense mutations, with only 7% being nonsense, 4% being splice site mutations while only 8% are small insertions and deletions (Soussi, 2007). All these mutations lead to the inactivation of p53. It either occurs through the disruption of its sequence-specific binding ability or through destabilization of its tertiary structure (Royds and Iacopetta, 2006).

The missense mutations are observed predominantly in the central DNA binding domain of the protein and represent more than 1 300 different single amino acid changes (Joerger and Fersht, 2007; Soussi, 2007). This is due to the presence of six mutational hotspots that have been identified within the gene (Royds and Iacopetta, 2006). Although so many missense mutations have been reported, only a few result in amino acid changes.

Tumour associated *Tp53* mutations are associated with a more aggressive phenotype and a worse prognosis (Royds and Iacopetta, 2006). Breast tumours expressing a high amount of p53 are more frequently estrogen receptor (ER) negative and progesterone receptor (PR) negative. They are characterized by a high proliferation rate, high histological and nuclear grade, aneuploidy and implicate a poorer survival (Lacroix *et al.,* 2006).

Tp53 mutations are present at a significantly higher frequency in breast carcinomas

arising in carriers of germline *BRCA* mutations than in sporadic carcinomas (Gasco *et al*., 2003). The loss of BRCA1 or BRCA2 function results in a p21 mediated G1 growth arrest that is in part p53 dependent. It is hypothesised that the loss of the wild type *BRCA1*/*2* allele originally present in cells of *BRCA* mutation carriers would activate the p53-checkpoint. Mutations in p53 could then lead to the abrogation of this checkpoint, resulting in the expression of a mutant BRCA protein lacking the ability to activate the checkpoint (Gasco *et al.,* 2003).

Genotype-phenotype correlations have been reported in *Tp53* germline mutation carriers (Olivier *et al*., 2003). The missense mutations located within the DNA-binding loops of the protein were associated with a higher prevalence of brain tumours and an earlier age at onset of BC, whereas missense mutations outside the DNA-binding loop were associated with a higher prevalence of adreno-cortical carcinomas (Olivier *et al*., 2003).

2.6.3 Polymorphisms of p53

Common germline polymorphisms have also been discovered within *Tp53* with most being SNPs affecting a single base. A large number of these natural variants are localized in the introns. The variant alleles of three well-characterized polymorphisms, an arginine to proline substitution at codon 72 (p.Arg72Pro), an intron 6 polymorphism (IVS6 + 62 G to A) and a 16 bp insertion in intron 3 have been associated with lower apoptotic indices and DNA repair capacity (Wu *et al*., 2002). These polymorphisms have been analyzed on a large scale and their distribution in the human population is well documented.

2.6.3.1 *Tp53* polymorphism at codon 72 (Arg72Pro)

A polymorphic site exists in exon 4 at nucleotide 215 in the *Tp53* gene (Matlashewski *et al.,* 1987). Both forms exist among human populations and are well represented in various populations. This SNP represents the most common polymorphism in *Tp53* and results in a non conservative change in the amino acid sequence between arginine (Arg) and proline (Pro) (Matlashewski *et al.,* 1987).

This SNP is located in a proline rich region, where the Pro72 amino acid constitutes one of five PXXP motifs resembling a SH3 binding domain (Langerød *et al.,* 2002).

It has been suggested that the codon 72 polymorphism may influence expression of *Tp53* since the substitution occurs within the transactivation domain. Codon 72 is located in the hydrophobic region of the protein which determines its conformation and DNA-binding and transcriptional activity, which is essential for growth suppression and apoptotic functions (Buyru *et al.,* 2003).

Beckman and co-workers (1994) were the first to study and document statistical differences in the allelic frequencies of the codon 72, arginine (72Arg) and codon 72 proline (72Pro) variants in human populations. They observed significant differences in the 72Pro allele frequencies among different ethnic groups. The 72Pro allele frequency has for instance been observed in approximately 60% of both the Black American and African populations, but only in 17-34% of Caucasians. Interestingly, Beckman noted that the 72Pro allele frequency increased in a linear manner in multiple populations as they neared the equator. This led him to hypothesize that the codon 72 polymorphism might have an impact on p53 function and that high exposure to UV light in populations near the equator led to natural selection for the 72Pro allele (Sjalander *et al.,* 1995).

The two polymorphic variants proved to have different biochemical properties such as differential binding to components of the transcriptional machinery. This affects their transcriptional ability (Thomas *et al.,* 1999). The p53 72Pro variant activates transcription much more efficiently than the 72Arg variant (Thomas *et al.,* 1999). Moreover, the p53 72Arg variant is much more susceptible to degradation by the HPV E6 protein. The p53 72Arg is more efficient in inducing cell death than the 72Pro variant due to its ability to localize to the mitochondria and induce apoptosis (Bonafè *et al.,* 2003; Dumont *et al.,* 2003). Pim and Banks (2004) postulated that the 72Pro variant induces cell-cycle arrest better than the 72Arg variant. Siddique and Sabapathy (2006) proved that 72Pro is also more efficient in specifically activating several p53-dependent DNA-repair target genes in several cellular systems. It was also shown that the p53 72Pro cells have significantly higher DNA-

repair capacity than those containing 72Arg. The functional differences between the two polymorphic variants suggest that their respective expression status may influence cancer risk (Siddique and Sabapathy, 2006). Published data highlights that both these polymorphic variants might have evolved for selectively regulating specific cellular functions.

2.6.3.1.1 Impact of the Arg72Pro on breast cancer risk

Many studies have been conducted to evaluate the potential role and association of the p53 Arg72Pro polymorphism regarding an increase in risk for different types of cancers. Unfortunately, most of these studies on BC made use of relatively small sample sets and yielded inconsistent, even contradictory results (Table 2.1).

Some authors referred to the presence of p53 72Pro allele in genotypes as a risk factor of BC development that could influence patient survival. Noma *et al.* (2004) observed a significant increased risk in the Pro/Pro genotype in ER positive BC development for the Japanese population, which was absent for ER negative BC cases. BC patients homozygous for the Pro/Pro genotype also seemed to be less sensitive to anthracycline-based chemotherapy (Xu *et al*., 2005) and may be associated with the development of acute skin toxicity after radiotherapy (Tan *et al.,* 2006). This allele was also indicated as a risk allele in the Russian (Suspitsin *et al.,* 2003), Slovakian (Franeková *et al.,* 2007), German (Wang-Gohrke *et al.,* 1998; Wang-Gohrke *et al.,* 2002), Japanese (Huang *et al.,* 2003), Swedish (Sjalander *et al.,* 1996) and American Caucasian (Weston *et al*., 1997) populations (Table 2.1).

Although Tommiska *et al.* (2005) did not find any association between the p53 codon 72 polymorphism and BC in the Finnish population they observed a significantly reduced survival for 72Pro homozygous BC patients. This has been confirmed by Arg/Pro heterozygous BC patients by Bonafè *et al.* (2003).

A high prevalence of the homozygous p53 72Arg genotype in BC patients were observed in the Caucasian women of New York (Keshava *et al.,* 2002), the Greek (Papadakis *et al.*, 2000; Kalemi *et al.,* 2005), Turkish (Buyru *et al.,* 2003), Israeli

Table 2.1 A summary of studies investigating the Arg72Pro polymorphism in BC patients. BC cases are highlighted in bold, dashes indicate no association with Arg72Pro, asterisk represents preliminary results - statistically insignificant.

References	Population	Study design	Participants (n)	Association with BC	Arg/Arg n (%)	Arg/Pro n (%)	Pro/Pro n (%)
Kawajiri *et al* ., 1993	Japanese	BC case-control	**93**	**-**	**37 (39.8)**	**51 (54.8)**	**5 (5.4)**
			347		144 (41.5)	165 (47.6)	38 (10.9)
Sjalander *et al* ., 1996	Swedish	BC case-control	**212**	**Pro**	**95 (44.8)**	**93 (42.8)**	**24 (11.3)**
			689		375 (54.0)	253 (36.7)	61 (9.72)
Weston *et al* ., 1997	American	Caucasian BC case-control	**65**	**Pro**	**32 (49.2)**	**27 (41.5)**	**6 (9.2)**
			117		72 (61.5)	42 (64.6)	3 (4.6)
		Hispanic BC case-control	**18**	**-**	**7 (38.8)**	**8 (44.4)**	**3 (16.6)**
			38		12 (31.5)	16 (42.1)	10 (26.3)
		African American BC case-control	**16**	**-**	**1 (6.25)**	**9 (56.25)**	**6 (37.5)**
			30		4 (13.3)	14 (46.6)	12 (40)
Wang-Gohrke *et al* ., 1998	German	BC case-control	**107**	**Pro**	**56 (52.3)**	**46 (42.9)**	**5 (4.67)**
			305		167 (54.75)	117 (38.36)	21 (6.88)
Khaliq *et al* ., 2000	Pakistani	BC patients	**41**	**-**	**13 (31.7)**	**18 (43.9)**	**10 (24.3)**
Papadakis *et al* ., 2000	Greek	BC case-control	**56**	**Arg**	**34 (61.0)**	**10 (18.0)**	**12 (21.0)**
			61		12 (26.0)	41 (67.0)	6 (10.0)
Wang-Gohrke *et al* ., 2002	German	BC case-control	**552**	**Pro**	**282 (51.0)**	**221 (40.0)**	**49 (8.8)**
			543		300 (55.2)	203 (37.38)	40 (7.3)
Langerød *et al* ., 2002	Norwegian	BC-tumour	**390**	**Arg**	228 (58,.5)	136 (34.8)	26 (6.6)

Table 2.1 Continued

References	Population	Study design	Participants (n)	Association with BC	Arg/Arg n (%)	Arg/Pro n (%)	Pro/Pro n (%)
Buyru *et al* ., 2003	Turkish	BC case-control	**115**	**Arg**	**64 (55.6)**	**39 (33.9)**	**12 (10.4)**
			51		10 (20.0)	32 (63.0)	9 (17.0)
Mabrouk *et al.* , 2003	Tunisian	BC case-control	**30**	**-**	**18 (60.0)**	**9 (30.0)**	**3 (10.0)**
			49		19 (39.0)	26 (53.0)	4 (8.0)
Bonafé *et al.* , 2003	Italian	BC patients	**67**	**Arg**	**32 (47.8)**	**29 (43.3)**	**6 (8.9)**
Suspitsin *et al* ., 2003	Russian	Bilateral BC case-control	**81**	**Pro***	**44 (54.0)**	**34 (42.0)**	**3 (4.0)**
		Unilateral BC case- control	**448**		**240 (54.0)**	**169 (38.0)**	**39 (9.0)**
			249		130 (52.2)	105 (42.2)	14 (5.6)
			144		77 (53.0)	54 (38.0)	13 (9.0)
Huang *et al* ., 2003	Japanese	BC patients	**200**	**Pro**	**64 (32.0)**	**100 (50.0)**	**36 (18.0)**
			282		114 (40.4)	138 (48.9)	30 (10.7)
Martin *et al* ., 2003	American	*BRCA1* and *BRCA2* BC patients	**84**	**Pro**	**51 (61.0)**	**28 (33.0)**	**5 (6.0)**
Noma *et al* ., 2004	Japanese	BC (ER positive)-control	**48**	**Pro**	**48 (44.9)**	**35 (32.7)**	**24 (22.4)**
			111		111 (50.9)	76 (34,9)	31 (14.2)
		BC (ER negative)-control	**40**		**40 (53.3)**	**30 (40.0)**	**5 (6.7)**
			111		111 (50.9)	76 (34,9)	31 (14.2)
Tommiska *et al* ., 2005	Finnish	BC-control	**858**	**-**	**459 (53.5)**	**336 (39.2)**	**63 (7.3)**
		Familial BC control	**923**		**478 (51.8)**	**385 (41.7)**	**60 (6.5)**
			733		403 (55)	278 (37.9)	52 (7.1)

Table 2.1 Continued

References	Population	Study design	Participants (n)	Association with BC	Arg/Arg n (%)	Arg/Pro n (%)	Pro/Pro n (%)
Ohayon *et al* ., 2005	Israeli	non-Ashkenazi-Jews BC-control	**24**	**Arg***	**15 (62.5)**	**8 (33.3)**	**1 (4.2)**
			107		24 (22.4)	70 (65.4)	13 (12.2)
		Ashkenazi-Jews BC-control	**108**		**74 (68.5)**	**32 (29.6)**	**2 (1.9)**
			60		30 (50.0)	24 (40.0)	6 (10.0)
Kalemi *et al* ., 2005	Greek	Familial BC-control	**42**	**Arg**	**26 (62.0)**	**13 (31.0)**	**3 (7.0)**
			51		10 (20.0)	32 (63.0)	9 (17.0)
Ma *et al* ., 2006a	Chinese	Sporadic BC-control	**404**	**Arg***	**149 (36.9)**	**178 (44.1)**	**77 (19.1)**
			472		150 (31.8)	222 (47.0)	100 (21.2)
Tan *et al* ., 2006	German	BC patient (radiotherapy)	**432**	**Pro**	**256 (59.2)**	**154 (35.6)**	**25 (5.78)**
Damin *et al* ., 2006	Brazilian	BC-control	**118**	**Arg**	**64 (54.2)**	**48 (40.7)**	**6 (5.1)**
			202		70 (34.6)	111 (54.9)	21 (10.3)
Osorio *et al* ., 2006	Spanish	*BRCA1* mutation carriers	**90**	**Pro**	**50 (60.0)**	**29 (35.0)**	**4 (5.0)**
		BRCA2 mutation carriers	**80**		**39 (55.0)**	**24 (34.0)**	**8 (11.0)**
Franeková *et al* ., 2007	Slovakian	BC-control	**91**	**Pro**	**49 (53.85)**	**34 (37.36)**	**8 (8.79)**
			156		92 (58.97)	55 (35.26)	9 (5.77)
Khadang *et al* ., 2007	Iranian	Sporadic BC-control	**221**	**-**	**83 (37.6)**	**29 (13.1)**	**109 (49.3)**
			205		75 (36.6)	40 (19.5)	90 (43.9)

Jewish (Ohayon *et al*., 2005) and Chinese populations (Ma *et al.,* 2006a). Langerød *et al.* (2002) reported a growth advantage of breast carcinoma cells carrying the *Tp53* 72Arg allele in a Norwegian population. The majority of those cancer cases had a somatic *Tp53* mutation in the tumour, which proved to be common in the 72Arg homozygotes (Langerød *et al.,* 2002; Noma *et al.,* 2004).

Studies were initiated using *BRCA1* and *BRCA2* mutation carriers to determine whether p.Arg72Pro could be a genetic modifier of BC risk (Table 2.1). Tommiska and co-workers (2005) observed that *BRCA2* carriers homo- or heterozygotic for the p53 72Pro allele are most likely to be diagnosed at a younger age. A similar trend was recorded by Martin *et al.* (2003) but the differences were not significant in both these studies. Martin *et al.* (2003) also documented that the presence of a 72Pro allele was associated with an earlier age of onset of BC in *BRCA1* mutation carriers. An association of the 72Arg homozygous genotype with multiple primary cancers or a family history of multiple primary cancers among *BRCA1/2* mutation carrier women was also suggested (Martin *et al*., 2003), but no such association was observed by Tommiska *et al.* (2005).

2.6.3.2 Polymorphism of intron 3 (g. 11951_11966dup)

There is growing evidence for a novel mechanism of gene regulation involving mutations in donor and acceptor splice sites of introns. These may be important in regulating gene expression in *Tp53* (Lacerda *et al*., 2005).

Of all the polymorphisms identified in the *Tp53* gene, the duplication and insertion of 16 bp in intron 3, together with the SNP in codon 72, has been the most frequently studied. Lazar *et al*. (1993) was the first to report the intron 3 polymorphism consisting of a 16 bp duplication and insertion. The repeat sequence is located between two identical 8 bp motifs that may be responsible for the generation of the insertion due to slipped strand mis-pairing during DNA synthesis (Cooper and Krawezak, 1991). This polymorphism proved to be common, for it was detected in a heterozygotic state in 28% of Caucasian individuals (Lazar *et al*., 1993).

BC case-control studies have reported a significant association of the 16 bp insertion allele and an increased BC risk (Runnebaum *et al*., 1995; Wang-Gohrke *et al*., 2002; Wirtenberger *et al*., 2006) whereas Sjalander et al. (1995) reported a protective risk. Wang-Gohrke *et al*. (2002) revealed a significant increase in BC risk for individuals heterozygous for the insertion and a borderline significant increase for the homozygous variant alleles.

This polymorphism has been reported to be in strong linkage disequilibrium with the intron 6 polymorphism, but not with the SNP at codon 72 (Wang-Gorhke *et al*., 1998). Although the functional relevance of the p53 intron 3 polymorphism is unknown, intronic sequences in this gene have been implicated in the regulation of gene expression and in DNA-protein interactions (Avigad *et al*., 1997). Therefore, this polymorphism may also be in linkage disequilibrium with other as yet unidentified genes, explaining its association with a distinctive phenotype.

2.6.3.3 Polymorphism of intron 6 (IVS6 G to A)

The polymorphism in intron 6 is represented by a G to A transition located 61 nucleotides downstream of exon 6. This single base change abolishes an *Msp*I restriction endonuclease site. Other intron 6 base substitutions have been reported indicating that introns may represent a potential hot spot for the occurrence of mutations (Lacerda *et al.,* 2005).

There are not many studies that investigated the association between the SNP in intron 6 and BC risk. The prevalence of the variant A allele was observed as a risk factor for BC development in the Israeli (Peller *et al*., 1995), German (Wang-Gohrke *et al.,* 2002), American Caucasian (Weston *et al.*, 1997) and the Slovakian populations (Franeková *et al.,* 2007). In contrast, no association was detected for the Russian (Suspitsin *et al.,* 2003), Swedish (Sjalander *et al.*, 1996) and the British populations (Mavridou *et al.,* 1998).

2.6.3.4 Haplotypes of p53

Since a haplotype constitutes the allele status of several polymorphisms on the same chromosome, its structure is reminiscent of its evolutionary history. This evolutionary history can be influenced by genetic mechanisms such as selection, mutation, genetic drift and admixture (Mahasneh and Abdel-Hafiz, 2004). Sjalander *et al*. (1995) indicated that inheritance of certain *Tp53* haplotypes, constructed from the codon 72, intron 3 and intron 6 biallelic polymorphisms, constitute a risk factor for BC.

In a pairwise haplotype estimation of Swedish and German patients, different allelic combinations were calculated for the three polymorphisms involved in the *Tp53* haplotype, occurring more frequently in the BC patients than in the controls (Sjalander *et al*. 1996; Wang-Gohrke *et al.*, 2002). The presence of the *Tp53* polymorphism in intron 3 (16 bp insertion) was linked to the absence of the SNP in p53 intron 6 and together they showed an increased risk for BC.

The precise opposite was observed for a Jordanian population, where the absence of *Tp53* intron 3 polymorphism together with the presence of the p53 intron 6 polymorphism were associated with BC (Mahasneh and Abdel-Hafiz, 2004). The exon 4 minor variant (72Pro) and intron 6 (the A allele), together showed an increased BC risk in the Slovakian population in women younger than 50 years (Franeková *et al*., 2007). The pairwise haplotypes of intron 3 (16 bp insertion) together with the exon 4 major variant (72Arg) and intron 3 (16 bp insertion) in combination with intron 6 were also more frequently observed in BC patients among the German (Wang-Gohrke *et al.*, 2002), American Caucasian (Weston *et al*., 1997) and Turkish populations (Buyru *et al*., 2007).

Osorio *et al*. (2006) observed that a specific haplotype involving the *Tp53* intron 3 polymorphism together with the minor variant in exon 4 (72Pro) modifies cancer risk in specifically *BRCA2* mutation carriers. This haplotype confers an increased risk for developing a first primary tumour before the age of 35. These results further confirmed that the variant allele (16 bp insertion) in combination with the

72Pro allele have a functional effect. The haplotype consisting of homozygous carriers for these variant alleles are more efficient at inducing apoptosis than those with genotypes that include at least one mutant 72Pro allele (Osorio *et al*., 2006).

A modifying effect of *Tp53* in *BRCA2* penetrance is not difficult to justify given the functional interaction of *Tp53* with both *BRCA1* and *BRCA2* (Jonkers *et al*., 2001). Cheung *et al*. (2004) suggested that Brca2 deficiency and down regulation of p53 can jointly promote mammary tumourigenesis. Together, the lack of BRCA2 function combined with a reduced effectiveness of p53 could promote an earlier development of a tumour. The fact that *Tp53* modifies *BRCA2* BC risk could be due to specific functional relationships between these two proteins (Cheung *et al*., 2004).

The expansion of the pairwise haplotype into an extended haplotype containing three SNPs proved to yield better results. They proved to be more informative than studying a single polymorphism in order to determine ethnic differences and associations between *Tp53* germline mutations and BC risk (Mahasneh and Abdel-Hafiz, 2004). The disadvantage of these extended haplotypes is that they require data collected from family studies.

Weston *et al*. (1997) developed a long range polymerase chain reaction (PCR) method to determine true extended haplotypes in diploid genomes. This method captures the combination of the alleles on each chromosome thereby eliminating the need to use statistical models to infer haplotypes which inherently introduces error.

Studies making use of the extended *Tp53* haplotype containing the polymorphisms in intron 3, intron 6 and exon 4 have observed an association with an increased risk of BC in postmenopausal women in Swedish (Sjalander *et al*., 1996), German (Wang-Gohrke *et al*., 1998) and American Caucasian populations (Weston *et al*., 1997; Weston and Wolff, 1998; Keshava *et al*., 2002). It is possible that the haplotype associated with carrying all three polymorphic alleles reflects a dose-effect due to the accumulation of several polymorphic alleles within one or more

genes involved in BC (Wang-Gohrke *et al*., 2002). This association was not observed for the Hispanic and African-American (Weston *et al.,* 1997), Pakistani (Khaliq *et al*., 2000) or in BC patients from Russia (Suspitsin *et al*., 2003).

The fact that these studies used different protocol and study designs hampers the comparison of results. Most of the studies did not have adequate power to detect an odds ratio of 1.5 with 80% statistical power at a 5% significance level for dominant allele. However, the analysis showed that the haplotype composed of all three variant alleles is associated with an increased BC risk.

2.7 Mouse double minute 2 homolog (*MDM2*) (OMIM 164785)

Two critical genes regulated to a large extent by *Tp53* are the *MDM2* proto-oncogene which modulates the trans-activating functions of wild type *Tp53* (Iwakuma and Lozano, 2003; Levine *et al*., 2006) and *WAF1*, a universal CDK-inhibitor (CDKI) implicated in mediating p53 dependent G1 arrest (Gartel and Tyner, 2002).

The protein encoded by *MDM2* is an onco-protein, since it is amplified in ~7% of all human cancers (Bond *et al.,* 2004). Malignant tumours, particularly breast tumours and soft tissue sarcomas, frequently exhibit over-expressed *MDM2* (Bond *et al.,* 2004). *MDM2* is over-expressed in 41% benign and 68% of malignant BC tumours and is strongly associated with the presence of the ER (Baunoch *et al.*, 1996). Overexpression of *MDM2* has also been correlated with *WAF1*. In BC cells, a correlation exists between the overexpression of *MDM2* and a lack of *WAF1* expression (Deb, 2003).

MDM2 was originally identified in spontaneously transformed Balb/cT3 mouse fibroblast cell lines. In these cells, *MDM2* was localized on a double minute chromosome and was amplified ~ 50-fold (Fakharzadeh *et al.,* 1991). Momand *et al*. (1992) characterized and identified the phosphoprotein with a molecular mass of 90 kDa that formed a complex with both the mutant and the wild-type p53 protein.

2.7.1 Gene structure and protein motifs

MDM2 is located on chromosome 12q13-14 and consist of 12 exons. The MDM2 protein is composed of 491 amino acids. *MDM2* contains two transcriptional promoter elements called P1 and P2 and transcription results in two different MDM2 polypeptides being formed. The shorter polypeptide acts as an activator of p53 and dominant negative inhibitor for the full length polypeptide (Perry *et al.,* 2000). A number of different *MDM2* splice variants exist of which some help control the activity of the full length protein (Bartel *et al*., 2002).

The N-terminus of the protein accommodates binding of MDM2 to the transactivation domain of p53 which down regulates its ability to activate transcription (Chen *et al*., 1993). Amino acids 25-109 of MDM2 form a hydrophobic pocket, which binds the N-terminus of p53 and hides it from the transcriptional machinery (Fig 2.6) (Iwakuma and Lozano, 2003). Other motifs present include NLS and NES (Fig 2.6). These signals shuttle MDM2 back and forth between the cytoplasm and the nucleus, another way by which p53 activity is tightly regulated (Iwakuma and Lozano, 2003). Besides mediating p53 degradation and transactivation, MDM2 also controls the location of p53, targeting it either to the cytoplasm or nucleus.

The RING-domain situated at the C-terminus of the protein co-ordinates the E3-ligase activity and ubiquitination of p53 (Fig 2.6) (Nakamura *et al*., 2000; Rodriguez *et al*., 2000). This domain is also required for efficient export of p53 into the cytoplasm. This function is normally performed by the NES in other proteins. MDM2 also mediates its own degradation through the same domain (Fang *et al*., 2000; Honda and Yasuda, 2000). The function of the zinc finger domain is currently still unknown.

The most important role of *MDM2* is the physiological regulation of the function of *Tp53* (Iwakuma and Lozano, 2003; Moll and Petrenko, 2003). *Tp53* activates many target genes, of which *MDM2* is one. The gene binds to the *MDM2* P2 promoter and transcriptionally up-regulates the expression of *MDM2* (Fig 2.7). In turn,

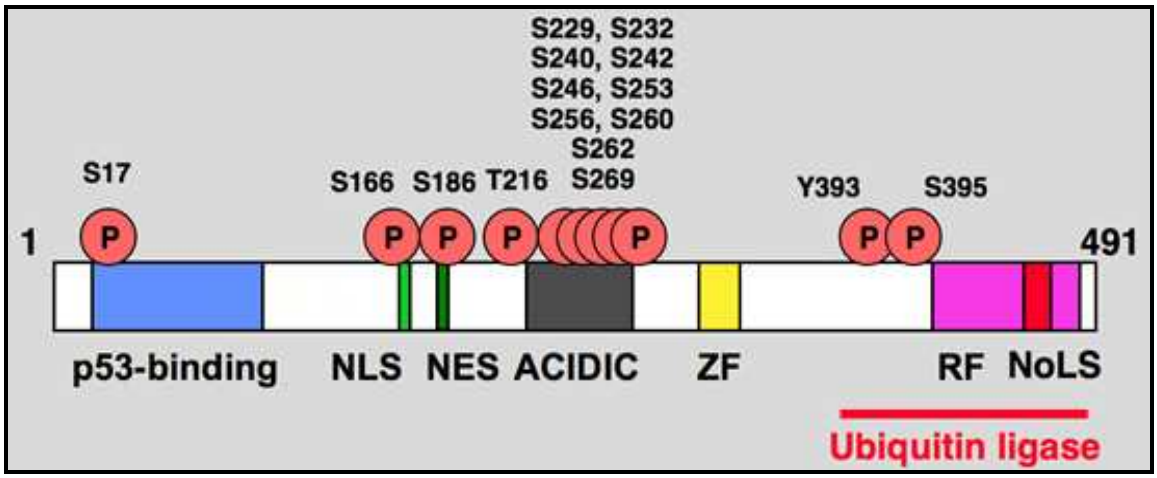


Figure 2.6 Structure of the MDM2 protein. Indicated are the p53 binding site, NLS, NES, Zinc finger domain, Ring finger domain and the nucleolar localization signal (NoLS) (http://p53.free.fr/p53_info/mdm_family.html).

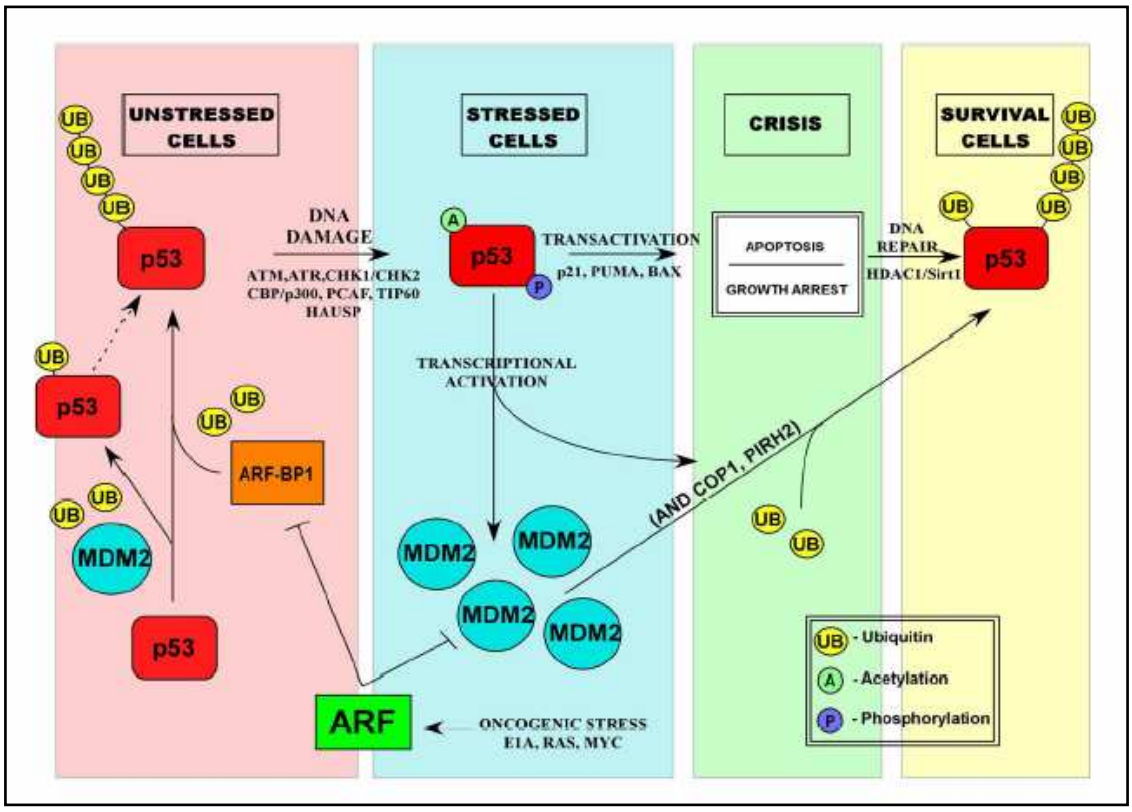


Figure 2.7 Schematic presentation of the role of *MDM2* in unstressed and stressed cells (Brooks and Gu, 2006).

MDM2 inhibits p53 activity by stimulating its degradation in the nucleus and cytoplasm, thereby blocking transcriptional activity and promoting its nuclear export.

A regulatory feedback loop exists between these two genes (Fig 2.7). In unstressed cells, the levels of MDM2 are low and it can only induce mono-ubiquitination of p53, which is not sufficient for the degradation of p53. When p53 is activated by stress, such as DNA damage, both p53 and MDM2 are phosphorylated, thereby preventing their interaction (Fig 2.7) (Brooks and Gu, 2003; Brooks and Gu, 2006). Oncogenes activate proteins which sequester MDM2 and import it into the nucleolus, thus preventing the degradation of p53 (Iwakuma and Lozano, 2003). Upon DNA damage, p53 becomes stabilized and is activated by posttranslational modifications and other signalling pathways that lead to expression of genes that are involved in cell survival (Appella and Anderson, 2001; Brooks and Gu, 2006).

When the stress situation is uplifted, the interaction between these genes returns to normal (Fig 2.7) (Iwakuma and Lozano, 2003). This auto-regulatory negative feedback loop is aimed at maintaining low cellular p53 levels in the absence of stress and plays an important role in regulating cell cycle progression and apoptosis (Fig 2.7) (Lacroix *et al*., 2006). This relationship furthermore limits the duration and severity of various p53-mediated biological responses after a non-lethal stress response. The existence of this auto-regulatory feedback loop adds a complex feature to the p53-MDM2 pathway and makes MDM2 one of the most important regulators of p53 activity (Moll and Petrenko, 2003; Levine *et al*., 2006).

2.7.2 Role of *MDM2* in growth regulation

Stress situations induce the overexpression of *MDM2* in a p53 dependent or independent pathway. In its overexpressed state, MDM2 induces growth arrest by halting the cell cycle at G1. This arrest will take place unless the cells bear a mutation that could disable the growth inhibitory domains of MDM2 (Moll and Petrenko, 2003).

This cell cycle inhibitory function of MDM2 serves as a protective mechanism of normal cells to prevent untimely growth proliferation normally or in response to abnormal tumorigenic signals (Moll and Petrenko, 2003). The normal growth arrest function of MDM2 is disabled in genetically damaged cells to an extent that the regulatory molecule turns into an oncoprotein generating further chromosomal abnormalities and oncogenesis. As MDM2 has three G1 arrest domains, it may take at least two or perhaps three errors to turn the growth regulatory molecule into an oncoprotein (Deb, 2003). This highly unlikely event is fairly frequent in cancer patients since the errors enable the damaged cell to evade the regulatory mechanism and confer selective growth advantage (Deb, 2003).

2.7.3 Polymorphism in the *MDM2* promoter (SNP309)

Bond and co-workers (2004) were the first to note significant variations in the p53 response in lymphocytes isolated from 50 healthy volunteers. This finding prompted them to analyze the *MDM2* gene for sequence polymorphisms that might contribute to this inter-individual variation in the p53 response. A functionally significant SNP was identified in the promoter of *MDM2* (T to G). The variant G allele is denoted as SNP309. This change creates a higher affinity binding site for the transcription factor Sp1, which is an important regulator of *MDM2* gene expression since higher levels of *MDM2* mRNA and protein result in the attenuation of the p53 pathway (Bond *et al*., 2004).

The variant G allele was observed at a frequency of 0.32, making this a common polymorphism (Bond *et al.*, 2004). Phelps *et al*. (2003) noted a consistent correlation between cell lines homozygous for the G allele and increased steady state levels of the MDM2 protein. The cell lines homozygous for the G allele of SNP309 were shown to have an attenuated p53 transcriptional and apoptotic response, due to the decreased ability of p53 to stabilize following DNA damage (Arva *et al*., 2005).

Bond *et al*. (2004) then analyzed individuals from families with LFS, who have one non-functional copy of the p53 gene and therefore have an attenuated p53

response. They hypothesized that the *MDM2* SNP might further attenuate the p53 pathway in these families. Analysis of 88 individuals from LFS families containing germline mutations in one allele of *Tp53* revealed that individuals who carried the G allele for SNP309 in either hetero- or homozygous state, showed a significantly earlier age at onset for all tumour types, with a minimum of 9 years earlier. Additionally, individuals homozygous for the G allele of SNP309 also had an increased occurrence of independent subsequent cancers (Bond *et al*., 2004).

The impact of this regulatory SNP is influenced by the ER. When ER status and hormonal levels were taken into account, the SNP309 resulted in a higher risk or aggressiveness in colon and breast cancer (Bond *et al*., 2006). The ER binds to the same region of the *MDM2* promoter that harbours the SNP309 locus. The presence of a G allele at this site has shown to alter the affinity of the co-transcriptional activator for multiple hormone receptors (Bond *et al*., 2006). They reported that the estrogen signalling pathway as well as tumour formation could also be affected, for an active estrogen signalling pathway is needed for the G allele to accelerate tumour formation.

2.7.3.1 Impact of the variant G allele of SNP309 on breast cancer risk

Several groups have reported that the G allele is associated with the attenuation of the p53 pathway, thereby enhancing an earlier age of BC onset (Table 2.2) (Bougeard *et al.,* 2006; Wasielewski *et al.,* 2006; Ruijs *et al.,* 2007; Yarden *et al*., 2007). It is possible that the impact of this polymorphism may differ for different tumour types or may depend upon the initiating molecular event that control tumour development. Three independent reports have shown that germline *Tp53* mutation carriers who possess the G allele of SNP309 were diagnosed with cancer on average 7-10 years earlier than those who were homozygous for the T allele (Bond *et al.,* 2004; Bougeard *et al.,* 2006; Ruijs *et al.,* 2007). In the Ashkenazi Jewish population, *BRCA1*/*2* carriers that were homozygous for the variant G allele had an increased risk of being diagnosed with early onset BC compared to hetero- and homozygous wild type alleles (Yarden *et al*., 2007). They also noted that the frequency of the variant G allele in the Ashkenazi Jewish population was

Table 2.2 A summary of studies investigating the *MDM2* SNP309 in BC patients. BC Cases are highlighted in bold, asterisk represents preliminary results-insignificant. Li-Fraumeni syndrome (LFS), Li-Fraumeni-like syndrome LFL, *Tp53*+ germline tumour suppressor.

References	Population	Study design	Participants (n)	Association with BC	T/T n (%)	T/G n (%)	G/G n(%)
Campbell *et al.* , 2006	British	BC-control	**351**	**No**	**132 (37.6)**	**160 (45.6)**	**59 (16.8)**
			258		105 (40.7)	111 (43.0)	42 (16.3)
Millikan *et al* ., 2006	American	African-Americans BC-controls	**767**	**No**	**594 (77.0)**	**158 (21.0)**	**15 (2.0)**
			680		542 (80.0)	121 (18.0)	17 (2.0)
		Caucasian BC-controls	**1270**		**516 (41.0)**	**573 (45.0)**	**181 (14.0)**
			1133		474 (42.0)	478 (42.0)	181(16.0)
Copson *et al* ., 2006	United Kingdom	*BRCA1* carriers-controls	**116**	**No**	**48 (41.4)**	**55 (47.4)**	**13 (11.2)**
			102		48 (47.0)	38 (37.3)	16 (15.6)
Wilkening *et al* ., 2006	German	Negative *BRCA1/BRCA2*- control	**549**	**No**	**218 (39.7)**	**243 (44.3)**	**88 (16.0)**
			1065		445 (41.8)	470 (44.1)	150 (14.1)
Bougeard *et al.,* 2006	French	61 LFS	**41**	**Yes**	**14 (30.0)**	**19 (31.0)**	**8 (13.0)**
Ma *et al* ., 2006b	Chinese	BC-control	**366**	**No**	**85 (23.2)**	**196 (53.6)**	**85 (23.2)**
		Benign BC-control	**236**		**55 (20.9)**	**131 (49.8)**	**77 (29.3)**
			605		145 (24.0)	308 (50.9)	152 (25.1)
Boersma *et al.* , 2006	American	African-Americans BC-control	**165**	**No**	**125 (75.8)**	**39 (23.6)**	**1 (0.6)**
			178		151 (84.8)	24 (13.4)	3 (1.68)
		Caucasian BC-controls	**125**		**60 (48.0)**	**42 (33.6)**	**23 (18.4)**
			136		60 (44.1)	63 (46.3)	13 (9.55)

Table 2.2 Continued

References	Population	Study design	Participants (n)	Association with BC	T/T n (%)	T/G n (%)	G/G n(%)
Wasielewski *et al.* , 2006	Dutch	All BC	**343**	**Yes**	**111 (32.3)**	**185 (54.0)**	**47 (14.0)**
		CHEK2 1100delC families	**20**		**4 (20.0)**	**15 (75.0)**	**1 (5.0)**
		BRCA1 families	**87**		**37 (42.5)**	**39 (45.0)**	**11 (13.0)**
		BRCA 2 families	**21**		**4 (19.0)**	**11 (52.0)**	**6 (29.0)**
		Non-mutant families	**215**		**66 (30.6)**	**120 (56.0)**	**29 (13.0)**
			126		38 (30.1)	67 (53.0)	21 (17.0)
Ruijs *et al.* , 2007	Finnish/Dutch	LFS (Tp53 +)	**11**	**Yes**	**2 (18.0)**	**8 (72.7)**	**1 (9.09)**
		LFS (Tp53 -)	**4**			**1 (25.0)**	**3 (75.0)**
		LFL (Tp53 +)	**22**		**15 (68.0)**	**7 (32.0)**	**0 (0)**
		LFL (Tp53 -)	**29**		**15 (51.7)**	**8 (27.5)**	**6 (20.6)**
		LFS Suggestive (p53+)	**2**				**2 (100)**
		LFS Suggestive (p53-)	**34**		**21 (61.7)**	**6 (17.6)**	**7 (20.5)**
		Negative *BRCA1/ BRCA2* (p53+)	**1**		**1 (100)**	**0(0)**	**0(0)**
		Negative *BRCA1/ BRCA2* (p53-)	**5**		**3 (60.0)**	**1 (20.0)**	**1 (20.0)**
		Dutch controls	230		94 (41.0)	109 (47.0)	27 (12.0)
		Finnish controls	551		185 (34.0)	259 (47.0)	107 (19.0)
Cox *et al* ., 2007	American	BC cases	**1267**	**No**	**519 (42.8)**	**544 (44.8)**	**150 (12.4)**
			1758		701 (42.1)	754 (45.3)	209 (12.6)
		BC cases	**317**		**137 (44.8)**	**130 (42.5)**	**39 (12.7)**
			634		257 (42.3)	273 (45.0)	77 (12.7)
Yarden et al., 2007	Ashkenazi Jewish	BRCA1/BRCA2 BC patients	**140**	**Yes**	**26 (17)**	**74 (53.6)**	**39 (28)**
		BRCA1/BRCA2 carriers	**120**		**28 (23.3)**	**58 (48.3)**	**34 (28.3)**
		Sporadic BC	**187**		**49 (26.0)**	**77 (41.0)**	**61 (33.0)**
			138		30 (22.0)	68 (49.0)	40 (29.0)

significantly different from the frequency published for the North American populations (Bond *et al*., 2004).

In contrast, several other groups have reported no association between SNP309 and BC or age of onset (Boersma *et al.*, 2006; Campbell *et al.,* 2006; Ma *et al.,*2006b; Millikan *et al.,* 2006; Wilkening *et al.,* 2006; Cox *et al.*, 2007), even in *BRCA1* germline mutation carriers (Copson *et al*., 2006) (Table 2.2).

Copson *et al*. (2006) suggested that the increased MDM2 levels associated with the G allele of SNP309 are insufficient to attenuate a response in *BRCA1* mutant breast cells, whereas in the LFS cohort studied by Bond *et al*. (2004) there is already a loss of one *Tp53* allele. Copson *et al*. (2006) further suggested that the transcription factors influenced by SNP309 could be cell type specific and therefore does not affect *MDM2* expression in breast tissue. This is however not true, since overexpression of *MDM2* has been found in up to 73% of BC cases (Buesmo-Ramos *et al.*, 1996) and in 25.8% LFS individuals (Bond and Levine, 2007).

Some studies reported a possible interaction between SNP309 and the codon 72 polymorphism in *Tp53* (Boersma *et al.*, 2006; Bougeard *et al.,* 2006). A strong interaction between SNP309 status and tumour p53 status appears to modify the association between p53 status and BC survival. p53 tumour status, as expressed by mutational status and the level of protein expression, was only associated with patients homozygous for the wild type of *MDM2* regarding outcome (Boersma *et al.*, 2006). This indicates that the variant allele acts as a dominant oncogene. The mechanism by which the variant SNP309 MDM2 protein exhibits an oncogenic function leads to increased MDM2 protein expression, abrogation of the p53 tumour suppressor function and formation of a transcriptionally inactive p53-MDM2 complex (Boersma *et al.*, 2006).

Bougeard *et al*. (2006) confirmed the impact of the *MDM2* SNP309 variant allele on the age of tumour onset in germline *Tp53* mutation carriers and suggested that this effect may be amplified by the presence of p53 72Arg alleles. Polymorphisms affecting p53 degradation therefore represents one of the rare examples of modifier

genetic factors that have a Mendelian type predisposition to cancer. An interaction between the SNP309 and Arg72Pro of p53 has been noted but no visible pattern of the risk is evident (Cox *et al*., 2007). Homozygosity for the codon 72Arg allele and heterozygosity at the SNP309 of *MDM2* showed an apparent inverse association with BC risk (Cox *et al*., 2007).

The *MDM2* SNP309 polymorphism has been shown to influence the age at onset of tumours in *Tp53* mutation carriers in a hormone-dependent manner. These examples show how a combination of mutations with intermediate penetrance in two tumour suppressor genes may result in atypical phenotypes (Bond *et al*., 2004; Bond *et al*., 2006). Families with a partially functional *Tp53* mutation may carry another intermediate penetrance mutation that might act in synergy with *Tp53* to promote cancer in tissues that are less typical or occur at a different age than the one in families that carry high-penetrance mutations.

2.8 Cyclin-dependent kinase inhibitor 1A (*WAF1*) (OMIM116899)

WAF1 encodes p21, a nuclear arginine-rich protein (El-Deiry *et al*., 1993). The p21 protein inhibits the activity of all CDKs preventing phosphorylation of their corresponding substrates and blocking cell cycle transition from the G1 to the S phase. This step prevents the replication of damaged templates by allowing appropriate DNA repair.

The p21 protein is an important post translational effector in the p53-specific pathway of cell cycle control (El-Deiry *et al*., 1993). Transcriptional activation of p21 by the up-regulated p53 tumour suppressor protein occurs via a p53 response element located 2.4 kb upstream of the *WAF1* transcription start site. This is in response to genotoxic stress and results in the arrest of cell cycle in G1 (El-Deiry *et al*., 1993).

p21 is a member of the Cip/Kip family of CKIs which also include p27 and p57 (Gartel and Tyner, 2002). These three CKIs contain a conserved region of sequence at the N-terminus that is required and sufficient for the inhibition of

cyclin/CDK complexes (Gartel and Tyner, 2002). The C-terminus regions are variable in length and function. They can bind and inhibit a broad range of cyclin/CDK complexes (Gartel and Tyner, 2002).

2.8.1 Gene and protein structure of *WAF1*

WAF1 is located on chromosome 6p21.2 and consists of three exons (El-Deiry *et al*., 1993). It is expressed in all human tissues and is located predominantly in the nucleus. The translational start codon is located at nucleotide 76 in exon 2 with the translational stop codon at nucleotide 567 in exon 3. Ninety percent of the second exon contains an open reading frame (El-Deiry *et al*., 1993). Human and mouse p21 share 79% identity at the amino acid level and contains two regions that are highly conserved and critical for protein function (El-Deiry *et al*., 1993). A putative zinc finger motif (not indicated on figure) has been identified between amino acids 13 and 41, as well as a potential NLS between amino acids 140-163 (Fig 2.8).

WAF1 encodes a 21 kDa protein comprising of 164 amino acids (Fig 2.8) (El-Deiry *et al*., 1993). The basic modular structure of the protein comprises of two binding domains. The N-terminus binds and inhibits cyclin/CDK complexes, whereas the second binding domain is a short sequence near the C-terminus between amino acid 144 to 151. This domain binds to the proliferating-cell nuclear antigen (PCNA) resulting in the inhibition of DNA replication (Chen *et al*., 1995; Gartel and Tyner, 2002). The PCNA binding motif overlaps with the NLS and the C-terminus cyclin binding site (Fig 2.8) (Chen *et al*., 1995). Either one of the N-terminus CDK or PCNA binding regions of p21 is sufficient to inhibit DNA replication when expressed in cells (Chen *et al*., 1995; Luo *et al*., 1995). The C-terminus domain may inhibit cell cycle progression independently of the N-terminus domain and thus contributes to the anti-proliferative activity of p21 (Luo *et al*., 1995).

2.8.2 Function of *WAF1*

The function of p21 during the cell cycle is to prevent progression of the cell cycle from G1 to the S phase by inhibiting the activity of CDKs (Gartel and Tyner, 2002).

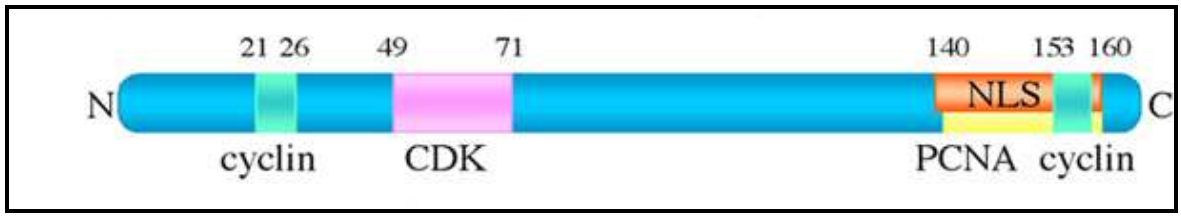


Figure 2.8 The structure of p21 protein with its protein interacting domains (http://AtlasGeneticsOncology.org/Genes/CDKN1AID139.html).

p21 acts as one of the effectors of p53 and functions to negatively control the cell cycle (Fig 2.4). In response to DNA damage, p53 up-regulates p21 expression leading to cell cycle arrest at the G1 checkpoint (Xiong *et al*., 1993; McKenzie *et al*., 1997). Alterations in the function of p53 results in the loss of p21 expression, although the p53-dependent pathways also regulate p21. p21 has been identified as a universal CDK inhibitor (Xiong *et al*., 1993; Lukas *et al*., 1997). The binding of p21 to the cyclin/CDK complexes prevents these protein complexes from phosphorylating and inactivating the retinoblastoma (RB) protein, a central regulator of G1-S progression *(*Xiong *et al.,* 1993; Lukas *et al*., 1997; McKenzie *et al.,* 1997). Inactivation of the retinoblastoma protein results in the release of the E2F protein which transcriptionally activates several genes necessary for cell cycle progression (Fig 2.4).

p21 also affects DNA replication by interacting with PCNA (Xiong *et al*., 1993; Gartel and Tyner, 2002). PCNA plays an essential role in DNA replication and different types of DNA repair including nucleotide excision repair, base excision repair and mismatch repair. Within the cell, p21 together with PCNA, CDKs and cyclins form a tetrameric complex in which the N-terminus of p21 binds CDK while the C-terminus binds PCNA (Fig 2.8) (Chen *et al.*, 1995). This interaction can block DNA synthesis (Lukas *et al.,* 1997; Gartel and Tyner, 2002). p21 is therefore an important downstream regulator of p53 and functions as a unique link for p53 to arrest the cell cycle in order for DNA repair to take place.

2.8.3 Interaction between MDM2 and p21

MDM2 is a negative regulator of *WAF1*, directly promoting its degradation. The MDM2 protein directly binds to p21 and facilitates its interaction with the 20S proteasome (Bornstein *et al*., 2003). MDM2 facilitates the proteasome-mediated degradation of p21, independent of both ubiquitin and p53. p21 plays an important role in tumour inhibition and chemo-sensitization induced by *MDM2* knockdown, indicating that negatively regulating p21 is one of the important p53-independent pathways contributing to MDM2 tumorigenic activity (Zhang *et al*., 2004).

2.8.4 Polymorphisms in *WAF1*

Mutations in *WAF1* have rarely been identified within human cancers, although various missense mutations have been reported. They occur more frequently in cancer patients than in healthy individuals suggesting a role in increased susceptibility for the development of some types of cancer (Mousses *et al.,* 1995; Facher *et al.,* 1997; McKenzie *et al.,* 1997). Some of the polymorphisms that have been described are Ser31Arg in exon 2, a SNP in the 3' untranslated region, Asp149Gly and the 5' region of intron 2.

2.8.4.1 The SNP in exon 2 (p.Ser31Arg)

The polymorphism at codon 31 arises from a C to A transversion at the third base of codon 31, resulting in an amino acid change of serine (Ser) to arginine (Arg) (Chedid *et al.,* 1994). The change results in the loss of one restriction site and the gain of another. Both polymorphic variants have very similar kinase inhibitory activity and growth suppression abilities and could be the reason why this polymorphism does not affect the structure or function of the protein (Chedid *et al.,* 1994).

Su *et al.* (2003) reported that this SNP does alter the expression of *WAF1*. It was observed in individuals who carried a copy of one p21Arg allele. These patients showed a 38% decrease in expression compared to individuals homozygous for the p21Ser genotypes (Su *et al*., 2003). Interestingly, individuals heterozygous for the codon 72 polymorphism in p53 and this p21 variant demonstrated a 57% reduction in p21 mRNA levels compared to individuals carrying the homozygous p53Arg and p21Ser alleles (Su *et al.,* 2003).

The frequency of the variant Arg allele varies considerably, ranging from 4 - 19% in Caucasians (Birgander *et al.,* 1996; Lukas *et al.,* 1997; Keshava *et al.,* 2002) to 50% in the Chinese (Birgander *et al.,* 1996). A relatively higher frequency of the variant Arg allele was observed for the African Blacks (29%) and Indians (16%), compared to the Caucasians (Birgander *et al.,* 1996). This demonstrates that the

geographic allele distribution patterns of p53 and p21 differ.

Studies on some cancers have described associations between the *WAF1* SNP in exon 2 and cancer risk. These include prostate and cervical adreno-carcinomas, cancers of the head and neck, endometrial and lung cancers (Mousses *et al*., 1995; Sjalander *et al*., 1996; Hachiya *et al*., 1999; Keshava *et al*., 2002). Although p21 expression may be linked with BC prognosis, the codon 31 polymorphism has not yet been definitely associated with an increased risk of BC (Mousses *et al*., 1995; Lukas *et al.,* 1997; McKenzie *et al.,* 1997). Discrepancies among these studies could be explained by inadequate sample sizes or inclusion of different ethnic groups in single studies (Keshava *et al.*, 2002).

An association has been determined between BC and the Ser31Arg polymorphism in African American and Latino populations, but the association was absent among American Caucasians (Keshava *et al*., 2002). Similarly, Powell *et al.* (2002) recorded no association of Ser31Arg in the Caucasian Australian BC patients.

The variant was three times more common in samples whose tumours lacked the PR. As PR is known to regulate the transcription of p21 in BC cells, it was suggested that the absence of PR might be due to the presence of the variant allele. Powell *et al.* (2002) suggested that the Ser31Arg genotype, through linkage to one or more progesterone sensitive polymorphisms in the promoter region, may provide selective pressure for the development of breast tumours having low progesterone levels. The Ser31Arg polymorphism in *WAF1* was strongly linked to negative PR status suggesting this variant may have functional significance for the progesterone signalling pathway in BC. These results add to growing evidence that genetic variants can influence not only the risk of BC but also the disease phenotype.

2.8.4.2 The polymorphism in intron 2 of *WAF1*

There is a possibility of a link between a polymorphism in the non coding region of *WAF1* in intron 2 and the Ser31Arg SNP. This polymorphism is situated 16 bp

downstream of the splice donor site of intron 2. This C to G transition is hypothesised to affect the mRNA splicing of *WAF1* (Powell *et al*., 2002). It was demonstrated that the C to G transition alone increase the risk of BC in the Caucasian Australian population. The SNP in intron 2 was more frequently detected in patients with well-differentiated tumours when compared to patients with histological undifferentiated tumours (Powell *et al.*, 2002).

2.8.4.3 Correlations between the Arg72Pro of p53 and Ser31Arg of p21

Since p21 is a downstream target of p53, some studies have investigated whether there is a correlation between the p53 polymorphisms and those in p21. Only two studies thus far have investigated a potential correlation between p53 codon 72 and p21 codon 31 with regards to BC risk. An interaction between the p21 Ser31Arg and p53 Arg72Pro polymorphism was not observed with regard to BC risk (Keshava *et al*., 2002; Tan *et al*., 2006).

2.9 Non-genetic modifiers

Environment and lifestyle factors can also modify the risk of BC and OVC among *BRCA* mutation carriers. It has been proposed that the risk of BC has increased among *BRCA* mutation carriers born in recent generations. This observation was based on the tendency observed in multiple-generation families (Antoniou *et al*., 2003). The penetrance of these mutations in recently born women is greater than those born before 1920 (Antoniou *et al*., 2003). The RR to develop BC in *BRCA1* carriers born after 1960 was 7.7 compared to a RR of 1 for those born before 1920 (Antoniou *et al*., 2003). This trend suggests that environmental factors with increasing prevalence could modify their RR.

Antoniou *et al.* (2003) suggested that the increase in risk for BC and OVC is due to the changing patterns of reproductive factors, such as age at first pregnancy, oral contraceptive use and breast feeding. Further environmental influences are suggested by the effect of country of origin on BC risk. Investigation of non-genetic factors has focused on factors known to affect BC risk and included reproductive

behaviour, hormonal exposure and lifestyle habits (Dumitrescu and Cotarla, 2005). In order to better define populations with a high BC risk, the use of both germline genetics and known environmental risk factors could prove beneficial in improving prevention strategies (Bond and Levine, 2007).

2.9.1 Reproductive factors

In the general population, reproductive factors including age at menarche, late age at first full-term pregnancy, null and low parity and late age at menopause are established risk factors for an increased risk of BC (Kelsey *et al.,* 1993; Key *et al*., 2001). These reproductive factors, including breastfeeding, have also been reported to influence risk among women with a hereditary predisposition to BC.

2.9.1.1 Age at menarche

In the general population, age at menarche is an established risk factor for development of BC (Kelsey *et al.,* 1993; Key *et al*., 2001). An early age at menarche is considered a positive predictor of BC risk. The age at which a woman experiences her first menstrual period is falling and has been proven to be influenced by various factors such as anthropometric measures, nutritional influences and a decrease in physical activity during childhood (Petridou *et al.*, 1996; Okasha *et al.*, 2001). Menarcheal age is directly determined by a girl's bone mass index (BMI) at age 18 and indirectly also by her physical activity during childhood. An increase in her BMI at age 18 will result in an earlier age at menarche, which in turn increases her risk to develop BC (Petridou *et al.*, 1996; Okasha *et al.*, 2001).

Various studies led to the identification of a statistically significant decrease in BC risk associated with later age at menarche among *BRCA1* mutation carriers. It has been reported that for every year of menarcheal delay, a 15% decrease in BC risk was observed. *BRCA1* carriers whose age at menarche was approximately 14 to 15 years had a 54% reduction in BC risk when compared to those with menarche at <11 years of age (Hulka, 2001; Kotsopoulous *et al*., 2005). A later age at

menarche implicates a decrease in the number of ovulatory cycles over a lifetime. This decrease in BC risk has not been observed for *BRCA2* mutation carriers (Kotsopoulous *et al*., 2005).

2.9.1.2 Pregnancy

The *BRCA1* gene plays a key role in the normal proliferation and differentiation of cells in the mammary gland. *BRCA1* expression is regulated during mammary gland development and increases during puberty and pregnancy in order to limit proliferation and promote differentiation (Mueller and Roskelley, 2003). During this time, it also acts as a caretaker of DNA recombination and the cell cycle.

The increase in *BRCA1* expression is due to an increase in estrogen levels. Higher estrogen levels decrease the activity of the estrogen receptor-α-mediated pathways, thereby suppressing cell proliferation (Cabanes *et al.*, 2004; Razandi *et al.*, 2004). Together, the increase in estrogen and BRCA1 levels during pregnancy further the differentiation of the terminal end buds and induces dramatic changes in the breast tissue, thereby conferring protection against the development of BC (Russo *et al.,* 1982).

Control of cell proliferation may be compromised in breast cells that harbour a *BRCA1* or *BRCA2* deleterious mutation (Cabanes *et al.*, 2004; Razandi *et al.*, 2004). *BRCA* mutation carriers carrying heterozygous mutant breast cells may be more susceptible to genotoxic carcinogens than normal breast cells during the period from menarche to first childbirth, when the breast cells are undifferentiated (Cabanes *et al.*, 2004).

The effect of parity on hereditary BC risk appears to differ for *BRCA1* and *BRCA2* carriers. In a large case-control study (Cullinane *et al*., 2005) it was reported that the risk of BC did not decrease with pregnancy in *BRCA1* carriers until four births were reached and offered a modest protective effect. An early first birth does not appear to be protective for BC risk among *BRCA1* carriers (Kotsopoulos *et al*., 2007). Among *BRCA2* carriers, there was a statistically significant increase in BC

risk with each additional pregnancy. In a case study, young age at first pregnancy delayed onset of BC in carriers (King *et al*., 2003). A study of 1601 carriers found that in women over 40 years of age, each full-term pregnancy reduced BC risk by 14%. For *BRCA2* mutation carriers with a later age of first pregnancies, an increase in BC risk was observed. In *BRCA1* carriers with first birth over the age of 30 years a lower risk of BC was observed compared to those with first birth before the age of 20 (Andrieu *et al*., 2006). Overall the effect of parity on BC in carriers is similar to that in sporadic BC.

2.9.1.3 Breast feeding

The manner in which breast feeding is associated with a reduced risk of BC is unclear. It may be related to developmental changes in the mammary gland during puberty and pregnancy or may affect breast estrogen levels. During the first part of pregnancy, the breast epithelium proliferates rapidly, where after the tissue undergoes a final differentiation during the final months. As a result, the parous breast contains a higher percentage of well-differentiated lobules than the nonpaorous breast (Russo *et al.*, 2001).

Lactation has both a direct and indirect affect on BC risk. A direct consequence of lactation involves the influence it has on the hormonal milieu, for breast fluid estrogen levels are suppressed for several years after breast feeding (Petrakis *et al.*, 1987). Breast feeding may also indirectly reduce the risk of BC by delaying the re-establishment of ovulation (Henderson *et al.*, 1981). Women who breast fed exclusively during the first 6 months postpartum have a low rate of ovulation, which reduce the number of lifetime ovulations.

Breast feeding has been shown to decrease a woman's risk of BC when associated with a longer duration (Shantakumar *et al*., 2007). Coogan *et al*. (1999) observed a decreased BC risk relating to lactation among South African women. He postulated that lactation causes differentiation of ductal epithelial cells, which is protective against carcinogens.

A case control study seeking an association between breast feeding and BC in *BRCA* mutation carriers reported a significant protective effect in women with *BRCA1* mutations, but not for *BRCA2* (Jernstrom *et al.*, 2004). *BRCA1* mutations carriers who breast fed for more than one year were 40% less likely to have BC than those who breast fed for a shorter period (Jernstrom *et al.*, 2004). Andrieu *et al.* (2006) did not observe any protective effect regarding breast feeding in *BRCA* carriers.

2.9.1.4 Oral contraceptives

The effect of oral contraceptives (OC) use on BC risk in carriers is difficult to assess because of changing patterns of use and altered OC formulations. No effect of OC on BC risk was determined for *BRCA2* carriers, although early - onset BC was increased for *BRCA1* mutation carriers in women who used OC before 1975, before age 30 years or for those who used for 5 years and more (Narod *et al*., 2002). Studies based on early onset BC including carriers found no general increase in BC with OC use. An increase in BC risk was observed with teenage OC use per year (< 20 years) (Jernstrom *et al*., 2005). In a study by Milne *et al*. (2005) there was a suggestion of decreased BC risk within *BRCA1* carriers. These studies suggest that for BC risk, OC have similar relative effects in carriers and non-carriers. These effects may be attenuated in newer, low-dose preparations, but the absolute effect may be higher in carriers.

2.9.1.5 Hormone replacement therapy

The use of hormone replacement therapy (HRT) in unaffected *BRCA* mutation carriers who opted for a prophylactic oophorectomy before natural menopause is somewhat controversial. This is due to the chance that these hormones may increase the risk for BC. Limited data are available to substantiate the preliminary indications that HRT increases the risk of BC in *BRCA* mutation carriers. Rouleau *et al*. (2004) observed that women at high risk for BC and/or OVC tend to avoid HRT when the presence of a *BRCA* mutation is either suspected or confirmed. Rebbeck *et al*. (2005) evaluated BC risk associated with HRT after bilateral

prophylactic oophorectomy in *BRCA* mutation carriers. They recorded that any type of HRT used after bilateral prophylactic oophorectomy did not significantly altered the reduction in BC risk associated with the surgery. The investigators claimed that short-term HRT and bilateral prophylactic oophorectomy was associated with a significant reduction in BC risk in *BRCA* mutation carriers.

2.9.2 Lifestyle modifiers

2.9.2.1 Smoking

Most studies have found no link between cigarette smoking and BC (Hamajima *et al*., 2002). Both active smoking and second-hand smoking have been suggested to increase the risk of BC in a number of studies that restricted the comparison group to women who report no exposure to second-hand smoke. This issue remains controversial.

Although the associations between smoking and BC are unclear, several observations have been published indicating it as a risk factor. Carcinogens found in tobacco smoke pass through the alveolar membrane (Yamasaki *et al.*, 1977) into the blood stream by means of which they are transported to the breast where they are metabolized and activated by mammary epithelium cells (Plant *et al.*, 1985). The higher prevalence of smoking-specific DNA adducts and *Tp53* mutations found in breast tissue of smokers compared with that in non-smokers supports the biological plausibility of a positive association between cigarette smoking and BC risk (Li *et al.*, 2002; Rundle *et al.*, 2002). However, various epidemiological studies have variably shown positive, inverse or null associations (Terry and Rohan, 2002). An additive interaction was shown between smoking, the presence of the *MDM2* SNP309 and the exon 4 SNP in *Tp53* in risk of oesophageal squamous cell carcinoma and lung cancers (Bond and Levine, 2007).

Explanations for the lack of consistency in previous studies include the possibility of biased results and the postulated “anti-estrogenic” effect of cigarette smoking (Baron *et al.*, 1990) since estrogen is a known risk factor for BC. Another factor

can include the way it was measured, namely qualitative versus quantitative measures. The fact that the various smoking measures are also correlated with each other complicates the differentiation of their independent effects, for example smokers of high intensity tend to be smokers of long duration and the latter tend also to have commenced smoking at an early age (Terry and Rohan, 2002).

Smoking can also have an influence on a woman's reproduction. Epidemiological data have repeatedly shown that women who smoke are at increased risk for a variety of reproductive problems, including ectopic pregnancies, delay to conception and infertility (Talbot and Riveles, 2005). Studies have indicated that the oviduct is targeted by smoke components in a manner that could explain some of the epidemiological data. The oviduct functions in picking-up ovulated oocytes, transporting gametes in opposite directions to the site of fertilization, providing a suitable environment for fertilization and early development, and transporting pre-implantation embryos to the uterus (Talbot and Riveles, 2005). Chemicals present in mainstream and sidestream cigarette smoke impair these oviductal functions, which results in reproductive problems.

2.9.2.2 Physical activity and diet

Evidence suggests that in women at average risk, physical activity and lack of obesity are protective against BC (McTierann *et al*., 2003; Dallal *et al*., 2007; Lahmann *et al*., 2007). High intake of vegetables and fruit in a female's diet has been found to decrease the risk of BC compared to women ingesting a low quantity of fibre. On the contrast, a case-control study indicated no protective effect regarding physical activity (Nkondjock *et al.*, 2004), but indicated an association between caloric intake and an increased risk for BC. A larger case-only study found that increased physical activity and the lack of obesity during adolescence were associated with significantly delayed BC onset in *BRCA1* and *BRCA2* carriers (King *et al.*, 2003).

2.9.2.3 Alcohol consumption

Alcohol consumption is consistently associated with an increased BC risk (Hamajima *et al*., 2002; Terry *et al*., 2006; Zhang *et al*., 2007). A meta-analysis of more than 40 epidemiologic studies suggested that the equivalent of 2 drinks a day may increase BC risk by 21%. This increased risk is dose-dependent and exists regardless of the type of alcoholic beverage consumed. Singletary *et al*. (2001) concluded that the most likely mechanism by which alcohol increases risk of BC is by increasing estrogen and androgen levels.

2.10 Mutation detection strategies

Numerous PCR based techniques have been developed for the detection of single base substitutions, small deletions or insertions. These include single-strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP) and DNA sequencing. Selection of an appropriate mutation detection method requires careful consideration. The method must be easy to perform and be cost-effective. The technique must also provide accurate results and be reliable. These factors are the basic requirements for the acceptance and implementation of new technologies.

2.10.1 Single strand conformational polymorphism (SSCP)

SSCP is based on the assumption that changes in the nucleotide sequence caused by mutations affect the conformation of DNA single strands and thus result in altered electrophoretic mobility (Jordanova *et al.*, 1997). Mobility depends not only on the size but also on sequence and most single base changes can be detected as mobility shifts. Using different conditions sensitivity can be increased. The PCR product is denatured, and separated strands adopt folded structures determined by their nucleotide sequences. A single base alteration is detected by SSCP when the folding of the single strand changes sufficiently to alter its electrophoretic mobility (Kozlowski and Krzyzosialc, 2001).

A mutation usually causes a mobility shift in electrophoresis and is so detected. Conformational change of the single stranded mutant DNA is believed to be the reason for the mobility shift. Conformation of single-stranded DNA is determined by intermolecular interactions which can change depending on physical conditions, such as temperature and ionic environment (Orita, 1989).

The most frequently claimed deficiency of the SSCP method is its variable mutation detection rate. The sensitivity of this method usually reported is about 80% when single conditions of electrophoresis are used and reaches 95% and more when several different conditions are applied for analysis of PCR products (Kozlowski and Krzyzosiak, 2001).

2.10.2 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) analysis was one of the first techniques to be widely used for detecting variation at the DNA sequence level (Botstein *et al*., 1980). It is a highly robust methodology with good transferability. The principle behind this technique is based on the possibility of comparing band profiles generated after restriction enzyme digestion in DNA molecules of different individuals. The DNA is cut into restriction fragments using suitable endonucleases, which only cut the DNA molecule where the specific DNA sequences are recognized by the enzymes (Botstein *et al*., 1980). Diverse mutations that might have occurred affect DNA molecules in different ways producing fragments of variable lengths. These differences in fragment lengths can be seen after gel electrophoresis. Disadvantages of this method include that large amounts of DNA are required and the technique is costly and time-consuming (Zhang *et al.*, 2005)**.**

2.10.3 DNA sequencing

Direct sequencing is considered as the gold standard method for mutation detection, however not necessarily accurate as unequal amplification of alleles in heterozygous samples have been reported (van der Heiden *et al.*, 2004). Most

other screening techniques such as SSCP still require DNA sequencing of detected variants to be able to identify the exact nucleotide change. The most serious disadvantage of direct sequencing is the high-cost involved.

In automated DNA sequencing dideoxynucleotides (ddNTPs), labelled fluorochromes, are used. The labels are incorporated into the ddNTPs and this is used to carry out chain termination. Alternatively the primers can be labelled with fluorochromes, which mean the same primers cannot be used for the initial amplification and the sequencing reaction. The four chain-terminated products are run on the same track of denaturing electrophoresis gel. Capillary electrophoresis utilizes liquid polymers in thin capillary tubes. Each product, with their base-specific dye is excited by a laser and the dye then emits light at its characteristic wavelength. A diffraction grating separates the emission, which are detected by a charged device and the sequence is interpreted by a computer (Sanger *et al.*, 1977).

Chapter 3

Patients and Methods

3.1 Patients

3.1.1 Index patients and relatives

A total of 60 *BRCA2* c.8162delG mutation carriers of Caucasian Afrikaner descent, both affected and unaffected with BC, were selected for the study. The *BRCA2* mutation positive subjects were retrospectively identified using patient records and pedigree information recorded over 10 years. Carriers represented families containing a minimum of three BC affected relatives. All BC patients were confirmed by means of hospital or pathology records or from death certificates.

In order to prevent biased results, only a single mutation positive BC patient and one unaffected carrier were selected per family. A family was defined as a pedigree consisting of three generations, with the index patient mostly representing the second generation. This allowed the usage of various lineages within an expanded family tree of for example six or seven generations.

The mutation carriers were divided into two groups of 30 individuals with the groups containing individuals affected with BC and unaffected carriers respectively. Each of the participants were stratified by age (5-year intervals) and matched according to age.

The *BRCA2* mutation carriers were contacted by an introductory letter (Appendix A), explaining the aim and protocol of the study and asking for their participation. Since all carriers received genetic counselling prior to their diagnostic testing, no additional counselling was required. Questions from participants regarding the study were answered telephonically.

3.1.2 Control participants

Individuals representing the control group were healthy Caucasian Afrikaner women without a family history of BC. The control group was selected to match the age of each individual (allowing for a maximum of five year variation) in the *BRCA2* carrier groups indicated in Table 3.1. The age at diagnosis was used for the affected patients. Control subjects were recruited with the aid of the South African National Blood Services in Bloemfontein, after obtaining permission.

3.1.3 Measurement

Each of the 30 groups consisted of four patients, namely a *BRCA2* mutation positive affected patient (MA), an age specific control (C1), a *BRCA2* mutation positive unaffected individual (UA) and its control (C2) (Table 3.1). The individuals in each group were numbered 1–1, 1–2, 1–3 and 1–4 , with 1–1 and 1–3 being the mutation positive carriers (affected and unaffected) and 1–2 and 1–4 the age specific controls. The term “BC patient” will refer to a mutation positive individual affected with BC. The term “case” will represent a currently healthy mutation positive individual.

All 120 participants were interviewed using an epidemiological questionnaire (Appendix B). The questionnaire included information pertaining to known environmental modifiers of BC risk, such as tobacco dependencies, contraceptive and pregnancy history.

3.1.4 Ethics

The proposed project was reviewed and approved by the Ethics Committee of the Faculty of Health Sciences of the University of the Free State in Bloemfontein (ETOVS 49/06). Permission was obtained from The Head of Clinical Services of Universitas Hospital, together with the Business Manager from the National Health Laboratory Service (NHLS), to approach and possibly involve patients attending clinics at the respective institutions (Appendix C and D). Permission was also

Table 3.1 Compilation of groups used in the study.

Group	*BRCA2* + Affected (MA)	Age at diagnosis	Control 1 (C1)	Current Age	*BRCA2* + Unaffected (UA)	Current Age	Control 2 (C2)	Current Age
1	MA18	24	C54	22	UA30	24	C59	28
2	MA14	29	C21	30	UA27	26	C7	31
3	MA23	30	C30	31	UA4	33	C33	32
4	MA11	35	C13	33	UA28	33	C34	33
5	MA17	35	C19	34	UA23	35	C11	35
6	MA5	36	C36	35	UA24	35	C16	35
7	MA29	37	C14	37	UA1	36	C35	37
8	MA12	38	C29	37	UA16	39	C4	38
9	MA27	40	C5	38	UA7	39	C23	38
10	MA3	40	C53	39	UA13	39	C25	39
11	MA22	41	C6	40	UA26	41	C8	41
12	MA19	41	C32	41	UA5	42	C37	41
13	MA15	42	C31	42	UA15	42	C28	43
14	MA16	42	C49	43	UA14	43	C52	44
15	MA25	42	C38	45	UA10	44	C62	44
16	MA6	43	C64	43	UA25	46	C55	46
17	MA30	44	C39	41	UA18	47	C65	51
18	MA10	44	C57	41	UA19	48	C61	51
19	MA13	45	C58	42	UA12	50	C63	54
20	MA7	47	C56	46	UA6	51	C20	48
21	MA8	50	C44	49	UA2	51	C40	51
22	MA20	50	C41	50	UA9	51	C18	51
23	MA28	51	C45	51	UA29	53	C2	52
24	MA4	52	C10	52	UA21	53	C15	53
25	MA2	54	C27	54	UA17	55	C17	55
26	MA24	54	C51	55	UA11	56	C46	55
27	MA9	55	C26	56	UA3	58	C47	57
28	MA26	55	C1	58	UA22	59	C12	58
29	MA21	57	C9	61	UA20	62	C22	61
30	MA1	60	C24	61	UA8	65	C42	62

obtained from the Business Manager of NHLS and the Head of the Division of Human Genetics to make use of the space and equipment of the Division of Human .Genetics (Molecular Laboratory) for the project (Appendix E).

All 120 participants gave written informed consent (Appendix F) at the end of the interview and were given unique sample numbers in order to perform the test blind and to maintain confidentiality.

3.2 Methods

3.2.1 DNA extraction

Peripheral blood (10–20 ml) was collected in ethylenediaminetetraacetic acid (EDTA) tubes and stored at -20°C. DNA was extracted from the lymphocytes according to an adapted salting out procedure (Miller *et al*., 1998). Once thawed, the cells were ruptured using a cold lysis buffer (0.3 M sucrose, 10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) pH 7.8, 5 mM $MgCl_2$, 1% (v/v) t-octylphenoxypolyethoxyethanol (Triton X-100)). The solution was centrifuged (20 min at 15 000 *g* at 4°C) where after the pellet was resuspended in 1x SET (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA), 10 $\mu g.\mu l^{-1}$ proteinase K and 1% (w/v) sodium dodecyl sulphate. The solution was incubated overnight at 37°C.

Equal volumes of phenol pH 8 (USB Corporation) and chloroform:isoamyl alcohol (24:1) were added to the solution and mixed thoroughly on an orbital shaker for 60 min at room temperature. Centrifugation for 10 min at 15 000 *g* at 15°C followed, where after the supernatant was transferred to a new tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the supernatant, mixed thoroughly and centrifuged as described above. The DNA was precipitated from the supernatant by adding 2 volumes ice-cold 100% (v/v) ethanol and sodium acetate pH 5.4 to a final concentration of 0.3 M. After 10 min at -20°C, the precipitated DNA was scooped from the solution, transferred to an eppendorf tube and washed with 70% (v/v) ethanol. The dried DNA was dissolved in $T._1E$ (10 mM Tris pH 7.6, 0.1 mM EDTA).

If no DNA was visible during precipitation, the tubes were incubated at -20°C for 24 h. The tubes were then centrifuged for 20 min (15 000 *g* at 4℃) to pellet the DNA, where after it was treated as described. DNA concentration and purity ($A_{260/280}$) were determined using spectrophotometry (NanoDrop® ND-100 Spectrophotometer v3.01, NanoDrop Technologies Inc). The NanoDrop® ND-100 Spectrophotometer v3.01 was operated according to the manufacturer's specifications. The DNA concentration was expressed as $ng.\mu l^{-1}$.

3.2.2 Molecular analyses of selected DNA polymorphisms

All PCR (polymerase chain reaction) reactions performed in this study had a final volume of 50 µl. Primers, deoxyribonucleotide triphosphates (dNTPs) and *Taq* DNA polymerase were obtained from Roche. Conditions were individually optimized for each primer set (Table 3.2). PCR was performed using the MyCycler™ (Biorad). A negative control was run with every PCR in order to indicate possible contamination. The control reactions contained all PCR reagents, except for the genomic DNA.

3.2.2.1 *BRCA2* c.8162delG

A baseline screen for the *BRCA2* disease causing mutation was performed for each individual to confirm their mutation status using SSCP. Exon 17 was amplified using two specific primers that anneal adjacent to the exon (Table 3.2). Each 50 µl PCR contained 300 ng template, 250 µM dNTPs (Roche), 20 pmol of each primer (Roche), 100 mM Tris-HCI pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCI and 1 U *Taq* DNA polymerase (Roche). The amplification regime was as follows: one cycle at 95℃ for 1 min, followed by 27 cycles at 94℃ fo r 1 min, 46℃ for 1 min and 72℃ for 1 min with a final elongation step at 72℃ for 10 min.

To each reaction, 10 µl loading buffer (95% (v/v) formamide, 1 mM EDTA pH 8, 0.05% (w/v) xylene cyanol FF, 0.05% (w/v) bromophenol blue) was added. Samples were denatured at 94℃ for 5 min, snap cool ed on ice for 5 min and then separated on a 10% (w/v) polyacrylamide gel (BioRad) (29 acrylamide: 1 bis-

Table 3.2 Oligonucleotides used in this study. Indicated are primer sequences, annealing temperatures and fragment lengths of the individual amplicons. Ta represents the optimal annealing temperature for each primer set.

Polymorphism	Forward Primer	Reverse Primer	Ta (℃)	Amplicon size (bp)
BRCA2 c. **8162delG**	**BR2F17**: 5'-CAGAGAATAGTTGTAGTTGTTGAA-3'	**BR2R17**: 5'-AGAACCTTAACCATACTGC-3'	46	306
Tp53 **intron 6 (*Nci* I)**	**F53**: 5'-CCTGAAAACAACGTTCTGGTAA-3'	**R53I6**: 5'-GCCAACCTAGGAGATAACACAG-3'	64	2069
Tp53 **intron 6 (*Nci* I)**	**F53**: 5'-CCTGAAAACAACGTTCTGGTAA-3'	**R53I6C**: 5'-CCCCCCTACTGCTCACCC-3'	64	1602
Tp53 **intron 6 (*Nci* I)**	**F53**: 5'-CCTGAAAACAACGTTCTGGTAA-3'	**R53I6T**: 5'-CCCCCCTACTGCTCACCT-3'	64	1602
Tp53 **(intron 3 and exon 4)**	**F53**: 5'-CCTGAAAACAACGTTCTGGTAA-3'	**R53E4**: 5'-TGTAGGAGCTGCTGGTGC-3'	58	258/274
MDM2 **(SNP309 T to G)**	**M2F**: 5'-GATTTCGGACGGCTCTCGCGGC-3'	**M2R**: 5'-CATCCGGACCTCCCGCGCTG-3'	64	121
WAF1 **codon 31**	**p21E2F**: 5'-CGCCATGTCAGAACCGGCT-3'	**p21E2R**: 5'-TTCCATCGCTCACGGGCC-3'	60	153
WAF1 **intron 2**	**p21I2F**: 5'-TCGCTCAGGGGAGCAGGCTGAA-3'	**p21I2R**: 5'-GAGAATCCTGGTCCCTTAC-3'	60	129

acrylamide, 3.3% cross linking) containing 1x TBE (0.089 M Tris, 0.089 M boric acid, 2 mM EDTA pH 8). The gels were run on a SE600 vertical electrophoresis system (Hoefer Pharmacia Biotech Inc) connected to a circulating low temperature waterbath. The temperature was recorded and documented before and after each run to ensure that the optimum running temperature was maintained. The gels were run using 1x TBE as running buffer at a constant 12℃
for 14 h at 10 volts per centimeter ($V.cm^{-1}$). Banding patterns were visualised by silver staining using the BDH Instaview silver stain kit (BDH Laboratory Supplies) according to the manufacturer's instructions. The gel was digitally captured and enhanced using KODAK EasyShare software.

3.2.2.2 *Tp53* haplotype

Three biallelic *Tp53* gene polymorphisms were analyzed using PCR (Weston *et al*., 1997). The polymorphisms used to construct the haplotype included a 16 bp duplication and insertion in intron 3 and two single base changes in exon 4 (Arg72Pro) and intron 6 (*Nci*I at nt 13494) respectively (Fig. 3.1 A, B, C and D).

For intron 6, a 2096 bp PCR product (exons 3-6) was amplified using the F53 and R53I6 primer combination (Table 3.2, Fig. 3.1 A). Each 50 µl reaction contained 100 ng template, 200 µM dNTPs (Roche), 25 pmol of each primer (Roche), 100 mM Tris-HCI pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCI and 1 U *Taq* DNA polymerase (Roche). The amplification regime was as follows: one cycle at 94℃ for 2 min, followed by 30 cycles at 94℃ for 30 sec, 64℃ for 40 sec and 72℃ for 45 sec with a final elongation step at 72℃ for 5 min. Aliquots (10 µl) of each reaction was subjected to restriction digestion with 10 U *Nci*I (New England BioLabs) in the presence of 50 mM potassium acetate, 20 mM Tris-acetate pH 7.9, 10 mM magnesium acetate and 1 mM 1,4-dithiothreitol (DTT) in a final volume of 15 µl. Digestion was for 3 h at 37℃. Following digestion , 15 µl of each reaction was separated on a 1% (w/v) agarose gel prepared with 1x TBE. The DNA was visualized by adding ethidium bromide (EtBr) to a final concentration of 0.5 $\mu g.ml^{-1}$. The running buffer used was 1x TBE. Samples were mixed with 5 µl loading buffer (0.25% (w/v) orange G, 40% (w/v) sucrose), loaded on the gel and separated at 20

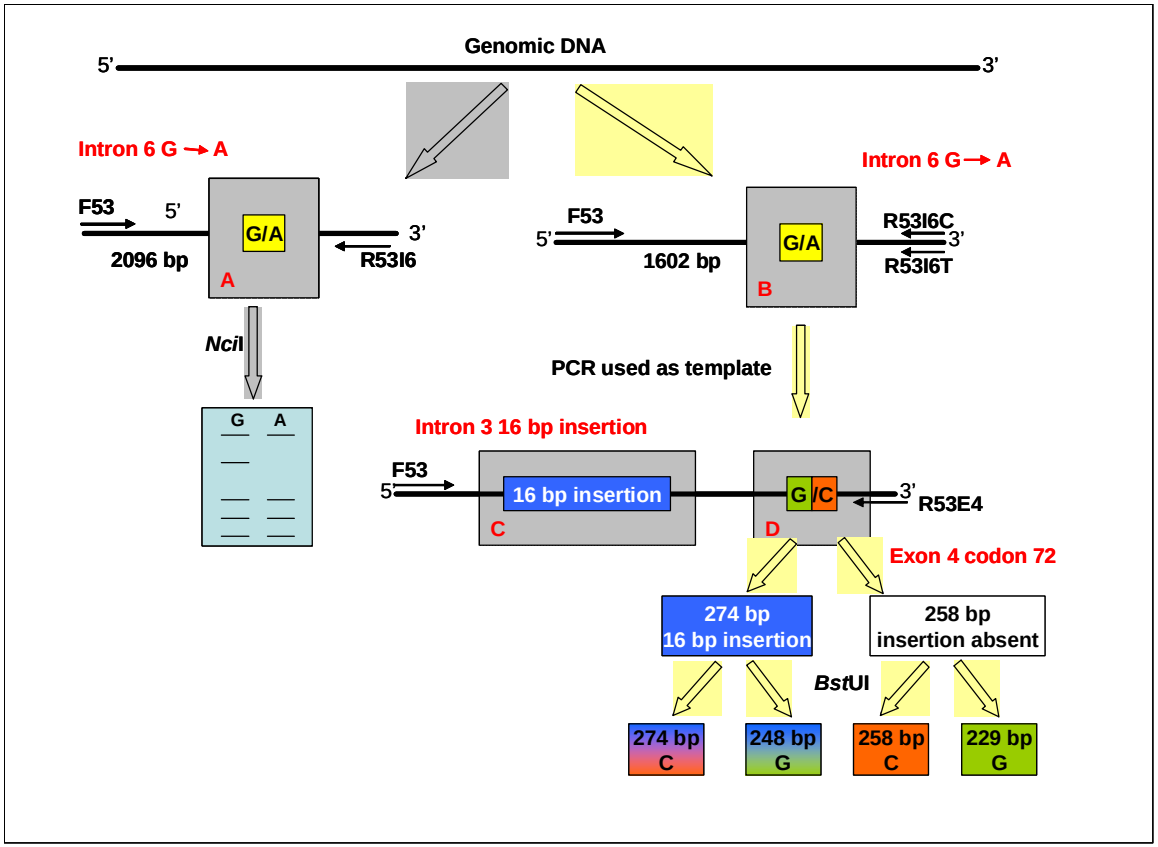


Figure 3.1 Construction of the *Tp53* haplotype. **A** *Nci*I digestion to detect polymorphism in intron 6 (G to A). **B** Detection of the polymorphism in intron 6 (G to A) using allele specific PCR. **C** Duplication and insertion of a 16 bp fragment in intron 3. **D** *Bst*UI digestion of exon 4 at codon 72 (G to C).

$V.cm^{-1}$. Migrated DNA fragments were analyzed with a Gel Doc™ XR gel documentation system using the Quantity One® 1-D analysis software (BioRad).

Two allele specific PCR reactions were performed for the intron 6 polymorphism (Fig 3.1 B) using the F53 primer in combination with the reverse R53I6C and R53I6T, respectively (Table 3.2). The reaction composition and amplification regime was as described previously with an annealing temperature of 64℃. Following PCR, samples were separated on a 1.5% agarose gel. A sample displaying both alleles was sequenced and thereafter used as a positive control in each run.

Allele specific PCR products (100x diluted, Fig 3.1 B) were used as templates to detect the other two polymorphisms of the *Tp53* haplotype. The primers used (F53 and R53E4) (Table 3.2) flank intron 3 (16 bp duplication and insertion) and exon 4 (Arg72Pro) (Fig 3.1 C & D). The PCR composition was as described for the allele specific PCR with the only differences being the addition of 200 mM tetramethylammonium chloride (TMAC) and an annealing temperature of 58℃. The DNA fragments were separated on a 3% (w/v) agarose gel. The resultant PCR products were either 274 or 258 bp in length, depending on the absence or presence of the 16 bp insertion in intron 3. Aliquots (10 µl) of each reaction were subjected to restriction digestion with 10 U *Bst*U1 (New England BioLabs) in the presence of 50 mM NaCl, 10 mM Tris-acetate pH 7.9, 10 mM $MgCl_2$ and 1 mM DTT in a final volume of 15 µl. Digestion was for 3 h at 60℃. DNA fragments were finally separated on a 3% (w/v) agarose gel.

3.2.2.3 *MDM2* polymorphism

MDM2 (SNP309) analysis was done according to Ma *et al*. (2006b) (Table 3.2). PCR was performed as described (3.2.2.2). The amplification regime was similar with the exception that 35 amplification cycles were used with a longer final elongation step of 10 min.

Aliquots (30 µl) of each reaction were subjected to restriction digestion with 30 U

*Pst*I (Fermentas) in the presence of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl and 0.1 $mg.ml^{-1}$ bovine serum albumin in a final volume of 40 µl. Digestion was for 3 h at 37℃. The DNA fragments were separated on a 3% (w/v) agarose gel.

3.2.2.4 *WAF1* polymorphism

WAF1 analysis was done according to Powell *et al*. (2002) (Table 3.2). PCR was performed as described (3.2.2.2) using 50 pmol of each primer (Roche). The amplification regime was as described (3.2.2.2) with an annealing temperature of 60℃ and 64℃ for *WAF1* intron 2 and *WAF1* codon 31, respectively.

SSCP analysis used for screening the *WAF1* codon 31 and *WAF1* intron 2 polymorphisms was performed as described (3.2.2.1), with the exception that the gel was run for 14 h at 16℃ at 10 $V.cm^{-1}$. Although a range of running temperatures (4°C, 12°C, 16°C and 20°C) were evaluated, the optimum temperature (16°C) revealed the most distinct banding patterns that allowed for the most accurate genotyping.

3.2.3 DNA Sequencing

DNA sequencing was performed to confirm the various identified genotypes and banding patterns. Each sample was re-amplified using the specific primers and purified using SigmaSpin Post-Reaction clean-up columns according to manufacturer's conditions. DNA fragments were sequenced bi-directionally, using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Electropherograms were analyzed by proprietary sequence analysis software (Chromas version 2.31, www.technelysium.com.au) followed by visual inspection and confirmation. The sequences were aligned LALIGN (www.ch.embnet.org/software/LALIGN) with the wild-type references for each gene. Translation of the DNA sequence was done using the ExPASy translate tool (http://au.expasy.org/tools/dna.html).

3.2.4 Statistical analysis

The statistical analyses were performed by the Department of Biostatistics at the University of the Free State. Results were summarized by frequencies and percentages (categorical variables) and means, standard deviations or medians (numeric variables). The analysis was initially performed between the controls and the *BRCA2* mutation carriers (Comparison 1). Odds ratios (OD) and confidence intervals (CI) using McNemar's test were calculated (Fleiss *et al*., 2003). There after the mutation carrier group was divided into the BC affected patients and unaffected carriers (Comparison 2). These two groups were analysed separately to evaluate the independent effect of each genotype with regards to BC risk.

Although McNemar's test bears a resemblance to the chi-square or Fisher exact propability test, it is more appropriate when using a matched case-control study design. McNemar's test assesses the significance of the difference between two correlated dependent proportions, where the two proportions are based on the same sample of subjects (http://faculty.vassar.edu/lowry/propcorr.html). The probabilities (P-values) observed, indicated whether the particular polymorphism deviated from Hardy-Weinberg expectation.

Chapter 4

Results

4.1 Epidemiology

A summary of the categorical characteristics of the entire sample population used for Comparison 1 (controls versus *BRCA2* mutation carriers) and 2 (*BRCA2* mutation positive BC patients and unaffected cases) is presented in Table 4.1. The estimated risks of BC associated with reproductive, pregnancy and smoking history are indicated. Only those results obtained from the questionnaire that showed any difference or required mentioning are listed.

The results of the statistical analysis of Comparison 1 and 2 indicated approximately similar usage of oral contraceptives and hormone therapy for the treatment of menopause among all participants (Table 4.1). The only difference observed was the lower percentage of *BRCA2* mutation carriers still using oral contraceptives (Comparison 1, 6.7% versus 18.3% for the controls). Currently none of the *BRCA2* mutation positive BC patients are using any contraceptives (Comparison 2).

A statistically significant difference was observed regarding nulliparity in the controls, for 40% of the controls compared to 13.3% for the mutation carriers have never been pregnant (Comparison 1) ($P = 0.0001$). This is also higher than the percentage observed for the entire sample population, for only 26.7% of the 120 participants were nulliparous. The percentages among the mutation carrier groups were similar (Comparison 2) (Table 4.1).

Parity as a characteristic had an involuntary influence on three other factors that were tested, namely the number of live births, the number of miscarriages and breast feeding. Statistically significant differences were observed between the control group and the mutation carriers (Comparison 1) for two of the characteristics, namely the number of live births ($P = 0.0002$) and the number of

Table 4.1 Categorical characteristics of controls and *BRCA2* mutation carriers (Comparisons 1 and 2).

Characteristic	Comparison 1 (controls versus *BRCA2* mutation carriers)			Comparison 2 (BC patients versus unaffected cases)		
	Controls n = 60 n (%)	*BRCA2* carriers n = 60 n (%)	*P* value	BC patients n = 30 n (%)	Unaffected cases n = 30 n (%)	*P* value
Reproductive history						
Oral contraceptive used (Q 3.1 & 3.3)						
Never used	7 (11.7)	9 (15.0)	0.2556	4 (13.3)	5 (16.7)	0.7055
Previous usage	42 (70.0)	47 (78.3)		26 (86.7)	21 (70.0)	
Current user	11 (18.3)	4 (6.7)		0 (0.0)	4 (13.3)	
Hormone treatment therapy for menopause (Q 5.1)						
Not used	35 (58.3)	34 (56.7)	0.7815	18 (60.0)	16 (53.3)	0.4142
Used	25 (41.7)	26 (43.3)		12 (40.0)	14 (46.7)	
Pregnancy history						
Parity (Q 3.5b)						
Nulliparous	24 (40.0)	8 (13.3)	0.0001	3 (10.0)	5 (16.7)	0.8318
1	5 (8.3)	3 (5.0)		1 (3.33)	2 (6.7)	
2	15 (25.0)	20 (33.3)		11 (36.7)	9 (30.0)	
3	11 (18.4)	14 (23.3)		8 (26.7)	6 (20.0)	
4	5 (8.4)	8 (13.3)		4 (13.3)	4 (13.3)	
5	0 (0.0)	5 (8.3)		3 (10.0)	2 (6.7)	
6	0 (0.0)	2 (3.4)		0 (0.0)	2 (6.7)	
Number of live births (Q 4.2b)						
Nulliparous	25 (41.7)	9 (15.0)	0.0002	4 (13.3)	5 (16.7)	1
1	5 (8.3)	4 (6.7)		2 (6.7)	2 (6.7)	
2	21 (35.0)	27 (45.0)		14 (46.7)	13 (43.3)	
3	8 (13.3)	11 (18.3)		4 (13.3)	7 (23.3)	
4	1 (1.7)	6 (10.0)		5 (16.7)	1 (3.33)	
5	0 (0.0)	2 (3.3)		1 (3.3)	1 (3.33)	
6	0 (0.0)	1 (1.67)		0 (0.0)	1 (3.33)	
Number of miscarriages (Q 3.5c)						
Nulliparous	49 (81.7)	44 (73.3)	0.2863	21 (70.0)	23 (76.7)	1
1	9 (15.0)	12 (20.0)		8 (26.7)	4 (13.3)	
2	2 (3.3)	1 (1.67)		0 (0.0)	1 (3.33)	
3	0 (0.0)	3 (5.0)		1 (3.3)	2 (6.7)	
Breast feeding						
Nulliparous	25 (41.67)	9 (15)	0.0082	4 (13.3)	5 (16.7)	0.9579
no	6 (13.33)	25 (15)		8 (26.7)	7 (23.3)	
yes	27 (45.0)	36 (60.0)		18 (60.0)	18 (60.0)	
Smoking history						
Active smoking (Q 2.1)						
Never	49 (81.7)	41 (68.3)	0.1167	22 (73.3)	19 (63.3)	0.3657
Ever	11 (18.3)	19 (31.7)		8 (26.7)	11 (36.7)	
Passive smoking (Q 2.7)						
Never	16 (26.7)	25 (41.7)	0.0947	14 (46.7)	11 (36.7)	0.3657
Ever	44 (73.3)	35 (58.3)		16 (53.3)	19 (63.3)	
Currently smoking (Q 2.5)						
Never	49 (81.7)	41 (68.3)	0.0627	22 (73.3)	19 (63.3)	0.343
No	3 (5.0)	13 (21.7)		6 (20.0)	7 (23.3)	
Yes	6 (13.3)	6 (10.0)		2 (6.7)	4 (13.4)	

women participating in breast feeding (P = 0.0082). Regarding Comparison 2, a disparity was observed among the number of BC patients that had one miscarriage (26.7%) versus that of the unaffected cases (13.3%) (Table 4.1).

The majority of the 120 women participating in this study have never smoked before (81.7% for the controls and 68.3% for the mutation carriers – Comparison 1) (Table 4.1). The percentages did not differ much among the mutation carriers, for both the BC patients and unaffected cases were similar (73.3% versus 63.3%) (Table 4.1). More mutation carriers were never exposed to secondary smoke during childhood in comparison with the controls (41.7% versus 26.7%). A trend was observed among the *BRCA2* mutation carriers as 21.7% did not currently smoke compared to only 5% of the controls (Comparison 1) (P = 0.0627). A similar tendency has also been observed for Comparison 2, as a low number of BC patients are currently still smoking (6.7% versus 13.4% in the unaffected cases).

The study design chosen implicated similar mean ages for both the control (44.5) and the mutation positive groups (44.4) (Table 4.2). The minimum and maximum ages for the mutation positive group (24 and 65 years respectively) were two to three years higher than that of the controls. Both groups started smoking at the young age of 22, although the mutation carriers smoked for a shorter period of time (4 years versus 15 years in the controls, P = 0.0083) and at a lower intensity (on average 5 cigarettes per day versus 15 for the controls, P = 0.0003). The mutation carriers tended to stop smoking at a younger age, namely 24 years (Table 4.2). Both groups were equally exposed to secondary smoking from birth to early adulthood which ceased when they left home.

Upon leaving home, most of the participants married and started using birth control at the age of 22 to 23. The average number of pregnancies (2) was identical for the two groups. Child bearing commenced on average two years earlier for the mutation positive group, namely at 24.1 years (SD $\pm$ 3.3) compared to 25.8 years (SD $\pm$ 4.4) for the controls. The average number of live births was 2 for the control and the mutation positive group (Table 4.2).

Table 4.2 Numerical variables for controls and *BRCA2* mutation carriers (Comparison 1). Highlighted values represent either the mean and standard deviation (SD) for symmetrical data or the median for skewed distributions.

	Comparison 1 (Controls vs *BRCA2* carriers)											
	Controls						*BRCA2* mutation carriers					
Variables	**n**	**Median**	**Mean**	**SD**	**Min**	**Max**	**n**	**Median**	**Mean**	**SD**	**Min**	**Max**
Age (Q 1)	60	43	**44.5**	**9.5**	22	62	60	43.5	**44.4**	**9.5**	24	65
Age first started smoking (Q 2.2)	11	21	**22**	**4.6**	14	31	19	20	**22.6**	**7.8**	16	49
P-value = 0.4600												
Number of years smoked regularly (Q 2.3)	11	**15**	15.5	7.9	4	31	18	**4**	7.8	7.9	1	30
P-value = 0.0083												
Number of cigarettes smoked per day (Q 2.4)	11	**15**	13.9	4.4	5	20	19	**5**	6.6	3.3	1	15
P-value = 0.0003												
Age when stopped smoking (Q 2.5b)	3	**27**	27	5	22	32	13	**24**	29	12	20	59
P-value = 0.5428												
During last year, number of cigarettes smoked per day (Q 2.6)	11	**15**	12.8	6.3	0	20	19	**5**	6.2	3.3	1	15
P-value = 0.0059												
Age when first exposed to household smoke (Q 2.9)	44	**0**	3.3	7.6	0	31	35	**0**	2.2	6.7	0	28
P-value = 0.0836												
Age when last exposed to household smoke (Q 2.10)	44	**22.5**	27	12.6	10	55	35	**18**	22.2	13.6	4	63
P-value = 0.0353												
Age when started using birth control (Q 3.2)	53	23	**23.5**	**3.6**	18	37	51	22	**22**	**4.2**	16	39
P-value = 0.0169												
Age when stopped using birth control (Q 3.4)	53	35	**35.1**	**7.7**	20	52	51	30	**30.2**	**6.6**	17	45
P-value = 0.0012												
Period of oral contraceptive use in months (Q 3.5)	52	**90**	121.6	95.2	1	336	51	**96**	99.1	75	6	276
P-value = 0.3221												
Number of pregnancies (Q 3.5b)	60	**2**	1.4	1.3	0	4	60	**2**	2.5	1.5	0	6
P-value = 0.0001												
Age at first birth (Q 4.1)	35	26	**25.8**	**4.4**	18	38	52	24	**24.1**	**3.3**	17	34
P-value = 0.0846												
Age at last birth (Q 4.2)	35	30	**29.9**	**4.5**	19	41	51	30	**29.7**	**3.7**	21	38
P-value = 0.8912												
Number of live births (Q 4.2b)	60	**2**	1.2	1.1	0	4	60	**2**	2.1	1.3	0	6
P-value = 0.0002												

The unaffected mutation positive group was on average two years older than the mutation positive BC patients (45.2 versus 43.7 years) (Table 4.3). Although both groups started smoking at a relatively young age (20.7 and 24 years respectively), the unaffected cases discarded the habit within 3 years compared to 8 for the BC patients (Table 4.3). The two groups were similar regarding the number of cigarettes smoked per day (5 per day) and the exposure to secondary smoke during childhood (Table 4.3).

On average, the BC patients had a three year shorter usage of oral contraceptives when compared to the unaffected cases (72 versus 108 months respectively). The average number of pregnancies, the age at first and last birth did not vary between the two groups (Table 4.3). An average of two live births was reported for both the BC patients and the unaffected cases (Table 4.3).

4.2 *BRCA2* c.8162delG baseline screen

All participants representing groups 1 – 30 (Table 3.1; page 68) were screened for the presence (mutation positive BC patients and unaffected individuals) or absence (controls) of the *BRCA2* c.8162delG mutation using SSCP in order to be included in the study. Two different SSCP banding patterns were observed (Fig 4.1 A). Control participants represented by lanes 2 and 4 had a SSCP pattern showing two bands. These individuals represented the wild type population, for they did not have a family history of BC. The SSCP pattern for the cases (1–1 and 1–3) represented in lanes 1 and 3 indicated a SSCP shift (Fig 4.1 A).

Amplification products of participants representing each banding pattern were sequenced and compared against the reference sequence obtained from OMIM NM_000059.3. Sequencing results confirmed that the SSCP pattern with two bands corresponded to the wild type DNA sequence (Fig 4.1 B). Sequence analysis of the cases exhibiting a SSCP shift indicated the presence of the single base deletion (G at position 8162) on one of the chromosomes (Fig 4.1 C). These results were in line with previous work describing the use of SSCP and DNA sequencing for the detection of this mutation (NC van der Merwe, personal

Table 4.3 Variable characteristics of BC patients and unaffected cases (Comparison 2). Highlighted values represent either the mean and standard deviation (SD) for symmetrical data or the median for skewed distributions.

	Comparison 2 (BC patients vs Unaffected cases)											
	BC patients						Unaffected cases					
Variables	**n**	**Median**	**Mean**	**SD**	**Min**	**Max**	**n**	**Median**	**Mean**	**SD**	**Min**	**Max**
Age (Q 1)	30	42.5	**43.7**	**8.8**	24	60	30	45	**45.2**	**10.2**	24	65
Age first started smoking (Q 2.2)	8	19.5	**20.7**	**3.6**	16	28	11	20	**24**	**9.8**	18	49
P-value = 0.7664												
Number of years smoked regularly (Q 2.3)	7	**8**	8.8	7.7	1	20	11	**3**	7.2	8.4	2	30
P-value = 0.7137												
Number of cigarettes smoked per day (Q 2.4)	8	**5**	6.1	3.4	1	10	11	**6**	7	3.4	3	15
P-value = 0.5211												
Age when stopped smoking (Q 2.5b)	6	**25.5**	30.1	10.7	20	47	7	**22**	28	13.8	21	59
P-value = 0.4720												
During last year, how many cigarettes smoked per day (Q 2.6)	8	**5**	5.8	2.9	1	10	11	**5**	6.5	3.7	2	15
P-value = 0.7956												
Age when first exposed to household smoke (Q 2.9)	16	**0**	4.3	9.5	0	28	19	**0**	0.3	1.6	0	7
P-value = 0.1873												
Age when last exposed to household smoke (Q 2.10)	16	**19**	28.1	16.7	12	63	19	**18**	17.2	7.6	4	40
P-value = 0.0458												
Age when started using birth control (Q 3.2)	26	22	**21.6**	**2.7**	16	27	25	21	**22.5**	**5.4**	16	39
P-value = 0.8793												
Age when stopped using birth control (Q 3.4)	26	29.5	**29.5**	**6.6**	17	45	25	30	**30.9**	**6.6**	23	44
P-value = 0.5024												
Period of oral contraceptive use in months (Q 3.5)	26	**72**	88.4	69.7	6	276	25	**108**	110.2	80.1	6	276
P-value = 0.3850												
Number of pregnancies (Q 3.5b)	30	**2.5**	2.6	1.3	0	5	30	**2**	2.5	1.7	0	6
P-value = 0.0001												
Age at first birth (Q 4.1)	27	23	**23.8**	**3.5**	17	34	25	25	**24.5**	**3.1**	19	29
P-value = 0.4119												
Age at last birth (Q 4.2)	26	29	**29.3**	**3.5**	24	38	25	31	**30.1**	**4**	21	37
P-value = 0.2761												
Number of live births (Q 4.2b)	30	**2**	2.2	1.3	0	5	30	**2**	2.1	1.4	0	6
P-value = 0.0002												

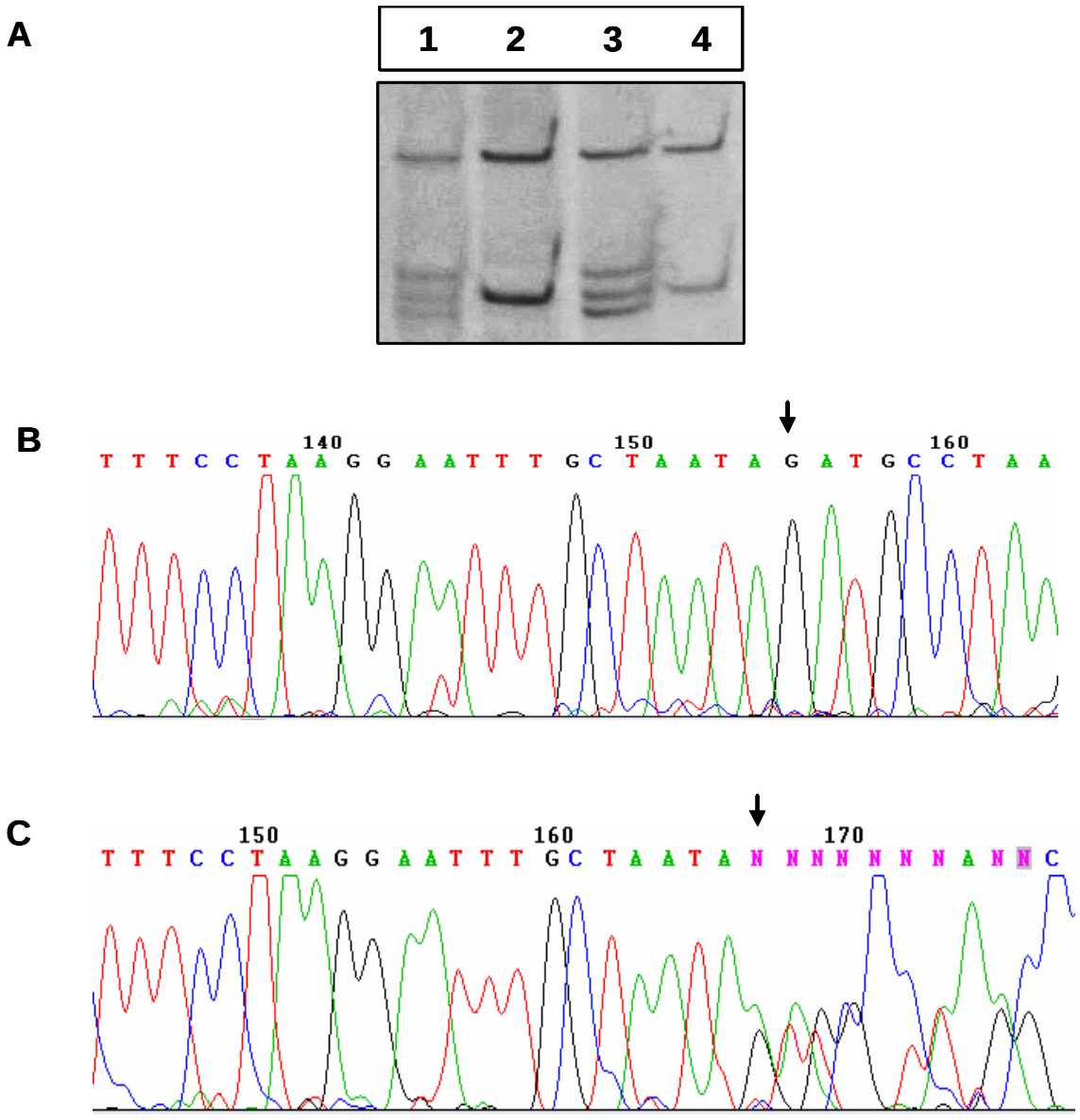


Figure 4.1 Baseline screen for the disease causing *BRCA2* c.8162delG mutation for all participants. **A** SSCP gel of participants screened for the *BRCA2* exon 17 mutation visualized by silver staining. Lane 1 – BC patient 1–1, lane 2 – control participant 1–2, lane 3 – unaffected mutation carrier 1–3 and lane 4 – control participant 1–4. **B** Double stranded sequencing results for control participant 1–4 with the normal banding pattern. **C** Double stranded sequencing results for BC patient 1–1, containing the *BRCA2* c.8162delG mutation. The position of the single base deletion is indicated by an arrow.

communication). The obtained results confirmed the placement of the individuals in their respective groups.

4.3 Compilation of a BC recombinant haplotype affecting BC risk in Caucasian Afrikaner women

Six polymorphisms representing three genes involved in the p53 pathway were selected and analyzed to determine any potential affect on BC risk in *BRCA2* mutation positive Afrikaner women.

4.3.1 *Tp53* gene analysis

Three polymorphisms in *Tp53* were analyzed using PCR, namely a SNP in intron 6 (IVS6+62 G to A), a 16 bp duplication and insertion in intron 3 (g.11951_11966dup) and a single base change in exon 4 (p.Arg72Pro). Amplification products of participants representing each of these polymorphisms were analyzed and a limited number were sequenced to confirm the three possible genotypes by comparison to the reference sequence (X54156) obtained from GenBank.

4.3.1.1 DNA analysis of the SNP in intron 6 (IVS6+62 G to A)

The DNA analysis for the SNP in intron 6 was performed according to two methods described by Weston *et al.* (1997), namely restriction digestion and allele specific PCRs. The first analysis involving restriction digestion was performed for a limited number of individuals only, with the main aim to identify positive controls for each of the genotypes. The actual screening for the presence of the SNP in all the participants was performed using the allele specific method.

For the initial analysis, a 2096 bp PCR product was generated using primers that flanked exon 3 to intron 6 (Table 3.2; page 71). After digestion with *Nci*I two banding patterns were observed when the digested PCR fragments were separated on an agarose gel (Fig 4.2).

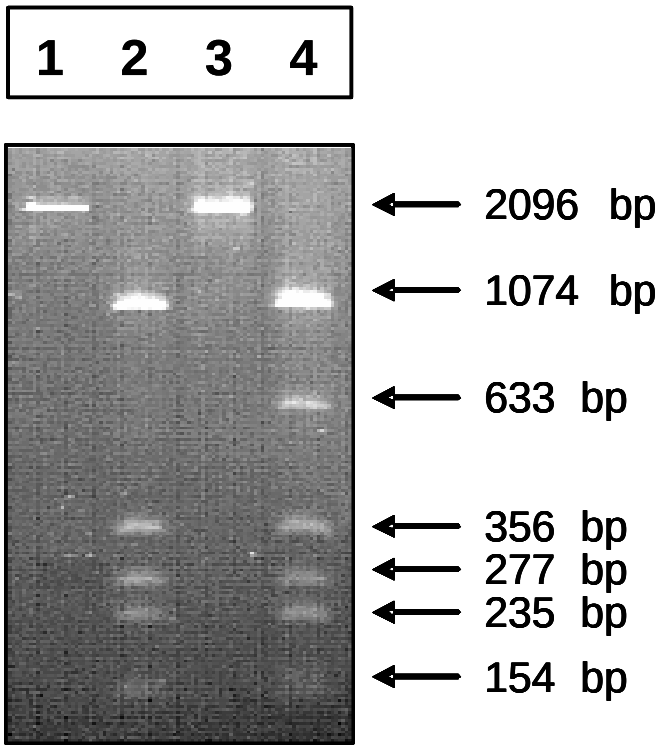


Figure 4.2 *Nci*I restriction analysis of the 2096 bp PCR amplified *Tp53* fragment separated on a 1.5% agarose gel, visualized by ethidium bromide. Undigested and digested amplification products of case 1–3 were loaded in lanes 1 and 2 respectively. Undigested and digested amplification products of control 1–4 were loaded in lanes 3 and 4. Fragment sizes are indicated by arrows.

Undigested amplification products of participants 1–3 and 1–4 were loaded in lanes 1 and 3 to indicate the optimal amplification of the PCR fragment. The most common restriction pattern observed was for the homozygous wild type (G/G) as seen for case 1–3 in lane 2 (Fig 4.2). This banding pattern was characterized by the presence of five bands 1074, 356, 277, 235 and 154 bp in size respectively (Fig 4.2). Individuals heterozygous for the variant (G/A) were identified based on the presence of six bands 1074, 633, 356, 277, 235 and 154 bp as observed for control 1–4 (Fig 4.2). Four fragments (1074, 633, 235 and 154 bp in size) indicating the absence of the restriction site, represented a homozygous variant (A/A). This banding pattern was not detected within the study population.

Construction of the *Tp53* haplotype commenced with the optimization of the allele specific PCR reactions (Table 3.2; page 71, Fig 3.1 A; page 73). These two separate PCR reactions using the same forward primer F53 in combination with one of two reverse primers, R53I6C and R53I6T respectively (Table 3.2; page 71), were optimized. The PCR components and regime were similar to that described by Weston *et al*. (1997), with the only exception being the annealing temperature. This was optimized using a gradient PCR (Fig 4.3 A). A final annealing temperature of 64°C proved to yield the most specific amplification product for both primer sets (Fig 4.3 A).

Both allele specific PCRs were simultaneously performed for all the participants within a group and run on the same gel, together with homozygous wild type (G/G) and heterozygous (G/A) controls. The genotype results for each individual were then scored using the corresponding lanes in the top and bottom gel (Fig 4.3 B and C). PCR amplification using the F53 and R53I6C primer pair revealed the presence of the G allele for all six individuals loaded in lanes 1 to 6 (Fig 4.3 B). The PCR using the F53 and R53I6T primer combination yielded PCR products for two participants only, namely control 19–2 in lane 1 and BC patient 20–1 in lane 4 (Fig 4.3 C). A case exhibiting an amplification product for the F53 and R53I6C primer set only was therefore homozygous (G/G) for the SNP in intron 6, while individuals containing amplification products for both PCR sets were heterozygous. (G/A) for the SNP (lanes 1 and 4) (Fig 4.3 B and C). No homozygotes for the A

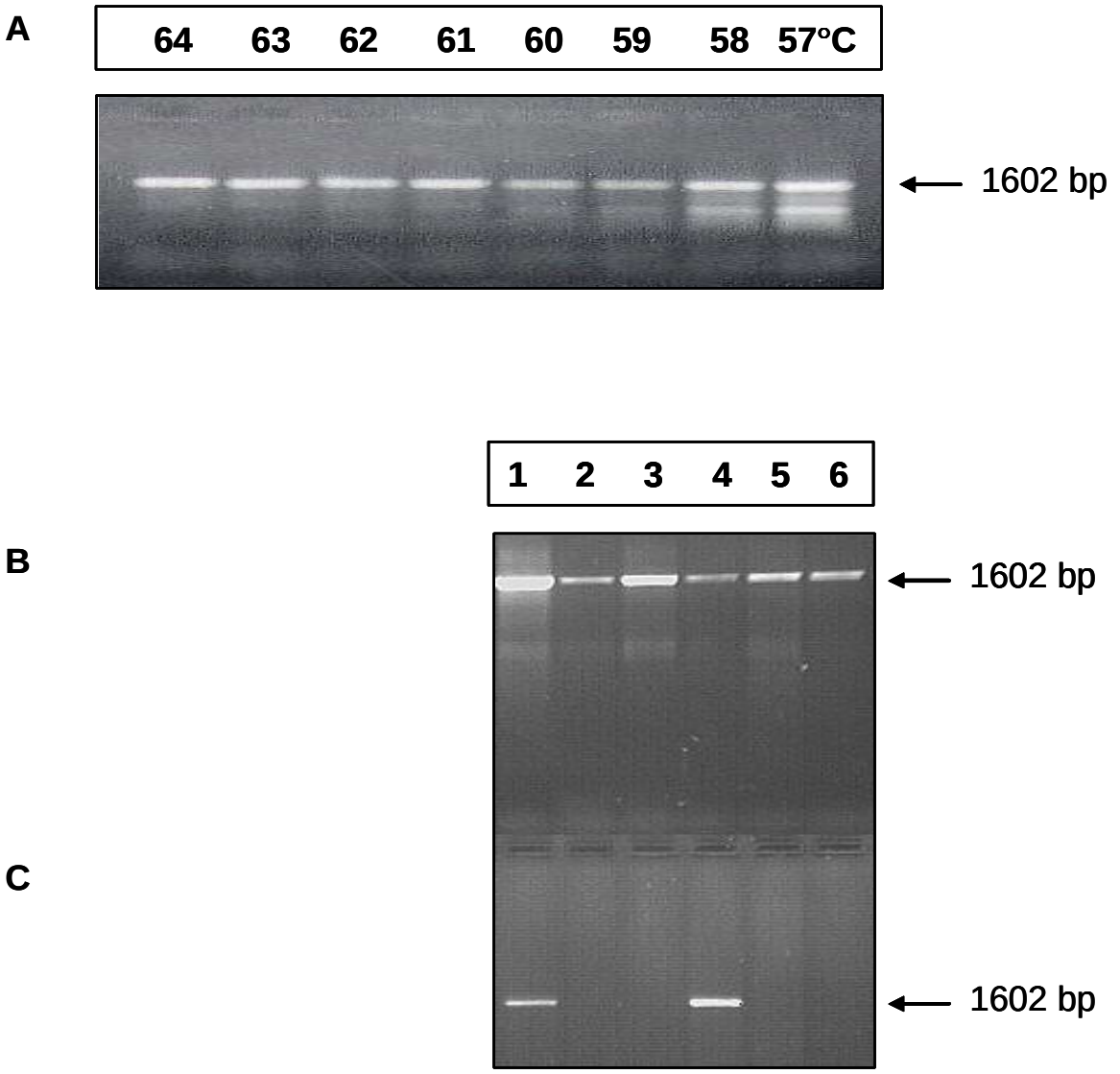


Figure 4.3 PCR amplification of the SNP present in *Tp53* intron 6, separated on a 1.5% agarose gel and visualized by staining with ethidium bromide. **A** Optimization of annealing temperatures for primer combination F53 and R53I6C, using gradient PCR. **B** PCR amplification of a 1602 bp fragment, representative of the G allele using the F53 and R53I6C primer combination. **C** PCR amplification of a 1602 bp fragment, representative of the A allele using the F53 and R53I6T primer combination. Lane 1 – control 19–2, lane 2 – case 19–3, lane 3 – control 19–4, lane 4 – BC patient 20–1, lane 5 – control 20–2 and lane 6 – case 20–3. Fragment sizes are indicated by arrows.

allele were observed in this study.

The results obtained from Comparison 1 (controls versus *BRCA2* mutation positive carriers) indicated a similar genotype distribution for the homozygous (G/G) (76.7% and 75%) and heterozygous participants (G/A) (23.3% and 25%) (Table 4.4). The frequencies were 88.3% for the G allele and 11.7% for the variant (A) in the controls and 87.5% and 12.5% in the mutation carriers respectively. No significant differences for the presence of this SNP in intron 6 were observed between the controls and mutation carriers (P>0.05).

Although no significant differences were observed during Comparison 1 regarding the number of heterozygotes observed, the genotype frequencies varied more between the mutation positive groups (Comparison 2). The frequency of the heterozygous genotype (G/A) was higher in the BC patient group (33.3%), with only 16.7% for the unaffected carriers. This observation was also reflected by the difference in the A allele frequencies observed for the two groups, namely 16.7% versus 8.3% but did not prove to be significant (P = 0.1655) (Table 4.4). The intron 6 SNP showed no deviations from Hardy-Weinberg expectations as the observed significance levels are above 5%.

4.3.1.2 DNA analysis of the intron 3 polymorphism (g.11951_11966dup)

The allele specific PCR products used to genotype the intron 6 polymorphism (4.3.1.1) served as templates for the detection of the polymorphism present in intron 3 (Fig 3.1 C; page 73). In the case of a heterozygous individual (intron 6 G/A), both allele specific PCR templates were used for the amplification of intron 3. For the homozygous major variant (G/G), only one reaction was used. Although a similar regime and PCR composition were used as described by Weston *et al.* (1997), further optimization was required since spurious amplification products were present that complicated analysis (Fig 4.4 A). The addition of 200 mM TMAC as a co-factor resulted in an increase in the specificity of the reaction and yielded only the correct amplification products (Fig 4.4 B).

Table 4.4 The allele and genotype distributions observed for the SNP in intron 6 of *Tp53.*

Frequency distribution	Comparison 1 (Controls vs *BRCA2* carriers)			Comparison 2 (BC patients vs Unaffected cases)		
	Controls n=60	Carriers n=60	OR (95% CI)	BC patients n=30	Cases n=30	OR (95% CI)
	n (%)	n (%)		n (%)	n (%)	
Allele (%)						
G	106 (88.3)	105 (87.5)	0.9 (0.32-2.46)	50 (83.3)	55 (91.7)	0.44 (0.10-1.59)
A	14 (11.7)	15 (12.5)	1.11 (0.40-3.12)	10 (16.7)	5 (8.3)	2.27 (0.62-10.0)
Genotype (%)						
G/G	46 (76.7)	45 (75.0)	0.9 (0.32-2.46)	20 (66.7)	25 (83.3)	0.44 (0.10-1.59)
G/A	14 (23.3)	15 (25.0)	1.11 (0.41-3.09)	10 (33.3)	5 (16.7)	2.25 (0.63-10.0)
A/A	0 (0)	0 (0)		0 (0)	0 (0)	
	P-value = 0.8185			**P-value = 0.1655**		
G/A+A/A	14 (23.3)	15 (25.0)	1.11 (0.41-3.09)	10 (33.3)	5 (16.7)	2.25 (0.63-10.0)
	P-value = 0.8185			**P-value = 0.1655**		

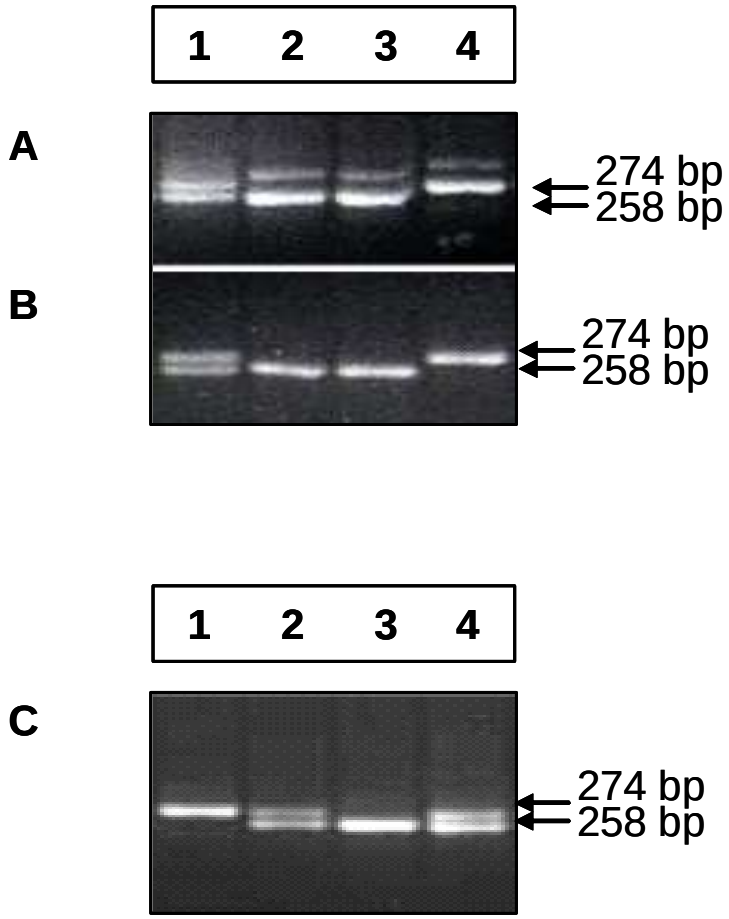


Figure 4.4 PCR amplification of the *Tp53* intron 3 polymorphism, separated on a 3% agarose gel and visualized by staining with ethidium bromide. **A** Amplification of intron 3 using primer combination F53 and R53E4 according to Weston *et al.* (1997). **B** PCR optimisation of intron 3 by the addition of 200 mM TMAC as co-factor. Lane 1 – case 6–3, lane 2 – control 6–4, lane 3 – BC patient 7–1 and lane 4 – control 7–2. **C** Results obtained for the polymorphism in intron 3. Lane 1 – control 14–4, lane 2 – BC patient 15–1, lane 3 – control 15–2 and lane 4 – control 15–4. Fragment sizes are indicated by arrows.

Amplification of the polymorphism in intron 3 resulted in two different sized DNA fragments (Fig 4.4 C). The individuals were scored for the presence or absence of this polymorphism based on the size and number of DNA fragments observed. The 258 bp fragment represented the wild type allele, whereas the 274 bp fragment denoted the variant allele carrying the 16 bp duplication (Fig 4.4 C). Participants displaying only the 258 bp fragment were homozygous for the wild type (w/w) as represented by control 15–2 in lane 3 (Fig 4.4 C). Individuals who were heterozygous were scored by the presence of both the 274 and 258 bp fragments as depicted in lanes 2 (BC patient 15–1) and 4 (control 15–4) (Fig 4.4 C). Only two participants were homozygous for the variant (v/v) and contained two copies of the 274 bp fragment (as indicated in lane 1 for control 14–4).

Both the 274 and 258 bp fragments were sequenced to verify the presence of the 16 bp duplication in individuals hetero- or homozygous for the variant (Fig 4.5). Case 3–3 containing the 258 bp fragment tested negative for the 16 bp duplication (Fig 4.5 A). Control 7–2 exhibiting two 274 bp fragments displayed the 16 bp duplication and insertion on both chromosomes (Fig 4.5 B).

The genotype and allele distributions for the intron 3 polymorphism are presented in Table 4.5. The majority of the controls and cases were homozygous for the wild type (w/w) representing 76.7% and 63.3% respectively. Homozygosity for the variant allele (v/v) was only observed for two control participants (Table 4.5). The only notable difference depicted was between the frequencies observed for the heterozygotes (36.6% in the mutation carriers versus 20.0% for the controls) (Table 4.5). This is to some extent reflected by the increase in frequency for the variant allele observed among the mutation carriers (18.3%) (Comparison 1, $P>0.05$). No significant differences ($P = 0.6374$) were observed among the genotype and allele frequencies in Comparison 2 (Table 4.5). The intron 3 polymorphism showed no deviations from Hardy-Weinberg expectations as the observed significance levels are above 5%.

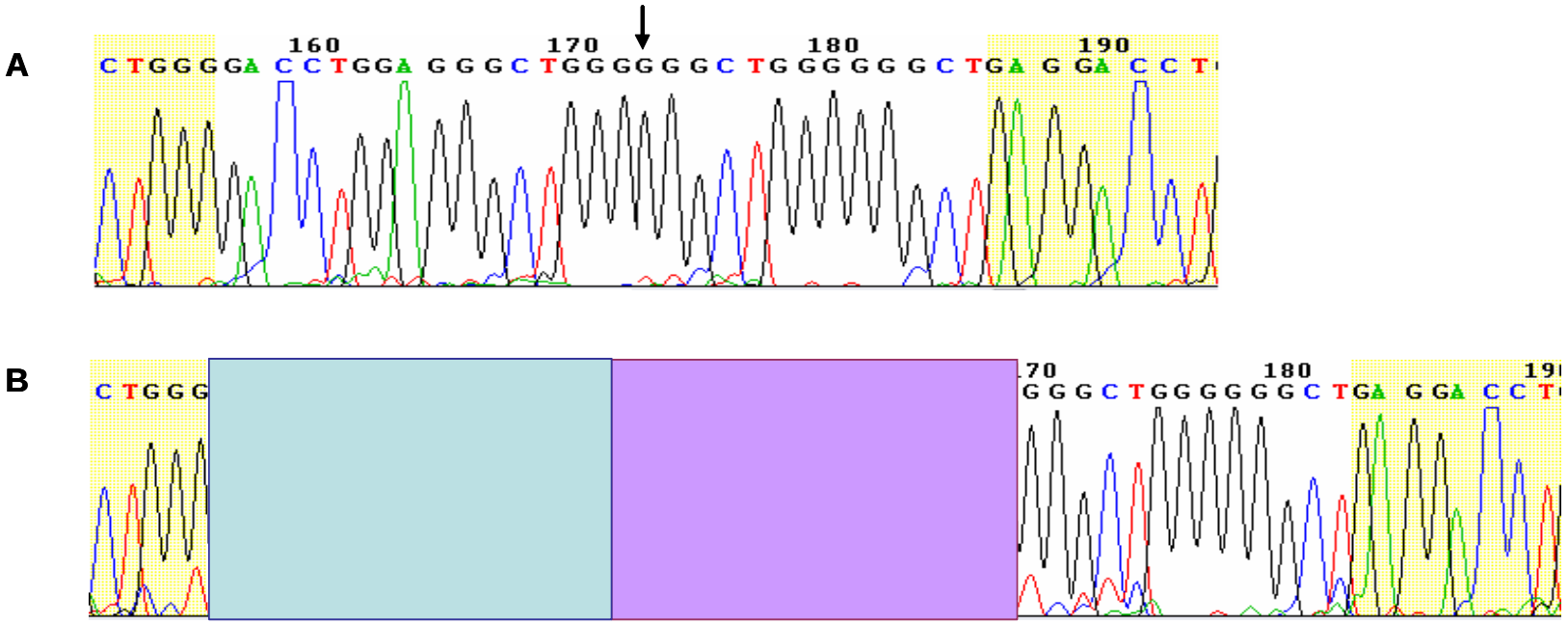


Figure 4.5 Sequence analysis of intron 3 of *Tp53* from two homozygous individuals. **A** Sequencing results for case 3–3, homozygous for the wild type allele. **B** Sequencing results for control 7–2, homozygous for the duplication. The duplicated 16 bases are highlighted in blue, with the position of the insertion indicated by an arrow in case 3–3 and highlighted in lilac for control 7–2.

Table 4.5 The allele and genotype distributions observed for the duplication in intron 3 of *Tp53.*

	Comparison 1 (Controls vs *BRCA2* carriers)			**Comparison 2 (BC patients vs Unaffected cases)**		
Frequency distribution	Controls n=60 n (%)	Carriers n=60 n (%)	OR (95% CI)	BC patients n=30 n (%)	Cases n=30 n (%)	OR (95% CI)
Allele (%)						
w	104 (86.7)	98 (81.7)	0.63 (0.25-1.47)	48 (80)	50 (83.3)	0.8 (0.27-2.25)
v	16 (13.3)	22 (18.3)	1.58 (0.68-4.0)	12 (20)	10 (16.7)	1.25 (0.44-3.70)
Genotype (%)						
w/w	46 (76.7)	38 (63.3)	0.5 (0.19-1.24)	18 (60.0)	20 (66.7)	0.8 (0.27-2.25)
w/v	12 (20.0)	22 (36.6)	2.43 (0.96-6.93)	12 (40.0)	10 (33.3)	1.25 (0.44-3.65)
v/v	2 (3.3)	0 (0)	0.33 (0.1-4.15)	0 (0)	0 (0)	
	P-value = 0.1179			**P-value = 0.6374**		
w/v+v/v	14 (23.3)	22 (36.6)	2 (0.84-5.40)	12 (40.0)	10 (33.3)	1.25 (0.44-3.65)
	P-value = 0.1025			**P-value = 0.6374**		

4.3.1.3 DNA analysis of the exon 4 polymorphism in *Tp53* (p.Arg72Pro)

Confusion exists regarding the reference sequence for *Tp53*. OMIM lists the wild type amino acid residue as Pro72Arg. This is opposed by the International Agency of Research in Cancer (IARC) p53 database, for the SNP is listed as Arg72Pro based on the GenBank genomic sequence X54156. For the purposes of this study, 72 Arg will be referred to as the major variant, since it is more prevalent in the Caucasian populations (Franeková *et al.*, 2007).

Restriction digestion with *Bst*UI of the remaining PCR product (4.3.1.2) was used to detect the SNP in exon 4 (Fig 3.1 D; page 73). When separated on an agarose gel four fragment sizes were detected (Fig 4.6). The results for this SNP were scored based on the digestion of the 274 and 258 bp fragments observed for intron 3. Undigested PCR products were loaded for each individual to assist with the scoring of the exon 4 results (Fig 4.6). The presence of the major variant (G) resulted in the digestion of the DNA fragments, whereas the minor variant (C) led to the abolishment of the restriction site. Digestion of the major variant in exon 4 on one or both chromosomes revealed a 245 bp and 229 bp, respectively. This was the case for control 6–2 depicted in lanes 1 and 2. This individual was heterozygous for the 16 bp duplication (274 and 258 bp) but homozygous for the major variant (digestion of both fragments).

The controls 6–4 and 2–2 in lanes 3, 4, 9 and 10 were scored as homozygous for the wild type allele (258 bp) in intron 3 and the major variant (229 bp) in exon 4 (Fig 4.6). Control 7–2 presented in lanes 5 and 6 was homozygous for both the intron 3 duplication (274 bp) as well as the minor variant in exon 4 (remained 274 bp). The fragment sizes for BC patient 2–1 in lanes 7 and 8 indicated that she was homozygous for the wild type allele in intron 3 (258 bp), but heterozygous for the exon 4 polymorphism (258 and 229 bp). Control 5–4 loaded in lanes 11 and 12 was heterozygous for intron 3 (274 and 258 bp), but homozygous for the minor variant in exon 4 (274 and 258 bp) (Fig 4.6).

DNA sequence analysis was performed to verify the data obtained from the

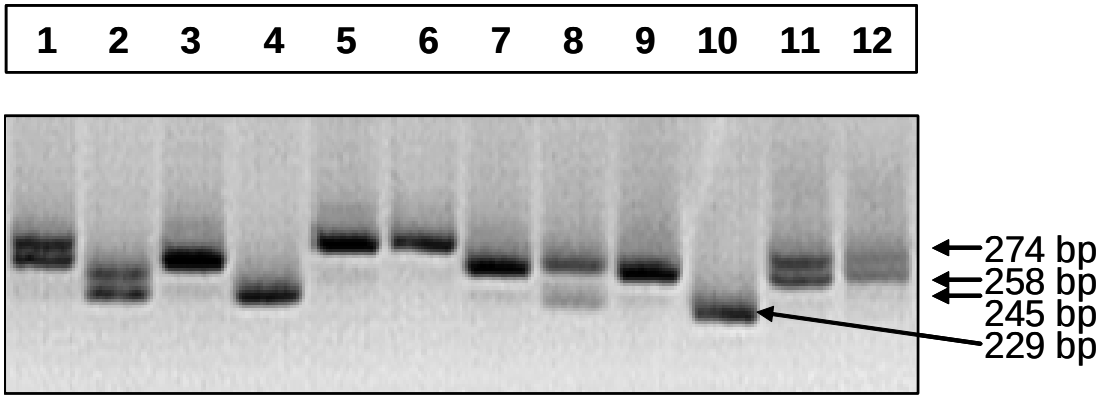


Figure 4.6 Detection of the polymorphism in exon 4 of *Tp53* codon 72 using *Bst*UI digestion separated on a 3% agarose gel, visualized by staining with ethidium bromide. Undigested PCR products were loaded in lanes 1, 3, 5, 7, 9 and 11 with the digested fragments in the remaining lanes. Lanes 1 and 2 – control 6–2, lanes 3 and 4 – control 6–4, lanes 5 and 6 – control 7–2, lanes 7 and 8 – BC patient 2–1, lanes 9 and 10 – control 2–2, lanes 11 and 12 – control 5–4. Fragment sizes are indicated by arrows.

restriction digestion. Three mutation carriers and two controls representing the various genotypes were sequenced using the reverse primer R53E4 (Table 3.2; page 71). The results confirmed BC patient 7–1 as being homozygous for the major variant (G/G) (Fig 4.7 A). The minor variant was present on both chromosomes for case 13–3, as evident in Fig 4.7 B. The nucleotide alignment indicated the mismatch caused by the presence of the SNP in case 13–3 (Fig 4.7 C).

Although it was not the intention to screen for new mutations in *Tp53*, a putative DNA change was detected in two of the five sequences, namely control 6–2 and case 6–3 (Fig 4.8 A and B). A single base change (T to G) was observed on one chromosome for each of these two patients at nt 12142. The DNA sequence was translated to determine the potential influence on the p53 polypeptide (Fig 4.9). The observed base change (T to G) resulted in a single amino acid change from valine to glycine at codon 73 (p.Val73Gly) (Fig 4.9).

The genotype and allele distributions for the *Tp53* polymorphism in exon 4 are presented in Table 4.6. No obvious differences were observed in the genotype frequencies for Comparison 1. The results obtained from Comparison 1 indicated a similar genotype distribution for the major variant (G/G) (48.3% and 45%), heterozygous participants (G/C) (41.7% and 43.3%) and minor variant (10% and 11.7%) (Table 4.6).

In contrast with this observation, a disparity was observed in Comparison 2 for the heterozygous genotype. The majority of BC patients proved to be heterozygous (53.3%), whereas most of the unaffected cases was homozygous for the major variant (G/G, 53.3%). The combined genotype frequencies also differed, as the variant allele was present in 63.3% of all the BC patients either in the hetero- or homozygous state, whereas it was present in only 46.6% of the unaffected cases (Table 4.6). Although the dissimilarity was interesting, it was not statistically significant ($P = 0.1967$). The SNP at exon 4 showed no deviations from Hardy-Weinberg expectations as the observed significance levels are above 5%.

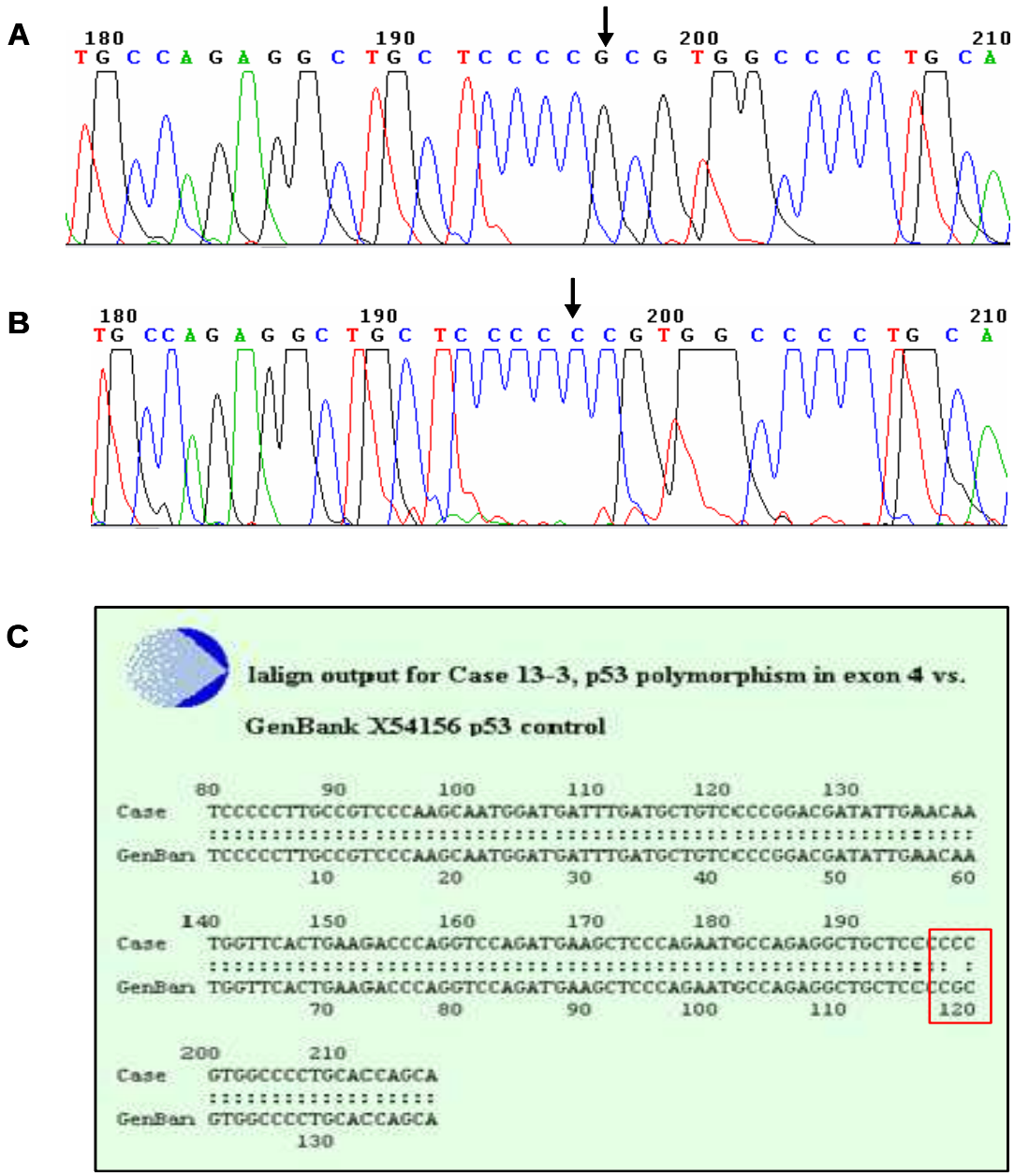


Figure 4.7 Sequence analysis of the SNP in exon 4 of *Tp53* using double stranded DNA. **A** Sequence results for BC patient 7–1 indicating homozygosity for the major variant (G) as depicted by the arrow. **B** Sequence results for case 13–3, homozygous for the minor variant (C/C). The position of the SNP is indicated by the arrow. **C** Nucleotide alignment of the sequencing results obtained for case 13–3 using the GenBank X54156 reference sequence. The mismatch is highlighted by the red block.

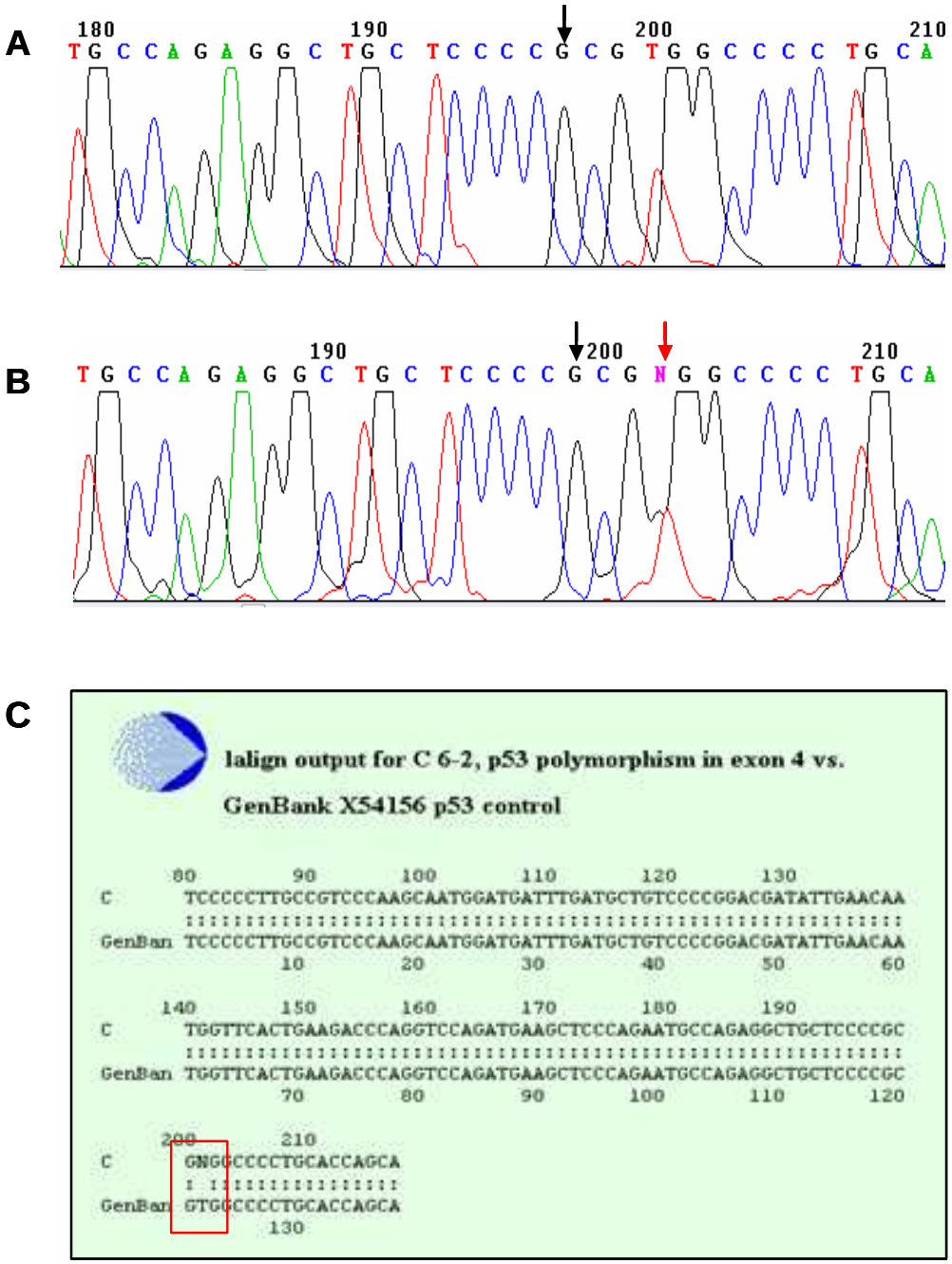


Figure 4.8 Detection of a putative new SNP in exon 4 of *Tp53*. **A** Sequence results for control 2–2, indicating homozygosity for the major variant (G/G, indicated by the black arrow) in exon 4. **B** Sequence analysis of 6–2, indicating homozygosity for the major variant at nt 12139, together with heterozygosity for a single base change (T to G) at nt 12142 as indicated by the red arrow. **C** Nucleotide alignment of sequencing results obtained for control 6–2 using the GenBank X54156 reference sequence. The putative mismatch is highlighted by the red block.

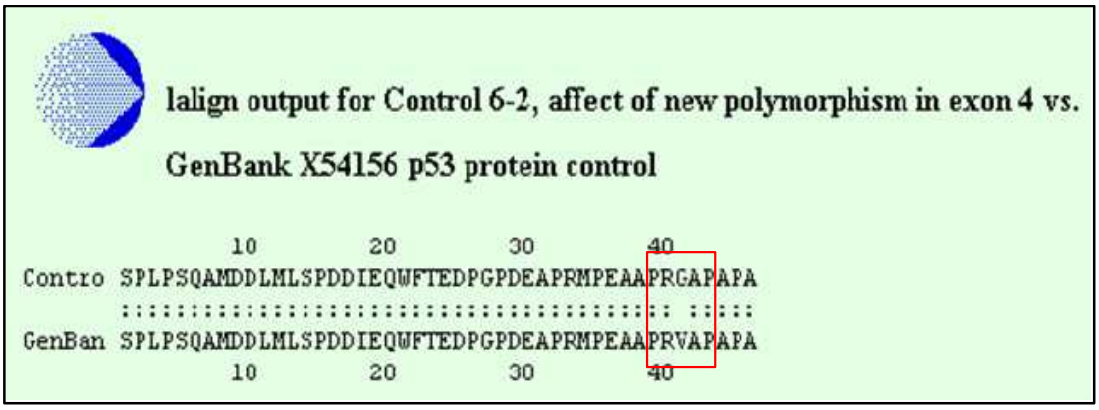


Figure 4.9 Amino acid alignment of exon 4 of *Tp53* for control 6–2 with the wild type (GenBank X54156). The amino acid change is highlighted by the red block.

Table 4.6 The allele and genotype distributions observed for the SNP in exon 4 of *Tp53*.

	Comparison 1 (Controls vs *BRCA2* carriers)			**Comparison 2 (BC patients vs Unaffected cases)**		
Frequency distribution	Controls n=60 n (%)	Carriers n=60 n (%)	OR (95% CI)	BC patients n=30 n (%)	Cases n=30 n (%)	OR (95% CI)
Allele (%)						
G	83 (69.2)	80 (66.7)	0.85 (0.43-1.69)	38 (63.3)	42 (70)	0.69 (0.26-1.75)
C	37 (30.8)	40 (33.3)	1.17 (0.59-2.32)	22 (36.7)	18 (30)	1.44 (0.57-3.83)
Genotype (%)						
G/G	29 (48.3)	27 (45.0)	0.86 (0.36-2.0)	11 (36.7)	16 (53.3)	0.5 (0.13-1.61)
G/C	25 (41.7)	26 (43.3)	1.09 (0.44-2.73)	16 (53.3)	10 (33.3)	2 (0.70-6.49)
C/C	6 (10.0)	7 (11.7)	1.14 (0.36-3.70)	3 (10.0)	4 (13.3)	0.8 (0.16-3.72)
	P-value = 0.9356			**P-value = 0.5473**		
C/G+ C/C	31 (51.7)	33 (55.0)	1.17 (0.50-2.76)	19 (63.3)	14 (46.6)	2 (0.62-7.46)
	P-value = 0.694			**P-value = 0.1967**		

4.3.1.4 Analysis of the *Tp53* haplotype

Analysis commenced with a pairwise comparison of all possible combinations of the three polymorphisms studied (Table 4.7). These were computed to determine the degree of non-random association between these SNPs. The polymorphisms have been expressed in the following order: intron 6, intron 3 and the SNP in exon 4. The alleles of each locus are designated as 1 for the wild type or major variant and 2 for the minor variant, either in the hetero- or homozygous state. For example, allele number 1 will represent the following with regards to the haplotype: homozygous for the restriction site in intron 6 (G/G), no insertion in intron 3 (w/w) and homozygous for the restriction site in exon 4 (G/G).

In Comparison 1, the assessment of the intron 6 (G to A) together with intron 3 (16 bp insertion) polymorphisms, revealed a minor dissimilarity for the 1 2 haplotype. This haplotype represented individuals homozygous for the intron 6 polymorphism (G/G) but heterozygous for the insertion in intron 3. This haplotype was observed in only 3.3% of the controls, whereas it was present in 11.67% of the mutation carriers. No differences were observed for the haplotypes involving the intron 6 together with exon 4 polymorphisms (Table 4.7). The third assessment (intron 3 insertion together with the SNP in exon 4) revealed insignificant disparities among the 1 1, 2 1 and 2 2 haplotypes for Comparison 1 (P = 0.7410).

However in Comparison 2, two noteworthy differences were observed for the 2 2 haplotype frequencies. The assessments were between intron 6 together with intron 3 and intron 6 together with exon 4. This haplotype represented individuals homo- or heterozygous for both of the variants. For both assessments, the frequencies of this haplotype were 33.3% in the BC patients versus 16.7% for the unaffected cases (Table 4.7).

The three polymorphisms were incorporated into a *Tp53* haplotype to study the risk associated with different polymorphisms within the same gene. The haplotypes were designated according to conventional nomenclature (Sjalander *et al.*, 1995) and described eight possibilities (1 1 1, 1 1 2, 1 2 1, 1 2 2, 2 1 1, 2 1 2, 2 2 1, 2 2

Table 4.7 Pairwise comparison and haplotype frequencies of the three *Tp53* polymorphisms.

Comparison 1 (Controls vs *BRCA2* carriers)					**Comparison 2 (BC patients vs Unaffected cases)**				
Comparison	Haplotype frequency				Comparison	haplotype frequency			
	1 1	1 2	2 1	2 2		1 1	1 2	2 1	2 2
	n (%)					n (%)			
Intron 6 vs intron 3					**Intron 6 vs intron 3**				
Controls n=60	44 (73.33)	2 (3.3)	2 (3.3)	12 (20.0)	BC Patients n=30	18 (60.0)	2 (6.7)	0 (0)	10 (33.3)
Mutation carriers n=60	38 (68.33)	7 (11.67)	0 (0)	15 (25.0)	Unaffected cases n=30	20 (66.7)	5 (16.7)	0 (0)	5 (16.7)
P-value = 0.6626					**P-value = 0.1354**				
Intron 6 vs exon 4					**Intron 6 vs exon 4**				
Controls n=60	29 (48.3)	17 (28.3)	0 (0)	14 (23.3)	BC Patients n=30	11 (36.7)	9 (30.0)	0 (0)	10 (33.3)
Mutation carriers n=60	27 (45.0)	18 (30.0)	0 (0)	15 (25.0)	Unaffected cases n=30	16 (53.3)	9 (30.0)	0 (0)	5 (16.7)
P-value = 0.9241					**P-value = 0.3810**				
Intron 3 vs exon 4					**Intron 3 vs exon 4**				
Controls n=60	28 (46.7)	18 (30.0)	1 (1.7)	13 (21.7)	BC Patients n=30	9 (30.0)	9 (30.0)	2 (6.67)	10 (33.3)
Mutation carriers n=60	22 (36.7)	16 (26.7)	5 (8.33)	17 (28.33)	Unaffected cases n=30	13 (43.3)	7 (23.3)	3 (10.0)	7 (23.3)
P-value = 0.7410					**P-value = 0.7206**				

2).

From the three biallelic polymorphisms, eight haplotypes and therefore 36 genotypes were possible. Among the 240 alleles analyzed per SNP in this study, six different haplotypes (1 1 1, 1 1 2, 1 2 1, 1 2 2, 2 1 2 and 2 2 2) were detected. Two haplotypes (2 2 1 and 2 1 1) were not observed, which reduced the number of genotypes identified to 25.

Approximately 42% of the study population (46.7% of the controls and 36.7% of the mutation carriers) exhibited the 1 1 1 haplotype and was therefore homozygous for all the wild type alleles (Table 4.8). A total of 22.5% (20% of the controls and 25% of the mutation carriers) of the study population had the 2 2 2 haplotype, representing hetero- or homozygosity for all three the variants. In Comparison 1 the 1 1 1, 1 1 2 and 2 2 2 haplotypes were the most frequent in the controls and mutation carriers. Among the mutation positive individuals the 1 2 1 and 1 2 2 haplotypes were observed at a higher frequency compared to the controls (8.3% versus 1.6% and 3.3% versus 1.6%).

Two dissimilarities were observed between the BC patients and the unaffected cases (Comparison 2) regarding the frequencies of each of the haplotypes observed (Table 4.8). A total of 30% of the BC patients had the 1 1 1 haplotype compared to 43.3% for the unaffected cases (Table 4.8). In contrast, the majority of BC patients (33.3%) had the 2 2 2 haplotype (depicting the presence of all variants) when compared to the unaffected cases (16.7%). These dissimilarities proved to be insignificant when tested (P = 0.7821).

4.3.2 DNA analysis of the SNP309 of *MDM2*

The amplification of the *MDM2* SNP309 was performed as described by Ma *et al*. (2006b) and yielded a PCR product of 121 bp. After *Pst*I digestion, the wild type T allele produced a single 121 bp fragment, whereas the variant G allele resulted in two fragments of 104 and 17 bp respectively. The 17 bp fragment was too small to visualize on the gel and the polymorphism was scored on the presence or absence

Table 4.8 Frequencies observed for the extended *Tp53* haplotype involving the polymorphisms in intron 6, intron 3 and exon 4.

Haplotypes	**Comparison 1 (Control vs *BRCA2* positive individuals)**			**Comparison 2 (BC patients vs Unaffected cases)**		
	Controls n=60 n (%)	Carriers n=60 n (%)	OR (95% CI)	BC patients n=30 n (%)	Cases n=30 n (%)	OR (95% CI)
111	28 (46.7)	22 (36.7)	0.63 (0.25-1.47)	9 (30.0)	13 (43.3)	0.64 (0.21-1.80)
112	16 (26.7)	16 (26.7)	1 (0.35-2.84)	9 (30.0)	7 (23.3)	1.33 (0.41-4.66)
121	1 (1.6)	5 (8.3)	3 (0.54-30.39)	2 (6.7)	3 (10.0)	0.75 (0.11-4.43)
122	1 (1.6)	2 (3.3)	1.5 (0.17-17.96)	0 (0)	2 (6.7)	0.33 (0.01-4.15)
212	2 (3.3)	0 (0)	0.33 (0.01-4.15)	0 (0)	0 (0)	
222	12 (20.0)	15 (25.0)	1.33 (0.52-3.58)	10 (33.3)	5 (16.7)	2.25 (0.63-10.0)
	P-value = 0.9928			**P-value = 0.7821**		

of the 104 bp fragment.

All three possible genotypes were observed (Fig 4.10 A). Control 16–2 was homozygous for the wild type T allele and produced a fragment size of 121 bp, indicating the absence of the restriction site. This result was confirmed by sequence analysis of a limited number of participants (Fig 4.10 B), using Ensemble accession number ENST 00000258148 as the reference sequence. Analysis revealed the presence of a T on both chromosomes. Case 16–3 and control 16–4 were heterozygous for the variant and displayed two DNA fragments, 121 and 104

bp in size respectively. Sequencing results for case 16–3 revealed the presence of both nucleotides at the polymorphic site (Fig 4.10 C). BC patient 16–1 was homozygous for the variant yielding a single 104 bp fragment (Fig 4.10 A). The genotype was confirmed as sequencing indicated the presence of a G on both chromosomes (Fig 4.10 D).

The genotype and allele frequencies for the SNP in *MDM2* are presented in Table 4.10. None of the associations proved to be significant (Table 4.9), but some were noteworthy. Although a higher frequency for the T allele was observed (61.7%), the majority of the controls (53.3%) proved to be heterozygous for the variant (T/G), with a further 11.7% being homozygous (G/G) (Table 4.9). In contrast, 50% of the mutation carriers were homozygous for the wild type (T/T) (Comparison 1). This contradiction was however not significant (P = 0.2923). This observation was reflected by the combined genotype frequencies for the variant (65% for the controls versus 50% for the mutation carriers, P = 0.1282).

In Comparison 2, unexpected results were obtained, indicating a higher combined variant (T/G and G/G) frequency amongst the BC patients when compared to the unaffected cases (56.7% versus 43.3%). The SNP309 of *MDM2* showed no deviations from Hardy-Weinberg expectations as the observed significance levels are above 5%.

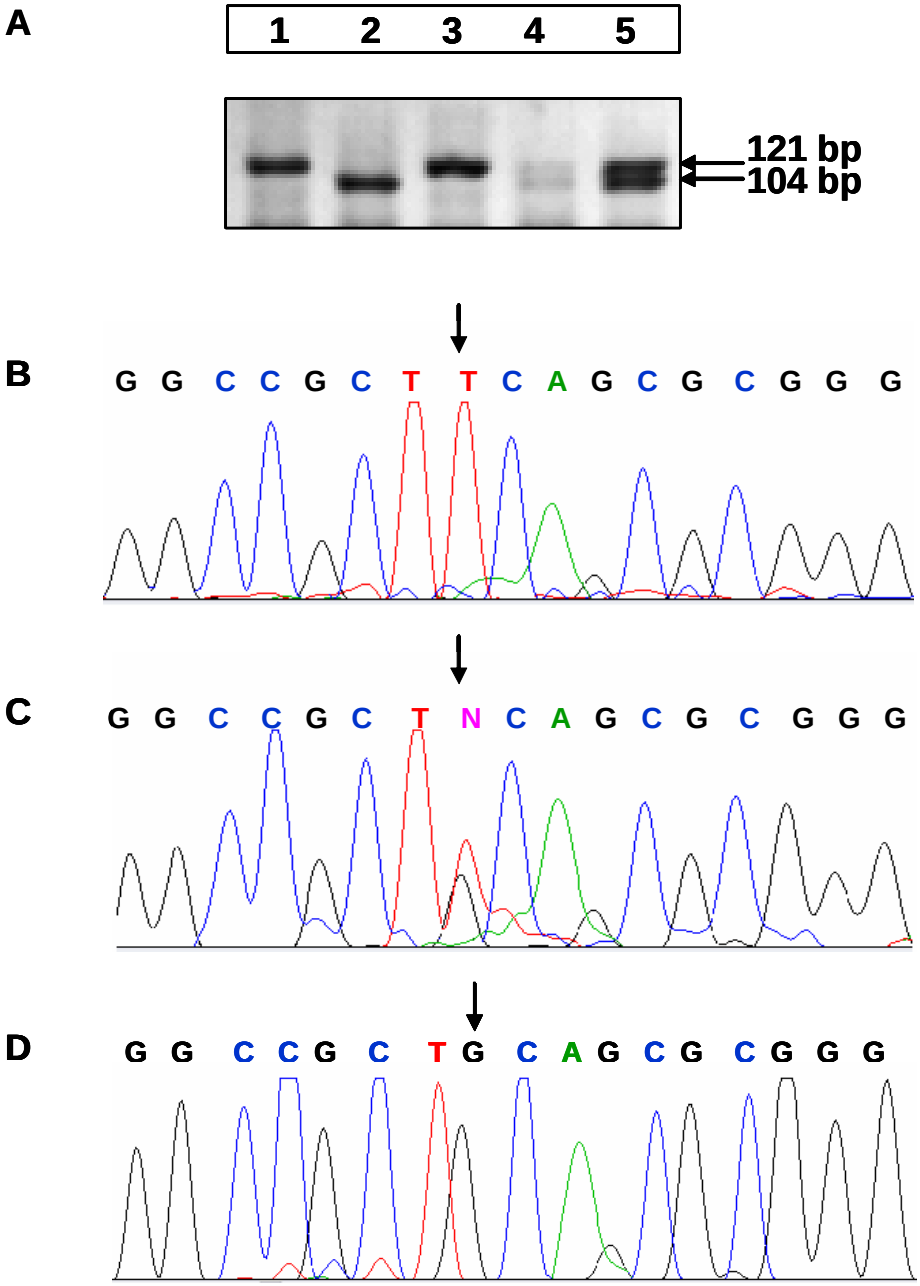


Figure 4.10 Analysis of the SNP309 of *MDM2*. **A** Detection of the SNP using *Pst*I digestion. Undigested PCR product was loaded in lane 1 with digested product in the remaining lanes. The products were separated on a 3% agarose gel and visualized by staining with ethidium bromide. Lane 2 – BC patient 16–1, lane 3 – control 16–2, lane 4 – case 16–3 and lane 5 – control 16–4. **B** Sequence results for control 16–2, indicating homozygosity for the wild type T allele. **C** Sequence results for case 16–3, indicating heterozygosity for both alleles. **D** Sequence results for BC patient 16–1, homozygous for the variant G allele. Fragment sizes and the polymorphic nucleotide are indicated by arrows.

Table 4.9 The allele and genotype distributions observed for the *MDM2* SNP309.

Frequency distribution	Comparison 1 (Controls vs *BRCA2* positive individuals)			Comparison 2 (*BRCA2* BC patients vs Unaffected cases)		
	Controls n=60 n (%)	Carriers n=60 n (%)	OR (95% CI)	BC patients n=30 n (%)	Cases n=30 n (%)	OR (95% CI)
Allele (n/%)						
T	74 (61.7)	86 (71.7)	1.69 (0.91-3.33)	40 (66.7)	46 (76.7)	0.53 (0.18-1.44)
G	46 (38.3)	34 (28.3)	0.59 (0.30-1.10)	20 (33.3)	14 (23.3)	1.86 (0.69-5.50)
Genotype (n/%)						
T/T	21 (35.0)	30 (50.0)	1.67 (0.82-3.66)	13 (43.3)	17 (56.7)	0.6 (0.18-1.82)
T/G	32 (53.3)	26 (43.3)	0.7 (0.33-1.46)	14 (46.7)	12 (40.0)	1.33 (0.41-4.66)
G/G	7 (11.7)	4 (6.7)	0.63 (0.16-2.17)	3 (10.0)	1 (3.3)	2 (0.29-22.11)
	P-value = 0.2923			**P-value = 0.5671**		
T/G+G/G	39 (65.0)	30 (50)	0.59 (0.27-1.23)	17 (56.7)	13 (43.3)	1.67 (0.55-5.8)
	P-value = 0.1282			**P-value = 0.3173**		

4.3.3 *WAF1* SNP analysis

4.3.3.1 DNA analysis of the exon 2 polymorphism (p.Ser31Arg)

SSCP was used to screen for the polymorphism in exon 2 according to Powell *et al.* (2002). This polymorphism involves a single base change (C to A) and results in an amino acid change at codon 31 with serine changing to arginine. For the study population two different banding patterns were observed (Fig 4.11 A). Sequencing of the DNA amplification products representing case 5–3 and control 6–2 with banding pattern 1 revealed the presence of the wild type allele (C) (Fig 4.11 B). Sequencing results for a limited number of PCR fragments representing individuals with banding pattern 2 indicated the presence of the variant on one chromosome (A) (Fig 4.11 C). None of the participants screened for this SNP was homozygous for the variant. The nucleotide sequence of the variant was aligned using NM_000389 as the reference sequence in order to verify the position of the base change (Fig 4.12).

The most abundant genotype observed for the participants in the study population was homozygous for the wild type (C/C) (Table 4.10) (Comparison 1 and 2). The heterozygous variant was extremely rare for it was present in only 6 individuals (5% of the study population). No differences for the genotype and allele frequencies were observed in either comparison (Table 4.10). The SNP of *WAF1* exon 2 showed no deviations from Hardy-Weinberg expectations as the observed significance levels are above 5%.

4.4.2 DNA analysis of the intron 2 polymorphism (IVS2+16 C to G)

SSCP was used to screen individuals for the polymorphism in intron 2 of *WAF1* (Powell *et al.*, 2002). This polymorphism involves a single base change (C to G) 16 bp downstream of the 5' splice site of intron 2.

Results of the SSCP analysis revealed three distinct banding patterns as indicated in Figure 4.13 A. The most common banding pattern (banding pattern 1) observed

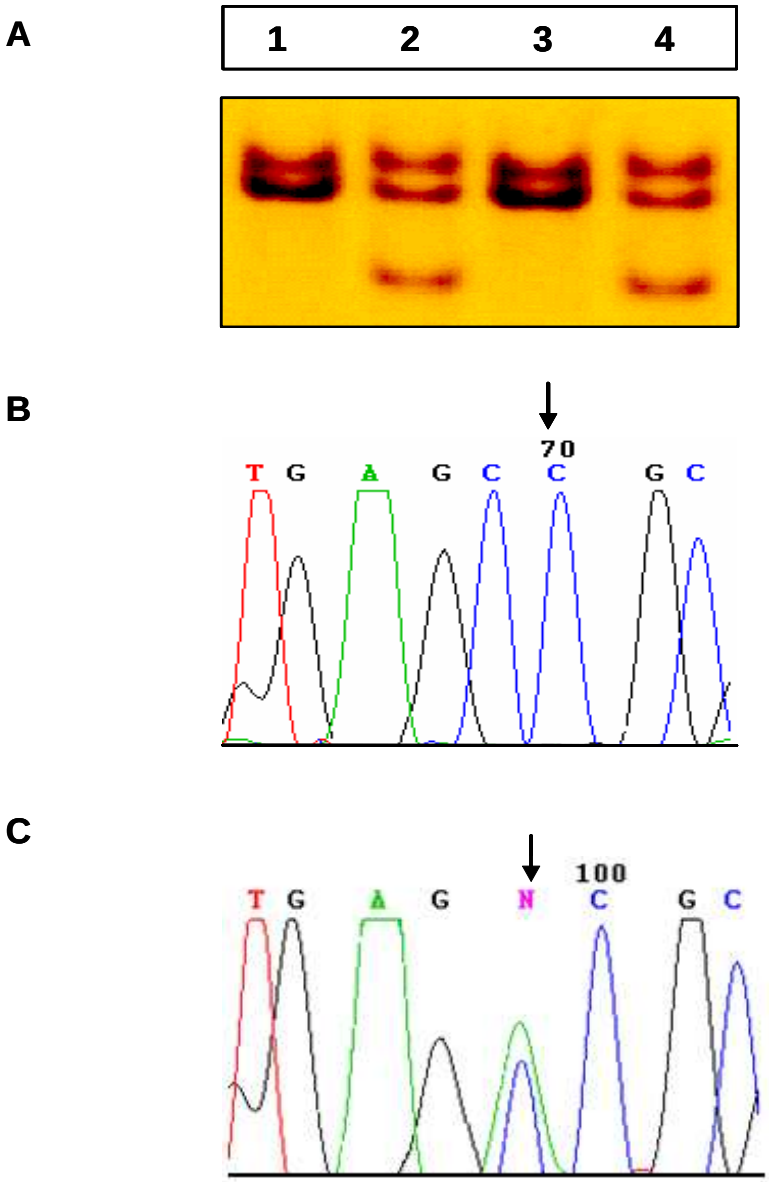


Figure 4.11 Analysis of the SNP in exon 2 of *WAF1*. **A** Detection of the SNP on a 10% silver stained poly-acrylamide gel (29:1). Lane 1 – case 5–3 (banding pattern 1), lane 2 – control 5–4 (banding pattern 2), lane 3 – control 6–2 and lane 4 – BC patient 23–1. **B** Sequence results for control 5–3 representing banding pattern 1, indicating homozygosity for the C allele (depicted by the black arrow). **C** Sequence results for BC patient 23–1, representing banding pattern 2, indicating heterozygosity for the two alleles (depicted by the black arrow).

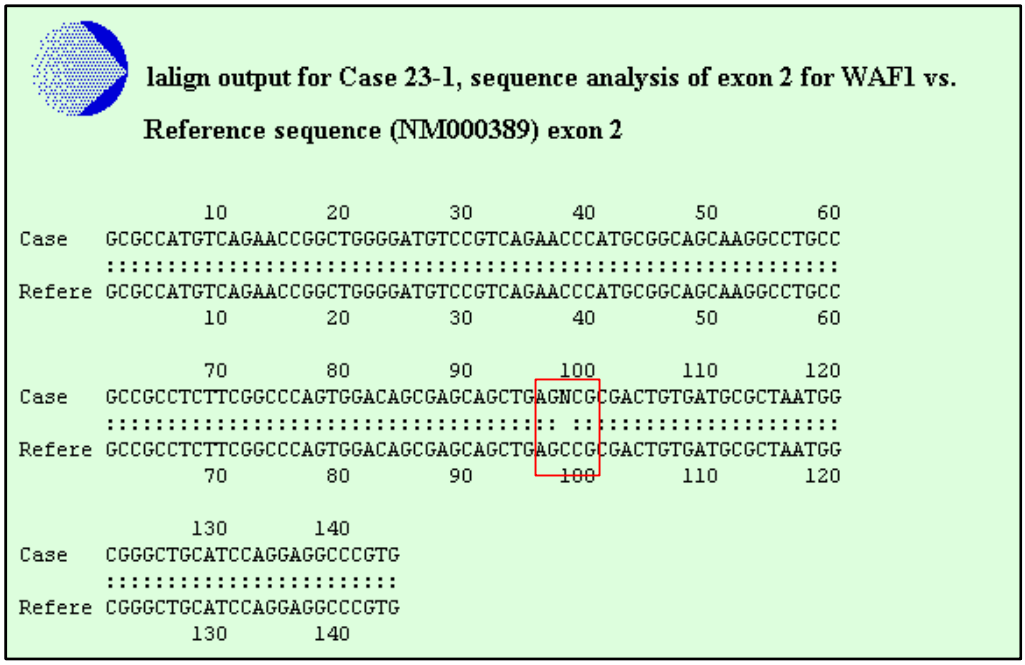


Figure 4.12 Nucleotide alignment of sequencing results obtained for BC patient 23–1 with the NM000389 reference sequence. The mismatch is highlighted by the red block.

Table 4.10 The allele and genotype distributions observed for the SNP in exon 2 of *WAF1* .

	Comparison 1 (Controls vs *BRCA2* carriers)			**Comparison 2 (BC patients vs Unaffected cases)**		
Frequency distribution	Controls n=60 n (%)	Carriers n=60 n (%)	OR (95% CI)	BC patients n=30 n (%)	Cases n=30 n (%)	OR (95% CI)
Allele (%)						
C	116 (96.7)	118 (98.3)	1.67 (0.32-10.73)	58 (96.7)	60 (100)	0.33 (0.01-4.15)
A	4 (3.3)	2 (1.67)	0.6 (0.09-3.08)	2 (3.3)	0 (0)	3.03 (0.24-100)
Genotype (%)						
C/C	56 (93.3)	58 (96.7)	1.67 (0.32-10.73)	28 (93.3)	30 (100)	0.33 (0.01-4.15)
C/A	4 (6.7)	2 (3.3)	0.6 (0.09-3.08)	2 (6.7)	0 (0)	3.03 (0.24-100)
A/A	0 (0)	0 (0)		0 (0)	0 (0)	
	P-value = 0.4142			**P-value = 0.2482**		
C/A+A/A	4 (6.7)	2 (3.3)	0.6 (0.09-3.08)	2 (6.7)	0 (0)	3.03 (0.24-100)
	P-value = 0.4142			**P-value = 0.2482**		

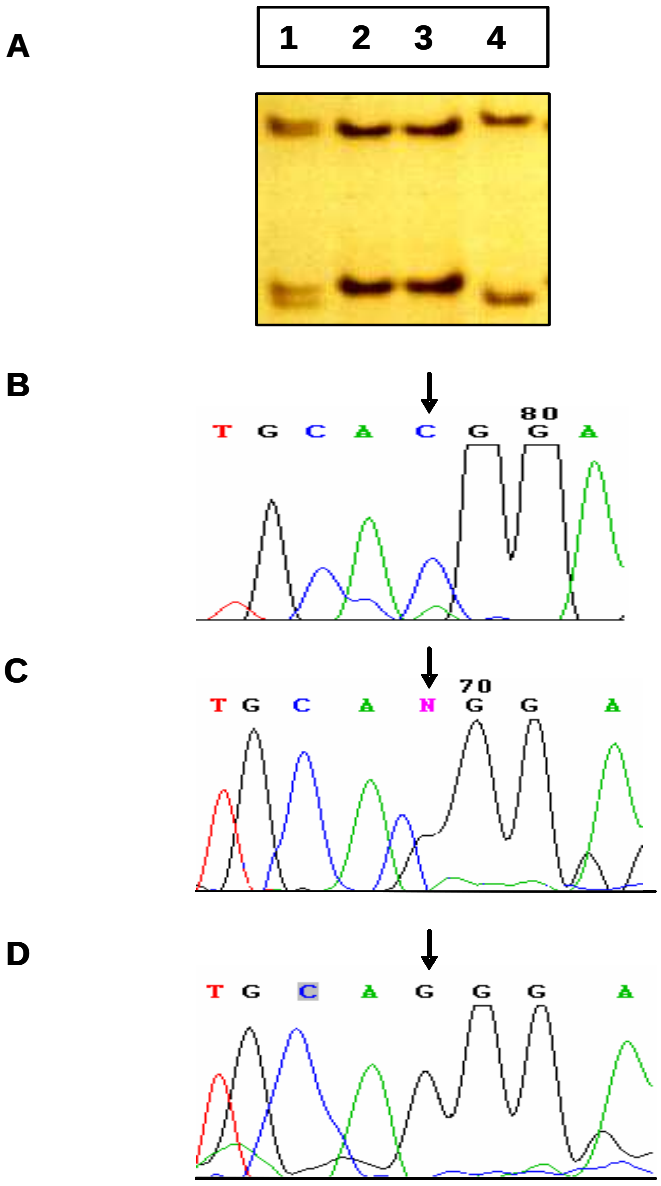


Figure 4.13 Analysis of the SNP in intron 2 of *WAF1*. **A** Detection of the SNP using a 10% silver stained poly-acrylamide gel (29:1). Lane 1 – BC patient 16–1 (banding pattern 3), lane 2 – control 16–2 (banding pattern 1), lane 3 – case 16–3 and lane 4 – control 16–4 (banding pattern 2). **B** Sequence results for control 16–2 representing banding pattern 1, indicating homozygosity for the C allele (depicted by the black arrow). **C** Sequence results for BC patient 16–1 representing banding pattern 3, indicated heterozygosity for both nucleotides. **D** Sequence results for control 16–4 representing banding pattern 2, indicating homozygosity for the G allele (depicted by the black arrow).

are represented by control 16–2 and case 16–3 in lanes 2 and 3 (Fig 4.13 A). A SSCP band shift was observed for both the upper and lower fragments when compared to banding pattern 1 and was scored as banding pattern 2. This banding pattern was observed for few participants including control 16–4 in lane 4, when compared to banding pattern 1 (Fig 4.13 A). The third banding pattern contained an additional band as evident for BC patient 16–1 (Fig 4.13 A).

DNA amplification products of individuals representing each banding pattern were sequenced. Sequencing results of a limited number of participants indicated that individuals with banding pattern 1 were homozygous (C/C) for the wild type allele (Fig 4.13 B). Individuals representing banding patterns 2 and 3 proved to be either heterozygous (C/G) or homozygous (G/G) for the variant allele (Fig 4.13 C and D).

The genotype and allele distributions for the polymorphism in intron 2 are presented in Table 4.11. No distinct differences were observed for the genotype and allele frequencies in Comparison 1 (Table 4.11) (P = 0.2971). The majority of the controls and mutation carriers were homozygous for the wild type (C/C) representing 66.7% and 58% respectively. Homozygosity for the variant allele (G/G) was only observed for 10 participants (Table 4.11).

Major differences were observed for the genotype and allele frequencies in Comparison 2. The majority of BC patients was heterozygous (46.7%) compared to only 20% for the unaffected cases. The difference in genotype frequencies tested statistically significant (P = 0.0244; OR, 5 (95% CI, 1.07-46.93)). The combined genotype (C/G and G/G) frequencies also differed, for it was observed in 60% of all the BC patients compared to 23.3% in the unaffected cases (Table 4.11). This association was also statistically significant (P = 0.0023). The GG genotype was significantly associated with BC risk among the *BRCA2* mutation carriers affected with BC (OR, 12 (95% CI, 1.78-512.97)). An insignificant value of linkage disequilibrium was observed for the SNP in intron 2 of *WAF1* (P = 0.0244).

Table 4.11 The allele and genotype distributions observed for the SNP in intron 2 of *WAF1.*

	Comparison 1 (Controls vs *BRCA2* carriers)			**Comparison 2 (BC patients vs Unaffected cases)**		
Frequency distribution	Controls n=60 n (%)	Carriers n=60 n (%)	OR (95% CI)	BC patients n=30 n (%)	Cases n=30 n (%)	OR (95% CI)
C	95 (79.2)	90 (75)	0.74 (0.34-1.55)	38 (63.3)	52 (86.7)	0.13 (0.01-0.53)
G	25 (20.8)	30 (25)	1.35 (0.64-2.94)	22 (36.7)	8 (13.3)	7.69 (1.88-100)
Genotype (%)						
C/C	40 (66.7)	35 (58.0)	0.64 (0.25-1.59)	12 (40.0)	23 (76.7)	0.08 (0.002-0.56)
C/G	15 (25.0)	20 (33.0)	1.5 (0.63-3.73)	14 (46.7)	6 (20.0)	5 (1.07-46.93)
G/G	5 (8.3)	5 (8.3)	1 (0.27-3.74)	4 (13.3)	1 (3.3)	2.5 (0.41-26.25)
	P-value = 0.2361			**P-value = 0.0244**		
C/G+G/G	20 (33.3)	25 (41.7)	1.56 (0.63-4.07)	18 (60.0)	7 (23.3)	12 (1.78-512.97)
	P-value = 0.2971			**P-value = 0.0023**		

4.4 Analysis of the BC recombinant haplotype

Analysis of the *Tp53* haplotype and polymorphisms in *WAF1* and *MDM2* resulted in the selection of the most informative genotypes that could influence BC risk among *BRCA2* mutation carriers. The polymorphisms included in the BC recombinant haplotype were selected based on disparities observed in allele frequencies (Suzuki *et al*., 2002; Soares *et al*., 2005).

The BC recombinant haplotype included the exon 4 SNP of *Tp53*, the *WAF1* polymorphism in intron 2 and the single base change in the promoter of *MDM2*. The alleles of each locus were designated as 1 for the wild type or major variant in the case of *Tp53* and 2 for the minor variant, either in the hetero- or homozygous state. Allele number 1 represents the following: homozygosity for the major variant in exon 4 (G/G), homozygosity for the wild type (C/C) in intron 2 and homozygosity for the wild type (T/T) in the promoter of *MDM2*. Allele 2 corresponded to the variant present in either the hetero- or homozygous states.

The BC recombinant haplotype was analyzed using a pairwise comparison of all possible combinations involving the three polymorphisms (Table 4.12). The allele frequencies for the assessments between *Tp53* exon 4 together with *WAF1* intron 2 and *Tp53* exon 4 together with *MDM2* SNP309 indicated no differences for Comparison 1 (Table 4.12). Two minor disparities were observed for the third assessment (*WAF1* intron 2 together with *MDM2* SNP309) for haplotypes 1 2 and 2 2.

The 1 2 haplotype was observed in 40% of the controls, but was present in only 23.3% of the mutation carriers (Table 4.12). The 2 2 haplotype was less common in the controls (25%) compared to the mutation carriers (40%) (Table 4.12). No significant associations were observed between the controls and mutation carriers for all three assessments.

Pairwise analysis of Comparison 2 revealed interesting results, for several differences were observed (Table 4.12). The results of the assessment between

Table 4.12 Pairwise comparison and haplotype frequencies of the SNPs included in the BC recombinant haplotype.

Comparison 1 (Controls vs *BRCA2* carriers)					**Comparison 2 (BC patients vs Unaffected cases)**				
Comparison	Haplotype frequency				Comparison	Haplotype frequency			
	1 1	1 2	2 1	2 2		1 1	1 2	2 1	2 2
	n (%)					n (%)			
***Tp53* exon 4 vs *WAF1* intron 2**					***Tp53* exon 4 vs *WAF1* intron 2**				
Controls n=60	20 (33.3)	9 (15.0)	20 (33.3)	11 (18.3)	BC Patient n=30	5 (16.7)	6 (20.0)	7 (23.3)	12 (40.0)
Mutation carriers n=60	17 (28.3)	10 (16.7)	18 (30.0)	15 (25.0)	Unaffected cases n=30	12 (40.0)	4 (13.3)	11 (36.7)	3 (10.0)
P-value = 0.8926					**P-value = 0.1812**				
***Tp53* exon 4 vs *MDM2* SNP309**					***Tp53* exon 4 vs *MDM2* SNP309**				
Controls n=60	9 (15.0)	20 (33.3)	12 (20.0)	19 (31.7)	BC Patient n=30	5 (16.7)	6 (20.0)	8 (26.6)	11 (36.7)
Mutation carriers n=60	13 (21.7)	14 (23.3)	17 (28.3)	16 (26.7)	Unaffected cases n=30	8 (26.6)	8 (26.6)	9 (30.0)	5 (16.7)
P-value = 0.7620					**P-value = 0.5015**				
***WAF1* intron 2 vs *MDM2* SNP309**					***WAF1* intron 2 vs *MDM2* SNP309**				
Controls n=60	16 (26.7)	24 (40.0)	5 (8.3)	15 (25.0)	BC Patient n=30	8 (26.6)	4 (13.3)	5 (16.7)	13 (43.3)
Mutation carriers n=60	21 (35.0)	14 (23.3)	9 (15.0)	24 (40.0)	Unaffected cases n=30	13 (43.3)	10 (33.3)	4 (13.3)	3 (10.0)
P-value = 0.5945					**P-value = 0.1871**				

Tp53 exon 4 together with *WAF1* intron 2 showed differences for the 1 1 and 2 2 haplotypes when BC patients were compared to unaffected cases. The haplotype representing the wild type alleles (1 1) was present in only 16.7% of the BC patients, whereas it was observed in 40.0% of the unaffected cases. The 2 2 haplotype was less common in the unaffected cases (10%) compared to the BC patients (40%). Although definite differences exist regarding the frequencies of the 1 1 and 2 2 haplotypes, it was not statistically significant (P = 0.1812).

The pairwise assessment between *Tp53* exon 4 together with *MDM2* SNP309 denoted only one disparity, namely for the 2 2 haplotype. The frequency of this haplotype was 36.7% in the BC patients compared to 16.7% for the unaffected cases (Table 4.12). The assessment as a whole was not statistically significant (P = 0.5015).

The third pairwise comparison (*WAF1* intron 2 together with *MDM2* SNP309) proved to be the most interesting, for various major disparities were observed between the two *BRCA2* mutation positive groups (Comparison 2). The most substantial dissimilarity was observed for the 2 2 haplotype, containing both the variants. This allele was observed at a frequency of 43.3% in the BC patients, whereas it was only present in 10.0% of the unaffected carriers. Two other haplotypes (1 1 and 1 2) were also noteworthy, for their frequencies varied with approximately 20% between the BC patients and the unaffected cases (Table 4.12). Despite the dissimilarities observed, the assessment was not statistically significant (P = 0.1871).

The haplotypes used for the extended analysis were designated according to original nomenclature and are described by eight possibilities (1 1 1, 1 1 2, 1 2 1, 1 2 2, 2 1 1, 2 1 2, 2 2 1 and 2 2 2). The polymorphisms have been expressed in the following order: SNP in exon 4 of *Tp53*, the polymorphism in *WAF1* intron 2 and SNP309 in *MDM2* in the promoter. The three biallelic polymorphisms resulted in a total of 36 genotypes observed. All eight different haplotypes were detected and their frequencies are presented in Table 4.13.

Table 4.13 Frequencies observed for the BC recombinant haplotype involving the SNP in *Tp53* exon 4, the polymorphism in intron 2 of *WAF1* and SNP309 in *MDM2* .

Haplotypes	**Comparison 1 (Control vs *BRCA2* positive individuals)**			**Comparison 2 (*BRCA2* BC patients vs Unaffected cases)**		
	Controls n=60 n (%)	Carriers n=60 n (%)	OR (95% CI)	BC patients n=30 n (%)	Cases n=30 n (%)	OR (95% CI)
111	8 (13.3)	8 (13.3)	1 (0.30-3.34)	3 (10)	5 (16.7)	0.33 (00.1-4.15)
112	12 (20)	9 (15.0)	0.7 (0.23-2.04)	2 (6.7)	7(23.3)	0.17 (0.004-1.37)
121	1 (1.6)	5 (8.3)	3 (0.54-30.39)	2 (6.7)	3 (10.0)	0.75 (0.11-4.43)
122	8 (13.3)	5 (8.3)	0.57 (0.12-2.25)	4 (13.3)	1 (3.3)	2.5 (0.41-26.25)
211	8 (13.3)	13 (21.7)	1.71 (0.62-5.14)	5 (16.7)	8 (26.7)	0.57 (0.12-2.25)
212	12 (20)	5 (8.3)	0.36 (0.08-1.23)	2 (6.7)	3 (10.0)	0.5 (0.01-9.60)
221	4 (6.7)	4 (6.7)	1 (0.23-4.35)	3 (10)	1 (3.33)	2 (0.29-22.11)
222	7 (11.7)	11 (18.3)	2.33 (0.53-13.98)	9 (30.0)	2 (6.7)	3.33 (0.86-18.85)
	P-value = 0.9983			**P-value = 0.9924**		

The frequency of the controls and mutation carriers (Comparison 1) that exhibited the 1 1 1 haplotype was identical (13.3%) (Table 4.13). These individuals were homozygous for all the wild type alleles and the major variant in *Tp53*. The mutation carrier group had a higher percentage individuals carrying the 2 2 2 haplotype (18.3% versus 11.7%). Other minor differences were observed for the 1 2 1 (1.6% in controls versus 8.3% in mutation carriers), 2 1 1 (13.3% versus 21.7%) and 2 1 2 haplotypes (20% versus 8.3%) (Table 4.13). The differences in haplotype frequencies tested statistically insignificant for Comparison 1 (P = 0.9983).

Since the BC recombinant haplotype was compiled to indicate a possible influence on BC risk, the dissimilarities observed in Comparison 2 between the mutation positive BC patients and the unaffected cases were of special interest (Table 4.13). The haplotype representing the wild type alleles (1 1 1) was more common among the unaffected carriers (16.7% versus 10%). Two major differences were noted between the two groups, namely the frequencies of the 1 1 2 haplotype (6.7% for the BC patients versus 23.3% in the unaffected cases) and the 2 2 2 haplotype (30% versus 6.7% in the unaffected cases) (Table 4.13). Although distinct differences were observed for the haplotype frequencies between the two groups, the assessment as a whole was insignificant (P = 0.9924).

Chapter 5

Discussion and Conclusion

Germline mutations in *BRCA2* confer a high risk for the development of BC in the Afrikaner population (NC van der Merwe, personal communication). A great deal of variability in BC development has been observed among individuals representing mutation positive families. It was suggested that genes affecting BC risk in the general population could presumably affect BC risk in *BRCA* mutation carriers (Antoniou *et al*., 2003; Dapic *et al*., 2005). Due to this, a number of studies have started evaluating previously identified SNPs in candidate genes and epidemiological factors to search for modifiers of penetrance (Antoniou and Easton, 2006; Levy-Lahad and Friedman, 2007). Shared environmental risk factors that cluster within families could contribute to some of the remaining unknown familial risk, although this contribution is less than 10% (Jonker *et al*., 2003). Therefore, the emphasis remains on the genetic factors.

5.1 Epidemiology

Familial BC has been extensively studied at the Division of Human Genetics at the UFS. This provided an important advantage for this retrospective study, for complete knowledge of the sampling frame was available. Comprehensive information was obtained from BC patients together with unaffected cases all carrying the founder Afrikaner *BRCA2* mutation, c.8162delG. The information gained from the interviews regarding reproductive risk factors, pregnancy history and life style habits made association studies possible.

The purpose of the epidemiological questionnaire was to evaluate potential risks associated with reproductive characteristics, pregnancy history and smoking among the *BRCA2* mutation carriers representing the Afrikaner. The questions addressing reproductive characteristics centred on the use of OC and HRT. Although OC use has been found to be associated with a modest increased risk of

BC (Ursin *et al.*, 1997), the potential association in the Afrikaner is also of interest since OC use may increase BC risk among *BRCA2* mutation carriers.

The use of these substances was similar for the individuals in Comparisons 1 and 2 (Table 4.1; page 78). Even though the majority of both the control participants and mutation carriers previously used OC, a reduction in the usage was observed (Table 4.1; page 78). Of all the individuals that previously used OC (70% of the controls and 78.3% of the *BRCA* mutation carriers) only 18.3% and 6.7% are still using it. For the mutation positive BC patients, OC use decreased from 86.7% to 0.0% (Table 4.1; page 78).

The observed tendency could be due to a number of factors, including the mean age of 44.5 for the entire study population (Tables 4.2; page 80 and 4.3; page 82). Only approximately a third of all the participants were 40 years or younger and would most likely still make use of some form of contraception. Family planning methods in SA seems to be changing from OC usage to intra uterine devices and female sterilization. Family planning methods vary considerably within and among population groups. Since the choice of a contraceptive method depends on the availability, cost and preferences, it could be that OC is no longer the family planning method of choice for the affluent Afrikaner female.

Another reason could be public awareness. Over the past three decades, the type and content of marketed OC formulations have changed remarkably, partially under pressure from the public (Doring *et al.*, 1993). The reason for the changes was early warnings from epidemiological studies that serious side effects may occur during OC use. Included were cardiovascular risk factors such as hypertension and hypercholesterolaemia (Gerstman, 1991; Torogood, 1991). The current assumption is that formulations with lower estrogen and progestogen content may be less dangerous (Haile *et al.*, 2006).

The search for an association between OC use and an elevated risk for developing BC could also have played a role (Grabrick *et al.*, 2000). As information, although sometimes controversial, becomes available, it catches the attention of the public

which could explain the drop in OC usage within the control population from 70.0% to 18.3%.

Although OC use is only weakly associated with BC risk in the general population, the association among women with a familial predisposition to BC is less clear. Conflicting findings have been published, some indicating an increased risk for *BRCA1* mutation carriers (Narod *et al.*, 2002), whereas others state an increase in risk for *BRCA2* mutation carriers who used OC for a period of five years or more (Haile *et al.*, 2006). Taking all the reports into account, it was concluded that the absolute risk for BC may still be higher in mutation carriers (Levy-Lehad and Friedman, 2007). This factor could also have contributed to the decrease in OC use, for the latest management programmes for *BRCA* mutation carriers focus on decreasing the levels of circulating estrogen in these women in an attempt to lower their BC risk (Allain *et al.*, 2007).

The affected *BRCA2* mutation carriers used in this study had either unilateral or bilateral surgery to remove their tumours. Some BC patients also elected to undergo an oophorectomy which induced natural menopause. None of these BC patients are currently using OC or HRT to alleviate the discomfort of natural menopause. Most of these patients have attended genetic counselling clinics and have been made aware of the danger of taking these supplements, since HRT has been shown to lead to a slightly higher risk for BC development with long-term use (Rossouw *et al.*, 2002). This is also applicable to the two unaffected mutation carriers who opted for prophylactic breast surgery. In an attempt to alleviate the discomfort of natural menopause, women should be made aware of the alternatives to hormone use that have been specifically manufactured for management of these symptoms (Mincey, 2003).

A woman's risk of developing BC depends on several factors, some of which are related to her natural hormones. Hormonal factors that increase the risk of BC include conditions that may allow high levels of hormones to persist for long periods of time. These include an early age at menarche (before age 12), a late age of menopause (after age 55), first live birth after age 30 and not having children at all

(Tryggvadottir *et al.*, 2003). Some of these reproductive factors such as parity and breast feeding have also been reported to influence risk among women with a hereditary predisposition to BC (Collaborative Group on Hormonal Factors in Breast Cancer, 2002; Jernstrom *et al.*, 2004; Andrieu *et al.*, 2006; Antoniou et al., 2006). Since the presence of a disease-causing *BRCA* mutation does not appear to influence a woman's age at menarche and the fact that menarcheal delay does not benefit the *BRCA2* mutation carriers used in this study, this question was not included in the questionnaire (Tryggvadottir *et al.*, 2003; Kotsopoulos *et al.*, 2005).

Several studies have investigated the effect of parity on BC risk among *BRCA2* mutation carriers, but the results were inconsistent. Some reported a protective effect against BC with an increase in parity (Beral *et al*., 2004; Antonio *et al.,* 2006), whereas others claim an increase in risk among *BRCA2* mutation carriers with each additional pregnancy (Cullinane *et al.*, 2005; Kotsopoulos *et al*., 2007). Since Comparison 1 and Comparison 2 were similar with regards to parity (Table 4.1; page 78) with the exception of nulliparity in the control individuals, no deductions could be made regarding BC risk and parity amongst the mutation carriers.

The prevalence of nulliparity in 40% of all the controls was remarkable and proved to be a statistically significant difference when compared to the mutation carriers (P = 0.0001) (Table 4.1; page 78). Various hypotheses were postulated why this was the case, as the control participants were selected based solely on the absence of a family history of BC. Since the study made use of a case matched control design, the average ages of the two groups were similar and could not explain the observed difference. One hypothesis is the fact that many of the control participants were recruited among the personnel of the UFS. Due to the fact that these women worked and most likely studied at a tertiary institution, they represented a more educated, well informed group of Afrikaner women, aimed at the professional sector. Several of these women in their thirties were single and might strive to obtain and maintain a professional life style rather than having a family.

Another factor could be the *BRCA2* mutation positive status of the women. These

individuals are well aware of their specific high risk of developing breast and other cancer types associated with the mutation. It could be that they were advised during their genetic counselling sessions to complete their families as soon as possible, in order for them to opt for preventative surgery such as an oophorectomy. Most of these individuals had close encounters during their childhood with breast and other cancers affecting their first degree relatives and therefore realized the threat. This hypothesis correlated with the ages observed at first birth for the mutation carriers which was on average two years earlier (mean 24.1 years SD ± 3.3) compared to the controls (mean 25.8 years, SD ± 4.4) (Table 4.2; page 80). The BC patients were on average also two years younger at the time of the birth of their first child, for the medians were 23 and 25 years respectively (Table 4.3; page 83).

A number of BC studies have searched for an association between parity and a positive family BC history but none has been reported (Sellers *et al*., 1992). The results of the current study agree with their findings, since no significant differences were observed regarding parity for Comparisons 1 and 2 except for the nulliparous percentage observed for the controls. It does not appear that being identified as a *BRCA2* mutation carrier prevented these women from having children (Table 4.1; page 78).

The significant differences observed in the number of pregnancies between the controls and the mutation carriers were automatically reflected in the number of live births observed, the number of miscarriages recorded and the number of individuals that breast fed due to a correlation between these factors (Tables 4.1; page 78, 4.2; page 80 and 4.3; page 82). Apart from the nulliparous women, most of the participants had an average of two live births (35% for the controls versus 45% for the mutation carriers) (Table 4.1; page 78). This was also the case for Comparison 2.

The majority of the parous women represented in Comparisons 1 and 2 breast-fed their newborn infants (Table 4.1; page 78). Although this characteristic was statistically significant (P = 0.0082), the results were biased by the high percentage

of controls that has never been pregnant. No differences were observed between the mutation positive BC patients and the unaffected controls, for 60% of all parous women breast fed (Table 4.3; page 82).

The presence of a disease-causing *BRCA* mutation may have an influence on the development of breast tissue. It has been reported that individuals with low levels of BRCA1 may have increased breast epithelial cell proliferation in response to the increased estrogen exposure during pregnancy (Jernstrom *et al.*, 2004). Russo *et al.* (2001) postulated that breast tissue from parous women with hereditary BC does not differentiate normally and resembles breast tissue from nulliparous women. The difference in tissue structure could influence lactation and therefore cancel the indirect protective effect of breast feeding. This has been proven for *BRCA1* mutation carriers (Jernstrom *et al.*, 2004), but seemed not to be the case for *BRCA2*. This study confirms this statement to some extent since only 26.7% of all the BC patients and 23.3% of the unaffected cases did not breast-feed their newborns (Table 4.1; page 78), nulliparous women excluded. Unfortunately the reasons for women not being able to breast-feed their infants were not ascertained.

The psychological effect of a BC diagnosis and subsequent treatment is well documented, revealing high levels of distress in 20-30% of BC patients during the first year after diagnosis (Schlich-Bakker *et al.*, 2007). Various factors can influence the degree of anxiety that is experienced by a woman after BC diagnosis or positive mutation test disclosure. These include age, personality characteristics, severely stressful cancer and non-cancer related life experiences, illness perceptions and many more (Burgess *et al.*, 2005; Millar *et al.*, 2005).

Active smoking as a lifestyle habit was more common within the mutation carrier group (31.7%) when compared to the controls (18.3%) (Comparison 1). This was an interesting although unexpected phenomenon, since these individuals normally are more conscious regarding life style factors that could increase their BC risk. All the above mentioned factors, including the close proximity of BC in their immediate family members, could have contributed to long-term psychological distress that could eventually have led to BC patients and unaffected cases taking up smoking.

Even though more mutation carriers once smoked actively, a greater percentage of them discarded the habit in comparison to the controls (P = 0.0627) (Table 4.1; page 78). The number of *BRCA2* mutation carriers currently still smoking declined from 31.7% to only 10.0% in comparison to a drop of only 5% for the controls (Comparison 1). This tendency was also observed for Comparison 2, for the number of BC patients and unaffected cases currently still smoking dropped from 26.7% and 36.7% to only 6.7% and 13.4% respectively.

According to the statistics released by Action on Smoking and Health (www.ash.org.uk) for the United Kingdom in November 2007, smoking prevalence is the highest in the 20 – 24 age groups for both men and women, whereas it is the lowest among people 60 years and older. According to these statistics, the only age group that recorded a fall in smoking prevalence for the period 2004 – 2005, was the 35 – 49 year age group.

This could also be the case for the Afrikaner population. Since the mean age of both the control and mutation carrier groups (44.5) fall into this age group, this tendency could also explain the drop in smoking prevalence observed in the current study (Table 4.1; page 78). Family responsibilities such as providing and caring for their loved ones are one of the top priorities of most women in this age group. It could be that due to complete commitment to their families, women and more specifically *BRCA* mutation carriers representing this age group are more willing to make changes in their life style in order for them to be there later on in life for their partners and children. This could also explain the drop in the number of current smokers among the mutation carriers.

The Action on Smoking and Health also reported a strong link between cigarette smoking and socio-economic status. The percentage of men and women in managerial and professional occupations that smoked tend to be lower (18% for men and 16% for women) than those in routine and manual occupations (32% for men and 29% for women). Professional women would also be more inclined to change their lifestyle for the better, based on the fact that they tend to be more aware, educated and well-read (Action on Smoking and Health, 2007).

The opposite scenario was observed within the study population regarding exposure to secondary smoke, for fewer mutation carriers were exposed to secondary smoke during childhood in comparison with the controls (58.3% versus 73.3%) (Table 4.1; page 78). Recent reviews of studies that have examined the association between passive smoking and BC risk suggested the possibility of a weak positive association (Khuder and Simon, 2000; Morabia, 2001). This was substantiated by the fact that nitrosamines and other carcinogens found in tobacco smoke appear to be more concentrated in passive smoke than in mainstream smoke. However, it has been argued that the general lack of an association between active smoking and BC risk makes any association with passive smoking implausible, given that women who are active smokers are also exposed to their own passive smoke (Khuder and Simon, 2000).

The evaluation of multiple epidemiological characteristics revealed various dissimilarities between the control participants representing the Afrikaner population and the *BRCA2* mutation carriers. The results obtained for parity, breast feeding, the number of miscarriages and the ages at first and last birth were unfortunately all biased due to the high percentage of nulliparous control participants. Although many of these characteristics were statistically significant within Comparison 1, no distinct differences were observed between the BC patients and the unaffected carriers (Comparison 2).

The results did indicate a decrease in the usage of OC within the Afrikaner population. This might be due to a change in family planning methods or public awareness of the negative side effects. The decrease in the mutation carriers could be due to informed decision-making during genetic counselling and altered management programmes to an attempt to reduce BC risk. Regarding smoking habits, the association between cigarette smoking and BC risk remains unclear.

Overall, the results suggest that these epidemiological characteristics probably do not influence BC risk in this study. These findings require confirmation in future extended studies. In addition to epidemiological studies, investigations are needed to unravel the molecular mechanisms underlying any effect of epidemiological

factors on BC risk.

5.2 Genotype analysis

Genetic factors such as mutations in *BRCA1* and *BRCA2* play a pivotal role in BC and OVC susceptibility, although their penetrance is incomplete (King *et al.*, 2003). This fact complicates genetic counselling and BC risk determination for individuals requesting *BRCA2* susceptibility testing, since estimates of penetrance range from 31-56% (Antoniou *et al.*, 2003). This study attempted to elucidate the residual risk observed within various *BRCA2* mutation positive families by investigating potential modifying polymorphisms present in genes involved in the *Tp53* pathway.

The *Tp53* pathway and *BRCA* genes seem to be functionally interrelated, since p53, BRCA1 and BRCA2 physically bind to each other. They are furthermore cooperatively involved with p21 (Jonkers *et al*., 2001; Cheung *et al*., 2004). BRCA2 shares a complex regulatory loop with p53 that is directly associated with cellular response to DNA damage. BRCA2 limits the length or severity of p53-mediated cell cycle arrest by inhibiting the transactivation activity of p53 (Wu *et al*., 2000).

The p53 pathway was chosen as a source of potential candidate modifiers due to its importance and function in maintaining the integrity of the genome. It is crucial for cell cycle control, DNA repair and apoptosis during cellular stress situations (Levine *et al*., 2006; Vousden and Lane, 2007). The selected genes, namely *Tp53*, *WAF1* and *MDM2*, are all involved in a mechanism controlling p53-mediated G1 cell cycle arrest (Levine *et al*., 2006).

The six SNPs selected for this study have been studied extensively, although not necessarily in such combination with each other or within the Afrikaner population. The analysis of Comparison 1 was used to determine the population frequencies of each of these SNPs. However, the results of Comparison 2 were vital for the identification of potential genetic modifiers of BC risk in order to explain the variation seen between individuals regarding BC susceptibility and disease progression.

Various studies investigated these potential variants involved in the p53 pathway with regards to BC risk (Tables 2.1; page 33 and 2.2; page 47). Comparison of these studies proved to be difficult, since most were performed using sporadic BC patients. Results of studies performed on specifically *BRCA2* mutation carriers are limited. The SNP in exon 4 of *Tp53* was only investigated in American (Martin *et al*., 2003), Finnish (Tommiska *et al*., 2005) and Spanish (Osorio *et al*., 2006) *BRCA2* populations. The results obtained by Martin *et al.* (2003) and Tommiska *et al*. (2005) did not prove any association between either of the variants and BC risk. On the other hand, Osorio *et al*. (2006) reported 72Pro as a statistically significant modifier of BC risk in *BRCA2* mutation carriers. All three studies reported an earlier age at onset and a reduced survival (Tommiska *et al*., 2005) in the case of *BRCA2* mutation carriers exhibiting the 72Pro allele.

For the *MDM2* SNP309, only two studies were performed using *BRCA2* carriers, namely the study by Wasielewski *et al*. (2006) for the Dutch population and Yarden *et al.* (2007) for the Ashkenazi Jewish population. Wasielewski *et al.* (2006) and others reported an acceleration of carcinogenesis for homozygosity of this SNP caused by enhanced MDM2 expression and activity (Bond *et al*., 2004; Bougeard *et al.,* 2006; Ruijs *et al.,* 2007). This finding was corroborated by Yarden *et al.* (2007) for they observed three times more mutation carrier BC patients exhibiting the GG genotype below the age of 52 years. Age 51 years and younger was selected as the cut-off since it is considered as the average age of menopause (Bond *et al.*, 2004). Both these studies reported no differences in the genotype frequencies of the controls compared to that of the mutation carriers. The high population frequencies therefore excluded that this polymorphism on its own is a BC susceptibility allele (Wasielewski *et al.*, 2006; Yarden *et al.*, 2007).

Regarding the polymorphisms in *WAF1*, no studies have as yet been performed for *BRCA2* mutation carriers. Powell *et al.* (2002) was the first to indicate that the polymorphism in intron 2 could affect BC risk. This study was performed on non-selected Australian BC patients.

Analysis of the three *Tp53* polymorphisms (intron 3 (g.11951_11966dup), intron 6

(IVS6+62 G to A), exon 4 (Arg72Pro)) indicated no significant differences between the control and mutation positive participants (Comparison 1). Of the three, only the Arg72Pro variant proved to differ between the BC patients and the unaffected carriers. The minor variant in either hetero- or homozygous state was more frequent in the BC patients, for the Pro allele was present in the majority of patients affected with BC (63.3% versus 46.6% for unaffected cases) (Table 4.6; page 100). On its own, this SNP was not significantly different between the two groups (P = 0.5473).

Although the SNP in exon 4 was statistically insignificant, the minor variant (72Pro) was observed in the majority of BC patients. Since various studies reported an earlier age at onset for *BRCA2* mutation carriers exhibiting the minor variant, this hypothesis was tested. The mean age of *BRCA2* mutation carriers affected with BC and homozygous for the 72Arg allele was 44.3 years (11 patients), heterozygous for 72Pro was 43.1 years (16 patients) and homozygous for the 72Pro was 45.3 (3 patients). Based on the obtained results, no earlier age at onset was observed for the mutation positive BC patients.

The Arg72Pro polymorphism, situated in a proline rich transactivation domain of the protein, has been extensively studied (Table 2.1; page 33). Although the 72Arg allele is reported to be more efficient at inducing apoptosis, the Pro allele is more capable in activating p53 dependant G1 arrest (Pim and Banks, 2004). Since the majority of unaffected cases (53.3%) were homozygous for the 72Arg allele compared to only 36.7% for the BC patients (Table 4.6; page 100), the lack of disease in these mutation carriers could be due to better induction of apoptosis. More efficient initiation of apoptosis would result in fewer cells, containing damaged DNA, proliferating by means of the cell cycle (Dumont *et al.,* 2003).

Even though pairwise comparisons between the three *Tp53* polymorphisms indicated similar frequencies for the controls versus the mutation carriers (Comparison 1) (Table 4.7; page 102), it indicated an interesting phenomenon amongst the mutation carriers (Comparison 2). The 1 1 haplotype representing all the wild type alleles was the most frequent haplotype observed for the unaffected

mutation carriers. In contrast, the 2 2 haplotype containing all of the variant alleles was the most common for the affected BC mutation carriers (Table 4.7; page 102). This tendency was observed for each of the three assessments. This observation was reflected by the decrease in the P-values from Comparison 1 to Comparison 2 for specifically the first two assessments (Table 4.7; page 102). The results of the pairwise analyses indicated that the addition of consecutive variants in the *Tp53* gene could have an influence on the development of the disease.

In the extended *Tp53* haplotype, a majority of controls (46.7%) in Comparison 1 displayed all three of the wild type alleles (1 1 1) (Table 4.8; page 104). In Comparison 2, the same scenario was observed for the unaffected cases, for the majority (43.3%) contained the 1 1 1 haplotype signifying all of the wild type alleles. The percentage of unaffected cases exhibiting any of the variants progressively became less as the number of variants increased, as 23.3% had a haplotype of 1 1 2, with only 16.7% being 2 2 2 (Table 4.8; page 104). For the BC patients, three main haplotypes were observed, namely 1 1 1 (30%), 1 1 2 (only positive for the variant in exon 4, 30%) and 2 2 2 (contained all of the variants, 33.3%). These observations correlated with findings described by various other authors. Weston *et al*. (1997, 1998) and Keshava *et al*. (2002) claimed that the haplotype composed of the three variant alleles (2 2 2) are associated with an increased BC risk among Caucasian populations. Unfortunately, the observations proved to be statistically insignificant (P = 0.7821).

MDM2 is a phosphoprotein that interacts with p53 and inhibits its activity (Iwakuma and Lozano, 2003; Moll and Petrenko, 2003). The polymorphism in the p53-response promoter of *MDM2* (Bond *et al.*, 2004) has been demonstrated to be associated with increased expression and a significantly earlier age of onset of BC (Bond *et al.*, 2004; Wasielewski *et al.*, 2006; Yarden *et al.*, 2007). This variant proved to be common within the Afrikaner population and was the only polymorphism that differed in frequency between the control and mutation carrier participants in Comparison 1 (Table 4.9; page 107). Although a higher frequency of the T allele was observed (61.7%), the majority of the controls (65%) proved to be either homo- or heterozygous for the variant (T/G) (P = 0.1282).

The high frequency of the variant G allele within the control population (0.65) (Table 4.9; page 107) for the Afrikaner, was corroborated by the findings of Wasielewski *et al.* (2006) and Yarden *et al.* (2007). These groups reported a high frequency of 0.53 and 0.54 for the variant allele respectively. Yarden and co-workers went as far as to suggest that the G allele is the major variant within the Ashkenazi-Jewish population. The Ashkenazi-Jewish population, together with the Dutch and the Afrikaner populations (current study), differs from the mixed North American population (Bond *et al.*, 2004). For this population, the frequency of the G allele was relatively low, namely 0.34 (Bond *et al.,* 2004).

Results obtained for Comparison 2 of the *MDM2* SNP309, indicated a higher frequency for the combined variant genotypes (T/G and G/G) observed among the BC patients when compared to the unaffected cases (56.7% versus 43.3%). Since only three BC patients were homozygous for the variant (G/G), it was futile to test whether the variant in *MDM2* was associated with an earlier age at onset in *BRCA2* mutation carriers as suggested in the literature (Wasielewski *et al.*, 2006; Yarden *et al.*, 2007). The three BC patients exhibiting the G/G genotype were diagnosed at the ages of 44, 45 and 47, which is well below the cut-off age of 51. This could preliminarily indicate an acceleration of carcinogenesis, but larger studies are needed.

The final gene included in this study namely *WAF1*, is a major downstream component of the *Tp53* pathway (El-Deiry *et al*., 1993). In contrast to the high mutation rate of *Tp53* in BC, the *WAF1* mutation rate is relatively low (McKenzie *et al.*, 1997). Two previously identified polymorphisms in this cell-cycle control gene were investigated. The single amino acid change in exon 2 of *WAF1* was not common within the Afrikaner population, for it was observed in only 6.7% and 3.3% of the controls and mutation carriers respectively (Table 4.10; page 111).

The splice site variant of intron 2 of *WAF1* was associated with an increased BC risk in the Afrikaner *BRCA2* c.8162delG BC patients (P = 0.0023, OR, 12 (95% CI, 1.78-512.97)). Although no distinct differences were observed in Comparison 1 (Table 4.11; page 114), a statistical significance (P = 0.0023) was proven for

Comparison 2. The majority of BC patients (60%) were either hetero- or homozygous for the variant allele compared to only 23.3% in the unaffected cases. The fact that this variant occurs more frequently in cancer patients than in healthy individuals (Table 4.11; page 114) (Powell *et al.*, 2002; Xi *et al.*, 2004), indicates a role in increased susceptibility for the development of BC (Powell *et al.*, 2002). The increased susceptibility could be due to the location of this polymorphism so close to the 5' splice site of intron 2, for it can influence mRNA splicing (Powell *et al*., 2002).

The results for the Afrikaner were corroborated by the findings of the only other study performed on BC patients. The investigation was done by Powell *et al.* (2002) for the Caucasian Australian population. Although they observed a lower combined variant frequency of 0.48 for BC cases compared to the Afrikaner's 0.60 (Table 4.11; page 114), they recorded a slightly increased BC risk of 1.4 associated with this SNP in intron 2 (P = 0.011).

A critical analysis of each of the six variants led to the compilation of a recombinant BC haplotype consisting of the three most prominent SNPs observed in the current study. Included were the Arg72Pro in p53, the SNP in intron 2 of *WAF1* and SNP309 in the promoter of *MDM2*. Their selection was mostly based on the results obtained from the initial (Tables 4.6; page100, 4.9; page 107, 4.11; page 114), pairwise (Table 4.7; page 102) and extended haplotype (Table 4.8; page 104) analyses. Arg72Pro proved to be the most informative of the three polymorphisms in *Tp53*, and together with the statistically significant SNP in intron 2 of *WAF1*, indicated discrepancies between the BC patients and the unaffected carriers. The polymorphism in the promoter of *MDM2* was not previously compared with any of the others. It was the only polymorphism that revealed distinct differences of borderline significance (P = 0.1282, OR, 0.59 (95% CI, 0.27-1.23)) (Table 4.9; page 107) between the control and mutation carrier participants (Comparison 1).

The results of the initial analysis of SNP309 in *MDM2*, namely the higher variant frequency observed within the control participants, were reflected in each of the pairwise analyses of Comparison 1 (Table 4.12; page 116). This was evident from

the high frequencies of control participants exhibiting the 1 2 and 2 2 haplotypes of the second and third assessments, with the *MDM2* SNP represented by the latter symbol. Apart from this observation, no obvious dissimilarities were observed between the control and mutation carrier participants (Table 4.12; page 116).

The associations observed for the pairwise assessments between the BC patients and unaffected carriers (Comparison 2) are worthy to be highlighted (Table 4.12; page 116). A similar tendency to that recorded for the *Tp53* pairwise analyses was observed (Table 4.7; page 102), for the 1 1 haplotype representing all the wild type alleles was the most frequent haplotype observed for the unaffected mutation carriers for assessments one and three. The BC patients consistently contained more variant alleles (2 2) compared to the unaffected cases (Table 4.12; page 116). In most cases, the percentages of unaffected cases displaying the wild type haplotypes (1 1) were approximately equal to the number of BC cases exhibiting the variant haplotypes (2 2) (Table 4.12; page 116). The associations observed within the pairwise analyses provided credibility to the selection of the most influential polymorphisms involved in the BC recombinant haplotype. This observation again was reflected by the decrease in the P-values from Comparison 1 to Comparison 2 for specifically two of the assessments (Table 4.12; page 116). The pairwise analyses indicated provisional interaction between the SNPs included in the BC recombinant haplotype.

The results obtained for the BC recombinant haplotype indicated no major differences within Comparison 1 (Table 4.13; page 118). In contrast to the results of the extended *Tp53* haplotype, the controls were not over represented by the wild type haplotype (1 1 1), for the analysis was again influenced by the high percentage of heterozygosity in the control participants of *MDM2* SNP309. It was reflected by the frequencies observed for 1 1 2, 1 2 2 and 2 1 2 (Table 4.13; page 118). If the high percentage of controls displaying the variant SNP309 allele is taken into account, it can be hypothesized that the G allele might be the major variant for the Afrikaner population. If this was the case, the majority of the controls would still exhibit the wild type haplotypes, namely 1 1 1 and 1 1 2 (together 33.3%) (Table 4.13; page 118).

The statistical analysis indicated no significant differences for the BC recombinant haplotype between the BC patients and the unaffected cases (P = 0.9924). This might be due to the influence of the SNP in *MDM2*, potentially conferring a protective effect for *BRCA2* mutation carriers (P = 0.1282, OR, 0.59 (95% CI, 0.27-1.23)). The inability to find any associations with the BC recombinant haplotype could also be due to the selection of the specific SNPs studied. Another factor that could have played a role was the high percentage of allelic heterogeneity observed for each of the individual polymorphisms.

The possibility of the BC recombinant haplotype influencing the aetiology of BC in some of the families was tested by applying the haplotype data. The pedigrees represented in each of the figures are families in which the *BRCA2* disease-causing mutation has been detected. Family 6 (Fig. 5.1) was included in order to illustrate the variability of penetrance in these families. The other two families were selected based on an equal number of BC affected patients and unaffected carriers tested.

As evident from the pedigrees, incomplete penetrance was most obvious within Family 6 (Fig 5.1). Although more family members have been affected with other cancer types, only two individuals were affected with BC, namely II:4 and III:3. The age at diagnosis of the only living BC patient (MA9) was 55, probably due to homozygosity for both the major variant 72Arg and the wild type of *MDM2*. This patient therefore tested negative for both alleles associated with an earlier age at onset of the disease.

The ages of the unaffected carriers (UA6, UA20 and UA21) in generation III vary from 51 to 62. The majority of unaffected cases within Family 6 seems to be either homo- or heterozygous for the wild type alleles (1 1 1) (Fig 5.1). Only two unaffected cases exhibited two or more of the variants in a heterozygous state (UA20 and UA21). The lack of haplotypes containing mostly variants could be protective within this family.

BC was the most common cancer type occurring within Family 11 (Fig 5.2). The ages at onset varied from as young as 36 years (MA5) to age 57 (MA21). The

Figure 5.1 Pedigree of Family 6. Indicated are sample and group numbers, ages at onset (dx), mutation status, date of death (where applicable) and symbol descriptions.

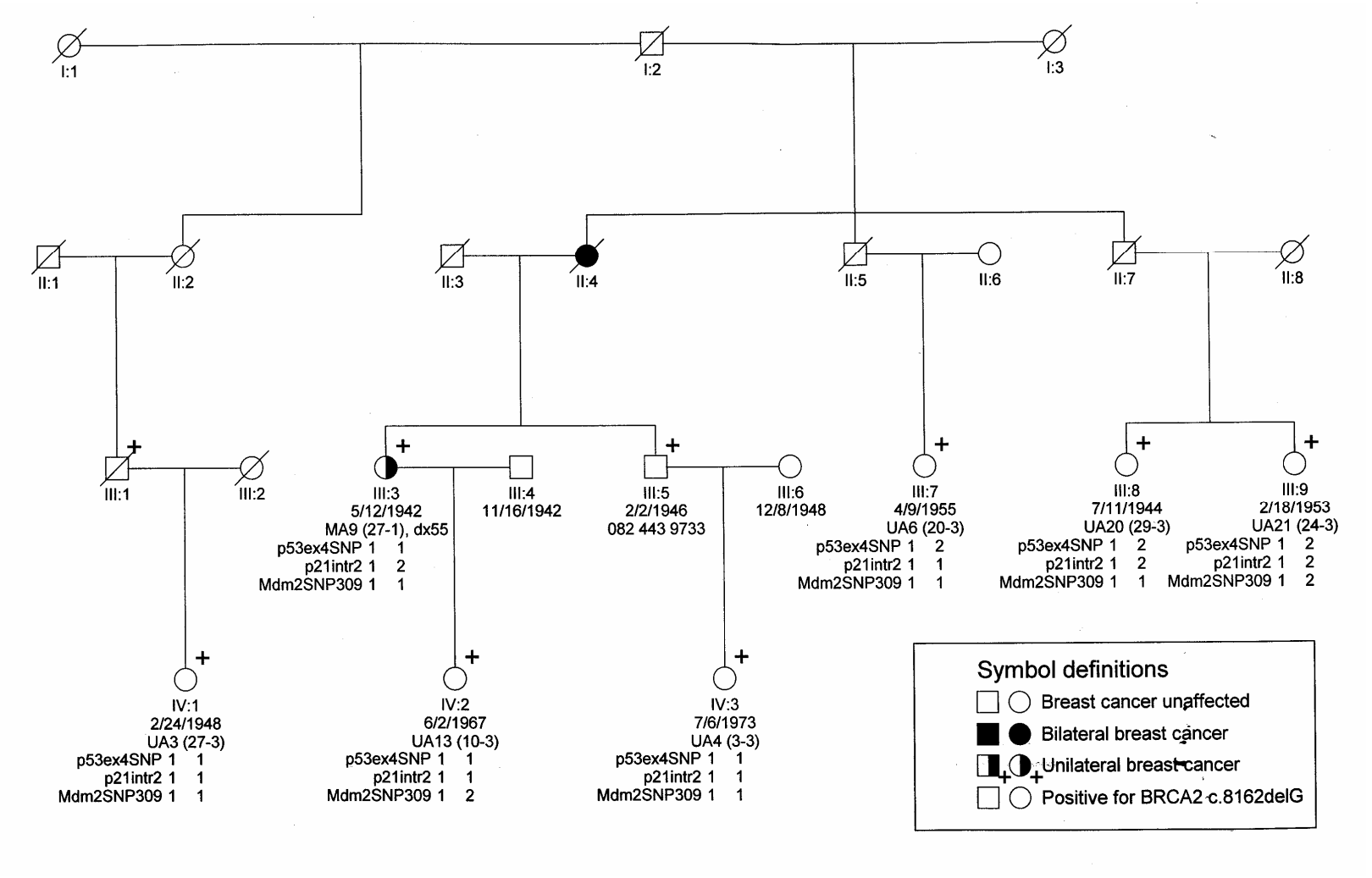
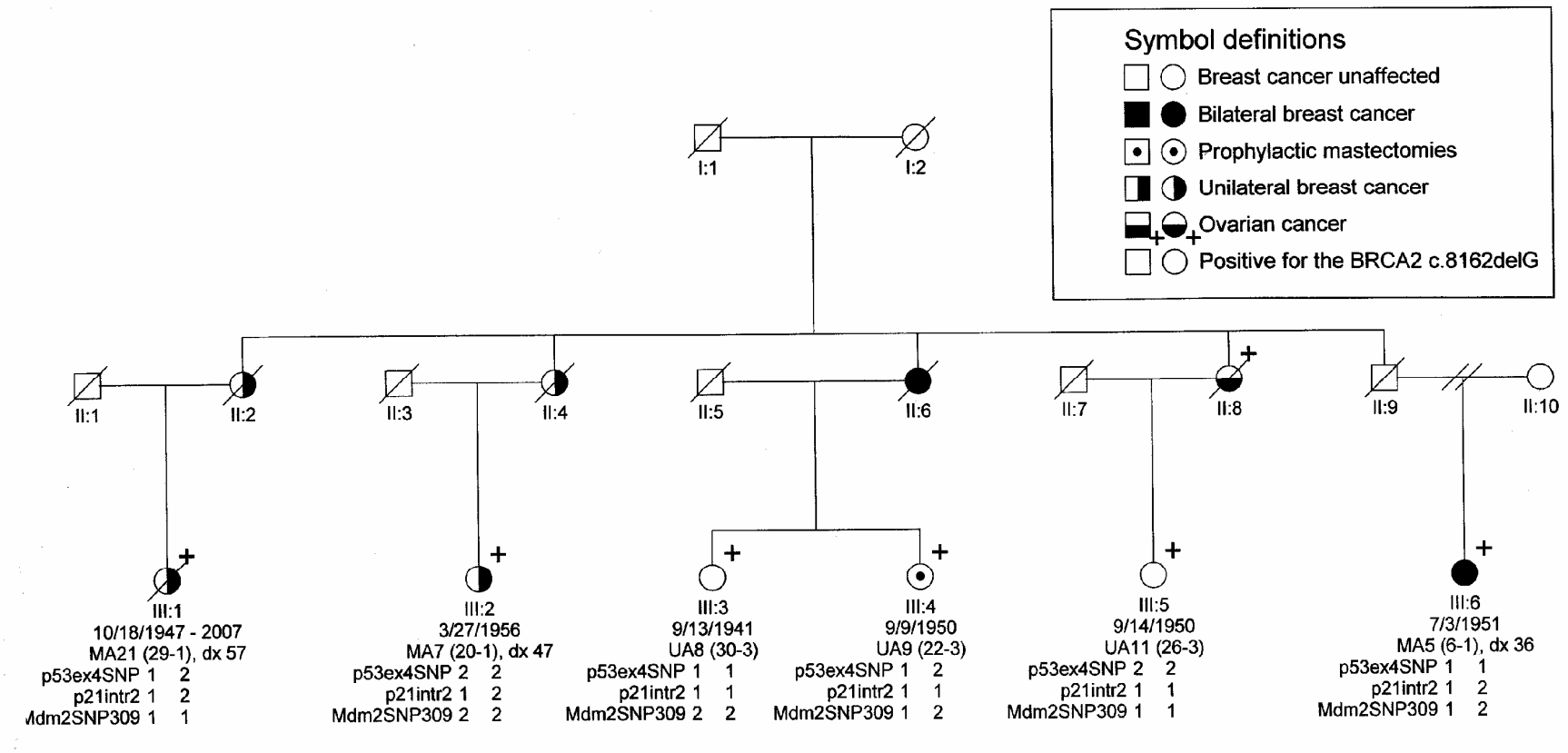


Figure 5.2 Pedigree of Family 11. Indicated are sample and group numbers, ages at onset (dx), mutation status, date of death (where applicable) and symbol descriptions.



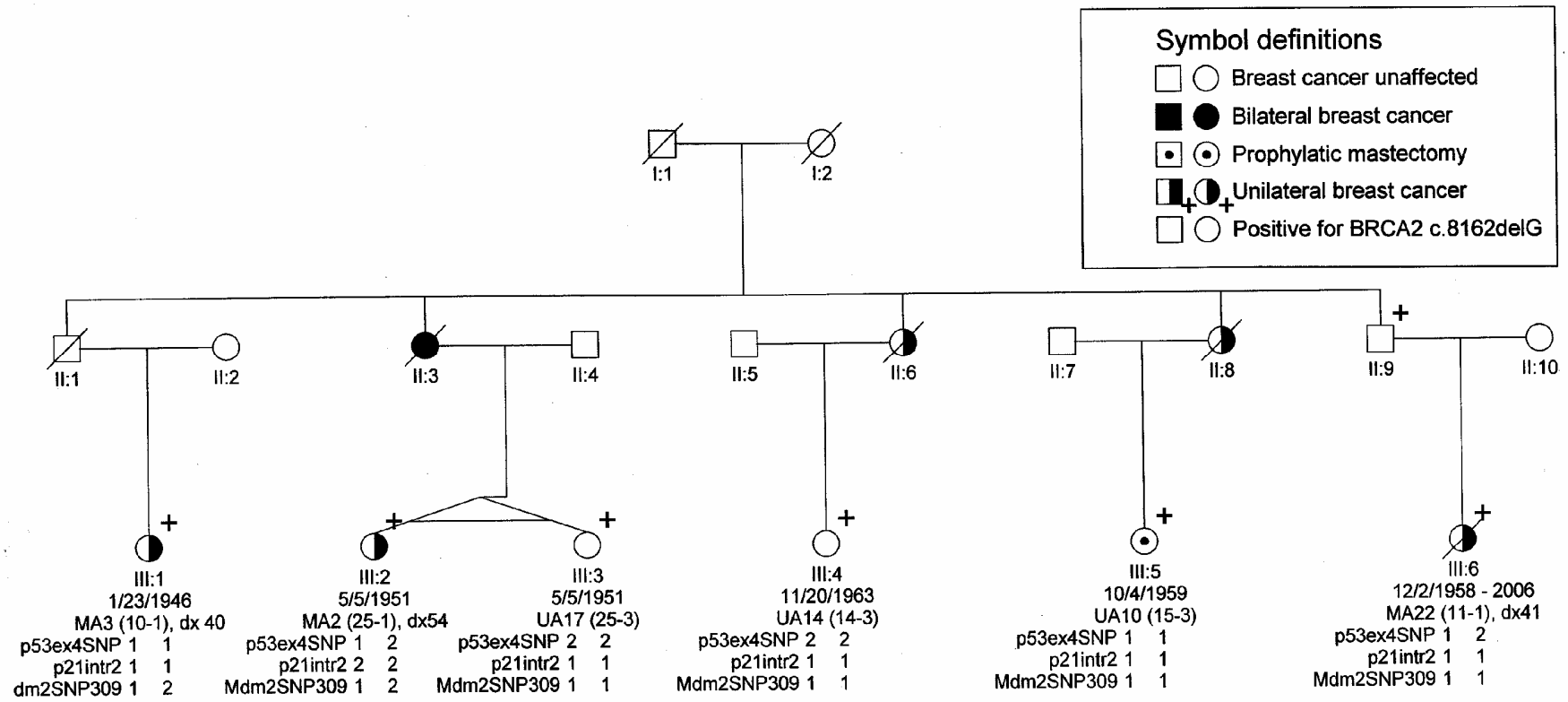
younger age at onset of MA7 could have been due to homozygosity for both the cancer acceleration alleles discussed for *MDM2* and *Tp53*. In comparison, BC patient MA21 diagnosed at age 57, did not exhibit any of the variant polymorphisms in a homozygous state. The cause of such an early age at diagnosis of MA5 could not be explained due to the presence or absence of any of these cancer accelerating polymorphisms. Since this patient was heterozygous for the most informative SNP observed in this study, she could have carried an additional BC due to the presence of the SNP in *WAF1*.

The ages of the three remaining unaffected cases are 65 (UA8) and 56 (UA9 and UA11). Although more of the variants were present in their BC recombinant haplotypes, the only universal feature common amongst these unaffected cases, was the absence of the informative SNP in intron 2 of *WAF1* (Fig 5.2). This corroborated to some extent the results described for Pedigree 6 (Fig 5.1).

The female representatives of Family 14 were also mainly affected with BC (Fig 5.3). The ages at onset varied from 40 (MA3) to 54 years (MA2). Interestingly, this family contained female twins (MA2 and UA17) that both tested positive for the mutation. The twins are currently 55 years old and differed to a great extent with regards to their BC recombinant haplotypes. The affected sister (MA2) was either homo- or heterozygous for all of the variant alleles, whereas her sister (UA17) was only homozygous for the variant in exon 4 of *Tp53* (Fig 5.3). The progressive accumulation of variants in MA2 in comparison with her sister could be the reason for her being affected. The absence of the SNPs in *WAF1* and *MDM2* in the unaffected sister could be the reason for her being unaffected thus far.

Regarding the other two patients (MA3 and MA22) (Fig 5.3), the results of the BC recombinant haplotype did not elucidate the role of these SNPs in the aetiology of the disease. These BC patients, as well as the three unaffected cases, were represented mostly by the wild type alleles, including the informative SNP in intron 2 of *WAF1*. The presence of BC within this family could not be attributed to a specific part of the BC recombinant haplotype, for there were no universal features among the three BC patients.

Figure 5.3 Pedigree of Family 14. Indicated are sample and group numbers, ages at onset (dx), mutation status and symbol descriptions.



The pairwise haplotype (Table 4.7; page 101) of 1 2 involving the *Tp53* 16 bp insertion in intron 3 and Arg72Pro was previously associated with an earlier age at onset in *BRCA2* mutation carriers (Osorio *et al.*, 2006). They suggested a risk effect for the 72Pro allele with the absence of the 16 bp insertion on the same chromosome. The opposite was also implicated, namely that the insertion presented a ‘‘protective’’ effect against the latter polymorphism (Sjalander *et al.*, 1995). This hypothesis was tested using the twins presented in Fig 5.3. The affected sister (MA2) was heterozygous for the 72Pro allele, but did not exhibit the 16 bp insertion in intron 3. The unaffected sister UA17 was also homozygous for the 72Pro allele, but was heterozygous for the 16 bp insertion. Therefore, the twins shared the 72Pro allele, but they differed regarding the presence of the 16 bp insertion in intron 3. The absence of the insertion in *Tp53* intron 3 in combination with the 72Pro allele could have resulted in an earlier age at onset for the affected sister, while a 2 2 pairwise haplotype (both variants) in the unaffected sister could have protected her from the effect of the 72Pro allele.

To test this hypothesis, the frequencies of the 72Pro allele in combination with the polymorphism in intron 3 were studied for all the affected patients. Fourteen of the 19 BC patients (73.7%) displaying the 72Pro allele were diagnosed before the age of 50. Of the fourteen, only six patients (31.6%) did not reveal the presence of the 16 bp insertion in intron 3. This indicated that the earlier onset of the disease could not have been completely attributed to the presence of the Pro allele, for most of these patients also had the polymorphism in intron 3 and was supposedly protected against an early age at onset. The lack of such an association could have been due to the relative small sample size. Larger studies will be needed to evaluate a possible modifying effect of the codon 72 polymorphism on the age at onset among *BRCA2* carriers.

The putative new DNA change observed for *Tp53* in exon 4 (Fig 4.8; page 98) was observed in two of five patients. The single base change (T to G) resulted in a single amino acid change from valine to glycine at codon 73. A PubMed search was performed to see whether this SNP has been described before. DNA alterations at codon 73 have only been reported once, namely a single base

deletion which resulted in a frameshift mutation. The deletion of a thymine was observed in colon-rectal cancer (Simms *et al*., 1998). It is therefore concluded that the putative mutation observed at codon 73 is novel. Future studies are however needed to determine its frequency within the Afrikaner population and to determine whether it plays a role in the aetiology of BC in the founder mutation carriers.

5.3 Conclusions

The present study was an initial investigation into the distribution of genotype frequencies for selected polymorphisms within the p53 pathway that could influence the penetrance of the South African Afrikaner founder *BRCA2* c.8162delG mutation. This study was the first to perform polymorphism association studies in the Afrikaner population in an attempt to elucidate the variation in penetrance of the major *BRCA2* founder mutation.

Of the six polymorphisms studied, only one proved to be statistically significant, namely the SNP in intron 2 of *WAF1.* This polymorphism seemed to explain the variation in penetrance for some of the families, but needs to be confirmed by further investigations. The BC recombinant haplotype proved to be uninformative for it did not explain any of the residual risk observed within these families. The study highlighted potential associations resulting from the pairwise analyses, although the results should be cautiously interpreted.

As the allelic heterogeneity was high within the Afrikaner population, polymorphism association studies for the dissection of such a complex trait were uninformative. The fact that the population association study was within a specific ethnic group all carrying the same founder mutation, provided strength to the study. The major weakness was the lack in patient numbers, since observations on a large scale are essential for reducing the risk of obtaining false positive results. The incorporation of this data by an organisation such as CIMBA could benefit all high risk individuals seeking susceptibility testing in the future, for a larger scale study could result in informative answers. Large scale genotyping of samples comparing mutation positive BC patients to healthy individuals with similar exposures could lead to

important breakthroughs. It will help us to understand gene to environment and gene to gene interactions as the mechanistic basis for the polygenic nature of BC.

Chapter 6

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6.1 General references

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Chapter 7

Summary

The aim of the study was an attempt to elucidate the variation in phenotypic expression observed within *BRCA2* c.8162delG mutation positive families by studying low penetrance modifiers. It involved incorporating environmental factors as well as various polymorphisms detected in critical genes of the *Tp53* pathway that regulate cell cycle arrest and apoptosis in the case of DNA damage. The investigated polymorphisms included three variants previously detected in *Tp53* (intron 3, exon 4 and intron 6), a polymorphism present in the promoter of *MDM2* and two SNPs identified in *WAF1* (intron 2 and exon 2).

The evaluation of a range of epidemiological characteristics revealed various dissimilarities between the control participants representing the Afrikaner population and the *BRCA2* mutation carriers. Although many of these characteristics were statistically significant for this comparison, no distinct differences were observed between the mutation positive BC patients and the unaffected carriers. The epidemiological study did not provide any specific characteristic associated with an increased or protective BC risk for the mutation carriers and did not explain the observed residual variation.

Of the six polymorphisms studied, only one proved to be statistically significant, namely the 5' splice-site variant in intron 2 of *WAF1.* This polymorphism seemed to explain the variation in penetrance for some of the families, but needs to be confirmed by further larger investigations. A BC recombinant haplotype was compiled using the three most informative variants, namely the polymorphism in the *MDM2* promoter, the 5' splice-site variant in intron 2 of *WAF1* and the SNP in exon 4 of *Tp53*. This haplotype proved to be uninformative as it did not reveal differences between the mutation carrier groups, namely the BC patients and unaffected carriers. The uninformative results could have been due to the high allelic heterogeneity present within the Afrikaner population. This made

polymorphism association studies for the dissection of such a complex trait extremely difficult.

The present study was an initial investigation into the distribution of genotype frequencies for selected polymorphisms within the p53 pathway that could influence the penetrance of the founder *BRCA2* c.8162delG mutation in South Africa. Although this study found various potential associations resulting from the pairwise analyses, the results should be interpreted with caution.

The incorporation of this data by an organisation such as CIMBA could benefit all high risk individuals seeking susceptibility testing in the future, for a larger scale study could result in informative answers. Large scale genotyping of samples comparing mutation positive BC patients to healthy individuals with similar exposures could lead to important findings. It will help us to understand gene to environment and gene to gene interactions as the mechanistic basis for the polygenic nature of BC.

Keywords: familial breast cancer, phenotypic expression, SNPs, modifiers, *Tp53*, *WAF1*, *MDM2*, epidemiological characteristics, penetrance, recombinant haplotype.

Chapter 8

Opsomming

Families waarin die siekte-veroorsakende *BRCA2* stigtersmutasie k.8162delG voorkom, verskil van mekaar ten opsigte van die ouderdom van aanvang. Hierdie fenotipiese variasie kan toegeskryf word aan verskeie faktore wat, alhoewel elkeen op sy eie ‘n geringe invloed het, gesamentlik die uitdrukking van die siekte kan veroorsaak. Hierdie faktore sluit in beide omgewingsinvloede en DNA polimorfismes in verskeie gene. Die doel van hierdie studie was om te bepaal watter moontlike omgewingstoestande en polimorfismes betrokke kan wees by hierdie variasie in aantastingsouderdom.

p53 speel ‘n essensiële rol in die beheer van die selsiklus deurdat dit die siklus kan onderbreek ten tye van stres of DNA skade. Die werking van die proteïen word gereguleer deur MDM2. Hierdie wisselwerking tussen die twee proteïene, tesame met p21, help om die selsiklus tot stilstand te bring sodat DNA herstel kan plaasvind. Indien onherstelbare skade voorgekom het, begelei p53 die sel na apoptose. As gevolg van die wisselwerking tussen die drie proteïene is ses poliformismes wat in die drie gene voorkom, ondersoek om te bepaal of hulle moontlike rolle kan speel in die voorkoms van die siekte in *BRCA2* mutasie-positiewe pasiënte. Drie polimorfismes wat in die *Tp53* geen voorkom is ondersoek, naamlik ‘n nukleotiedverandering in intron 6, ‘n duplikasie en invoeging van 16 nukleotide in intron 3 en ‘n nukleotiedverandering in ekson 4. Die *WAF1* polimorfismes is beide nukleotiedveranderings wat onderskeidelik in die oorgang tussen die nie-koderings en die koderingsgebied van ekson 2, asook in ekson 2 self, geleë is. Die laaste polimorfisme is ‘n nukleotiedverandering wat geleë is in die promoter van *MDM2*.

Die epidemiologiese studie het hoofsaaklik gefokus op verskillende reproduktiewe en swangerskapsfaktore wat moontlik ‘n bydrae kon lewer tot die ontwikkeling van borskanker in mutasie-positiewe vrouens. Alhoewel die resultate van hierdie studie

verskille tussen die kontrolegroep en mutasie-positiewe individue uitgelig het, was die demografie van die *BRCA2* positiewe borskankerpasiënte dieselfde in vergelyking met die onaangetaste *BRCA2* individue. Die epidemiologiese resultate het daarop gedui dat nie een van die faktore wat ondersoek was, verbind kon word met ‘n verhoogde of verlaagde risiko vir die ontwikkeling van borskanker in die mutasie-positiewe draers nie.

Die statistiese ontleding van die genotiperingsdata het slegs een polimorfisme as ‘n moontlike rolspeler opgelewer, naamlik die polimorfisme in intron 2 van *WAF1*. Hierdie polimorfisme kon die variasie in die vorming van borskanker in sommige families tot ‘n groot mate verklaar. Verdere studies is egter nodig om hierdie waarneming te bevestig. Die drie mees beduidende polimorfismes, naamlik die in *MDM2*, die polimorfisme in intron 2 van *WAF1* en die polimorfisme in ekson 4 van *Tp53*, is saamgevoeg om ‘n borskanker-haplotipe saam te stel. Die statistiese ontleding van die saamgestelde haplotipe het geen beduidende verskille tussen die *BRCA2* positiewe borskanker pasiënte en die *BRCA2* onaangetaste individue aangedui nie. Die gebrek aan statisties betekenisvolle resultate kan toegeskryf word aan die hoë persentasie heterogenisiteit wat in die Afrikaner bevolking teenwoordig is. Die hoë aantal heterosigote het die analise van so ‘n komplekse interaksie bemoeilik.

Hierdie studie was die eerste waarin die effek van ‘n verskeidenheid omgewings- en genetiese faktore op die uitdrukking van borskanker in *BRCA2* Afrikaner individue getoets is. Alhoewel daar verskeie moontlike invloede geïdentifiseer is, moet verdere uitgebreide studies egter gedoen word om vals positiewe resultate te voorkom. Die inkorporering van hierdie data by ‘n organisasie soos CIMBA kan egter tot voordeel van alle hoë risiko mutasiedraers strek, deurdat dit kan bydrae tot die ontrafeling van geen-tot-omgewings en geen-tot-geen interaksies wat betrokke is by die poligeniese ontwikkeling van borskanker.

Sleutelwoorde: oorerflike borskanker, fenotipiese uitdrukking, polimorfismes, modifiseerders, *Tp53*, *WAF1*, *MDM2*, epidemiologiese faktore, penetrasie, borskanker- haplotipe.

Appendix A

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INFORMATION FOR PATIENTS

Research project – Search for genetic modifiers of BRCA2 cancer risk and penetrance

Breast cancer is a disease caused by a **complex combination of genetic and environmental factors**. It is one of the most common types of cancer affecting women in the Western world. The life-time risk for developing cancer of the breast ranges from 1 in 13 in white females to 1 in 81 in black females, with a life-time risk of 1 in 36 for all South African women. This means that one of every 36 women in South Africa will develop breast cancer.

For some women the risk is higher. Approximately 5% of breast cancers are inherited. The risk for developing breast cancer increases to as high as 87% if you have been **identified as a *BRCA* mutation carrier**. This does not necessarily imply that you will develop cancer, only that there is an increased risk. The proportion of mutation positive individuals who will manifest the disease (be affected) is referred to as the penetrance of the specific mutation. Penetrance estimates for disease-causing mutations in *BRCA2* were initially relatively high, but has been determined for breast cancer as being 60% by age 70 years, and 40% or lower for ovarian cancer.

We have identified various *BRCA* mutations in our South African Afrikaner families, who all had a positive family history of breast cancer. We have observed a **high degree of variation** within and among the families regarding the cancer types present within each family, as well as the age at onset of the breast cancer cases. This variation of breast cancer risk and age at onset among women who carry the same *BRCA2* mutation suggest the existence of environmental and genetic factors that influence the penetration of this mutation. These factors are called genetic modifiers or variants. These variants can include a single genetic change in another gene or environmental causes such as smoking and the use of oral contraception.

The identification of such modifiers is important for providing accurate risk assessments for carriers who face difficult choices regarding prophylactic mastectomy and oopherectomy. Some potential genetic and non-genetic modifiers have been identified internationally, although the results are conflicting. Due to the founder effect of these mutations in the Afrikaner, we consider it essential to test these modifying variants, in order to define their role in our current *BRCA2* mutation carriers.

What is needed from you to participate in our study? We need the following:

- give us written consent to include you in our study after hearing what the project is about
- allow us to draw 10 milliliters of whole blood
- answer a few questions

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Geagte pasiënt **01/06/06**

Bekendstelling van 'n verdere navorsingsprojek op oorerflike borskanker

Ons weet dat borskanker veroorsaak word deur 'n komplekse kombinasie van genetiese- en omgewingsfaktore en dat dit een van die algemeenste kanker tipes in vroue van die Westerse wêreld is. Die borskanker risiko varieer van 1 in 13 vir blanke vrouens, tot 1 in 81 vir swart vrouens, met 'n gemiddelde van 1 in 36 vir die Suid-Afrikaanse vrou.

Vir sekere vroue soos u is die risiko heelwat hoër (tot so hoog as 87% in *BRCA* mutasie draers). U het reeds op diagnostiese vlak kliniese raadgewing ontvang en is deur ons laboratorium getoets vir die teenwoordigheid van siekte-veroorsakende mutasies in die oorerflike borskanker gene, *BRCA1* en *BRCA2*. Die genetiese toetse het daarop gedui dat u wel 'n *BRCA2* mutasie draer is.

Ons benodig u hulp. Ons vind dat positiewe mutasie draers binne 'n spesifieke familie nie noodwendig op dieselfde ouderdom siek word nie, of dat party nooit aantasting toon nie, selfs al is hulle reeds op 'n gevorderde ouderdom. Met ander woorde die penetrasie van hierdie spesifieke mutasie varieer tussen individue binne dieselfde familie en selfs tussen verskillende families. Wanneer daar verwys word na die penetrasie van 'n mutasie, word die aantal mutasie draers wat wel die siekte ontwikkel gereflekteer in die persentasie. Internasionaal word aanvaar dat die penetrasie van mutasies in *BRCA2* redelik hoog is, naamlik 60% vir borskanker teen die ouderdom 70.

Die variasie tussen en binne een familie is 'n aanduiding dat ander faktore (geneties sowel as omgewingsfaktore) ook moontlik 'n rol kan speel in die uiteindelike ontwikkeling van borskanker in 'n mutasie draer, m.a.w. op die penetrasie van die mutasie. Hierdie onbekende of modifiserende faktore kan enige iets insluit, van 'n genetiese polimorfisme in 'n ander geen tot 'n omgewingsfaktor soos rookgewoontes of die gebruik van orale voorbehoed.

Die identifisering van hierdie modifiserende faktore is baie belangrik aangesien dit die risiko-bepaling van mutasie draers meer akkuraat kan maak. Dit sal u, die pasiënt in staat stel tot beter besluitneming rakende voorkomende behandeling soos profilaktiese mastektomie en ovarektomie. Internasionale navorsers het reeds 'n paar moontlike modifiserende faktore uitgewys, alhoewel die resultate baie kontrasterend is vir die verskillende bevolkingsgroepe en nasionaliteite. Ons voel dat die navorsing van waarde

kan wees vir die Afrikaner bevolking, omdat dit so 'n unieke populasie is wat stigterseffekte toon.

Wat word van u benodig om deel te wees van hierdie studie? Ons benodig die volgende:

- ingeligte toestemming om u deel te maak van die studie (sal geskied nadat u breedvoerig oor die studie ingelig is)
- 'n nuwe/vars bloedmonster (10 milliliter bloed vir DNA ekstraksie)
- antwoorde op 'n algemene vraelys rakende hormoongebruik, obstetries en reproduktiewe geskiedenis (sal later gedoen word)

Daar sal geen kostes verbonde wees aan u deelname nie, aangesien dit 'n navorsingsprojek is. Indien ons wel betekenisvolle antwoorde tydens die uitvoer van die studie kry, sal dit aan u bekend gemaak word via u huisdokter of ons eie genetiese raadgewer. Indien daar ander vroulike familielede binne u onmiddelike familie is wat onlangs gediagnoseer is met borskanker waarvan ons nie weet nie, sal ons dit hoog op prys stel as u hulle kontak besonderhede aan ons kan deurgee. Ons is bereid om hulle kosteloos te toets vir die siekte-veroorsakende mutasie. Ons stel ook belang in onaangetaste positiewe vroulike mutasie-draers. Hulle vorm ook deel van ons projek.

Ek hoop dat u ons versoek gunstig sal oorweeg, aangesien dit ook vir u eie kinders en die nageslag voordeel kan inhou. Die mediese veld as geheel kan by u deelname baat vind. Ons uiteindelike doel is om die risiko bepaling en diagnostiese toetsing van hierdie spesifieke mutasie te verfyn. Dit sal ons instaat stel om u vrae beter te beantwoord en meer sinvolle siftingsmetodes of voorkomende prosedures voor te stel.

Ek gaan oor twee weke telefonies met u kontak maak om u antwoord te verneem. Indien u wel wil deelneem, sal ons dan reëlings tref vir die inligtings-gesprek, die teken van die ingeligte toestemmingsbrief en die trek van die bloed.

Baie dankie by voorbaat.

Die uwe

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THE UNIVERSITY OF THE FREE STATE

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The effect of known genetic polymorphisms as potential genetic modifiers of breast cancer risk and penetrance in *BRCA2* Afrikaner woman: Questionnaire

Reference number: _____

1. What is your date of birth? _____

2. Smoking

2.1. Has there ever been a time when you smoked at least one cigarette per day for three months or longer? ___ Yes ___ No
If no, continue with 2.8

2.2. At what age did you **FIRST** start smoking cigarettes **REGULARLY** (that is, at least one cigarette per day for three months or longer? _____ Years

2.3. For how many years in total had you smoked cigarettes **REGULARLY**? _____ Total years

2.4. Over the time when you smoked **REGULARLY**, how many cigarettes did you smoke in a day? _____ cigarettes/day

2.5. Are you currently smoking **REGULARLY**? ___ Yes ___ No
If no, at what age did you stop
_____ years

2.6. Over the last year, on average how many cigarettes have you smoked in a day? _____ cigarettes/day

2.7. At any time in your life, did any member of your household, including caregiver, smoke in your presence? ___ Yes ___ No

2.8. What was your relationship to the person who smoked? _____

2.9. How old were you when you were first exposed to your household member's smoke? _____ Age
_____ Age

2.10. How old were you when you were last exposed to your household member's smoke?

2.11. For how many years (from age 10 years) were you exposed to their smoke? _____Years

3. Reproductive History

3.1. Have you ever used birth control pills or other hormonal contraceptives (implants or injections)? ___ Yes ___ No
If no, go to 4.1

3.2. Age when first used birth control pills? ___ Years

3.3. Are you currently taking birth controls pills or hormones? ___ Yes ___ No

3.4. Age when last took birth control pills? _____

3.5. In total, for how many weeks, months or years had you taken birth control pills or hormones? _____ Weeks
_____ Months

4. Pregnancy History

4.1. How many **PREGNANCIES** did you have? _____ Pregnancies
If none, go to 5.1

4.2. How old were you when your **FIRST** (live) child was born? _____ Age

4.3. How old were you when your **LAST** (live) child was born? _____ Age

4.4. How many **LIVE BIRTHS** did you have? _____

4.5. Did you ever breastfeed a child for one month or more? ___ Yes ___ No

5. **Menopause and Hormone Replacement Therapy**

5.1. Have you ever taken estrogens, progesterone or other female hormones for menopause? The preparation may be pills, injections, skin patches, vaginal creams or vaginal suppositories? ___ Yes ___ No

UNIVERSITEIT VAN DIE VRYSTAAT
FAKULTEIT GESONDHEIDSWETENSKAPPE, MEDIESE SKOOL
Afdeling Mensgenetika, NHLS

The effect of known genetic polymorphisms as potential genetic modifiers of breast cancer risk and penetrance in *BRCA2* Afrikaner woman: Vraelys

Verwysingsnommer: _____

1. Geboortedatum: _____

2. Rookgewoontes:

2.1. Was daar ooit 'n tyd in u lewe wanneer u **ten minste** een sigaret/dag vir drie maande of langer gerook het? ___ Ja ___ Nee
Indien nee, gaan na 2.7

2.2. Op watter ouderdom het u **GEREELD** begin rook (dws een sigaret per dag vir ten minste drie maande)? _____ Jaar

2.3. Vir hoeveel jaar het u **gereeld** gerook? _____ Jaar

2.4. Hoeveel sigarette het u per dag gerook? _____ sigarette/dag

2.5. Rook u **tans**? ___ Ja ___ Nee
Indien nee,op watter ouderdom het u opgehou? _____ Jaar

2.6. **Gemiddeld**, hoeveel sigarette het u gedurende die laaste jaar per dag gerook? _____ sigarette/dag

2.7. Het enige familielid of persoon binne die huishouding gerook tydens u kinderjare? ___ Ja ___ Nee

2.8. Wat was u verhouding met die betrokke persoon? _____

2.9 Hoe oud was u tydens u **eerste blootstelling** aan sekondêre rook? _____ Oud

2.10. Wat was u ouderdom tydens u **laaste blootstelling** aan sekondêre rook? _____ Oud

2.11. Vir hoeveel jare **in totaal** was u blootgestel aan sekondêre rook? _____ Jare

3. Obstetriese geskiedenis

3.1. Het u al ooit enige vorm van voorbehoed gebruik (orale voorbehoed/inspuitings/inplantings)? ___ Ja ___ Nee
Indien nee, gaan na 4.1

3.2. Ouderdom tydens eerste gebruik van voorbehoed? ___ Jaar

3.3. Maak u tans gebruik van enige voorbehoed? ___ Ja ___ Nee

3.4. Op watter ouderdom het u die gebruik daarvan gestaak? _____ Jaar

3.5. Vir hoeveel maande of jare **in totaal** het u enige van 3.1 gebruik? _____ Maande
_____ Jare

4. Reproduktiewe geskiedenis

4.1. Hoeveel keer was u al ooit swanger? _____ Keer

Indien geen, gaan na 5.1

4.2. Hoe oud was u met u eerste kind (lewend) se geboorte? _____ Jaar

4.3. Hoe oud was u met u laaste kind (lewend) se geboorte? _____ Jaar

4.4. Hoeveel kinders (lewend) is gebore? _____

4.5. Het u ooit u kind vir een maand of langer geborsvoed? ___ Ja ___ Nee

5. **Menopouse en Hormoon Vervangings-terapie**

5.1. Het u al ooit estrogeen, progesteroon of ander vroulike hormone vir die behandeling/voorbereiding van menopause gebruik? (insluitend pille, inspuitings, vel plakkers, vaginale room of vaginale setpille)

___ Ja ___ Nee

Appendix C

FREE STATE PROVINCE

Ref. no.: H4/3/2

20 March 2006

Dr. NC van der Merwe
Principal Investigator
Division of Human Genetics
Faculty of Health Sciences
University of the Free State
BLOEMFONTEIN
9300

Dear Dr. van der Merwe

PERMISSION FOR USING LABORATORY SPACE AND BLOOD SAMPLES FROM CLINIC PATIENTS

Your letter dated 10 March 2006 in the abovementioned refers.

Herewith permission for the mentioned project to be done at Universitas Academic Hospital on condition that approval is obtained from the Ethics Committee.

No findings can be published without permission of the Chief Executive Officer.

Yours sincerely

DR NIC R J VAN ZYL
HEAD: CLINICAL SERVICES

DR NRJ VAN ZYL
2006 -03- 20
HEAD: CLINICAL SERVICES
UNIVERSITAS ACADEMIC HOSPITAL

Head: Clinical Services, Dr. NRJ van Zyl, Universitas Tertiary Hospital • P/Bag X20660, Bloemfontein 9300 •
Tel: 051-405 2866 • Fax: 051-444 0792, e-mail: vanzylnr@fshealth.gov.za • Room 1129, 1st Floor,
Universitas Tertiary Hospital, Bloemfontein

Appendix D

NATIONAL HEALTH LABORATORY SERVICE

Office of the Business Manager
UNIVERSITAS ACADEMIC LABORATORIES
PO BOX 339(G3)
C/O: CHEMICAL PATHOLOGY
1st FLOOR
BLOCK C
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF FREE STATE
BLOEMFONTEIN
9301

20 March 2006

Dr. NC van der Merwe
Human Genetics
NHLS Universitas Laboratories

Dear Dr. van der Merwe,

Your request for permission to use laboratory facilities and blood samples from clinic patients (Research Project on familial breast cancer)

Your request to use laboratory space and equipment for the research project is granted on condition that:

1) UFS Ethics Committee approval is obtained
2) That the DNA extracted from all blood specimens is anonymised and destroyed after use according to Ethics Committee instructions
3) The NHLS is acknowledged in all publications
4) Funding for analyses (reagents, kits & other consumables) is obtained through a research funding agency or a k-project.

Wishing you well in your research endeavours.

Sincerely,

Prof. H. Pieters
Business Manager

Appendix E

NATIONAL HEALTH LABORATORY SERVICE

Office of the Head of Human Genetics
UNIVERSITAS ACADEMIC LABORATORIES
C/O: HUMAN GENETICS
GROUND FLOOR
BLOCK C
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF FREE STATE
PO BOX 339(G11)
BLOEMFONTEIN
9300

24 March 2006

Dr NC van der Merwe
Medical scientist and principal investigator
Division of Human Genetics/NHLS
Faculty of Health Sciences
UFS
Bloemfontein

Dear Dr van der Merwe,

Re: Permission for using laboratory space and blood samples from clinic patients

You are hereby granted permission to use the space and equipment of the Division of Human Genetics' Molecular Laboratory. The patients attending the Genetics clinic do not fall under my jurisdiction or that of the NHLS. You will have to obtain informed consent from each individual patient attending the clinic. Please inform and obtain permission from the specialist in charge, Dr BD Henderson, as well as Dr Nic van Zyl, Head: Clinical Services, Universitas Hospital, under whose jurisdiction all non-private patients attending the clinic, falls.

Please note that neither the standard "consent form for DNA analysis and storage" nor "patient's informed consent for genetic testing of BRCA1 and BRCA2 genes" provide for the permission you are seeking. A specific consent form outlining the research needs to be designed (as required by the Ethics Committee). You will also have to seek the same consent from the previously determined *BRCA2* mutation positive patients, since the purpose of this investigation is different from the original, i.e., the identification of the essential disease causing mutations.

Yours sincerely,

Prof Stander Jansen
Head: Division of Human Genetics

☎ 051- 405 3047 Fax: 051- 444 1161 PO BOX 339 (G11)

THE UNIVERSITY OF THE FREE STATE

FACULTY OF HEALTH SCIENCES, SCHOOL FOR MEDICINE

Division of Human Genetics, NHLS

INFORMED CONSENT for DNA analysis and storage

PROJECT: Search for genetic modifiers of BRCA cancer risk and penetrance

1. I, _____,
   (ID number) _____ confirm that I was invited to participate in this research project of the Division of Human Genetics from the University of the Free State and NHLS, on familial breast cancer.
2. It has been explained to me that:
   a. the project forms part of an attempt to more accurately define the risk and penetrance of BRCA mutations.
   b. blood (10ml) will be collected by venepuncture from my arm for DNA/RNA extraction
   c. I may experience discomfort during the venepuncture
   d. the genetic material obtained will be used for genetic analysis
   e. should any informative results be obtained, I choose to be informed via my doctor or the Clinician/Genetic Counselor of the Division of Human Genetics: Yes ☐ No. ☐
3. I know that the project will take approximately three years to be completed.
4. It has been explained to me that participation will be of benefit to the field of medical genetics in the future, and could therefore be beneficial for my children.
5. I request that no portion of the sample be stored for later use for other disorders ☐ **OR** I request that a portion of the sample be stored indefinitely for research purposes (other disorders), subject to the approval of the University of the Free State (Faculty of Health Sciences) Ethics Committee, provided that any information from such research will remain confidential and anonymous. ☐
6. I agree to provide comprehensive information regarding epidemiological factors such as my lifestyle, reproductive history, etc.
7. I authorize / do not authorize my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the Division of Human Genetics, UFS.

7. I have been informed that:
   a. there are risks and benefits associated with genetic analysis and storage of biological material and these have been explained to me.
   b. the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
   c. the genetics laboratory is under an obligation to respect and maintain medical confidentiality .
   d. genetic analysis may not be informative for some families or family members.
   e. even under optimal conditions, current technology of this type is not perfect and could lead to incorrect results.
   f. where biological material is used for research purposes, there may be no direct benefit to me.
8. I understand that I may withdraw or modify my consent for any aspect of the above at any time without this affecting my future medical care.
9. All of the above has been explained to me in a language that I understood and my questions has been answered by: _____

Adress:

_____

_____

Tel. (_____) _____ Cell: _____

Signature: _____ Date: _____

UNIVERSITEIT VAN DIE VRYSTAAT **FAKULTEIT GESONDHEIDSWETENSKAPPE, SKOOL VIR GENEESKUNDE**

Afdeling Mensgenetika, NHLS

INGELIGTE TOESTEMMING vir ondersoeke op en die bewaar van DNA

NAVORSINGSPROJEK: – Ondersoek van genetiese polimorfismes in ander gene wat die penetrasie en risiko van 'n BRCA2 siekte-veroorsakende mutasie kan beinvloed

1. Ek, _____, (ID nommer) _____ bevestig dat ek uitgenooi is om deel te neem aan bogenemelde navorsingsprojek van die Afdeling Mensgenetika (Universiteit van die Vrystaat/NHLS) op oorerflike borskanker.
2. Daar is aan my verduidelik dat:
   a. die projek deel uitmaak van 'n studie waarin daar gepoog sal word om die risiko en penetrasie van die Suid-Afrikaanse BRCA mutasies meer akkuraat te bepaal
   b. bloed (10ml) uit die voorarm versamel sal word vir DNA/RNA ekstraksie
   c. die proses van bloedtrek effense ongemak mag meebring
   d. die genetiese materiaal gebruik sal word vir genetiese analises
   e. indien enige betekenisvolle resultate verkry word, ek daarvan in kennis gestel sal word via my huisdokter of die Genetiese raadgewer van die Afdeling Mensgenetika: Ja ☐ **Nee** ☐
3. Ek weet dat die projek ongeveer drie jaar sal neem om te voltooi.
4. Daar is verder aan my verduidelik dat deelname aan die projek kan bydra tot die uitbouing van mediese kennis, wat moontlik voordele vir my kinders kan inhou.
5. Ek versoek dat geen gedeelte van die monster vir latere gebruik geberg mag word nie ☐ **OF** ek versoek dat 'n gedeelte van die monster onbepaald geberg mag word vir navorsingsdoeleindes onderhewig aan die goedkeuring van die Etiekkomitee van die Fakulteit Gesondheidswetenskappe, Universiteit van die Vrystaat, op voorwaarde dat enige inligting voortspruitend uit hierdie navorsing vertroulik en anoniem sal bly. ☐
6. Ek stem in om so volledig moontlik inligting rakende epidemiologiese faktore soos lewenswyse, reproduktiewe geskiedenis, ens. te verskaf.
7. Ek magtig / magtig nie my geneesheer(e) (SKRAP WAT NIE VAN TOEPASSING IS NIE) om toepaslike kliniese besonderhede aan die Afdeling Mensgenetika, UV beskikbaar te stel.
8. Ek is ingelig dat:
   a. daar risiko's en voordele verbonde is aan die genetiese ondersoeke en berging van biologiese material en dat dit aan my verduidelik is
   b. die ondersoekprosedure spesifiek is tot die oorerflike toestand hierbo gemeld en nie die volledige genetiese samestelling van 'n individue kan bepaal nie
   c. die genetika laboratorium verplig is om mediese vertroulikheid te respekteer en te handhaaf .
   d. genetiese ondersoeke nie vir alle families of familielede antwoorde kan verskaf nie
   e. selfs onder optimale toestande die bestaande tegnologie feilbaar is en tot foutiewe resultate kan lei
   f. waar biologiese materiaal vir navorsingsdoeleindes aangewend word, dit geen direkte voordele vir my mag inhou nie.
9. Ek verstaan dat ek te enige tyd my toestemming mag terugtrek of wysig sonder inbreuk op my toekomstige mediese sorg.
10. Al die bogenoemde aspekte is aan my verduidelik in 'n taal wat ek verstaan en my vrae is beantwoord deur: ____**Dr NC van der Merwe**_____

Adres: _____

_____

Tel. (_____) _____ Sel: _____

Handtekening: _____ Datum: _____