Molecular Screening of the South African Indian Population for *BRCA1* and *BRCA2* Using High Resolution Melting Analysis

by

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Declaration

I certify that the dissertation hereby submitted for the degree M.Med.Sc at the University of the Free State is my independent effort and has 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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HMVE Combrink

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“The great thing about science is that you can get it wrong over and over again because what you're after - call it truth or understanding - waits patiently for you. Ultimately, you'll find the answer because it doesn't change."

-Dudley Herschbach

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Summary

The lifetime risk for developing breast cancer within the Indian population of South Africa is one in 17. Disease causing mutations in *BRCA1/2* increase the risk of developing this disease by up to 80%. The main objective of this study was to screen this unique population for mutations in *BRCA1/2.*

This was achieved by optimising High Resolution Melting Analysis (HRMA) as the screening technique for the smaller exons while the Protein Truncation Test (PTT) was used to screen exon 11 for *BRCA1/2* respectively. In order to optimise HRMA, a full *BRCA1/2* screen was performed on 24 patients from four different South African ethnic groups using Single-Stranded Conformation Polymorphism/ Heteroduplex Analysis (SSCP/HA). These results were compared to a HRMA screen performed on the same patients. No differences were observed between the sensitivity of the three techniques and the turnaround time (TAT) was considerably less for HRMA.

The entire cohort used in this study came from 50 unrelated South African Indian patients. A full *BRCA1/2* screen was performed on these patients. A total of nine different pathogenic mutations were detected. Four of the disease causing mutations (*BRCA1* c.1360_1361delAG, p.Ser454Terfs; c.3593T>A, p.Leu1198Ter and *BRCA2* c.5279C>G, p.Ser1760Ter; 5563C>G, p.Ser1855Ter) were detected using PTT, whereas the other five mutations (*BRCA1* 185delAG, p.Leu22_Glu23LeuValfs; c.191G>A, p.Cys64Tyr; c.5365_5366delGCinsA, p.Ala1789_Ile1790LeuTrpfs and *BRCA2* c.9435_9436delGT, Val3145_Phe3146=fs; c.8754+1G>A, IVS21+1G>A) were detected using HRMA. Three unrelated patients were carriers of the splice site mutation found within *BRCA2* exon 21.

The research that was conducted, contributed to the knowledge pool for predictive testing in the clinical setting of South Africa and gave insight into possible diagnostic tests that could be designed for this population.

Keywords: Indian Population, *BRCA1/2*, Optimising, HRMA, Screening.

Opsomming

Die risiko onder die Indiër bevolking van Suid-Afrika om borskanker te ontwikkel was een in 17. Siekteveroorsakende mutasies in *BRCA1/2* het die risiko om hierdie siekte te ontwikkel met tot 80% verhoog. Die hoofdoelwit van hierdie studie was om sifting van *BRCA1/2* in hierdie unieke bevolkingsgroep te doen.

Dit is bereik deur High Resolution Melting Analysis (HRMA) as die siftingsmetode vir kleiner eksons te optimiseer, terwyl Protein Truncation Test (PTT) gebruik is om ekson 11 vir *BRCA1/2* te sif. Om HRMA te optimiseer is volle *BRCA1/2* sifting uitgevoer op 24 pasiënte vanuit 4 verskillende Suid-Afrikaanse etniese groepe deur middel van Single-Stranded Conformation Polymorphism/ Heteroduplex Analysis (SSCP/HA). Hierdie resultate is met HRMA vergelyk wat op dieselfde pasiënte uitgevoer is. Geen verskille is opgemerk tussen die sensitiwiteit van die drie tegnieke nie en die omkeertyd was aansienlik korter vir HRMA.

Die hele studiegroep het bestaan uit 50 onverwante Suid-Afrikaanse Indiër pasiënte. Volle *BRCA1/2* sifting is uitgevoer op hierdie pasiënte. ‘n Totaal van nege verskillende patogeniese mutasies is ontdek. Vier van die siekteveroorsakende mutasies (*BRCA1* c.1360_1361delAG, p.Ser454Terfs; c.3593T>A, p.Leu1198Ter en *BRCA2* c.5279C>G, p.Ser1760Ter; 5563C>G, p.Ser1855Ter) is ontdek deur middel van PTT, terwyl die ander vyf mutasies (*BRCA1* 185delAG, p.Leu22_Glu23LeuValfs; c.191G>A, p.Cys64Tyr; c.5365_5366delGCinsA, p.Ala1789_Ile1790LeuTrpfs en *BRCA2* c.9435_9436delGT, Val3145_Phe3146=fs; c.8754+1G>A, IVS21+1G>A) ontdek is deur middel van HRMA. Drie onverwante pasiënte was draers van die mutasie wat in *BRCA2* ekson 21 ontdek is.

Die navorsing wat gedoen is het bygedra tot die kennis vir voorspellingstoetsing in die kliniese omgewing van Suid-Afrika en het insig gelewer van die moontlike diagnostiese toetse wat ontwerp kan word vir hierdie bevolkingsgroep.

Sleutelwoorde: Indiër bevolking, *BRCA1/2*, Optimisering, HRMA, Sifting.

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Abbreviations

Evidence-based Network for the Interpretation of Germline Mutation Alleles	**ENIGMA**
Ataxia Telangiectasia Mutated Kinase	**ATM kinase**
Breast Cancer	**BC**
Base Pairs	**bp**
Breast Cancer Information Core	**BIC**
Breast Cancer Susceptibility Gene 1	***BRCA1***
Breast Cancer Susceptibility Gene 2	***BRCA2***
Deletion	**del**
Deoxyribonucleic Acid	**DNA**
Deoxyribonucleotide Triphosphate	**dNTP**
Diagnosis	**dx**
Dithiothreitol	**DTT**
Double Stranded DNA	**dsDNA**
Ethylenediaminetetraacetic Acid	**EDTA**
Ethanol	**EtOH**
Frame shift	**fs**
Global Minor Allelic Frequency	**MAF**
Gregorian Calendar	**GC**
Heteroduplex Analysis	**HA**
High Resolution Melting Analysis	**HRMA**

Human Genome Variation	**HGVS**
2-Amino-2-Hydroxymethyl-1,3-Propanediol	**Tris**
Insertion	**ins**
National Center for Biotechnology Information	**NCBI**
Ovarian Cancer	**OVC**
Polyacrylamide Gel Electrophoresis	**PAGE**
Protein Truncation Test	**PTT**
Quantitative Polymerase Chain Reaction	**qPCR**
Serine Cluster Domain	**SCD**
Serine/Threonine-Protein Kinase	**ATR kinase**
Single Nucleotide Polymorphism	**SNP**
Single Stranded Conformation Polymorphism	**SSCP**
Single Stranded DNA	**ssDNA**
Sodium Dedocyl Sulphate	**SDS**
South Africa	**SA**
Turnaround Time	**TAT**
Unclassified Variant	**UV**

Chapter 1

Introduction to the study

1.1 Introduction

According to the Global Burden of Cancer Study (GLOBOCAN), 14.1 million new cancer cases and 8.2 million cancer related deaths were reported for 2012. These statistics indicated that approximately 32.6 million people above the age of 15 were diagnosed with cancer over 5 years. Of the total number of cases reported, 11.9% were breast cancer (BC) or BC related (Bray *et al*., 2013; Ferlay *et al*., 2013).

Disease causing mutations in the BC susceptibility gene 1 (*BRCA1*) and BC susceptibility gene 2 (*BRCA2*) increases the risk of developing BC by up to 80% (Claus *et al*., 1996). The main challenges regarding screening for mutations in these genes is the turnaround time (TAT) and a lack of population specific diagnostic information (Feliubadaló *et al*., 2013).

The general aims of this study were to screen the *BRCA1* and *BRCA2* genes within the Indian population of South Africa (SA) by using more effective molecular screening techniques. The more effective techniques include High Resolution Melting Analysis (HRMA) and the Protein Truncation Test (PTT), while moving away from the time consuming Single Stranded Conformation Polymorphism (SSCP) and Heteroduplex Analysis (HA). This study provided insight into the mutation profile of these two genes for this population, as the population has not been exclusively studied for these genes before in SA.

Chapter 2

Literature review

2.1 Introduction to the Indian population of South Africa

2.1.1 Historical background

The first recorded Indians in SA arrived during the Dutch colonial era in 1652 Gregorian Calendar (GC) (Mayson, 1855) (Table 2.1). A total of 1 195 Indian slaves (including people from Bangladesh) were initially brought to SA, according to the arrival records (Bradlow and Cairns, 1978). As SA was under British rule at the time, approximately 80% of the slaves (a total of 16 000 individuals) imported to SA during 1690 GC to 1725 GC were of Indian descent (Worden, 1985; Carl-Heinz, 1994). The practice of importing slaves to SA ended in 1838 GC (De Beer, 1996). By 1860 GC the Natal English colonial authorities entered into an agreement with British ruled India to import people from the Indian sub-continent as indentured workers to serve the economic needs of the colony. These two occurrences resulted in SA Indians residing in mostly the Western Cape and Kwazulu-Natal region where they were completely integrated into the Cape White and Coloured communities by 1880 GC (Vishnu and Morrell, 1991).

In total, approximately 150 000 Indian workers arrived over a period of 5 decades (Wright, 1831; Reddy, 1991). The modern SA Indian community are largely descendants from individuals who arrived in SA from 1860 GC onwards as indentured workers as well as migrations between mainland India and SA. They speak Tamil, Telugu and Hindi, with the majority being Hindu with Christians and Muslims amongst them. In 1910 GC, a quarter of the men returned to India, but 73.15% stayed behind to make SA their home (Green, 2008). The majority of the SA Indian community still reside in the vicinity of Durban on the East Coast of SA. Due to this, Durban is the city that has the highest concentration of people from India, outside of India itself, in the world (Dickinson, 2015).

Table 2.1 Summary of the historic account between India and SA from 1652 – 1910 (Wright, 1831; Reddy, 1991; Green, 2008).

Date	Description of migration event	Number of people involved
1652	First reported individuals from mainland India and Bangladesh arrive in the Cape as slaves.	1 195
1690 - 1725	Slave migration from India to SA.	12 800
1838	Slave trade between SA and India ended.	< 40 000
1860	Indians from sub-continent to work as indentured labourers to serve the economic needs of SA.	> 40 000
1880	The Indians residing in SA became totally integrated with the Coloured and Cape white communities.	> 90 000
1860 - 1910	Indentured workers to work on the sugarcane plantations of Natal Colony as well as serve other economic needs within SA.	> 150 000

2.1.2 Peopling the SA Indian population

The SA Indian population is comprised of people from different castes, cultures and different ethnic groups due to globalisation (Shah *et al*., 2011). This unique population group is an admixture of individuals from mainland India, neighbouring countries such as Bangladesh, as well as SA Coloured and other local SA ethnic groups as suggested by Vishnu and Morrell (1991).

Populations are normally genetically grouped by means of haplotype analysis. Haplotypes are a set of genetic markers located on a single chromosome that progeny inherit. A haplotype analysis is a process whereby genetic markers are assigned to individuals in a specific population, which is used comparatively to other individuals of the same or different populations (Gichohi-Wainaina *et al*., 2015). This type of analysis could determine how closely related the sampled individuals are against the population they were tested against (Duminil *et al*., 2015). In a recent study conducted by Isaacs *et al*. (2013), titled '*The reconstruction of major maternal and paternal lineages of the Cape Muslim population*', it was shown that the Indian Muslim communities of SA have a very unique haplogroup which cannot be clearly defined. These data correlated with a study from Roychoudhury *et al*. (2001). In this study they correlated unique haplogroups from various parts of India in an attempt to group the Indian population of mainland India. They too, were un-successful. A separate study reported that grouping the Indian population was challenging and not yet effective enough to perform (Mastana, 2014).

From these studies there was no concrete method to group or measure the genetic heritage of the Indian population (Reich *et al*., 2009). It also meant that even though a person might belong to a certain language group, religion or caste from India, that they should be looked at as a separate genetic group and that these groups were mixed within one another (De Wit *et al*., 2010). This led to the assumption that the SA Indian population is a unique ethnic group which could not be compared to a single frame of reference such as groups from mainland India, the SA Coloured population, Malaysian, Bangladesh or even the Pakistani population. This population has a unique local gene pool, which was looked at within the context of SA as a unique population group in this study.

2.2 Familial Breast Cancer

2.2.1 Mammary carcinoma

BC (BC MIM #114480) is predominantly referred to as cancer of the breast epithelial tissue. The mammary gland (breast) consists of various cell types (Stewart *et al*., 2015). These include connective tissue, adipose tissue and lobules inside the physiologically normal mammary gland (Figure 2.1). A collection of lobules make a lobe and the connection between the nipple and the lobes is the link that transports milk from the lobes to the nipple (Kalimuthu *et al*., 2015). The ducts that mediate liquid transport between these systems are aligned with buciodal epithelial cells, which in turn are surrounded by myoepithilial cells (Zhang *et al*., 2015). The abnormal proliferation of either buciodal or myoepithelial epithelial cells inside the breast is normally a result of BC.

Somatic BC and familial BC fall within the category of BC (Pfeifer *et al*., 2014). Somatic BC occurs in individuals without any prior family history for the disease and is random between individuals where the clinical features differed (Molyneux *et al*., 2014). Some of the risk factors for somatic BC include: age, gender, exposure to radiation, certain hormone levels, physical tissue damage, diet, various lifestyle choices such as a lack of exercise and inadequate amounts of sleep (Thomson *et al*., 2014).

The specific causes of somatic BC are not known, but these risk factors have been extensively studied (Ahern *et al*., 2014; Neilson *et al*., 2014; Santen *et al*., 2014). In contrast, familial BC refers to individuals who inherited a predisposition to the disease (Maxwell *et al*., 2014). The single most significant contributor towards an individual's risk is the number of first degree relatives that have been affected with the disease (family history for the disease) (Anderson *et al*., 2014). Figure 2.2 illustrates a pedigree for an individual that has three family members affected with the disease to indicate a high-risk individual.

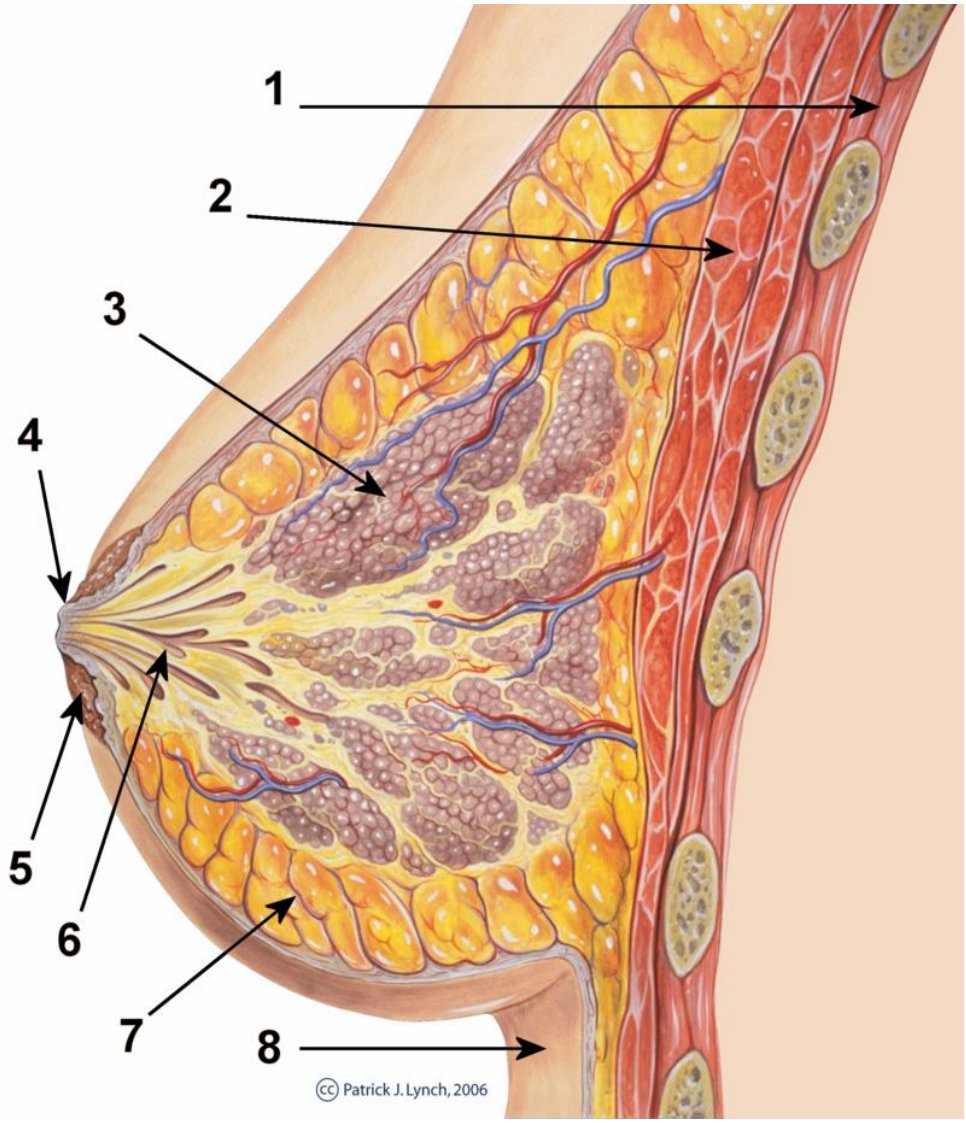

Figure 2.1 Schematic representation of a female mammary gland (Lynch, 2006). **1**. Chest wall. **2**. Muscles. **3**. Lobules. **4**. Nipple. **5**. Areola. **6**. Milk duct. **7**. Fatty tissue. **8**. Skin. The main tissues such as the lobules, breast lobe as well as the connecting tubes of the breast are outlined and indicated with arrows.

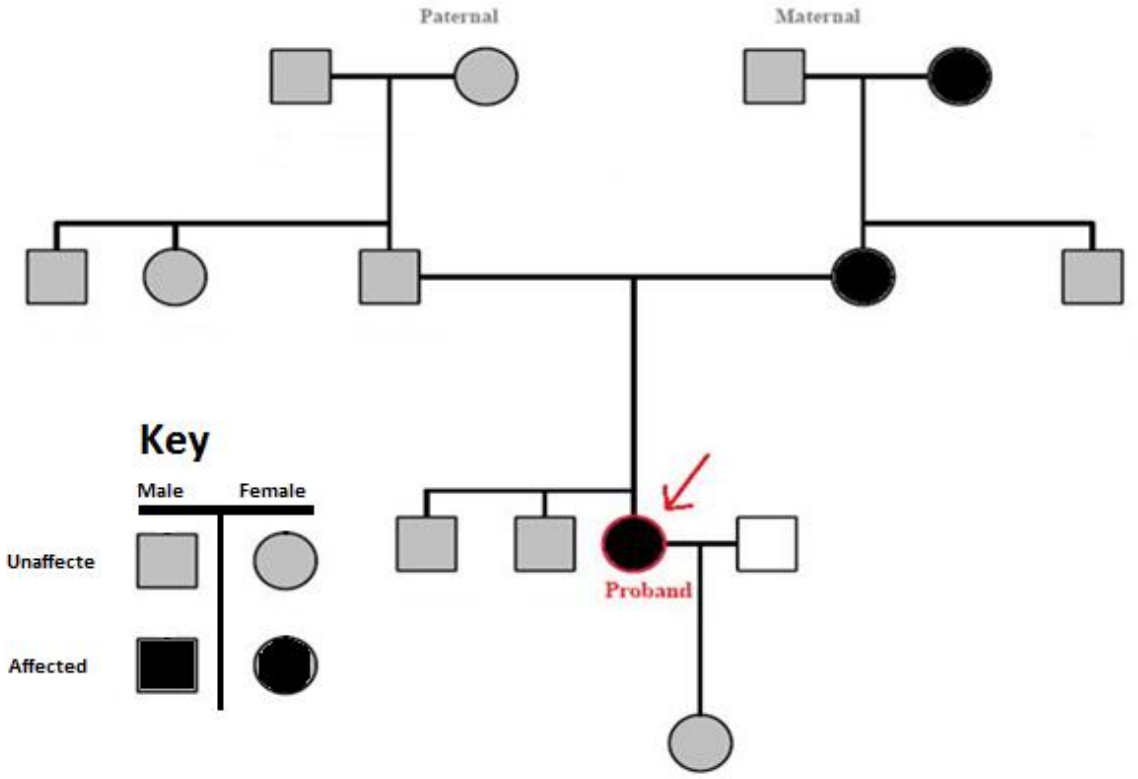

Figure 2.2 Example of a pedigree indicating a high-risk family. The red arrow indicated the affected patient with BC.

Roughly five to ten percent of women with BC have a genetic predisposition caused by germline mutations present in genes involved in either DNA or structural repair within the breast and ovarian tissue (Boyd, 2014). Two of the genes that greatly influence an individual's risk of developing BC are *BRCA1* and *BRCA2* (Pedroni *et al*., 2014). Germline mutations within these genes increases a woman's risk of developing BC by 60 - 80% and ovarian cancer (OVC) by 20 - 40% (Domchek *et al*., 2010).

2.2.1.1 Familial BC susceptibility gene 1 (*BRCA1* OMIM 113705)

BRCA1 plays various critical roles in cell cycle checkpoint control, DNA repair, as well as maintaining genomic stability within certain nuclear pathways (Hu *et al*., 2014). The gene was discovered in 1990 and increases the predisposition to BC in cases where the function of the protein has been altered (Hall *et al.*, 1990). *BRCA1* is located on the long arm of chromosome 17q21 (Figure 2.3 A) (Dacheva *et al*., 2015). The gene consists of 24 exons of which 22 exons were coding (Figure 2.3 B). Apart from the large exon 11 located in the middle of the gene, all the exons are relatively small. The 22 coding exons transcribe a 7.22 kb mRNA molecule that encodes for a 1 863 long amino acid chain of approximately 220 kDa (Easton *et al*., 1993).

2.2.1.2 Familial BC susceptibility gene 2 (*BRCA2* OMIM 600185)

BRCA2 is responsible for the regulation of genes that actively repair single and double stranded DNA (ssDNA and dsDNA) breaks (Rytelewski *et al*., 2014). The gene is located on the long arm of chromosome 13q12.3 (Figure 2.3 C) (Bershadskii, 2011). The gene was discovered in 1994 by Stratton and Wooster. They proved that *BRCA2* consists of 27 exons, which encode a protein of 3 418 amino acids (Figure 2.3 D) (Wooster *et al*., 1995). *BRCA2* shows a similarity to *BRCA1*, due to the presence of an extremely large exon 11 located in the middle of the gene (Figure 2.3. D).

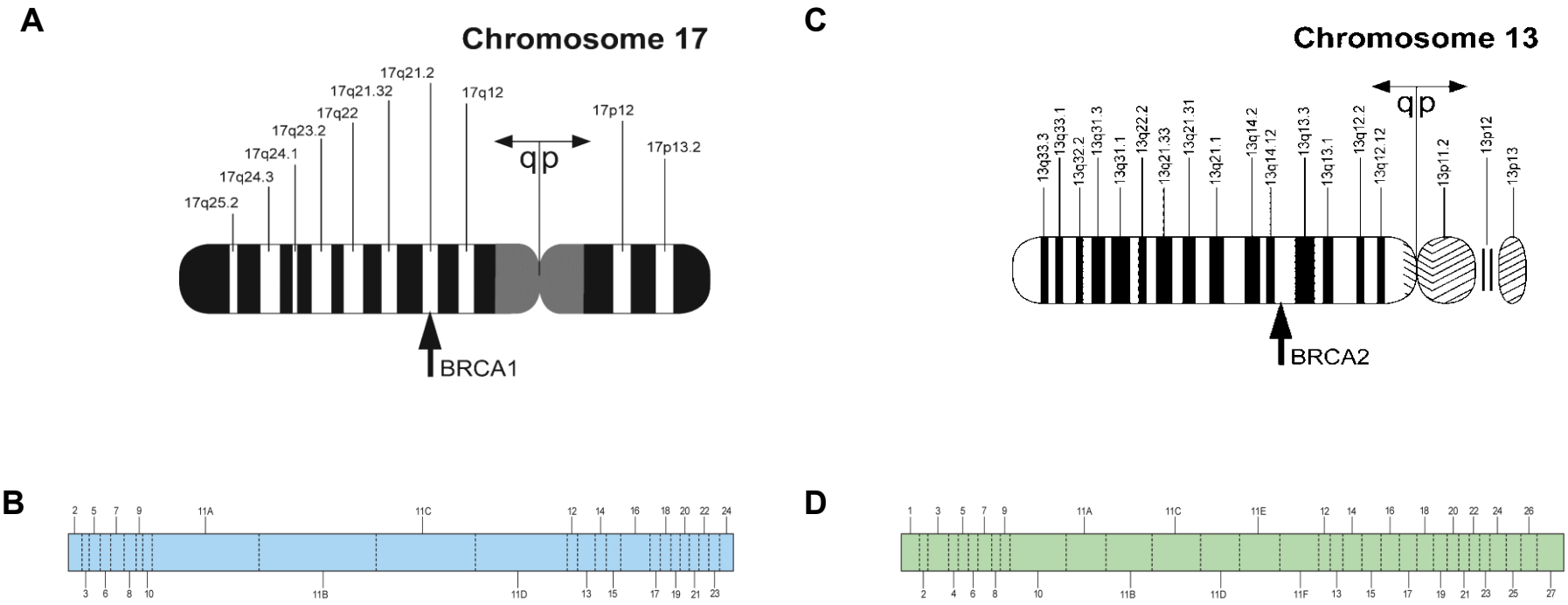

Figure 2.3 Schematic representation of *BRCA1* and *BRCA2*. **A**. *BRCA1* located on the long arms of chromosome 17 (highlighted by the arrow). **B**. Indication of the gene structure of *BRCA1*, showing 24 different exons (Easton *et al*., 1993). **C**. *BRCA2* located on the long arms of chromosome 13 (as indicated by the arrow). **D**. Indication of the gene structure of *BRCA2*, showing 27 different exons (Wooster *et al*., 1995).

2.2.2 The BRCA protein complex

The *BRCA1/2* genes are known as caretaker genes because of their role in maintaining DNA repair in somatic cells, as well as their involvement during meiotic DNA breaks (Hatchi *et al*., 2015). The genes translate two functional proteins that work together in DNA repair (Aleskandarany *et al*., 2015). The BRCA1 protein has three main functional domains (Meza *et al*., 1999). These domains are known as the zinc-finger domain, serine cluster domain (SCD) and the BRCT (BRCA1 C Terminus) domains (Figure 2.4 A).

The zinc-finger domain is located within *BRCA1* exon 2. This domain consists of approximately 60 amino acids with eight metal binding residues inside (Calderon *et al*., 2014). This motif interacts with a homologous region that is located inside the BARD1 protein (Shi and Manley, 2015). Four peptide helices (two alpha-helices from the BRCA1 and t,he BARD1 protein) merge together to form a heterodimerization boundary that stabilises the BRCA1-BARD1 heterodimer compound, an essential domain required in tumour suppression (Wiener *et al*., 2015). This domain is also an important site for ubiquitin E3 ligase, a small regulatory protein that cascades several biochemical pathways responsible for correct signalling as well as assisted DNA repair (Berndsen and Wolberger, 2014).

The second critical area located within this protein is SCD, located within *BRCA1* exons 11, 12 and 13 (Lu *et al*., 2015). This domain encodes a polypeptide between 1 280 and 1 524 AA within the BRCA1 protein (Takada *et al*., 2015). The SCD site is phosphorylated by both Ataxia telangiectasia mutated kinase and Serine/Threonine-protein kinase (ATM/ATR kinase) (Zhang *et al*., 2014). The SCD is ultimately responsible for detecting DNA breaks and localisation to damaged DNA sites. The BRCT motifs are shown to actively bind phosphorylated proteins involved in the DNA damage response (Na *et al*., 2014). Furthermore the BRCT domain of *BRCA1* interacts with Checkpoint kinase 1 (Chk1), a protein translated from the *CHEK1* gene. The function of BARD1 is to guide BRCA1 to the damaged DNA site. The combination of the BRCT-Chk1 interaction directed the DNA damage response (Wu *et al*., 2015a; Wu *et al*., 2015b).

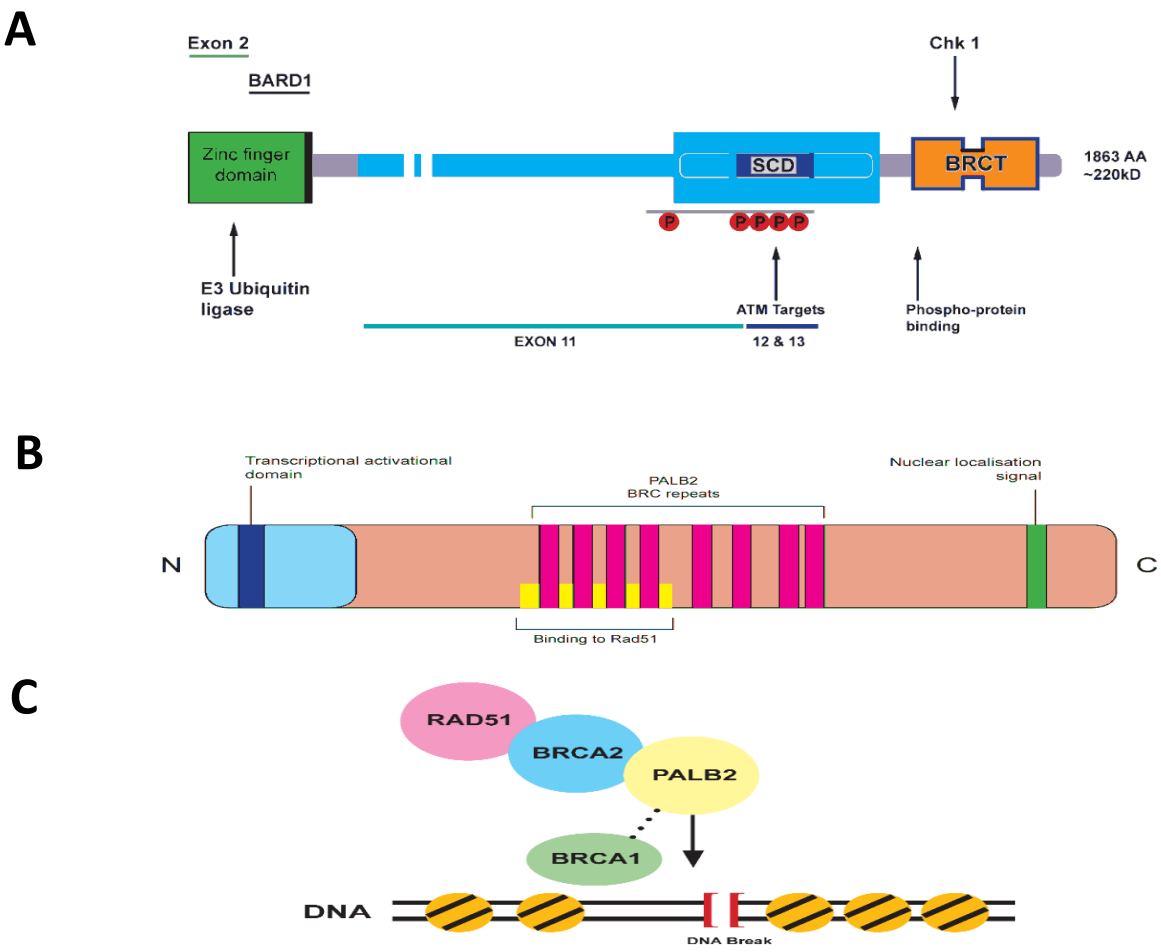

Figure 2.4 Schematic representation of the BRCA1 and BRCA2 protein functional domains (adopted from: Berndsen and Wolberger, 2014; Takada *et al*., 2015). **A**. Indicated are the functional domains found on the BRCA1 protein. **B**. Indicated are the main functional domains of the BRCA2 protein. **C**. Indicated are the binding between RAD51, PALB2, BRCA1 and BRCA2.

BRCA2 has a different function compared to BRCA1. BRCA2 binds to ssDNA and actively interrelates with RAD51, a nuclear recombinase that fuels a process of homologous recombination (Shahid *et al*., 2014). In order for RAD51 to localise to the dsDNA break, binding is facilitated by the formation of a protein complex. The binding to RAD51 is enabled by the BCR repeats that are located within the BRCA protein (Zhu *et al*., 2013) (Figure 2.4 B). The BRCA1-PALB2-BRCA2 complex actively assists RAD51 to bind to a DNA break (Orthwein *et al*., 2015). PALB2 is a localiser protein for BRCA2 and forms a chimera protein (piBRCA2) that promotes DNA strand binding (Ancot *et al*., 2015). All of these interactions are necessary in assisting DNA repair (Figure 2.4 C).

2.2.3 Different types of nucleotide variation for *BRCA1/2*

For a genetic variation to be pathogenic, it needs to translate a polypeptide that alters or inhibits the function of the BRCA protein complex (Couch *et al*., 2014). Furthermore, different populations of people have a different genetic makeup for *BRCA1/2* mutations (Hall *et al*., 2009). Although other types of mutations exist, the mutation screening techniques used were efficient at detecting 6 major types.

The first type of mutation is known as a missense mutation. This mutation changes one base pair (bp) of DNA, resulting in a substitution of one amino acid for another in the polypeptide chain (Kamburov *et al*., 2015). The second type of mutation is known as a nonsense mutation. Similar to a missense mutation, this type of DNA mutation creates a premature stop codon truncating the protein at that location (Styrkarsdottir *et al*., 2013).

The third type of mutation is called an insertion mutation. This type of mutation is brought by the introduction of an additional nucleotide or several nucleotides to the genetic sequence (Brakeleer *et al*., 2013). The fourth type of mutation is called a deletion. This mutation alters the number of bp by removing a nucleotide or section from the genetic sequence (Cancer Genome Atlas Network, 2012).

The fifth type of mutation, named a duplication mutation, occurs when a nucleotide or section of DNA is copied one or more times (Kais *et al*., 2012). The sixth type of mutation, named a frameshift mutation, is the result of additional or removed DNA to the genetic sequence. This causes a shift in the reading frame of the gene. Insertions, deletions and duplications may cause frameshift mutations (Zick *et al*., 2015).

All of the abovementioned mutations could have resulted in an altered function of the BRCA1/2 protein. An unclassified variant (UV) is a genetic difference that has not been classified in terms of its function on the protein (Shirts *et al*., 2013). Understanding which pathways and parts of the *BRCA1/2* genes were involved in tumour suppression was important for the mutation analysis of UVs.

2.3 Mutation screening

2.3.1 Laboratory molecular screening techniques

BRCA1/2 has different coding regions that differ in size and nature (Figure 2.3). The screening of these genes requires different laboratory techniques to accurately test each region.

2.3.1.1 Single-Stranded Conformation Polymorphism/ Heteroduplex Analysis

Combined Single-Strand Conformation Polymorphism and Heteroduplex Analysis (SSCP/HA) were techniques that screened the genomic regions for mutation detection (Mohyuddin *et al*., 2015). SSCP is a screening technique that detects single base changes in ssDNA. HA performed the same function as SSCP with the exception that HA detects changes in dsDNA (Hestekin *et al*., 2011). SSCP works on the principle that ssDNA folds onto itself, creating molecular stability in the absence of a second strand of DNA. When the ssDNA folds onto itself, it creates a molecular structure of a certain shape and size.

The optimal fragment size for SSCP/HA is between 150 - 300 bp. When multiple samples are run on an Acrylamide electrophoresis gel, the single strands migrate at the same speed on the gel. The moment there is a sample with a nucleotide difference in the genetic code then that sample's ssDNA folded differently from the rest of the samples *in vitro*. This specific molecular difference influenced the electrophoretic mobility of that sample. This change is observed once the molecular products are run on the Acrylamide gel followed by silver staining. For the HA, the polymerase chain reaction (PCR) products are denatured and re-annealed creating a mixture of two homoduplexes. If a sample has a genetic difference within the amplified region, two homoduplexes and two heteroduplexes are formed in a heterozygous sample. Heteroduplexes have a more distorted structure as compared to homoduplexes. This distorted structure causes the heteroduplexes to migrate slower than homoduplexes. The HA analysis has drawbacks in detecting single base changes but is very effective at detecting insertions and deletions (Nataraj *et al*., 1999).

SSCP/HA are labour intensive techniques that takes more than 24 hours to get a result. Moreover, these techniques are limited to the amount of exons that are run each day. This was only a screening technique and the exact nucleotide differences that were observed between samples had to be confirmed. In order to screen the large genomic regions of *BRCA1/2* exon 11, the Protein Truncation Test (PTT) was used.

2.3.1.2 Protein-Truncation Test

PTT is used to screen the large genomic regions of *BRCA1/2* exon 11 by translating a protein *in vitro* for an amplified PCR product (Kast *et al*., 2014). The PCR products used in this technique have an additional eukaryotic promoter sequence attached to the fragment to promote expression of the RNA target within the amplified product. PTT is limited to detecting only changes that impact the formation of the protein. These changes include all mutations that have a deleterious effect on the protein. When a sample has a truncated protein within one of the BRCA1/2 protein products, then the effect of that truncation is pathogenic (Alattraqchi *et al*., 2012).

PTT thus served a dual purpose at 1) detecting deleterious mutations and 2) functionally assessing the formation of the protein product for that fragment. Once a truncated protein was recognised with PTT, the exact location of the mutations still had to be identified (Hogervorst *et al*., 1995). In this study SSCP/HA was performed on the sub-fragments of the PTT fragments that contained the truncated protein. As each PPT fragment was large (more than 1300 bp), SSCP/HA fragments (between 150 -300 bp) were used to screen the large region (Garvin, 1997).

2.3.1.3 High Resolution Melting Analysis

HRMA is a molecular screening technique that uses fluorescence as a function of temperature to identify nucleotide differences between samples of the same genomic region (Rudnicka *et al*., 2014). This post-qPCR technique is performed in the presence of a saturating dye (Ng *et al*., 2014). The saturated dye only binds to dsDNA and leaves the molecule into the surrounding solution once it becomes ssDNA due to an increase in temperature. This temperature ramp typically occurs by increasing the temperature of the qPCR product from 60ºC to 96ºC over a five-minute period by taking several acquisitions (Thomsen *et al*., 2012).

As DNA in the sample changes from dsDNA to ssDNA, the rate of denaturation and subsequent change in fluorescence is recorded. If the genomic region is identical for all the samples, then the dsDNA melts at the same rate and intensity, giving identical fluorescence intensity for all the samples. The melt from dsDNA to ssDNA is directly dependant on the melting temperature (Tm) of the sample (Nemcova *et al*., 2015). Each nucleotide within the oligonucleotide contributes a different kind of stability to the amplicon (Fischer *et al*., 2015). If there is a nucleotide difference within one sample, then the Tm of that sample is influenced. The change in Tm causes the intensity of the fluorescence at a specific temperature to differ and the difference is detectable on the equipment. Figure 2.5 illustrates the different steps involved in the HRMA from the point the samples start melting to the point that the dsDNA dissociates into ssDNA.

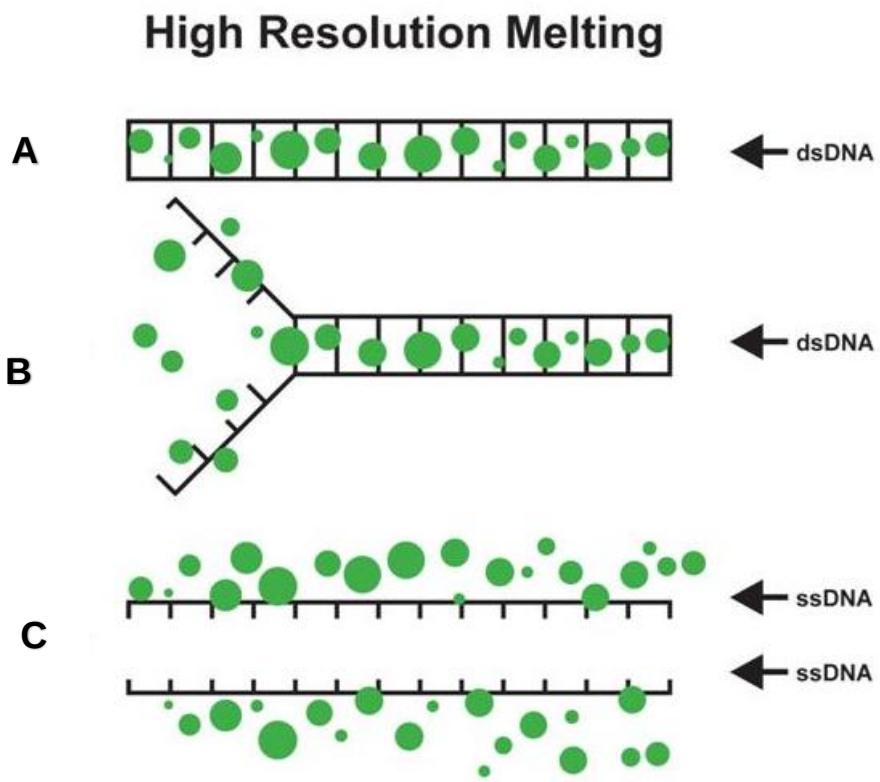

Figure 2.5 Representation of the saturated dsDNA dye as the reaction heats up (Schematic representation by the researcher, adopted from Roche laboratory manual). **A**. When the reaction is at 60ºC. **B**. When the reaction temperature increases from 60ºC to 96ºC. **C**. When the dsDNA completely dissociates into ssDNA and no dye is bound to the PCR product.

The HRMA captures and records the data of the samples, and focuses on the melt curve itself for the analysis. The melt curve is the position (in degrees) at which the dsDNA melts to ssDNA. Software initially grouped the melt curve into a negative value.

This negative value is an inverse graph of the melt curve. Next, the parameters regarding what specific temperatures within a specific range to work on was defined. This ensured that the analysis was only performed on the specific temperatures in which the dsDNA melted into ssDNA. After that the amount of background signal was reduced by what was known as a temperature shift. The temperature shift excluded a percentage of data that were generally found towards the end of the melt curve when the samples started to become homogenous within the solution. This small percentage of background noise normally ranged from 2.5 to 5%. After the parameters were established, the difference plot was calculated. The difference plot of the HRMA indicated which sample was statistically different than the rest. In the event that multiple mutant forms were present as well as the ancestral allele, the difference plot differentiated the samples that were genetically different (Erali and Wittwer, 2010). This method used a saturating DNA dye to detect sequence variants within a targeted region without the use of fluorescently labelled probes or primers (Lipsky *et al*., 2001; Kwok and Chen, 2003; Graham *et al*., 2005).

As HRMA is only a screening technique, the technique does not give the genotype of a sample based on the difference plot (Wittwer, 2009). If a sample was grouped as genetically different on the difference plot, sequencing was performed to confirm the specific nucleotide discrepancy within that specific sample. With HRMA there were no post-reaction gel electrophoresis steps like SSCP/HA and PTT (Gady *et al*., 2009). This technique was performed within one reaction, reducing contamination during the experiment (Reed *et al*., 2007).

2.3.2 Computer based analysis

Several online databases exist (Breast cancer information core (BIC) and Evidence-based Network for the Interpretation of Germline Mutation Alleles (ENIGMA)) that

document and record new disease causing mutations as well as polymorphisms that do not contribute towards a genetic predisposition to BC (https://enigmaconsortium.org/wp-content/uploads/2016/01/ENIGMA_Rules_2015-03-26.pdf) (Gelbart, 1998; Tatusova, 2016). The nature of each *BRCA1/2* mutation that altered the protein function differed in terms of the type of mutation involved. This proved difficult to establish the clinical implications of UV. Computer based testing was used as an investigative tool to provide insight into the clinical significance of an UV.

In order to understand the function of a genetic variant within *BRCA1/2* it was important to speculate the probability that a genetic variant was clinically significant. Some of the fastest ways of determining the clinical significance of a genetic variant from a speculative perspective was the use of guidelines, online tools and databases. The classification of variants for pathogenicity was based on the guidelines of ENIGMA and the BIC. Both these consortiums aimed to establish the clinical significance of UV found within the *BRCA1/2* genes (ENIGMA, 2015; NIH, 2015). The multi-evidence guidelines proposed by ENIGMA were universal and standardised approaches that assessed the pathogenicity of UV.

The Reference SNP cluster ID (rsid) are accession numbers assigned to SNPs so that researchers may universally access clusters of data for specific genetic variation. The rs number of a genetic variant refers to its rsid, and each rs number has a unique designation that refers to specific variants within the human genome (example: rs123456). When researches identify a new genetic variant, the data is reported to the NCBI dbSNP database and there is only one rsid assigned for each variant (McDonagh *et al*., 2015). The statistics and data regarding each variant, such as population group, frequency, and relevant data on the nature of the variant, were included in the rsid. The rsids of specific variants are found in the technical report section of scientific articles and sources.

One of the variant calling techniques used in this study was the use of global minor allelic frequency (MAF). All of the MAF scores were analysed by comparing the data to the 1000 Genome project phase 1 genotype database. The 1000 Genome project phase 1 genotype data consists out of full genome sequences from 1 094

individuals worldwide. MAF refers to the frequency, in a given population, of a specific allele. This statistical calling differentiates common polymorphisms from rare variants (1000 Genomes Project Consortium, 2015). All MAF scores are given in two parts. The first part of the score indicates which minor allele was detected, and at what percentage this variant was detected. The second part of the score indicates how many times this variant was observed. If a sample has an MAF score of T = 0.4570/1000 then the data represents that the minor allele "T" had a frequency of 45.70 % in the 1000 Genome phase 1 genotype data. In other words, the specific variant (T) was observed 1 000 times for 1 094 individuals in the population group (of 2 188 chromosomes) (National Center for Biotechnology Information, 2015).

The final online tool that was used for the analysis of UV was ENSEMBL. ENSEMBL was a collaborative project between several companies that designed software that stored, grouped and annotated genetic variants within eukaryotes (Yates *et* al., 2015). The genetic variants in this study were run within the above mentioned databases for analogies within genes of a similar nature or function to determine a possible effect on the protein. This type of analysis alone was not strong enough to support the pathogenicity (According to the ENIGMA guidelines) of the variant and the computational screening needed to be coupled with laboratory testing and publications to prove the clinical significance. If the databases indicated, however, that a variant was most likely to be benign, then no further laboratory testing was needed according to the guidelines.

2.4 Objectives of this study

The objectives of this study were to screen the *BRCA1* and *BRCA2* genes within the Indian population of SA by using more effective molecular screening techniques. The first part of the study was to decrease the TAT of *BRCA1/2* screening by introducing HRMA. The second part of the study was to screen 50 unrelated individuals who were at risk for Familial BC, so that new information about this diverse population group may be used in diagnostic testing.

Chapter 3

Optimisation and validation of HRMA as a mutation screening technique for *BRCA2*

3.1 Introduction

The electrophoretic detection of conformational changes in PCR-amplified DNA molecules are the basis of SSCP and HA (Orita *et al.,* 1989). The analysis of ssDNA was the basis of the SSCP analysis, whereas HA focused on heteroduplex formation between sections of dsDNA. Both these techniques are considered sensitive and inexpensive in detecting sequence variation (Sekiya, 1993).

The eventual combination of SSCP and HA into a single experiment proved to be ideal for traditional mutational analysis (Axton and Hanson, 1998). The combined method relied on the fact that a significant proportion of a PCR product re-annealed under SSCP conditions before electrophoresis (Ravnlk-Glavač *et al.,* 1994). The capturing of ssDNA and dsDNA on the same gel allowed data from both techniques to be gathered simultaneously. This resulted in a higher detection rate and an increase in sensitivity (Axton *et al.*, 1997).

Disadvantages of these techniques included large sample sizes. There needs to be a minimum of six samples per experiment, as the samples themselves serve as the controls in the experiment (Jaeckel *et al*., 1998). The techniques were also labour intensive, extremely time consuming and were always accompanied by additional methods for gel visualisation, such as silver staining (Hayashi and Yandell, 1993).

Rapid advances in technology resulted in the development of real-time based qPCR and the introduction of HRMA (De Leeneer *et al*., 2008). As a mutation detection technique, the main advantages included its close-tube system, and that no post-PCR gel electrophoresis was required (Reed and Wittwer, 2004; Jones *et al.*, 2008).

The aim of this part of the study was to optimise and implement HRMA as a mutation detection technique for *BRCA2* by using the LightCycler 480 II thermocycler (Roche), as well as to compare and validate the obtained results with the combined SSCP/HA methods to test sensitivity and specificity of HRMA.

3.2 Materials and Methods

3.2.1 Patients

3.2.1.1 Sample used for Conventional PCR and qPCR optimisation

DNA from a single individual was used to optimise the conventional PCR and qPCR reactions of the various *BRCA2* HRMA primer sets. Blood was voluntarily given after signing informed consent. This individual did not have a positive family history of breast and/or other cancer types.

3.2.1.2 Samples used for validation of HRMA

Twenty-four women affected with BC with a positive family history (a minimum of two affected family members) were used for the validation of HRMA (Table 3.1). As part of this study, these patients were also screened for *BRCA2* exons 2 - 9 and 12 - 27 using the combined SSCP/HA approach. All band shifts were sequenced and the nucleotide differences documented. These results were stored so that it could be later compared to the *BRCA2* HRMA screen, to validate the technique.

3.2.1.3 Ethical considerations

The project proposal was presented to and approved by a Postgraduate Evaluation Committee of the Faculty of Health Sciences, School of Medicine, University of the Free State. Once approved, ethical approval was obtained from the Ethics Committee of the Faculty of Health Sciences of the University of the Free State (ECUFS 107/2014, Appendix A). Authorization to perform the study was granted by the acting Business Manager of the National Health Laboratory Service and the Head of the Division of Human Genetics for using their facilities (Appendix B).

Table 3.1 Patients used for HRMA optimisation.

Sample number	Internal reference number	Population group
1	CAM2303	African
2	CAM2298	African
3	CAM2278	African
4	CAM2325	African
5	CAM2306	African
6	CAM2297	African
7	CAM2514	African
8	CAM2295	Afrikaner
9	CAM2372	Afrikaner
10	CAM2329	Afrikaner
11	CAM2318	Afrikaner
12	CAM2273	Afrikaner
13	CAM2271	Afrikaner
14	CAM2465	Indian
15	CAM2361	Indian
16	CAM2282	Indian
17	CAM2291	Indian
18	CAM2319	Coloured
19	CAM2304	Coloured
20	CAM2267	Coloured
21	CAM2369	Coloured
22	CAM2243	Coloured
23	CAM2332	Coloured
24	CAM2330	Coloured

3.2.2 Methods

3.2.2.1 DNA Extraction Methods

Peripheral blood (10 - 20 ml) was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Three different DNA extraction methods that used peripheral blood were done to determine whether the DNA extraction method had an influence on HRMA results.

3.2.2.2 DNA Extraction using the Promega Wizard Extraction Kit

Peripheral blood (10 - 20 ml) was mixed with the provided cell lysis solution according to the volumes indicated on the package insert (Promega Corp., Madison, WI). The hypotonic solution was left for 10 min at room temperature to lyse the cells, where after it was centrifuged for 2 min at 4000 *g*. The supernatant was discarded and 300 µl nuclei lysis solution added to the leucocyte pellet. After vortexing for 20 sec to loosen the pellet, a protein precipitation solution was added to the mix. The tube was inverted repeatedly to mix the sample and centrifuged for 3 min at 4 000 *g* at room temperature.

The supernatant was transferred to a new tube containing 400 µl 100% isopropanol. The sample was mixed gently for 10 min, whereafter it was centrifuged for 3 min at 4 000 *g*. The pellet was washed with 70% (v/v) ethanol. After the centrifuge step for 3 min at 4000 *g,* the pellet was left to dry. The precipitated DNA was rehydrated in 50 µl $T_{.1}E$ buffer (10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) pH 8.0, 0.1 mM EDTA) and stored at -20°C.

3.2.2.3 DNA Extraction using the Salting Out Method

DNA was extracted using the salting out procedure of Miller *et al*. (1988). Peripheral blood (10 - 20 ml) was transferred to 50 ml tubes and stored at -20°C for a minimum of 4 h. Once frozen, the blood was thawed by slowly rotating the tubes on an orbital shaker.

Lysis buffer (10 mM Tris, 0.3 M sucrose, 5 mM $MgCl_2$, 1% (v/v) t-octylphenoxypolyethoxyethanol (Triton X100)) was added and the mixture placed on ice for 10 min. The mixture was centrifuged for 20 min at 1 914 *g* at 4°C after which the supernatant was discarded. The pellet was re-suspended in 10 $\mu g.\mu l^{-1}$ proteinase K, 1% (w/v) sodium dodecyl sulphate (SDS), 1 X SET (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA). The samples were incubated overnight at 37°C, whereafter 5 mM NaCl was added. The solution was agitated vigorously for 15 sec, after which the tubes were centrifuged at 1 914 *g* at 15°C for 15 min. After centrifugation, the supernatant was transferred to a new tube containing 20 ml 100% EtOH. DNA was precipitated for a minimum of 15 min after which the DNA was transferred to a 1.5 ml Eppendorf tube containing 1.2 ml 70% (v/v) EtOH. The DNA was washed for a minimum of 2 h. DNA was finally pelleted by centrifugation for 10 min at 4 000 *g* at room temperature and the supernatant discarded. The DNA was left to air dry, after which it was dissolved in 100 µl $T._1E$.

3.2.2.4 DNA Extraction using Phenol/Chloroform Method

This extraction method was adopted from Kramvis *et al*. (1996). The first three steps of the phenol/chloroform method are similar to that described for the salting out method (3.3.1.2). After the overnight incubation period, 7 ml of phenol and chloroform:isoamyl alcohol (24:1) were added to the lysed solution. The tubes were gently rolled on an orbital shaker for 60 min, whereafter it was centrifuged for 15 min at 1 914 *g* at room temperature. The supernatant was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added. The sample was mixed for another 60 min, after which it was centrifuged for 15 min at 1 914 *g*. The supernatant was transferred to a tube containing 1 ml 0.3 M NaAc and 40 ml 100% ethanol and placed on the orbital shaker for 10 min. The precipitated DNA was transferred to a 1.5 ml tube containing 70% (v/v) EtOH. The tubes were slowly shaken for a minimum of 60 min whereafter it was centrifuged for 5 min at 4 000 *g* at room temperature. The supernatant was discarded and the dried pellet dissolved in 100 µl $T._1E$ buffer.

3.2.2.5 Ensuring DNA Quality and Quantity

As DNA quality was critical for HRMA, the quantity and purity of the extracted DNA was determined with the NanoDrop®ND-100 Spectrophotometer (v3.01, NanoDrop Technologies) according to the manufacturer's instructions. The concentration of the sample was expressed as ng.µl^{-1}. The purity of the extracted DNA was also determined. The ratio of absorbance at 260 nm and 280 nm (ideally ~1.8) was used to determine the presence of contaminants such as proteins or phenol. The ratio of absorbance at 230 nm and 260 nm (ideally ~2.0 - 2.2) was used to determine the presence of contaminants (Technical Bulletin, NanoDrop - http://www.nanodrop.com/Library/T042-NanoDrop-spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf). The stock DNA was diluted to a concentration of 50 ng.µl^{-1} in T.$_1$E for the initial optimisation of the conventional PCR reactions.

3.2.3 PCR reactions

3.2.3.1 Primer Sets for *BRCA2*

The sequences of 35 primer sets used for qPCR of *BRCA2,* excluding exons 10 and 11, were obtained from the Centre of Human and Clinical Genetics, Leiden University Medical Centre, Leiden, The Netherlands (Table 3.2). The primers were homologous to the reported *BRCA2* gene sequence (accession MIM 600185, Genbank accession number U43746). An M13 primer tail was added to both the forward and reverse primer sequences to allow direct sequencing analysis. The primer lengths varied from 19 to 30 nucleotides, excluding the M13 tail, and produced amplicons of between 155 and 400 bp. The sequence of each set was tested for specificity using genome sequence database analyses such as BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The primer sets were manufactured and HPLC purified by Gibco® (Life Technologies). Each primer set was diluted from stock in a T.$_1$E buffer to a final concentration of 20 µM. Each primer set was further diluted to 3 µM for PCR reactions as suggested by van der Stoep *et al.* (2008).

Table 3.2 *BRCA2* primer sets used in the HRMA. Primer sequences were obtained from van der Stoep (personal communication) for mutation screening using HRMA of all *BRCA2* exons excluding exons 10 and 11. Indicated are the 5' - 3' sequence with the M13 sequence indicated in bold.

Primer name	Primer sequence (5' - 3')	Annealing temperature and number of cycles	Expected fragment size in bp
HRM BR2ex2F	**TGTAAAACGACGGCCAGT**TTCCAGCGCTTCTGAGTTTT	61/40	264
HRM BR2ex2R	**CAGGAAACAGCTATGACC**TGGGTTTTTAGCAAGCATTTTT		
HRM BR2ex3.1F	**CAGGAAACAGCTATGACC**TCTTTAACTGTTCTGGGTCACAA	61/40	285
HRM BR2ex3.1R	**TGTAAAACGACGGCCAGT**GAGATTGGTACAGCGGCA		
HRM BR2ex3.2F	**TGTAAAACGACGGCCAGT**CAACAATTACGAACCAAACCTAT	61/40	209
HRM BR2ex3.2R	**CAGGAAACAGCTATGACC**TGCCTAAATTCCTAGTTTGTAGT		
HRM BR2ex4F	**TGTAAAACGACGGCCAGT**AAGAATGCAAATTTATAATCCAGAGT	61/40	285
HRM BR2ex4R	**CAGGAAACAGCTATGACC**TTCTACCAGGCTCTTAGCCA		
HRM BR2ex5F	**TGTAAAACGACGGCCAGT**CCAGCAGCTGAAATTTGTGA	60/40	355
HRM BR2ex5R	**CAGGAAACAGCTATGACC**AAAAGGGGAAAATTGTTAAGTTTTA		

Primer name	Primer sequence (5’ - 3’)	Annealing temperature and number of cycles	Expected fragment size in bp
HRM BR2ex6F	**TGTAAAACGACGGCCAGT**AAAACTTAACAATTTTCCCCTTTTT	60/40	192
HRM BR2ex6R	**CAGGAAACAGCTATGACC**TGCCTGTATGAGGCAGAATG		
HRM BR2ex7F	**TGTAAAACGACGGCCAGT**TCCTTAATGATCAGGGCATTTC	61/40	225
HRM BR2ex7R	**CAGGAAACAGCTATGACC**TGACAATTATCAACCTCATCTGC		
HRM BR2ex8F	**TGTAAAACGACGGCCAGT**TGTGCTTTTTGATGTCTGACAAA	60/40	308
HRM BR2ex8R	**CAGGAAACAGCTATGACC**GAGACAGCAGAGTTTCACAGGA		
HRM BR2ex9F	**TGTAAAACGACGGCCAGT**TAAGGGGGGACTACTACTATATGTGC	61/40	280
HRM BR2ex9R	**CAGGAAACAGCTATGACC**GAGATCACGGGTGACAGAGC		
HRM BR2ex10.1F	**TGTAAAACGACGGCCAGT**TTCTATGAGAAAGGTTGTGTAGAATAAT	60/40	400
HRM BR2ex10.1R	**CAGGAAACAGCTATGACC**GCTACATTTGAATCTAATGGATCAGTAT		
HRM BR2ex10.2F	**TGTAAAACGACGGCCAGT**AAACCAAGTGAAAGAAAAATACTCATTTGT	63/40	373
HRM BR2ex10.2R	**CAGGAAACAGCTATGACC**ATCTCTCTTATTTACCACTGTTTCCTC		
HRM BR2ex10.3F	**TGTAAAACGACGGCCAGT**GCCACGTATTTCTAGCCTACC	61/40	399
HRM BR2ex10.3R	**CAGGAAACAGCTATGACC**GCCACGTATTTCTAGCCTACC		
HRM BR2ex10.4F	**TGTAAAACGACGGCCAGT**GTCCAAATTTAATTGATAATGGAAGC	61/40	314
HRM BR2ex10.4R	**CAGGAAACAGCTATGACC**CACAGAAGGAATCGTCATCTA		
HRM BR2ex12F	**TGTAAAACGACGGCCAGT**ATTTTTGTTTAACATTTAAAGAGTCAATAC	60/40	281
HRM BR2ex12R	**CAGGAAACAGCTATGACC**GAGGTCAGAATATTATATACCATACCTA		

Primer name	Primer sequence (5’ - 3’)	Annealing temperature and number of cycles	Expected fragment size in bp
HRM BR2ex13F	**TGTAAAACGACGGCCAGT**ACAGTAACATGGATATTCTCTTA	61/40	189
HRM BR2ex13R	**CAGGAAACAGCTATGACC**AAACGAGACTTTTCTCATACTG		
HRM BR2ex14.1F	**TGTAAAACGACGGCCAGT**ATTCCTAAATATTTATATGTGTACTAGTCA	60/40	390
HRM BR2ex14.1R	**CAGGAAACAGCTATGACC**TTACTATCATCAGAGCCATGTC		
HRM BR2ex14.2F	**TGTAAAACGACGGCCAGT**ACAAGAAATGAAAAAATGAGACACT	63/40	357
HRM BR2ex14.2R	**CAGGAAACAGCTATGACC**GGGAAAACCATCAGGACATTAT		
HRM BR2ex15F	**TGTAAAACGACGGCCAGT**GCCAGGGGTTGTGCTTTTA	61/40	284
HRM BR2ex15R	**CAGGAAACAGCTATGACC**CTCTGTCATAAAAGCCATCAG		
HRM BR2ex16F	**TGTAAAACGACGGCCAGT**TTTGGTAAATTCAGTTTTGGTTTG	61/40	379
HRM BR2ex16R	**CAGGAAACAGCTATGACC**GCCAACTTTTTAGTTCGAGAGA		
HRM BR2ex17F	**TGTAAAACGACGGCCAGT**TTGAATTCAGTATCATCCTATGTGG	61/40	353
HRM BR2ex17R	**CAGGAAACAGCTATGACC**GTGGGATGGCAACTGTCACT		
HRM BR2ex18.1F	**TGTAAAACGACGGCCAGT**TTTAAACAGTGGAATTCTAGAGTCACA	61/40	284
HRM BR2ex18.1R	**CAGGAAACAGCTATGACC**TCTAACTGGGCCTTAACAGCATA		
HRM BR2ex18.2F	**TGTAAAACGACGGCCAGT**TCTAGCAATAAAACTAGTAGTGCAGATA	61/40	283
HRM BR2ex18.2R	**CAGGAAACAGCTATGACC**AAACTTCTAGAATTTAACTGAATCAATG		
HRM BR2ex19.1F	**TGTAAAACGACGGCCAGT**ATGAAAACTCTTATGATATCTGTAATAGAA	61/40	210
HRM BR2ex19.1R	**CAGGAAACAGCTATGACC**ATTACATCAACACAACCAACAT		

Primer name	Primer sequence (5’ - 3’)	Annealing temperature and number of cycles	Expected fragment size in bp
HRM BR2ex19.2F	**TGTAAAACGACGGCCAGT**CTCTGCCCTTATCATCGCTT	61/40	175
HRM BR2ex19.2R	**CAGGAAACAGCTATGACC**GGCAAGAGACCGAAACTCC		
HRM BR2ex20F	**TGTAAAACGACGGCCAGT**CCTGGCCTGATACAATTAACT	60/40	276
HRM BR2ex20R	**CAGGAAACAGCTATGACC**AGTCTCTAAGGACTTTGTTCTCA		
HRM BR2ex21F	**TGTAAAACGACGGCCAGT**TTTTAGTTGCTTTTGAATTTACAG	61/40	262
HRM BR2ex21R	**CAGGAAACAGCTATGACC**TCCTGTGATGGCCAGAGAGT		
HRM BR2ex22F	**TGTAAAACGACGGCCAGT**ACATTAACCACACCCTTAAGAT	61/40	395
HRM BR2ex22R	**CAGGAAACAGCTATGACC**TCATTTTGTTAGTAAGGTCATTTTT		
HRM BR2ex23F	**TGTAAAACGACGGCCAGT**CAAACATTTAAATGATAATCACTTCTTCC	61/40	285
HRM BR2ex23R	**CAGGAAACAGCTATGACC**GGAGATTCCATAAACTAACAAGC		
HRM BR2ex24.1F	**TGTAAAACGACGGCCAGT**TTTATGGAATCTCCATATGTTGA	61/40	155
HRM BR2ex24.1R	**CAGGAAACAGCTATGACC**CCTATTAGGTCCACCTCAG		
HRM BR2ex24.2F	**TGTAAAACGACGGCCAGT**CAGCAAATTTTTAGATCCAGAC	63/40	174
HRM BR2ex24.2R	**CAGGAAACAGCTATGACC**CTGGTAGCTCCAACTAATCAT		
HRM BR2ex25.1F	**TGTAAAACGACGGCCAGT**TTCTTGCATCTTAAAATTCATCTAACAC	60/40	211
HRM BR2ex25.1R	**CAGGAAACAGCTATGACC**CCTGATTTGGATTCTGGTCG		
HRM BR2ex25.2F	**TGTAAAACGACGGCCAGT**AGGACATTATTAAGCCTCATATGTTAATTG	61/40	244
HRM BR2ex25.2R	**CAGGAAACAGCTATGACC**GCTATTTCCTTGATACTGGACTGT		

Primer name	Primer sequence (5’ - 3’)	Annealing temperature and number of cycles	Expected fragment size in bp
HRM BR2ex26F	**TGTAAAACGACGGCCAGT**TGGGTTTGCAATTTATAAAGCAG	63/40	254
HRM BR2ex26R	**CAGGAAACAGCTATGACC**CAGAATATACGATGGCCTCCA		
HRM BR2ex27.1F	**TGTAAAACGACGGCCAGT**TTTCAATGAAAAGTTACTTTGATTTAGTT	61/40	400
HRM BR2ex27.1R	**CAGGAAACAGCTATGACC**GTCATCTGAGGAGAATTCAGT		
HRM BR2ex27.2F	**TGTAAAACGACGGCCAGT**TTGTGGCACCAAATACGAA	61/40	397
HRM BR2ex27.2R	**CAGGAAACAGCTATGACC**AACTGGAAAGGTTAAGCG		

3.2.3.2 PCR reaction for High Resolution Melting Analysis

Each 10 µl PCR reaction contained 30 ng genomic DNA, 0.3 µM of each primer and 4 µl LightScanner® Mastermix (BioFire Diagnostics Inc, Salt Lake City, UT). Thermal cycling conditions for a 96-well plate entailed the following: pre-incubation of one cycle at 95°C for 10 min (ramp rate of 4.4°C/s), followed by amplification steps consisting of 40 cycles at 95°C for 10 sec (ramp rate of 4.4°C/s), primer dependent annealing temperature for 15 sec (ramp rate of 2.2°C/s) and 72°C for 10 - 25 sec, depending on the length of the amplicon (ramp rate of 4.4°C/s) (van der Stoep *et al.*, 2008). The amplified products were separated on a 2% (w/v) agarose gel to inspect the quality and specificity of each PCR product. The gels were run with 1x Tris-Borate-EDTA buffer (0.089 M Tris pH 8, 0.089 M boric acid, 2 mM EDTA) at 120 V for 40 min.

3.2.3.3 High Resolution Melting Analysis

An immediate high resolution melt followed amplification and consisted of a single cycle starting at 95°C for 1 min (ramp rate of 4.4°C/s) to 40°C for 1 min to allow heteroduplex formation (ramp rate of 2.2°C/s), whereafter the actual melting (Tm) was achieved by gradually increasing the temperature from 60°C to 95°C. Single acquisitions were recorded during the elongation step of amplification, but were continuous (25 acquisitions per °C) during the high resolution melt. The final phase of the HRM consisted of cooling for 10 sec at 40°C (ramp rate of 4.4°C/s). Each of the primer sets was optimised using this protocol as stipulated (Roche Diagnostics, Mannheim, Germany).

3.2.4 Combined Single-Strand Conformation Polymorphism/ Heteroduplex Analysis

The amplification protocol for conventional SSCP/HA PCR entailed one cycle at 95°C for one min, followed by 32 cycles at 94°C for 45 sec, optimal annealing temperature for 1 min and 72°C for 45 sec, with a final elongation step at 72°C for 10 min (ramp rate of 4.4°C/s).

Each 50 µl PCR reaction contained 300 ng template DNA, 20 mM exon specific primers, 250 µM deoxyribonucleotide triphosphate, 100 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl and 1 U *Taq* DNA polymerase. For each reaction, 10 µl loading buffer (95% (v/v) formamide, 0.05% (w/v) xylene cyanol FF, 0.05% (w/v) bromophenol blue, 1 mM EDTA (pH 8.0)) was added post-PCR amplification. The reactions were denatured at 94°C for 5 min and snap-cooled on ice for 5 min. The samples were run on a polyacrylamide gel (PAGE) (37.5 acrylamide:1 bis - acrylamide, 2.7% cross linking) containing 1 x TBE buffer overnight on a SE600 vertical electrophoresis system (Hoefer Pharmacia Biotech Inc.). The system was attached to a temperature regulating water bath. The optimal running temperature varied from 12° - 17°C and depended on the size of the appropriate PCR fragment. Gels were electrophoresed for a minimum of 16 h at a constant voltage of 260 – 280 V.

The gel was suspended in 1 M dithiothreitol (DTT) containing 0.5 M KOAc (pH 4.5) for 10 min, followed by 30 sec in deionized water. This was followed by a submerged gel in 0.1% (w/v) silver nitrate solution for 10 min, rinsing for 1 min in deionized water, the gel was developed with 1.5% (w/v) $NaCO_3$, 0.155% (v/v) formaldehyde until bands were visible (Pinar *et al*., 1997; Bassam *et al*., 1991). The reaction was stopped using 0.01 M citric acid for 10 min. The gel was rinsed in distilled water digitally captured on the Bio-Rad Gel documentation system (Bio-Rad Laboratories Inc., Hercules, CA).

3.2.5 Sanger Sequencing

The product of the HRMA was bi-directionally sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Scientific Corp, Waltham, MA). Each 10 µl sequencing reaction contained 3 µl PCR product, 1 µl Ready Reaction mix, 3.2 mM primer, 2 µl BigDye® sequencing buffer and 4 µl PCR product. The amplification regime was: one cycle at 96°C for 1 min, followed by 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, with a final holding temperature at 4°C. The sequenced products were precipitated by adding 5 µl 125 mM EDTA and 60 µl 100% ethanol, followed by a 30 min incubation step at room temperature in

the dark. After centrifugation at 4 000 *g* for 30 min at room temperature, the supernatant was aspirated, the pellet washed once with 70% (v/v) ethanol followed by an air dry step, which was dissolved in 100 µl $T._1E$ buffer.

Hi-Di™ formamide buffer was added to the pellet, whereafter it was denatured for 5 min at 96°C and the tubes snap-cooled in a cold block (0 - 5°C). The pellets were suspended by pipetting, and analysed on an ABI Genetic Analyzer. The electropherograms were analyzed by visual inspection using proprietary sequence analysis software (Chromas version 2.31, www.technelysium.com.au). The sequences were aligned to the reference sequences (NM_00059.3 for *BRCA2*) with LALIGN (www.ch.embnet.org/software/LALIGN), whereafter translation was performed with the Expasy translate tool (http://au.expasy.org/tools/dna.html).

3.3 Results

3.3.1 Optimisation of qPCR and High Resolution Melting Analysis

DNA was extracted from a single individual using three different DNA extraction methods. This sample served as the sole source of genomic DNA that illustrated the effect of various components on the optimisation and analysis of HRMA.

The three different extraction methods gave different amounts of DNA ranging from 14.7 to 18.0 µg for the Promega Wizard extraction to 84.6 µg for the phenol:chloroform method (Table 3.3). The $OD_{260/230}$ ratio was the best for the salt extraction (average of 2.21), whereas the Promega Wizard extraction had the worst quality parameters (average of 2.02). For the $OD_{260/280}$ value, both the salt and phenol:chloroform methods performed excellent and had an average of 1.9, whereas the average value for the Promega Wizard extraction was 1.85. All the primers pairs for *BRCA2* (Table 3.2) were tested for optimal annealing temperature and reaction composition as proposed by van der Stoep *et al*. (2008). An example of the optimal PCR products of three exons, namely exon 2, 22 and 24, can be seen in Figure 3.1.

Table 3.3 DNA quantity and quality for different extraction methods, repeated in triplicate.

DNA Extraction method	Final µg	$OD_{260/230}$	$OD_{260/280}$
Salt extraction	65.08	2.20	1.89
Salt extraction	71.84	2.18	1.90
Salt extraction	71.51	2.26	1.91
Phenol:Chloroform extraction	72.34	2.21	1.89
Phenol:Chloroform extraction	82.32	2.17	1.90
Phenol:Chloroform extraction	84.65	2.19	1.90
Promega Wizard extraction	17.76	1.96	1.79
Promega Wizard extraction	18.08	2.02	1.84
Promega Wizard extraction	14.71	2.09	1.92

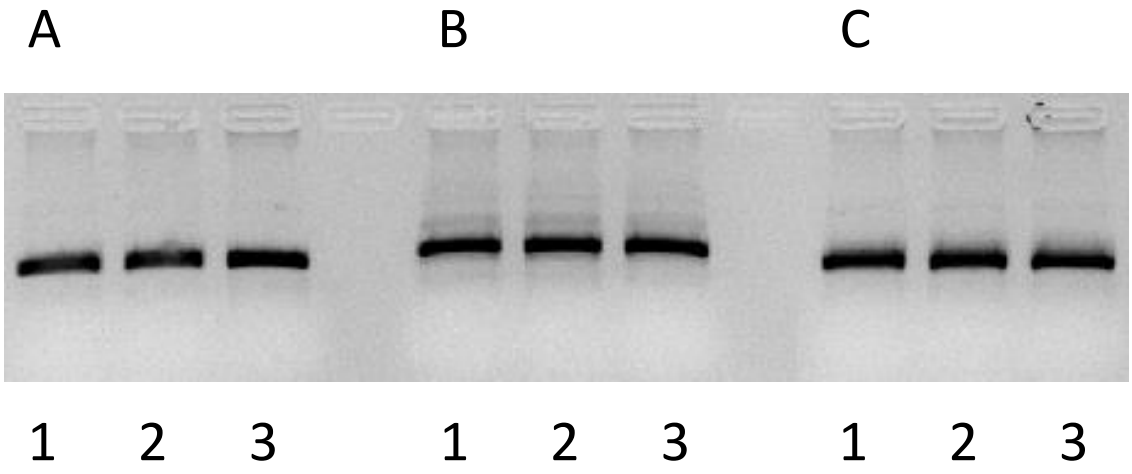

Figure 3.1 Agarose gel visualization of conventional PCR products for *BRCA2,* repeated in triplicate for each of the three extraction methods. **A**. PCR product for exon 2 in one individual repeated in triplicate. **B**. PCR product for exon 22 of one individual repeated in triplicate. **C**. PCR product of exon 24 of one individual repeated in triplicate.

The qPCR regime, as published within the package insert of the High Resolution Melting Dye designed for the LC480 II from Roche (Roche Diagnostics) proved to be optimal. An example of the amplification regime for qPCR that was adjusted to have a crossing threshold (Ct) between 30 and 50 can be seen in Figure 3.2 with exons 5 and 7.

The normalisation, temperature shift, as well as the difference plot was derived from the negative calling of the HRMA. The negative curve was captured during qPCR with the acquisitions taken from 60°C to 96°C for a 5 min period at a rate of 25 acquisitions per 1°C. The criteria for normalisation composed of moving the gliders to the area just before and after the melt, and adjusting them in such a way that the two bars were 1°C apart on both sides.

The temperature shift was adjusted to exclude the last 3% of the melt curve data. This adjustment was performed on all the different experimental parameters tested for *BRCA2*. The difference plot was performed using the default sensitivity settings and algorithm used in the analysis software. If a fluorescence deviation of 1.5 units from the baseline occurred, the sample was called as different from the rest based on the difference in fluorescence intensity.

Three experiments were performed to test and illustrate the following factors, namely i) the effect of additional $MgCl_2$, ii) the influence of the DNA dilution buffer (water versus $T._1E$) with the addition or omission of $MgCl_2$ and iii) varying DNA amounts in each reaction. The Mastermix contained $MgCl_2$ at a concentration of 1.5 mM.

Figure 3.3 showed the influence of additional 1 mM $MgCl_2$ for three DNA extraction methods diluted in $T._1E$ for a single individual. The samples for the Promega Wizard method and phenol:chloroform methods were grouped on the baseline (Figure 3.3 Aii).

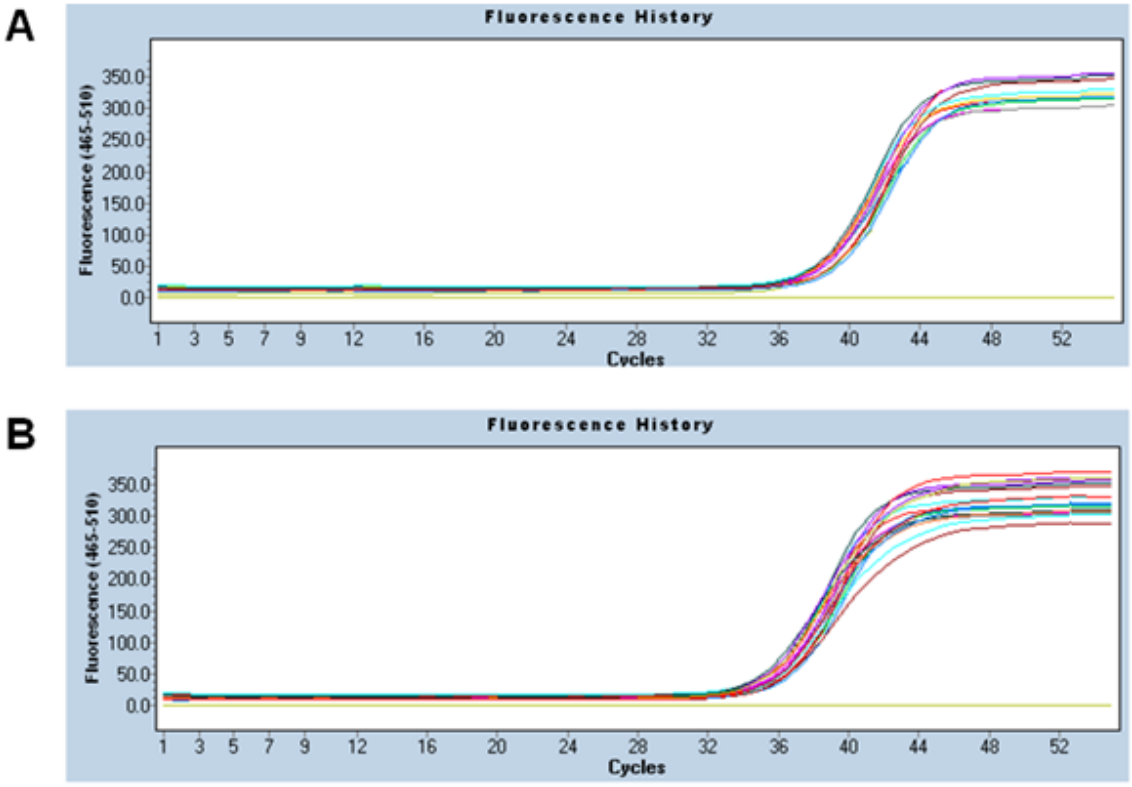

Figure 3.2 qPCR and number of cycle differences. **A**. qPCR of *BRCA2* exon 5. **B**. qPCR of *BRCA2* exon 7 showing that the Ct of the exons was approximately 38 with 10 additional cycles were added to the amplification regime.

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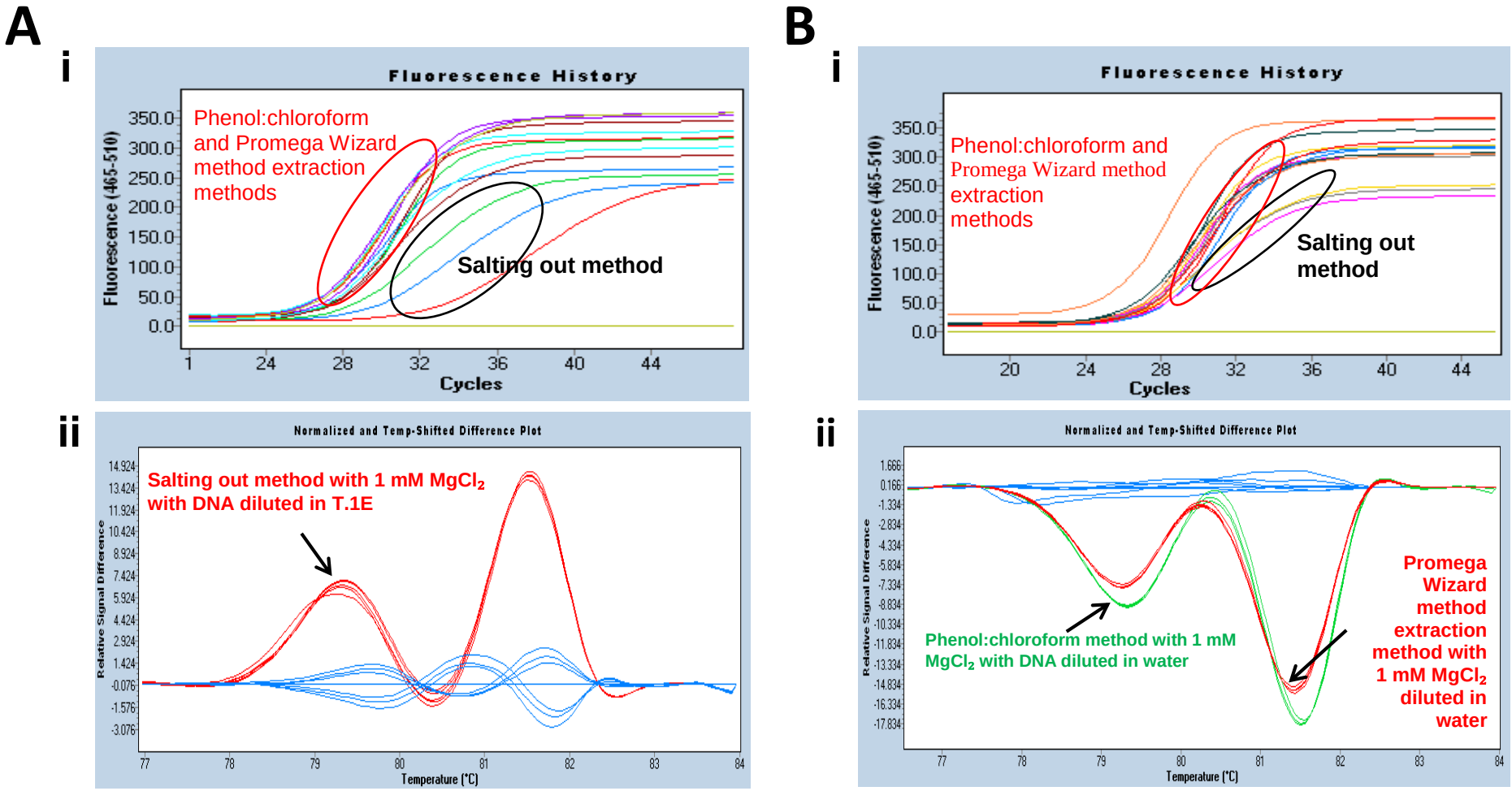

Figure 3.3 HRMA results for *BRCA2* exon 2 for a single individual, using differently extracted genomic DNA samples. Comparison of the effect of the DNA elution buffer on the HRMA results, with and without the addition of 1 mM $MgCl_2$. **A**. Differently extracted DNA samples diluted in $T._1E$, with the addition of 1 mM $MgCl_2$. i. The qPCR amplification plot. ii. HRMA difference plot indicating the presence of a distinctly different group from the base line (false positive), namely the red curve (salt extracted DNA diluted in $T._1E$ with the addition of 1 mM $MgCl_2$). **B**. Differently extracted DNA samples diluted in water, without the additional 1 mM $MgCl_2$. i. The qPCR amplification plot. ii. HRMA difference plot indicating the presence of two distinctly different groups from the base line (false positives), namely green (Phenol:cloroform extracted DNA diluted in water) and red curves (Promega Wizard method extraction diluted in water).

Figure 3.3 showed the influence of additional 1 mM MgCl for three DNA extraction methods diluted in water. Although the water diluted DNA for the Promega Wizard method and phenol:chloroform methods amplified successfully (Fig 3.3 Bi), these two extraction methods delivered false positive results on the difference plot in the presence of 1 mM $MgCl_2$ (green and red curves - Figure 3.3 Bii). These curves had a relative signal difference of more than 7.334 units.

Since Figure 3.3 illustrated that additional $MgCl_2$ affected HRMA for the different DNA extraction methods, an experiment was performed to determine the influence of the DNA dilution buffer. Two dilution solutions were compared, namely water versus $T._1E$ using DNA extracted with the DNA salt extraction. In this experiment, no additional $MgCl_2$ was added to any of the reactions. The dilution of the DNA in either of the two solutions did not influence the HRMA (Figure 3.4), and all the reactions were grouped on the baseline with no false positive curves present in the difference plot (less than 1.5 relative signal difference from the baseline).

Next, the effect of different DNA amounts on HRMA were evaluated. Three different DNA dilutions were prepared that contained a final amount of 30 ng, 60 ng and 100 ng respectively in each reaction separately. All the dilutions were prepared using water with no additional $MgCl_2$. No distinct differences were observed (Figure 3.5), except for a low degree of variation in the crossing threshold of the various reactions within the amplification curve (Figure 3.5 A). All the samples grouped together and melted in a similar way (Figure 3.5 B - D). No differences were observed in the difference plot, as all the reactions grouped close to the baseline (less than 1.5 relative signal difference from the baseline) (Figure 3.5 E).

3.3.2 Testing optimisation of HRMA

In order to test optimisation, a set of 24 BC patients (Table 3.1) were selected and screened for *BRCA2* using SSCP/HA. An example of an exon with no genetic variation was *BRCA2* exon 2.

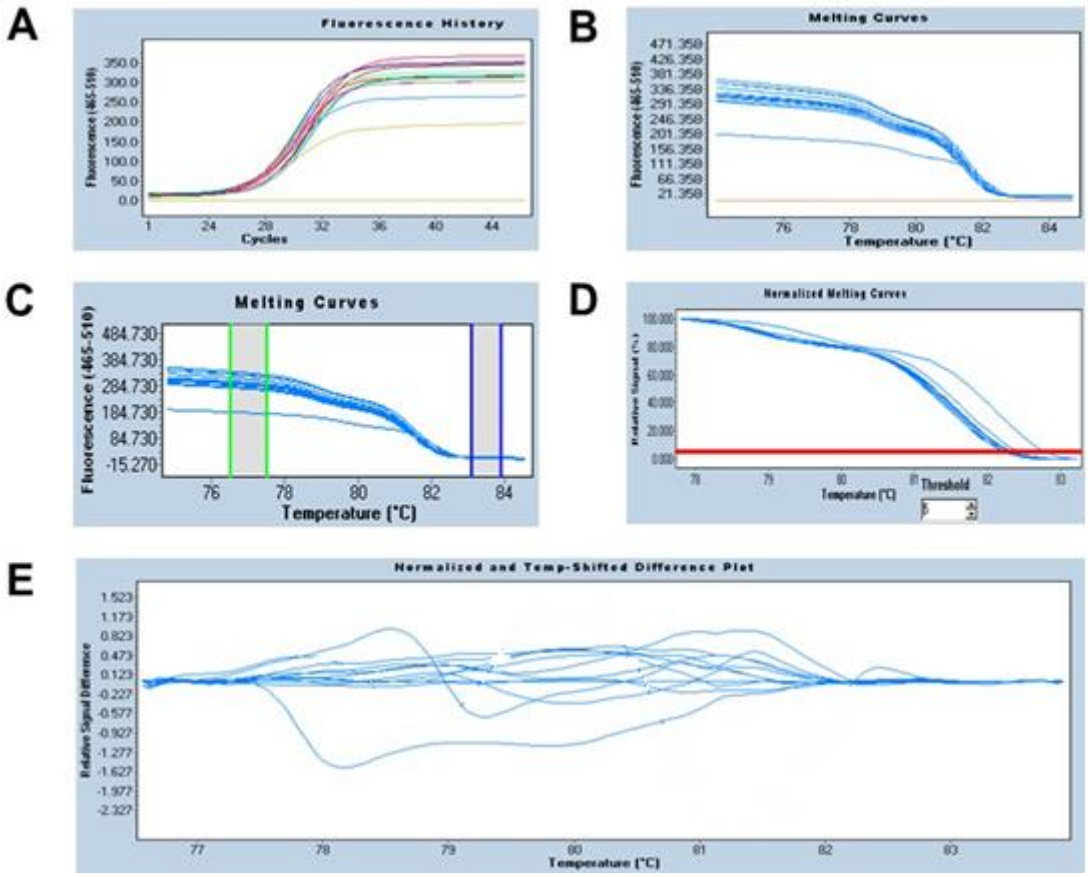

Figure 3.4 Effect of different dilution buffers on HRMA. **A**. The qPCR amplification curves. **B**. The negative calling. **C**. Normalisation of amplification curves. **D**. The temperature shift. **E**. The HRMA difference plot for the experiment with all samples grouping on the baseline with a relative signal difference of less than 2.3 units.

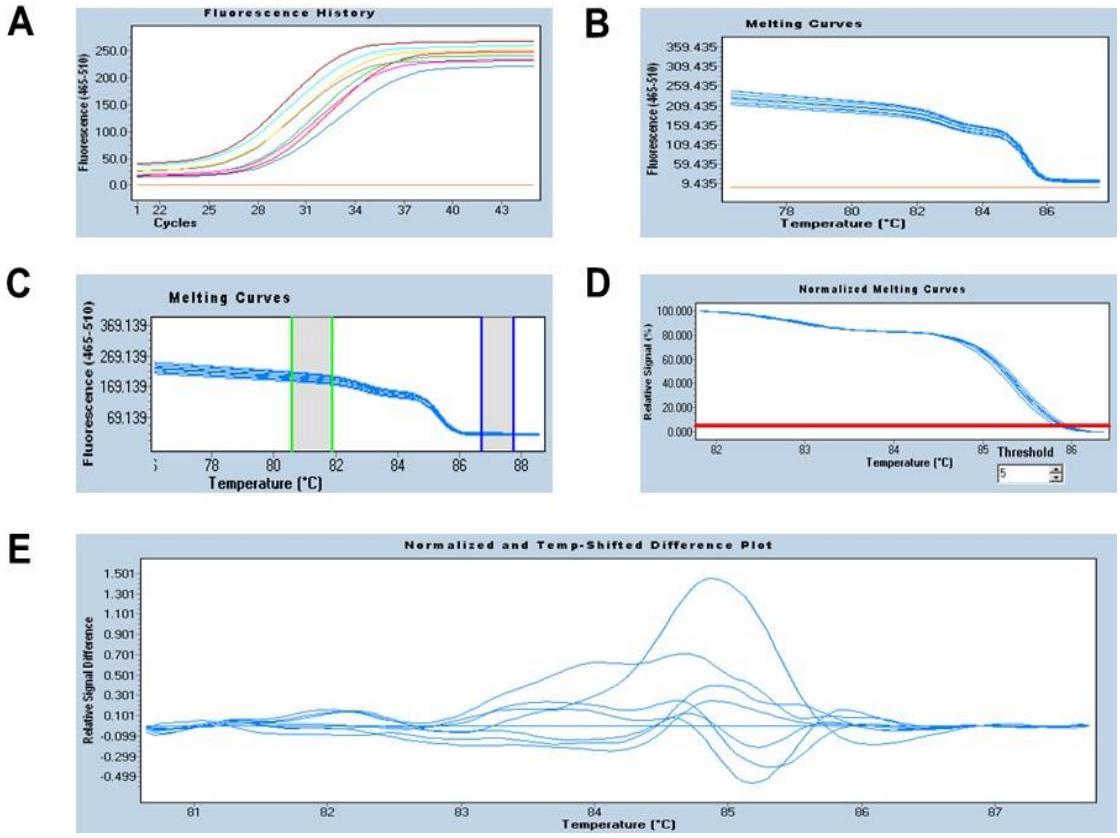

Figure 3.5 HRMA results for DNA samples extracted with the salting out method, diluted in water using different DNA amounts (30 ng, 60 ng and 100 ng). **A**. The qPCR amplification curves. **B**. The negative calling. **C**. Normalisation of amplification curves. **D**. The temperature shift. **E**. The HRMA difference plot for the experiment, with all samples grouping on the baseline with a relative signal difference of less than 1.5 units.

All the reactions grouped together and melted in exactly the same way (Figure 3.6). No differences were observed in the difference plot (Figure 3.6 E), as all the samples grouped close to the baseline with less than 1.5 relative signal difference. All the quality parameters (3.4.1), such as using salt extracted DNA with no additional $MgCl_2$, diluted in water with a final amount of 30 ng DNA were used in the rest of the HRMA reactions.

3.3.3 Validation of HRMA as Mutation Screening technique

To validate whether the optimised HRMA regime was able to detect true variants, an experiment was conducted that included a known *BRCA2* exon 2 variant (*BRCA2* c.-26G>A, g.32890572G>A) against individuals who were previously screened and tested negative for this variant (Figure 3.7). The sample highlighted in red (Figure 3.7 E) contained the variant while all the samples in blue were DNA from individuals who did not contain the variant.

HRMA was then performed on 24 patients that were screened for *BRCA2* exons 2 - 9 and 12 - 27. The results from these three complete tests (SSCP/HA and HRMA) were compared against one another to validate the results for HRMA. All exons that exhibited band shifts for any of the techniques were sequenced to determine the precise DNA alteration.

An example of an exon that had homozygous (c.8755-66 (CC)), heterozygous (c.8755-66T>C) and the wild type (c.8755-66 (TT)) form within a genetic region was exon 22. Figures 3.8 showed the identification of these alleles on both SSCP/HA and HRMA.

Table 3.4 summarised the data of the 24 patients that were screened using SSCP/HA as well as HRMA. The data presented in this table showed that the same genetic variation that was detected with SSCP/HA, was also detected with HRMA. There were no differences in the detection of genetic variants between the two techniques.

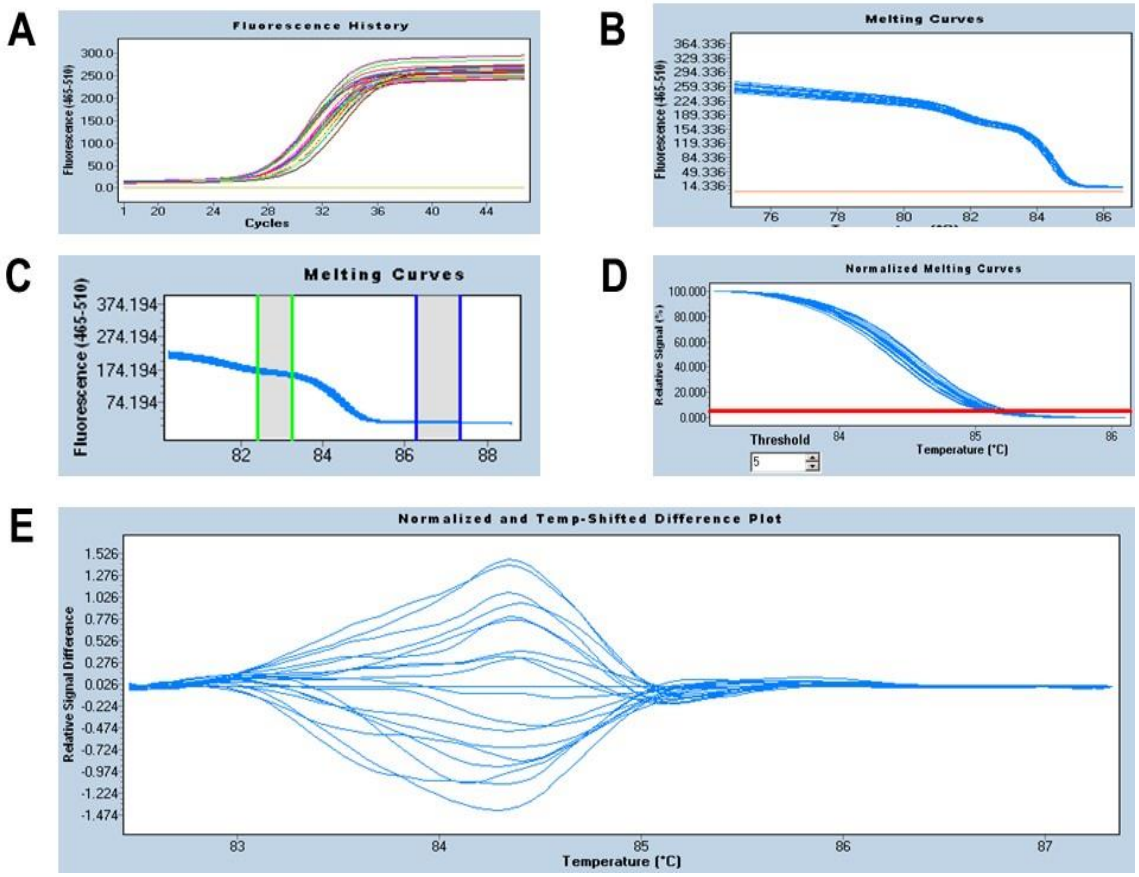

Figure 3.6 HRMA results for *BRCA2* exon 2 from 24 previously screened samples. **A**. The qPCR amplification curve. **B**. The negative calling. **C**. Normalisation of the amplification curves. **D**. The temperature shift. **E**. The HRMA difference plot for the experiment, with all samples grouping on the baseline with a relative signal difference of less than 1.5 units.

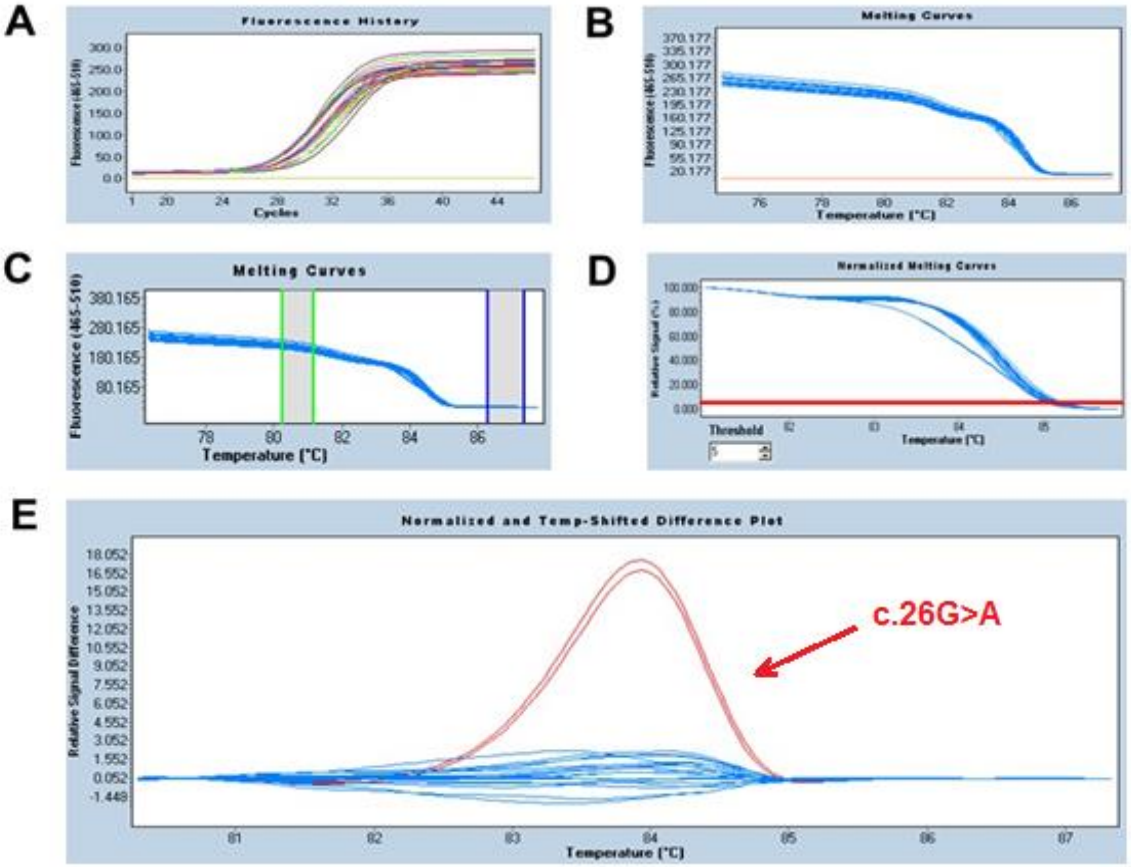

Figure 3.7 HRMA results for exon 2 for the *BRCA2* c.26G>A variant. **A**. The qPCR amplification curve. **B**. The negative calling. **C**. Normalisation of the amplification curves. **D**. The temperature shift. **E**. The HRMA difference plot for the experiment, with the variant (red curve) grouping separate from the samples on the baseline, with a relative signal difference of ~17.0 units.

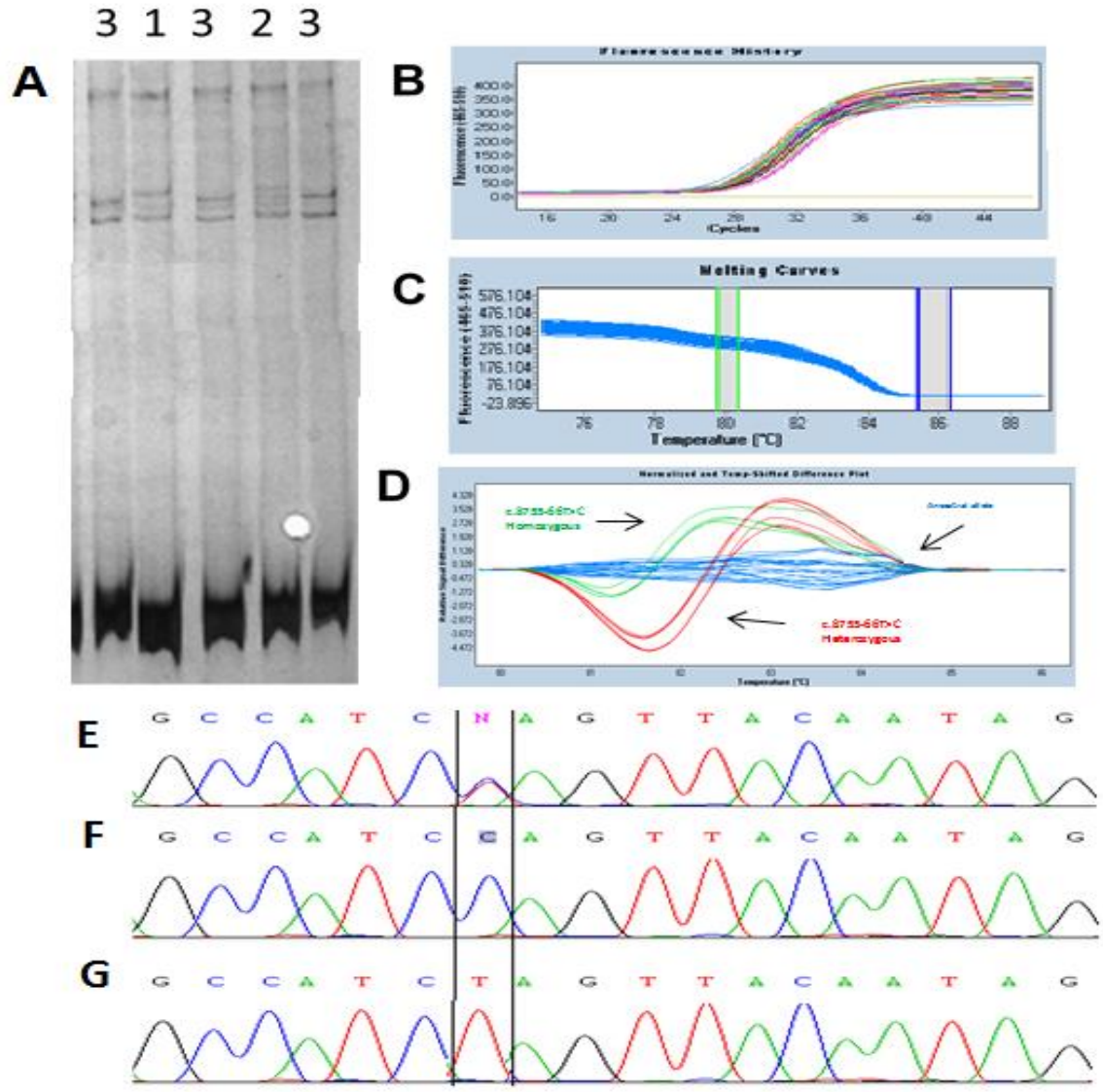

Figure 3.8 HRMA for *BRCA2* exon 22 on the study group. **A**. SSCP/HA indicating all three genotypes 1) homozygous wild 2) heterozygous and 3) homozygous mutant. **B**. The amplification curves. **C**. Normalisation of the amplification curves. **D**. Difference plot indicating all three genotypes with a relative signal difference less than 4.4 units. **E**. DNA sequence indicating the presence of a heterozygote (T/C). **F**. DNA sequence indicating the homozygous mutant allele (C/C). **G**. DNA sequence indicating the ancestral allele (T/T).

Table 3.4 Comparison of known SSCP/HA results against HRMA results. Each of the genetic variations identified were listed in the table as well as the *BRCA2* exon that had the variation. SSCP/HA and HRMA results were identical.

Exon	Variation detected with SSCP/HA	Variation detected with HRMA	n/Patients	Similarity in %
2	c.-26G>A	c.-26G>A	4	100
3	c.68-7delT	c.68-7delT	1	100
5 and 6	c.476-37A>G	c.476-37A>G	1	100
7	c.517-4C>G	c.517-4C>G	4	100
8	c.681+56C>T	c.681+56C>T	1	100
12	c.6842 -73T>A	c.6842 -73T>A	5	100
16	c.7618-17G>T	c.7618-17G>T	1	100
17	c.7806-14T>C	c.7806-14T>C	1	100
21	c.8754+1G>A	c.8754+1G>A	2	100
22	c.8755-66T>C	c.8755-66T>C	13	100
25	c.9257-16T>C	c.9257-16T>C	1	100
27.2	c.10023C>T, p.Glu3344= c.9976A>T, p.Lys3326Ter	c.10023C>T, p.Glu3344= c.9976A>T, p.Lys3326Ter	4	100

3.4 Discussion

3.4.1 PCR Amplification of *BRCA2*

The purity and quantity of the DNA extractions used in this study fit Raso and Biassoni's (2014) description of optimal DNA conditions used for PCR, qPCR, as well as HRMA. The visualisation of the agarose gel that contained the amplified DNA (Figure 3.1), showed that DNA from three extraction methods amplified using the specified parameters as suggested by van der Stoep *et al.* (2008). All the *BRCA2* exons amplified using the suggested annealing temperatures as well as optimal PCR conditions as used in Table 3.2.

3.4.2 HRMA reaction for *BRCA2*

According to the Roche Laboratory manual (http://www.gene-quantification.com/ LC480-Technical-Note-01-HRM.pdf), $MgCl_2$ had an influence on calling of samples for HRMA. False positive results were observed on HRMA when the same DNA was used from three different extraction methods, which was observed with additional $MgCl_2$. In Figure 3.3 A(i) and B(i), there were two distinct groups. The first group with a higher fluorescence intensity in the qPCR using the samples extracted using the phenol:chloroform and the Promega Wizard Kit.

All the DNA samples that were extracted with the salt extraction had distinctly lower fluorescence intensity. Differences were further observed in Figure 3.3 A(ii) and B(ii) where the samples that were extracted using the DNA salt extraction had different shapes on the graph as compared to the other two extraction methods. It was demonstrated from these experiments that the salt extraction method without the addition of additional $MgCl_2$ yielded the most consistent Ct values and difference plots. Samples extracted with phenol:chloroform or the Promega Wizard Kit yielded better results with additional $MgCl_2$

in terms of the qPCR grouping (Ct values) and HRMA. These results correlated with Aihara *et al*. (2012).

The Promega Wizard DNA extraction and phenol:chloroform extractions in the presence of additional $MgCl_2$ was not consistent in fluorescent emission compared to salt extracted DNA diluted in water. Samples from the DNA salt extraction gave reliable results with regards to the fluorescent emissions and Ct values without additional $MgCl_2$. This DNA extraction method was the preferred extraction method at the Department of Human Genetics, University of the Free State, Bloemfontein, South Africa.

Another experiment measured the effect of diluting salt extracted stock DNA in water and $T._1E$, without additional $MgCl_2$ in the reaction. The difference between the samples that were diluted in $T._1E$ and water were negligible on the qPCR proving that the two different DNA elution buffers did have a significant influence on HRMA. Rouleau *et al*. (2009) also noted that different buffers did not have an effect on HRMA. Different DNA amounts were tested using DNA extracted with the salt extraction. Similar results were obtained using the HRMA and qPCR.

The average Ct for *BRCA2* was determined to be at 28 cycles which fell well within the specifications of the laboratory manual (Roche Applied Science - http://www.gene-quantification.com/LC480-Technical-Note-01-HRM.pdf). Additionally, each experiment was ran for 40 cycles to ensure adequate amplification. The software analysed the fluorescence of the sample as a function of time and the data for the difference plot were calculated from the initial negative graph. The consistency of this graph in terms of the sample grouping was an indication of the uniformity of the protocols used in handling the DNA (Nomoto *et al*., 2006; Montgomery *et al*., 2007a).

An experiment was performed with only the DNA salt extraction method without the addition of $MgCl_2$ diluted in the water for individuals that had the ancestral allele for *BRCA2* exon 2 (Figure 3.6). This test was to confirm that if all the samples were treated the same way, then all the samples should group together on the difference plot for the

HRMA. No variation was observed during this experiment and all the samples had a fluorescence difference of between -1.4 and 1.5 units, well below the requirement to call a sample as an outlier (Montgomery *et al*., 2007b). Following this experiment, another was performed on the same cohort, but with an additional sample, run in duplicate, that had a known polymorphism for *BRCA2* exon 2. The experiment showed that the sample was detected by HRMA as being genetically different than the other samples (Vandersteen *et al*., 2007).

It may therefore be concluded that samples used for HRMA should be extracted with the DNA salt extraction method, the stock DNA must be diluted in water, 30 ng DNA must be used and no additional $MgCl_2$ must be added when using the LightScanner® mastermix on the LightCycler 480 II thermocycler (Roche).

3.4.3 SSCP vs HRMA

BRCA2 exons 4, 9, 15, 18.2, 23 and 24 were not included in Table 3.4 because no variation was detected using either SSCP/HA or HRMA. Of the 624 individual reactions for the HRMA, 2.7% (17 wells) gave false positive results and were bi-directionally sequenced to investigate the nature of the result. No nucleotide discrepancies were found within those samples. To be clear, as each sample was performed in duplicate, the false positives were never observed for the same sample twice. Also, the false positives amplified differently, with a higher or lower intensity, than the other samples. This observation could be seen as the experiment progressed. The false positive results could have been attributed to pipetting errors, or chemicals that were not adequately mixed within the reaction. Wojdacz and Dobrovic. (2007) noted that false positive results could occur if additional errors such as if homogeneousness is not consistent for all the samples. Although we experienced false positive results, they were however within acceptable ranges, and there was a sequence performed on each of the false positive samples. In the entire initial screen, there were no experiments performed on SSCP/HA that differed from HRMA, and vice versa. Both of these techniques gave outliers, or differences, for the same samples. Sequencing confirmed that all of the nucleotide

variations between SSCP/HA, and the HRMA, correlated 100% with one another. We could confirm that we yielded the same results from using HRMA, than SSCP/HA. The biggest difference was TAT.

The SSCP/HA analysis was performed at a rate of 3 exons per day for 24 samples, estimating the time for a *BRCA2* screen at 9 days, if all the experiments worked. The HRMA was performed at a rate of 2 exons per 1.5 h, a total of 10 exons per day for 24 samples, estimating the time for a *BRCA2* screen at 2.5 days if no samples were repeated. These differences and tested parameters resulted in HRMA being the preferred method to use in screening for nucleotide variation of *BRCA1/2* in this study. These results correlate with a study performed by Bardien *et al.* (2009), where SSCP/HA and HRMA were used to test the same genetic difference, with the difference that HRMA had a much faster detection rate.

Chapter 4

Molecular screening of *BRCA1* and *BRCA2* within the Indian population of SA

4.1 Introduction

Out of the 50.59 million South Africans, 2.5% (1.3 million) are of Asian origin. The majority of Asian women in SA are of Indian descent (Sitas *et al.*, 1998; Statistics South Africa 2011). Within this group, BC was ranked as the most common cancer (Vorobiof *et al*., 2001). The lifetime risk for developing BC within the Indian population of SA is one in 17 (National Health Laboratory Service, 2009).

Prior to this study, the Indian population of SA was mostly unexplored regarding *BRCA1*/2 testing and no information regarding this dynamic and mixed population was available in terms of familial BC. In addition to this, no diagnostic panel exists for this population. The aims of this study were to screen high-risk familial BC families for deleterious mutations within two high-risk genes and to try and construct a diagnostic population directed mutation panel for use as a diagnostic tool.

4.2 Materials and Methods

4.2.1 Patients

A total of 50 unrelated SA Indian patients were included in this study (Table 4.1). The selection criteria was based on one of the following: each individual had a positive family history for cancer ranging from high risk (two or more affected family members) to moderate risk (one to two affected individuals within the immediate family).

Table 4.1 Patients used in the cohort. Indicated is the unique internal number of the index patient, the age at diagnosis (dx), whether the patient represented a high risk, moderate risk or low risk family and the extent of the family history.

Number	Age of onset (dx)	High, moderate or low risk	Family history
CAM2108	dx33	High risk	Mother BC, Sister OVC (dx47; dx?)
CAM2113	dx29	Moderate risk	Grandmother BC, Paternal Aunt Lung Cancer (dx50; dx40)
CAM2184	dx50	Moderate risk	Maternal Aunt BC, Maternal Cousin BC (dx40; dx60)
CAM2245	dx55	High risk	Mother BC, Maternal Aunt BC, 2 Paternal Aunts BC (dx?; dx?; dx?; dx60)
CAM2247	dx35	High risk	2 Maternal Aunts BC, Mother BC (dx?; dx60; dx65)
CAM2251	dx81	High risk	3 Sisters BC (dx?; dx?; dx?)
CAM2261	dx30	Moderate risk	Mother BC, Maternal Cousin BC (dx?; dx?)
CAM2291	dx58	Moderate risk	2 Sisters BC (dx29; dx41)
CAM2331	dx49	Moderate risk	Paternal Aunt BC (dx?)
CAM2333	dx30	Low risk	No Family History for BC
CAM2335	dx35	Moderate risk	Maternal Aunt BC, Maternal Cousin BC (dx?; dx42)
CAM2336	dx46	Moderate risk	Sister BC (dx?; dx?; dx35)
CAM2338	dx35	High risk	3 Paternal Aunts BC (dx52, dx40, dx40)
CAM2339	dx38	Moderate risk	Paternal Aunt BC, Maternal Aunt Stomach Cancer (dx60; dx65)

Number	Age of onset (dx)	High, moderate or low risk	Family history
CAM2359	dx48	Moderate risk	Mother, Sister BC (dx60; dx38)
CAM2360	dx48	Moderate risk	Mother, Maternal Aunt BC (dx33; dx?)
CAM2465	dx40	High risk	Mother OVC, 3 Maternal Aunts BC (dx? dx?; dx?; dx?)
CAM2481	dx37	Moderate risk	Maternal Cousin BC, Mother Lung Cancer (dx37; dx72)
CAM2513	dx31	Moderate risk	Maternal Great aunt BC, Paternal Uncle Colon Cancer (dx50; dx56)
CAM2551	dx44	High risk	Mother BC, Paternal Aunt BC, Paternal Grandmother BC (dx30; dx62; dx?)
CAM2601	N/A	Moderate risk	Paternal Cousin BC, Paternal Aunt BC (dx?; dx?)
CAM2644	dx21	Low risk	Unknown
CAM2645	dx59	High risk	Sister BC, Maternal Cousin BC, Maternal Aunt BC (dx?; dx30; dx40)
CAM2646	dx39	Moderate risk	Mother BC, Maternal Grandmother BC (dx?; dx?)
CAM2648	dx34	Moderate risk	Maternal Aunt BC, Maternal Cousin BC (dx40; dx60)
CAM2651	dx56	High risk	2 Sisters BC, Paternal Aunt BC (dx61; dx65; dx71)
CAM2695	dx45	High risk	Mother OVC, Maternal Aunt Kidney Cancer Father Throat Cancer (dx55; dx?; dx62)
CAM2699	dx48	Moderate risk	2 Sisters BC (dx45; dx40)

Number	Age of onset (dx)	High, moderate or low risk	Family history
CAM2708	dx48	Moderate risk	Mother BC, Maternal Aunt (dx?; dx?)
CAM2715	dx70	Moderate risk	2 Sisters BC (dx?; dx72)
CAM2737	dx29	Moderate risk	Mother BC, Maternal Uncle Lung Cancer (dx38; dx40)
CAM2738	dx23	Moderate risk	Mother BC, Maternal Great Aunt OVC (dx75; dx60)
CAM2750	dx32	Moderate risk	2 Paternal Aunts BC (dx?; dx60)
CAM2752	dx34	High risk	3 Sisters OVC (dx40; dx46; dx50)
CAM2758	dx50	Moderate risk	Mother, Paternal Aunt BC (dx32; dx66)
CAM2770	dx33	High risk	Mother BC, Paternal Aunt OVC, Paternal Grandfather Leukaemia (dx?; dx?; dx?)
CAM2780	dx66	Moderate risk	2 Sisters with BC (dx55; dx66)
CAM2781	dx30	Low risk	Not indicated
CAM2806	dx56	High risk	2 Sisters, Maternal Great Aunt, Paternal Cousin BC (dx43; dx57; dx61; dx68)
CAM2807	N/A	Moderate risk	Maternal Aunt, Maternal Grandmother BC (dx?; dx?)
CAM2809	Dx60	Low risk	Aunt BC (dx?)
CAM2819	dx41	Moderate risk	Paternal Mother, Great Aunt BC (dx60; dx70)
CAM2821	dx48	High risk	Mother BC, Aunt BC, 2 Uncles Leukaemia (dx68; dx39; dx50; dx60)
CAM2822	dx32	Moderate risk	Sister BC, Cousin BC (dx38; dx38)

Number	Age of onset (dx)	High, moderate or low risk	Family history
CAM2826	dx67	High risk	2 Sisters BC, Brother BC (dx?; dx?; dx?)
CAM2827	dx39	Moderate risk	Great Aunt BC, Second Cousin BC (dx?; dx30)
CAM2831	dx29	Moderate risk	Mother BC (dx?)
CAM2862	dx33	Moderate risk	Half Sister BC (dx?)
CAM2885	dx28	Moderate risk	Sister BC, Grandmother BC (dx28; dx70)
CAM2890	dx39	High risk	Mother BC, Father Stomach Cancer, Maternal Grandmother BC (dx?; dx?; dx?)

The patient had to be of Indian descent. Individuals with an early age at onset for BC (dx below 31) with no family history were included as low risk individuals.

Two pre-symptomatic individuals (CAM2601; CAM2807) were included for screening based on their family history and the absence of a living affected family member. The suitability of each patient was established from the demographics indicated on the patient's request form. Each request form was accompanied by a family pedigree and letter of consent.

4.2.2 Ethical considerations

The project was approved by the Ethics Committee of the Faculty of Health Sciences of the UFS in Bloemfontein (ECUFS 107/2014, Appendix A). All patients were interviewed and counselled by the genetic counsellor or a genetic nurse in collaboration with Dr Buccimazza at Inkosi Albert Luthuli Hospital in Durban. The counsellor at Inkosi Albert Luthuli Hospital made use of the proposed and propagated protocol of the SA Genetic Counselling Association (Appendix C).

This protocol has been constructed specifically for individuals requesting information regarding familial/hereditary BC and the involvement of the *BRCA1* and *BRCA2* genes. It served as a guideline for genetic counsellors nationally as to what should be transferred to individuals requesting information regarding hereditary BC, and aims to structure these counselling sessions according to a standardized format.

At the end of the counselling session, each participant gave written, informed consent (Appendix D), whereafter blood was drawn and sent to Bloemfontein for analysis. The Division of Human Genetics at the UFS had no direct contact with the patients participating in this project. Once the blood arrived, the sample was given a unique internal reference number (CAM number) to ensure confidentiality of the information. Permission was obtained from the Acting Business Manager of the NHLS and the Head

of the Division of Human Genetics for use of the SANAS accredited laboratory space and equipment for analysis (Appendix B).

4.2.3 DNA extraction

DNA was extracted using the salting out method as described in Chapter 3 (3.3.1.2). As DNA quality was proven to be critical for HRMA, the quantity and purity of the extracted DNA was determined by using the Nanodrop (3.3.1.4). The stock DNA was diluted in water to 15 ng.μl^{-1} for HRMA analysis, whereas the dilution for SSCP/HA was 50 ng.μl^{-1}.

4.2.4 Protein Truncation Test

PTT was used to screen the larger genomic regions, such as *BRCA1* exon 11 and *BRCA2* exon 11 (Figure 2.3 and 2.4). Seven overlapping primer sets were used to amplify these exons, three for *BRCA1* exon 11 and four for *BRCA2* exon 11 (Table 4.2).

Each forward primer contained a T7 promoter sequence, which acted as a eukaryotic translation initiation sequence. A total of 300 ng genomic DNA was added to a total reaction volume of 25 µl containing 1 U Takara Ex *Taq* (Otsu, Japan), 250 µM dNTPs, 1.5 µM $MgCl_2$, 4 µM of each primer, 50 mM KCl and 100 mM Tris-HCl pH 7.2.

The amplification regime included an initial 95°C for 1 min followed by 35 cycles of 93°C for 4 min, 55°C for 1 min and 72°C for 3 min and a final extension step at 72°C for 5 min. 10 µl of each amplified product was separated on a 2% (w/v) agarose gel in 1 X TBE buffer to confirm amplification. An *in vitro* transcription/translation reaction was performed for each of the amplified fragments, using the TnT® T7 Quick Coupled Transcription/Translation System from Promega Corp.

Table 4.2 Primer sets used for the screening of exon 11 for *BRCA1* and *BRCA2* using PTT. Each of the forward primers contained a T7 promoter (indicated in bold) to initiate the *in-vitro* transcription and translation process.

Primer name	Primer sequence (5'-3')	Annealing temperature
BRCA1 exon 11 Fragment 1F	**CGCTAATACGACTCACTATAGGAACAGACCACCATGG**CTTGTGAATTTTCTGAGACGG	55.0°C
BRCA1 exon 11 Fragment 1R	CAGGAAACAGCTATGACATGAGTTGTAGGTTTCTGCTGTG	
BRCA1 exon 11 Fragment 2F	**CGCTAATACGACTCACTATAGGAACAGACCACCATGG**ACAATTCAAAAGCACCTAAAAAG	55.0°C
BRCA1 exon 11 Fragment 2R	CAGGAAACAGCTATGACAACCCCTAATCTAAGCATAGCATTC	
BRCA1 exon 11 Fragment 3F	**CGCTAATACGACTCACTATAGGAACAGACCACCATGG**CACCACTTTTTCCCATCAAGTC	55.0°C
BRCA1 exon 11 Fragment 3R	CAGGAAACAGCTATGACATTATTTTCTTCCAAGCCCGTTCC	
BRCA2 exon 11 Fragment AF	**CGCTAATACGACTCACTATAGGAACAGACCACCATGG**TGCATTCTTCTGTGAAAAGAAGC	55.0°C
BRCA2 exon 11 Fragment AR	GCACTTCAAATGTACTCTTCTGC	
BRCA2 exon 11 Fragment BF	**CGCTAATACGACTCACTATAGGAACAGACCACCATGG**TAAAGCAGCATATAAAAATGACTC	55.0°C
BRCA2 exon 11 Fragment BF	GATCTTTTCATCACGTTCGG	
BRCA2 exon 11 Fragment CF	**CGCTAATACGACTCACTATAGGAACAGACCACCATGG**TTGATGGCAGTGATTCAAG	55.0°C
BRCA2 exon 11 Fragment CF	TGCTACATTCATCATTATCTAGAGAG	
BRCA2 exon 11 Fragment DF	**CGCTAATACGACTCACTATAGGAACAGACCACCATGG**ATGACAAAAATCATCTCTCCG	55.0°C
BRCA2 exon 11 Fragment DF	AACTGACTACACAAAAATGGCTG	

Each reaction contained 5 µl PCR product, 8 µl TnT® T7 Quick master mix, 0.5 µl PCR enhancer and 0.6 µl of 10 $mCi.ml^{-1}$ L-[S^{35}] methionine (PerkinElmer Inc, Boston, MA). The reactions were incubated at 30°C for 90 min. Laemmli buffer [0.05% (w/v) bromophenol blue, 10% (w/v) SDS, 0.05 M Tris-HCl pH 6.8, 1 M DTT] and β-Mercaptoethanol at a ratio 0.2:100, was added to stop the reaction, followed by denaturation at 95°C for 5 min.

The samples were loaded on a 12% (w/v) SDS-PAGE gel [(0.38 M Tris-HCl pH 8.8, 0.0625% (w/v) SDS) with a 4% (w/v) stacking gel (0.13 M Tris-HCl pH 6.8, 0.005% (w/v) SDS)] together with a Benchmark™ Prestained Protein Ladder (Thermo Fischer Scientific Corp). The peptides were separated (15 min at 120 V/cm^{-1} through the stacking gel, followed by 90 min at 220 V) with precooled 25 mM Tris, 192 mM glycine, 0.1% (v/v) SDS running buffer (Bio-Rad Laboratories Inc., Hercules).

The SDS-PAGE gels were fixed with 10% (v/v) acetic acid, 65% (v/v) isopropanol for 15 min, rinsed in tap water and placed on the orbital shaker for 15 min in Amplify (GE Healthcare, UK). The SDS-PAGE gels were dried under vacuum (60°C) for a minimum of 2 h and exposed to X-ray film overnight. The X-ray film was developed until the X-ray was fully visible and the bands were clear.

4.2.5 Single-Stranded Conformation Polymorphism/ Heteroduplex Analysis

To determine the position of a specific mutation within a truncated PTT fragment, SSCP/HA (3.3.3) was used with smaller multiple overlapping primer sets to screen the genomic area represented by the PTT fragment. The primer sets were obtained from the BIC (Table 4.3) (National Human Genome Research Institute, 2015). Silver staining and visualization was performed as described elsewhere (3.3.3). Once the specific primer set that contains the mutation was identified, DNA from the specific samples were sequenced (3.3.4).

Table 4.3 SSCP/HA primer sets used for the analysis of certain sections within *BRCA1* and *BRCA2* exons 11.

Primer name	**Primer sequence (5'-3')**	**Size of fragment**
BRCA1 exon11 DF	GCCAAAGTAGCTGATGTATTGG	173 bp
BRCA1 exon11 DR	CGCTTTAATTTATTTGTGAGGG	
BRCA1 exon11 PF	AACTTAGAACAGCCTATGGGAA	196 bp
BRCA1 exon 11 PR	AACAAGTGTTGGAAGCAGGG	
BRCA2 exon 11 SF	AACCAGAAAGAATAAATACT	238 bp
BRCA2 exon 11 SR	TCCTCAACGCAAATATCTTCAT	
BRCA2 exon 11 TF	TTTCCAAAGTAATATCCAATGTA	205 bp
BRCA2 exon 11 TR	ATTTTTGATTTATTCTCGTTGTT	

4.2.6 High Resolution Melting Analysis

A total of 56 primer sets representing both *BRCA1* and *BRCA2* were used for HRMA to screen each patient for the presence of variants. Collectively, 22 HRMA primer sets representing *BRCA1* (Table 4.4) and 34 sets representing *BRCA2* (Table 3.2) were used to screen both genes (3.3.2.3).

4.2.7 Computer Based Analyses

The analysis of UV with an unknown clinical significance was conducted using a multiple evidence based approach. The variant classification criteria used in the screening and identification process of defining the pathogenicity of the genetic difference in this study was the ENIGMA and BIC guidelines.

The specific classification criteria on the pathogenic nature of disease causing and benign can be found on the ENIGMA *BRCA1/2* Gene Variant Classification Criteria that was published online (http://enigmaconsortium.org/documents/ publications/ENIGMA_ Rules_2015-03-26.pdf). The multi-evidence guidelines proposed by ENIGMA are therefore a universal and standardised approach to assess the pathogenicity of UV.

4.3 Results

4.3.1 Genetic variation observed within *BRCA1*

Mutation screening was performed using a combination of mutation screening techniques, which included PTT, SSCP/HA, HRMA and DNA sequencing. The coding regions of *BRCA1* exons 2 - 10 (excluding the non-expressed exon 4) and 12 - 24 were screened using HRMA, whereas PTT was used for the screening of exon 11. HRMA was performed in duplicate for each sample. The duplicate results served as an internal control (3.5.2).

Table 4.4 Mutation screening of *BRCA1* exons using HRMA. Indicated are the primer names for each exon (van der Stoep *et al.,* 2008), the 5' - 3' sequence with the M13 sequence indicated in capitals, annealing temperatures, the number of cycles used and the expected size of the specific fragments in bp.

Primer name	Primer sequence (5' - 3')	Annealing temperature (°C)/ and number of cycles	Expected fragment size in bp
HRM *BRCA1* ex2F	**TGTAAAACGACGGCCAGT**GAAGTTGTCATTTTATAAACCTTT	59 /45	294
HRM *BRCA1* ex2R	**CAGGAAACAGCTATGACC**TGTGTCTTTTCTTCCCTAGTATGT		
HRM *BRCA1* ex3F	**TGTAAAACGACGGCCAGT**TTGAGGCCTTATGTTGACTCAG	66/45	347
HRM *BRCA1* ex3R	**CAGGAAACAGCTATGACC**TGAAATGGAGTTGGATTTTTCG		
HRM *BRCA1* ex5F	**TGTAAAACGACGGCCAGT**TTCATGGCTATTTGCCTTTTG	59/40	292
HRM *BRCA1* ex5R	**CAGGAAACAGCTATGACC**TGATGAATGGTTTTATAGGAACG		
HRM *BRCA1* ex6F	**TGTAAAACGACGGCCAGT**GGTTTTCTACTGTTGCTGCATCT	59/45	318
HRM *BRCA1* ex6R	**CAGGAAACAGCTATGACC**GAAAGTAATTGTGCAAACTTCCTG		
HRM *BRCA1* ex7F	**TGTAAAACGACGGCCAGT**GGGTTTCTCTTGGTTTCTTTGA	59/50	279
HRM *BRCA1* ex7R	**CAGGAAACAGCTATGACC**AGAAGAAGAAAACAAATGGTTT		
HRM *BR*CA1 ex8F	**TGTAAAACGACGGCCAGT**TTCAGGAGGAAAAGCACAGAA	66/45	320
HRM *BRCA1* ex8R	**CAGGAAACAGCTATGACC**CACTTCCCAAAGCTGCCTAC		

Primer name	Primer sequence (5' - 3')	Annealing temperature (°C)/ and number of cycles	Expected fragment size in bp
HRM *BRCA1* ex9F	**TGTAAAACGACGGCCAGT**ACCCTTTTAATTAAGAAAACTTTTAT	63/50	217
HRM *BRCA1* ex9R	**CAGGAAACAGCTATGACC**AAAGAGAGAAACATCAATCCT		
HRM *BRCA1* ex10F	**TGTAAAACGACGGCCAGT**TGGTCAGCTTTCTGTAATCGAA	59/50	318
HRM *BRCA1* ex10R	**CAGGAAACAGCTATGACC**AAGGTCCCAAATGGTCTTCA		
HRM *BRCA1* ex12F	**TGTAAAACGACGGCCAGT**CAGCAAGTTGCAGCGTT	63/45	251
HRM *BRCA1* ex12R	**CAGGAAACAGCTATGACC**ATACATACTACTGAATGCAAAGGAC		
HRM *BRCA1* ex13F	**TGTAAAACGACGGCCAGT**AATGGAAAGCTTCTCAAAGTATT	63/45	341
HRM *BRCA1* ex13R	**CAGGAAACAGCTATGACC**CCTTACTCTTCAGAAGGAGAT		
HRM *BRCA1* ex14F	**TGTAAAACGACGGCCAGT**CTAACCTGAATTATCACTATC	63/45	348
HRM *BRCA1* ex14R	**CAGGAAACAGCTATGACC**GTGTATAAATGCCTGTATGCA		
HRM *BRCA1* ex15F	**TGTAAAACGACGGCCAGT**CTTTCACAATTGGTGGCG	63/45	346
HRM *BRCA1* ex15R	**CAGGAAACAGCTATGACC**CCAGAATATCTTTATGTAGGATTCAG		
HRM *BRCA1* ex16AF	**TGTAAAACGACGGCCAGT**GACCAGAACTTTGTAATTC	59/45	296
HRM *BRCA1* ex16AR	**CAGGAAACAGCTATGACC**CCCAGCAGTATCAGTAGTAT		
HRM *BRCA1* ex16BF	**TGTAAAACGACGGCCAGT**AAAGTTGCAGAATCTGCCC	63/45	273
HRM *BRCA1* ex16BR	**CAGGAAACAGCTATGACC**CATAAAACTCTTTCCAGAATGTTG		
HRM *BRCA1* ex17F	**TGTAAAACGACGGCCAGT**ACTAGTATTCTGAGCTGTGTGC	66/45	249
HRM *BRCA1* ex17R	**CAGGAAACAGCTATGACC**CCTCGCCTCATGTGGTT		
HRM *BRCA1* ex18F	**TGTAAAACGACGGCCAGT**CTTTAGCTTCTTAGGACAGCA	63/55	242
HRM *BRCA1* ex18R	**CAGGAAACAGCTATGACC**AAATGCAATTCTGAGGTGTTA		

Primer name	Primer sequence (5' - 3')	Annealing temperature (°C)/ and number of cycles	Expected fragment size in bp
HRM *BRCA1* ex19F	**TGTAAAACGACGGCCAGT**TTGTGAATCGCTGACCTCTCT	66/45	247
HRM *BRCA1* ex19R	**CAGGAAACAGCTATGACC**GGTGCATTGATGGAAGGAAG		
HRM *BRCA1* ex20F	**TGTAAAACGACGGCCAGT**CTGGCCTGAATGCCTTAAAT	61/50	266
HRM *BRCA1* ex20R	**CAGGAAACAGCTATGACC**CAGAGTGGTGGGGTGAGATT		
HRM *BRCA1* ex21F	**TGTAAAACGACGGCCAGT**AGATTTTCCTTCTCTCCATTCC	63/45	227
HRM *BRCA1* ex21R	**CAGGAAACAGCTATGACC**CCATCGTGGGATCTTGCTTA		
HRM *BRCA1* ex22F	**TGTAAAACGACGGCCAGT**TCCCATTGAGAGGTCTTGCT	66/45	333
HRM *BRCA1* ex22R	**CAGGAAACAGCTATGACC**GAGAAGACTTCTGAGGCTAC		
HRM *BRCA1* ex23F	**TGTAAAACGACGGCCAGT**ATGAAGTGACAGTTCCAGTAG	63/45	225
HRM *BRCA1* ex23R	**CAGGAAACAGCTATGACC**GTGATAAACCAAACCCATGC		
HRM *BRCA1* ex24F	**TGTAAAACGACGGCCAGT**CCTAGTCCAGGAGAATGAATTGA	63/55	282
HRM *BRCA1* ex24R	**CAGGAAACAGCTATGACC**CTGGAAAGGCCACTTTGTAA		

4.3.1.1 *BRCA1* Pathogenic mutations detected using PTT

Two prematurely truncated peptides of ~ 26 and ~ 37 kDa were detected for *BRCA1*, namely one for CAM2360 in PTT fragment 1 (Figure 4.1 A) and another for CAM2251 in PTT fragment 2 (Figure 4.2 A). To determine the position of each of these mutations, smaller overlapping SSCP/HA primers spanning the respective PTT areas, were amplified.

For the truncated fragment detected for CAM2360, SSCP/HA fragment D was used, whereas fragments P was used for CAM2251. A SSCP and HA band shift was observed (Figure 4.1 B). DNA sequencing revealed the deletion of two base pairs at position c.1360 in exon 11 (Figure 4.1 C - D). The mutation *BRCA1* c.1360_1361delAG,p.Ser454Terfs (g.41246187_41246188delCT) resulted in a shift in the reading frame, which created a premature stop codon.

For the prematurely truncated protein in fragment 2 (CAM2251, Figure 4.2 A), the position of the mutation was confirmed to be within SSCP fragment P (Figure 4.2 B). DNA sequencing revealed the presence of a single base change (T>G) resulting in the creation of a nonsense mutation resulting in a premature stop codon. The mutation was identified as *BRCA1* c.3593T>A,p.Leu1198Ter (g.43091938A>T).

4.3.1.2 Coding variants detected with HRMA for *BRCA1*

HRMA was performed for *BRCA1* exons 2 - 10 and exons 12 - 24. A total of ten different variants were identified of which three were synonymous mutations. The most common was *BRCA1* c.4308T>C,p.Ser1436Ser (g.41234470A>G).

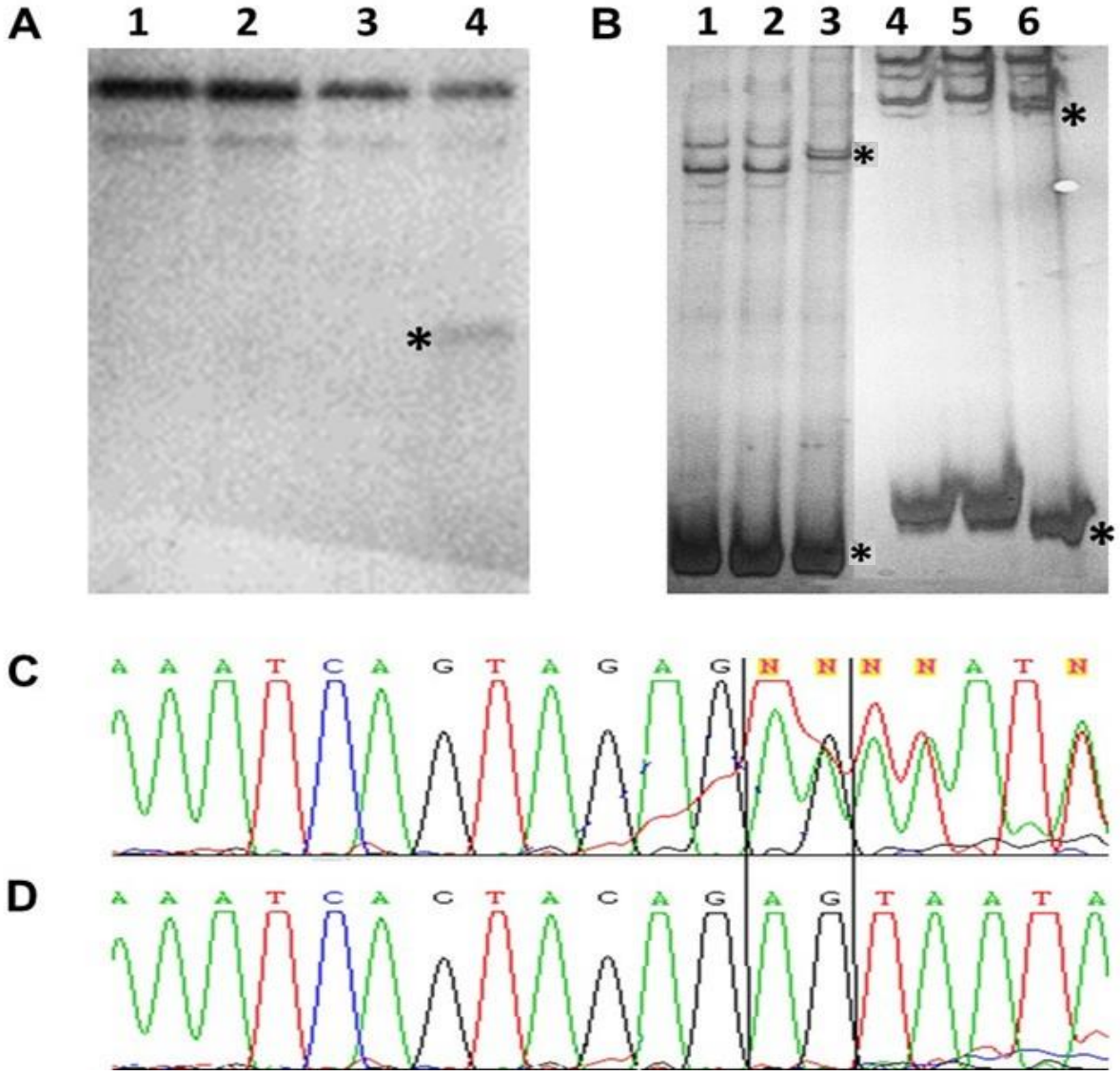

Figure 4.1 Identification and designation of *BRCA1* c.1360_1361delAG,p.Ser454Terfs (g.41246187_41246188delCT). **A**. SDS page gel exhibiting *in-vitro* transcription/translated peptides for *BRCA1* fragment 1. Loaded in lane 1 is CAM2338, with CAM2339 in lane 2, CAM2359 in lane 3 and CAM2360 in lane 4. **B**. The position of the mutation was determined by using smaller overlapping SSCP/HA primer sets. Indicated are the results for primer sets C (lanes 1 - 3) and D (lanes 4 - 6). The products loaded in lanes 1 and 4 represent CAM2338, with CAM2359 in lanes 2 and 5 and CAM2360 in lanes 3 and 6. The difference in SSCP/HA pattern is visible in lane 3 and 6, as indicated by the asterisk. **C**. DNA sequencing results for CAM2360, indicating the creation of a frame-shift due to the deletion of two base pairs (AG) as indicated. **D**. DNA sequencing results for CAM2359, which represented a normal control.

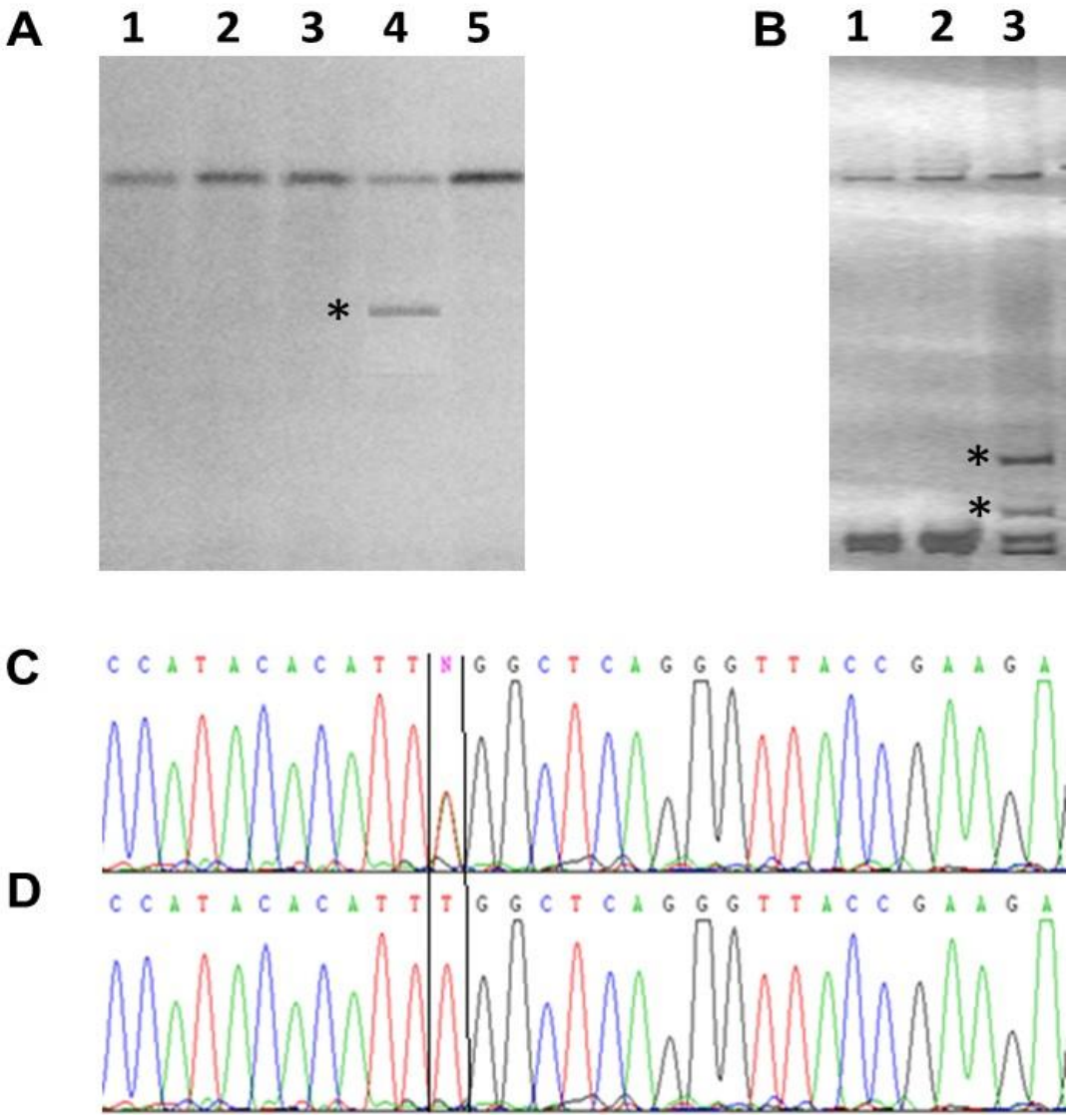

Figure 4.2 Identification and designation of *BRCA1* c.3593T>A,p.Leu1198Ter (g.43091938A>T). **A**. SDS page gel exhibiting *in-vitro* transcription/translated peptides for *BRCA1* fragment 2. Loaded in lane 1 is CAM2184, with CAM2245 in lane 2, CAM2247 in lane 3, CAM2251 in lane 4 and CAM2261 in lane 5. **B**. The position of the mutation was determined by using smaller overlapping SSCP/HA primer sets. Indicated is the result for primer set P (lanes 1 - 3). The products loaded in lane 1 represent CAM2247, with CAM2261 in lane 2 and CAM2251 in lane 3. The difference in SSCP/HA pattern is visible in lane 3, as indicated by the asterisks. **C**. DNA sequencing results for CAM2251, indicating the presence of a single base change (T>G) at position cd.3593 in exon 11 as indicated. **D**. DNA sequencing results for CAM2261, which represented a normal control.

This variant within exon 13 was found in 14 Indian BC patients (CAM2184, CAM2247, CAM2331, CAM2335, CAM2644, CAM2645, CAM2651, CAM2738, CAM2758, CAM2780, CAM2781, CAM2806, CAM2807 and CAM2821) and was present in both the homozygous (CAM2651) and heterozygous form (CAM2247) (Figure 4.3 A - F). The other synonymous *BRCA1* c.591C>T,p.Cys197Cys (g.41249263G>A) variant was observed for CAM2750 in exon 9 whereas *BRCA1* c.4812A>G,p.Glu1604Glu variant was detected in exon 16 (CAM2750).

Three missense mutations were detected within the coding region of *BRCA1*. *BRCA1* c.191G>A,p.Cys64Tyr (g.43106477C>T) was found in one individual (CAM2699) and caused an amino acid change from cysteine to tyrosine (Figure 4.4). The second mutation detected in this study was *BRCA1* c.4535G>T,p.Ser1512lle (g.43074471C>A) observed for CAM2465 within exon 9 and involved an amino acid change from serine to isoleucine. The common coding variant *BRCA1* c.4837A>G,pSer1613Gly (g.41223094T>C) also situated within exon 16 (Figure 4.5 A - C) was present in nine patients (CAM2247, CAM2333, CAM2644, CAM2645, CAM2738, CAM2748, CAM2806, CAM2807 and CAM2821) and was an amino acid change from a serine to a glycine. This variant was also detected in the homozygous and heterozygous form (Figure 4.5 D - F). CAM2738 proved to be heterozygous (A/G) (Figure 4.5 D) and CAM2821 homozygous for the variant (Figure 4.5 E).

Two frameshift mutations were identified within *BRCA1* using HRMA. The first represented the deletion of two base pairs in exon 2 for CAM2770 (Figure 4.6). The deletion resulted in the creation of a frameshift and a premature truncation of the associated protein product. The mutation was identified as the Ashkenazi Jewish founder mutation *BRCA1* 185delAG,p.Leu22_Glu23LeuValfs (g.41276047_41276048delCT) (Figure 4.6 A - E). The difference plot for this mutation is shown in Figure 4.6 C. The second frameshift mutation *BRCA1* c.5365_5366delGCinsA, p.Ala1789_Ile1790LeuTrpfs (Figure 4.7 A - E) was detected within exon 22 for CAM2261.

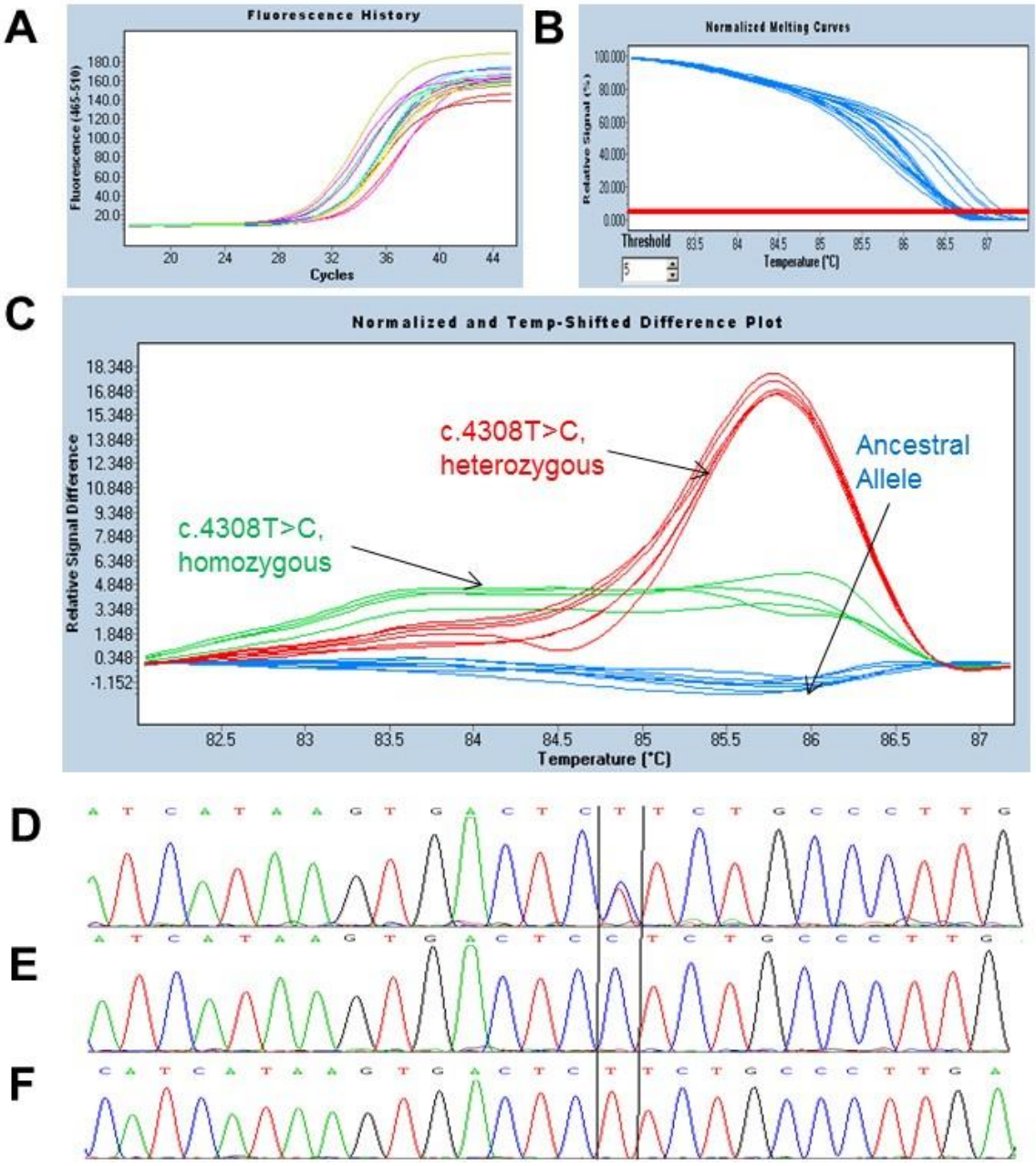

Figure 4.3 Results for the common synonymous variant *BRCA1* c.4308T>C,p.Ser1436Ser (g.41234470A>G). **A**. The qPCR amplification curve. **B**. Normalisation of the amplification curves. **C**. Difference plot indicating all three genotypes with a relative signal difference less than 18.3 units. **D**. DNA sequence indicating the presence of a heterozygote (T/C). **E**. DNA sequence indicating the homozygous mutant allele (C/C). **F**. DNA sequence indicating the ancestral allele (T/T).

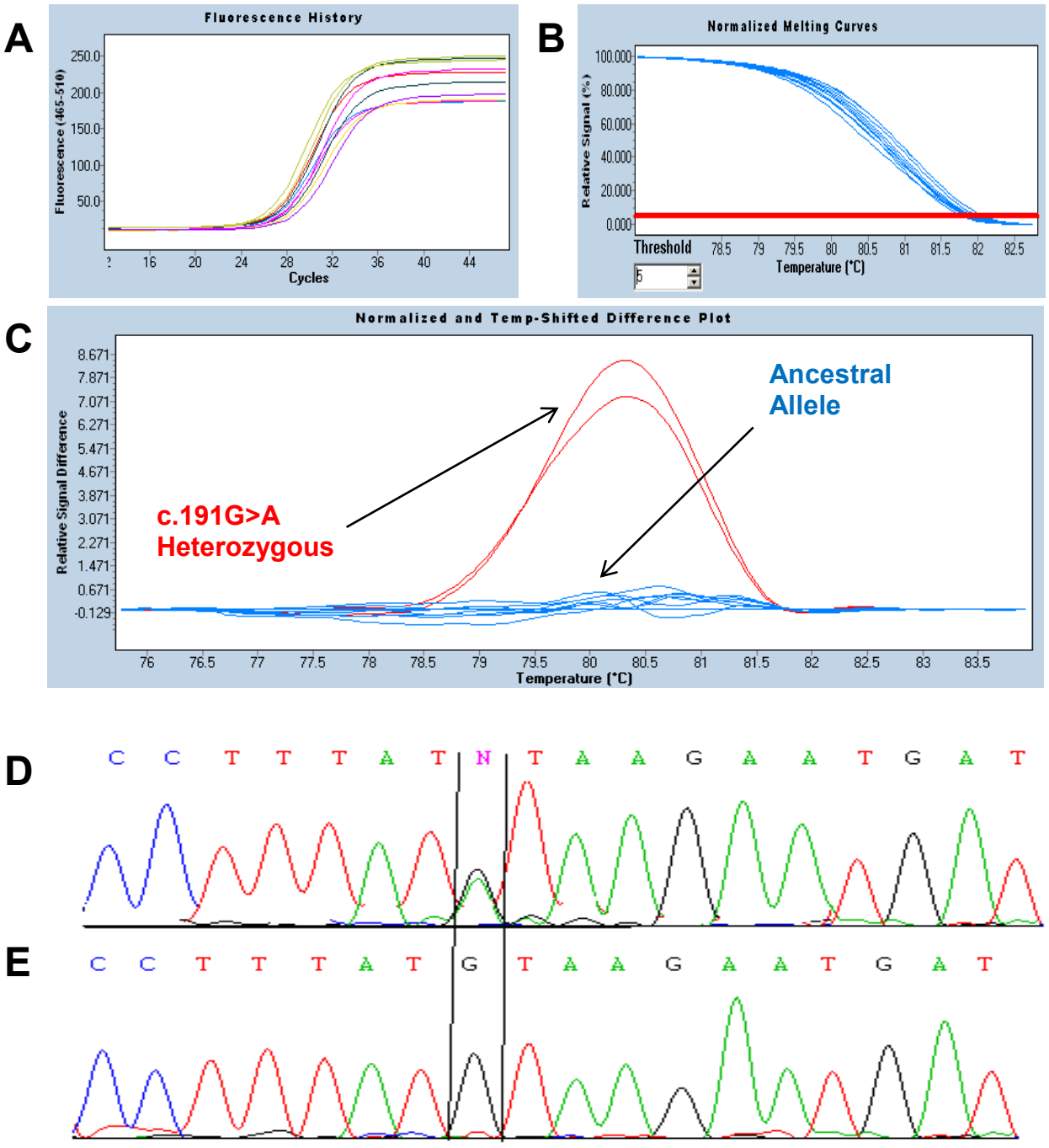

Figure 4.4 Results for the missense variant *BRCA1* c.191G>A,p.Cys64Tyr (g.43106477C>T). **A**. The qPCR amplification curve. **B**. Normalisation of the amplification curves. **C**. Difference plot indicating both genotypes with a relative signal less than 8.6 units. **D**. DNA sequence indicating the presence of a heterozygote (G/A). **E**. DNA sequence indicating the ancestral allele (G/G).

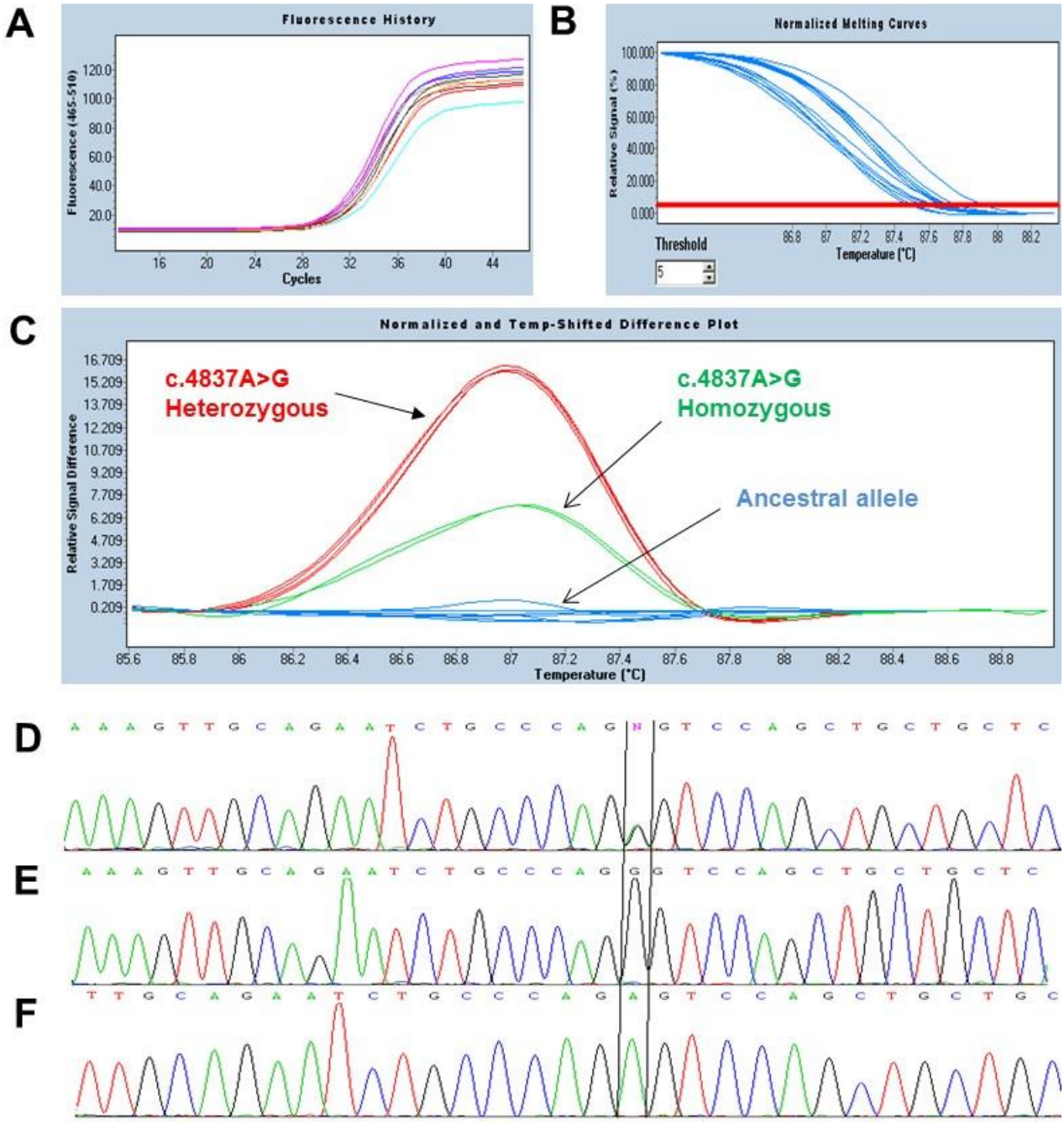

Figure 4.5 Results for the missense variant *BRCA1* c.4837A>G,p.Ser1613Gly (g.41223094T>C). **A**. The qPCR amplification curve. **B**. Normalisation of the amplification curves. **C**. Difference plot indicating all three genotypes with a relative signal less than 16.7 units. **D**. DNA sequence indicating the presence of a heterozygote (A/G). **E**. DNA sequence indicating homozygous mutant allele (G/G). **F**. DNA sequence indicating the ancestral allele (A/A).

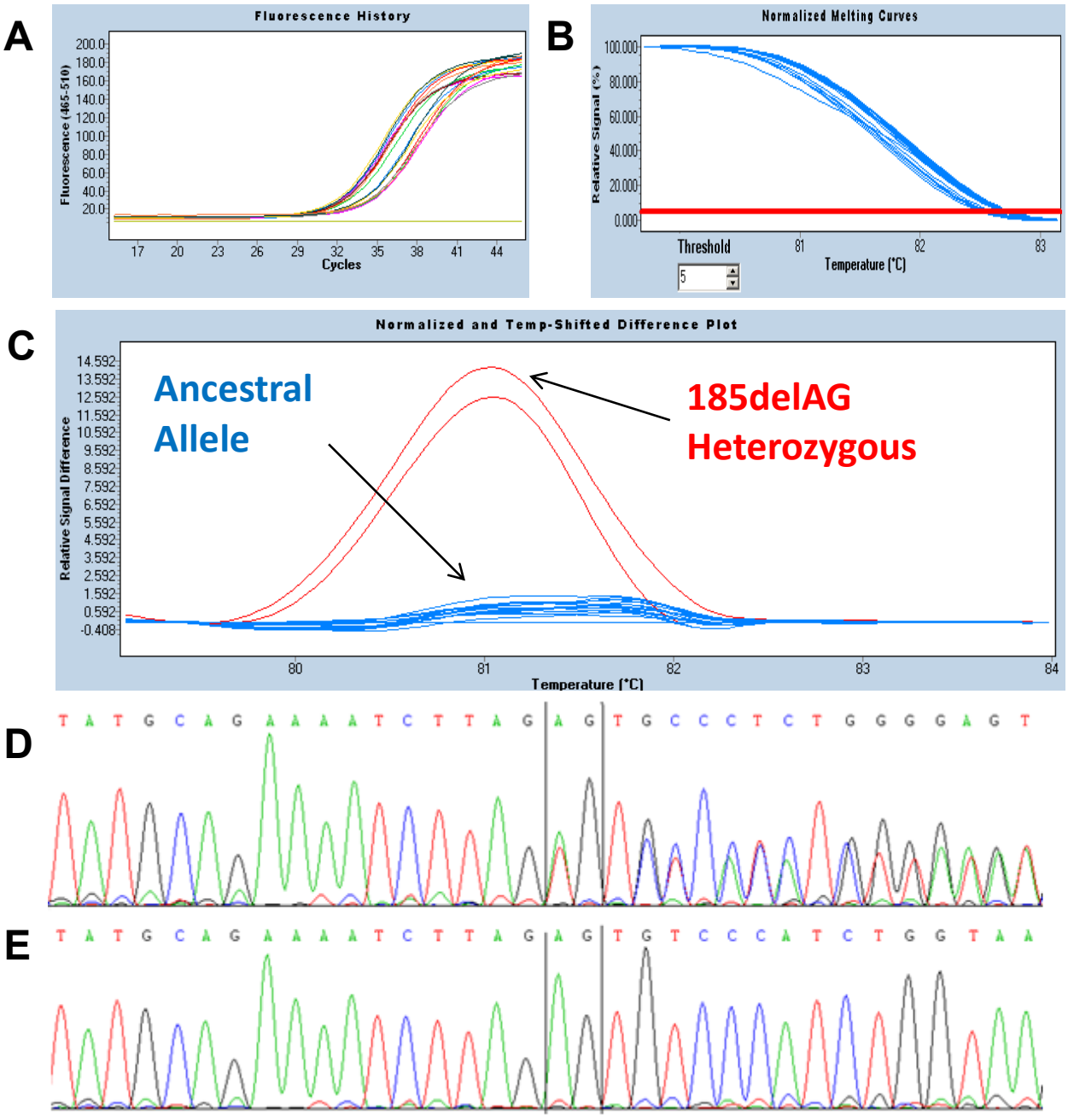

Figure 4.6 Results for the deleterious frameshift mutation 185delAG, p.Leu22_Glu23LeuValfs (.41276047_41276048delCT). **A**. The qPCR amplification curve. **B**. Normalisation of the amplification curves. **C**. Difference plot indicating both genotypes with a relative signal less than 14.5 units. **D**. DNA sequence indicating the presence of a heterozygote (delAG/AG). **E**. DNA sequence indicating the ancestral allele (AG/AG).

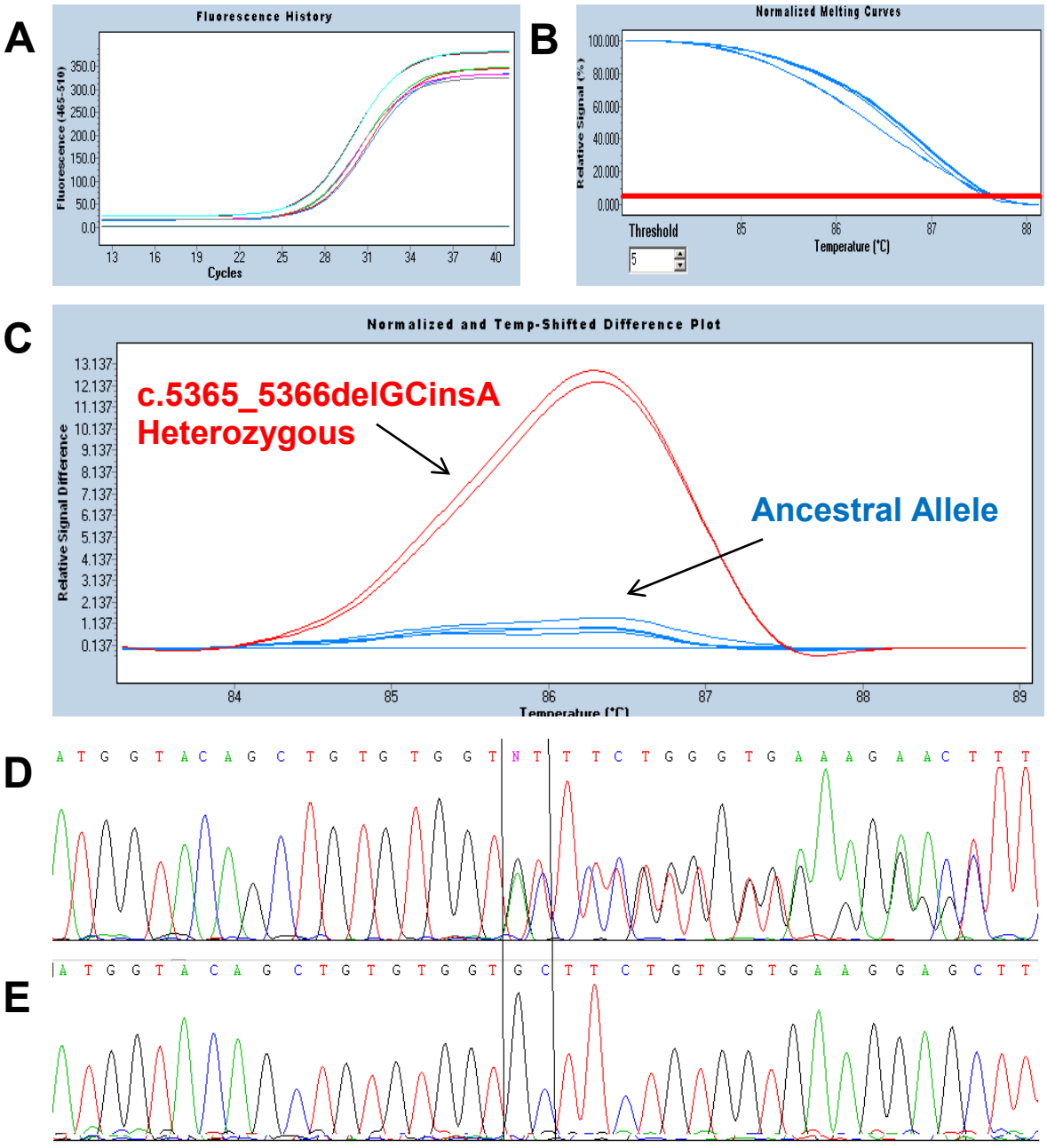

Figure 4.7 Results for the deleterious frameshift mutation c.5365_5366delGCinsA, p.Ala1789_Ile1790LeuTrpfs (Novel). **A**. The qPCR amplification curve. **B**. Normalisation of the amplification curves. **C**. Difference plot indicating both genotypes with a relative signal less than 13.1 units. **D**. DNA sequence indicating the presence of a heterozygote (delGCinsA/GC). **E**. DNA sequence indicating the ancestral allele (GC/GC).

Table 4.5 A summary of all the coding variation detected within *BRCA1.*

Exon	BIC designation	AA designation	n x/50	HVGS	rsid	Clinically significant
2	185delAG	p.Leu22_Glu23LeuValfs	1	g.41276047_41276048delTC	rs80357713	Yes
5	c.191G>A	p.Cys64Tyr	1	g.43106477C>T	rs55851803	Yes
9	c.591C>T	p.Cys197Cys	1	g.41249263G>A	rs1799965	No
11 A	c.1360_1361delAG	p.Ser454Terfs	1	g.41246187_41246188delCT	rs80357969	Yes
11 D	c.3593T>A	p.Leu1198Ter	1	g.43091938A>T	rs397509085	Yes
13	c.4308T>C	p.Ser1436Ser	14	g.41234470A>G	rs1060915	No
15	c.4535G>T	p.Ser1512Ile	1	g.43074471C>A	rs1800744	No
16	c.4837A>G	p.Ser1613Gly	9	g.41223094T>C	rs1799966	No
16	c.4812A>G	p.Glu1604Glu	1	g.41223119T>C	rs28897693	No
22	c.5365_5366delGCinsA	p.Ala1789_Ile1790LeuTrpfs	1	None	Novel	Yes

The DNA change was clearly indicated by the difference plot (Figure 4.7 C) and confirmed by the sequencing results (Figure 4.7 D - E). A summary of the genetic differences detected within the coding regions of *BRCA1* is shown in Table 4.5.

4.3.1.1 Intronic variation observed within *BRCA1*

A total of 10 intronic variants were identified. *BRCA1* c.442-34C>T (g.41251931G>A) was the most common and was detected for five individuals (CAM2184, CAM2339, CAM2465, CAM2750 and CAM2781) (Figure 4.8). This variant was detected in the heterozygous allele (C/T).

The non-coding variant *BRCA1* c.302-41T>C (g.41256319A>G), located 41 bp from the coding region of *BRCA2* exon 6 was only found within two individuals (CAM2807, CAM2821) as a heterozygous polymorphism. The UV c.5075-13T>G (g.41215981A>C) is found 13 bp downstream from the coding region of *BRCA1* exon 18. This UV was only detected within one individual (CAM2291).

One non-coding deletion c.548-58_548-58delT (g.41249364delA) found 58 bp from the coding region of *BRCA1* exon 9 was detected within four individuals (CAM2108, CAM2113, CAM2184 and CAM2331). The rest of the *BRCA1* upstream variants *BRCA1* c.-19-115T>C (g.41276247A>G), *BRCA1* c.4485-64C>G (g.41226602G>C), *BRCA1* c.5153-73T>C (g.41215463A>G), were detected in the same individual (CAM2184).

Three downstream intron variants were detected in the intervening sequence between different coding regions, among which *BRCA1* c.5152+28T>A [(g.41215907A>T) CAM2108, CAM2113], *BRCA1* c.5152+73G>A [(g.41215818C>T) CAM2108, CAM2113] and *BRCA1* c.547+44A>G [(g.41251788T>C) CAM2113] were UV. Table 4.6 is a summary of each non-coding *BRCA1* variant that was detected using HRMA.

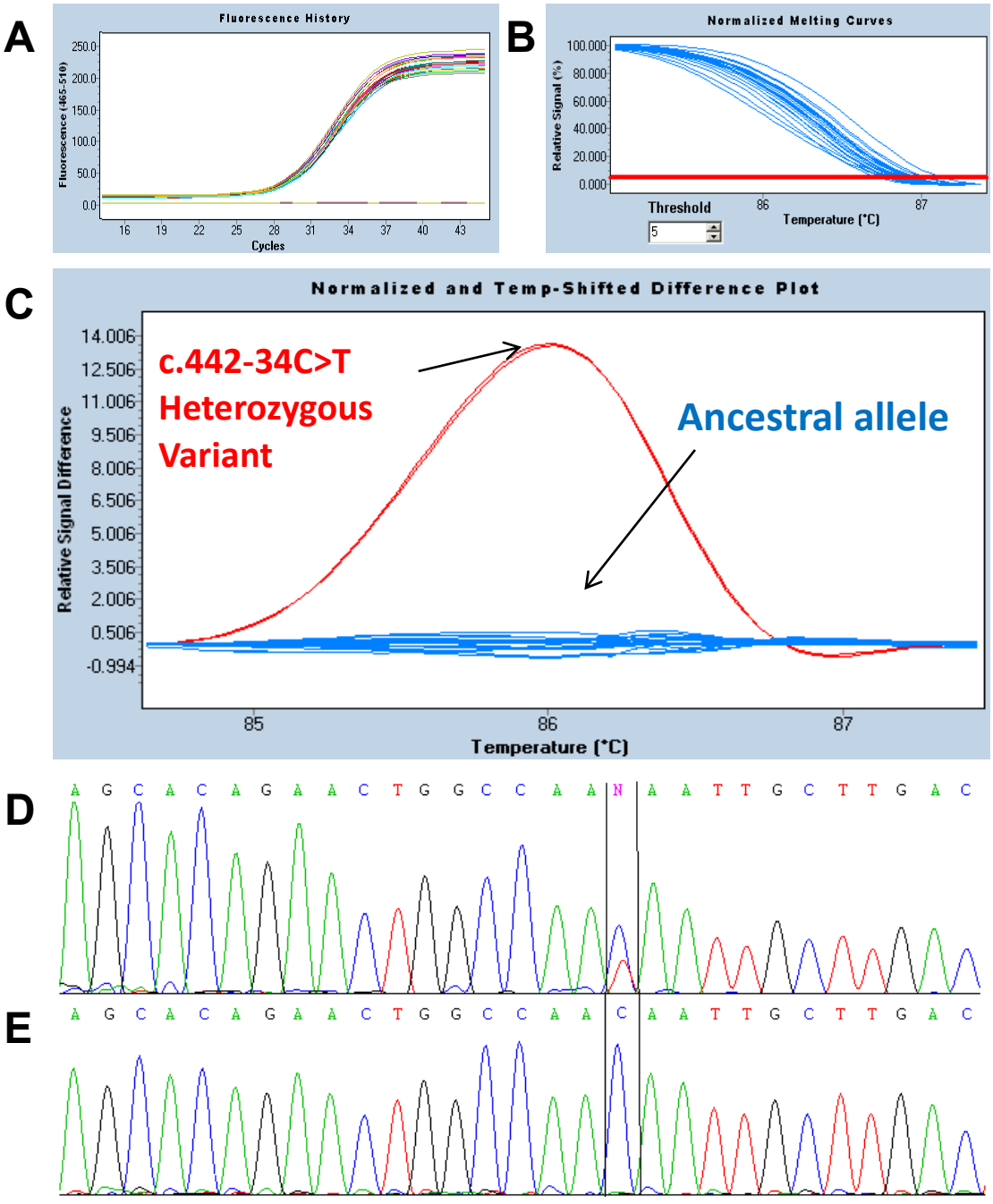

Figure 4.8 Results for the non-coding polymorphism c.442-34C>T (g.41251931G>A). **A**. The qPCR amplification curve. **B**. Normalisation of the amplification curves. **C**. Difference plot indicating both genotypes with a relative signal less than 13 units. **D**. DNA sequence indicating the presence of a heterozygote (C/T). **E**. DNA sequence indicating the ancestral allele (C/C).

Table 4.6 A summary of all the intronic variation detected within *BRCA1.* This table indicated the exon, BIC designation, IVS Designation, how many patients in the study had the difference (n x/50), HVGS nucleotide number, rs number and the clinical significance of each genetic difference.

Exon	HVGS cDNA	BIC designation	n x/50	HVGS	Rsid	Clinically significant
I-1	c.-19-115T>C	IVS1-115T>C	1	g.41276247A>G	rs3765640	No
I-6	c.302-41T>C	IVS6-41T>C	2	g.41256319A>G	NA	No
I-7	c.442- 34C>T	IVS7-34T>C	7	g.41251931C>T	rs799923	No
I-8	c.548-58_548-58delT	IVS8-58delT	4	g.41249364delA	NA	No
I-8	c.547+44A>G	IVS8+44A>G	1	g.41251788T>C	NA	UV
I-14	c.4485-64C>G	IVS14-64C>G,	1	g.41226602G>C	NA	No
I-17	c.5152+28T>A	IVS17+28T>A	2	g.41215907A>T	NA	UV
I-17	c.5075-13T>G	IVS17-13T>G	1	g.41215981A>C	NA	UV
I-18	c.5153 - 73T>C	IVS18-73T>C	1	g.41215463A>G	NA	UV
I-18	c.5152+73G>A	IVS18+73G>A	2	g.41215818A>G	NA	UV

UV: Unclassified Variant

NA: Not Applicable

4.3.2 Genetic Variation Observed within *BRCA2*

BRCA2 was screened using a combination of PTT, HRMA and DNA sequencing. PTT was used for exon 11, whereas the coding regions of *BRCA2* exons 2 - 10 and 12 - 27 were screened using HRMA. Each sample served as an internal control for the other.

4.3.2.1 *BRCA2* Pathogenic mutations detected using PTT

Two prematurely truncated peptides of ~ 49 and ~ 64 kDa were detected for *BRCA2*, namely one for CAM2695 in PTT fragment D (Figure 4.9 A) and another for CAM2359 in PTT fragment D (Figure 4.10 A). To determine the position of each of these mutations, a series of smaller overlapping SSCP/HA primers spanning the appropriate PTT areas, were used. For the truncated fragment detected for CAM2695 (Figure 4.9 A), SSCP/HA fragment S was used, whereas fragments T was used for CAM2359 (Figure 4.10 B).

A HA band shift was observed for *BRCA2* SSCP primer S (Figure 4.9 B). DNA sequencing revealed a single base change at *BRCA2* c.5279C>G,p.Ser1760Ter (g.32339634C>G) (Figure 4.9 C).

The second *BRCA2* mutation detected with PTT was identified by a HA band shift in the SSCP/HA analysis. DNA sequencing revealed the mutation as *BRCA2* 5563C>G, p.Ser1855Ter (g.32914127G>A) (Figure 4.10 C). Both these mutations prematurely stop the translation of the protein by encoding a premature stop codon.

4.3.2.2 Coding variants detected with HRMA for *BRCA2*

The most common coding variant for *BRCA2* was the *BRCA2* c.7242A>G, p.Ser2414Ser (g.32355095A>G) synonymous polymorphism. Three individuals (CAM2335, CAM2481 and CAM2651) were carriers for this polymorphism.

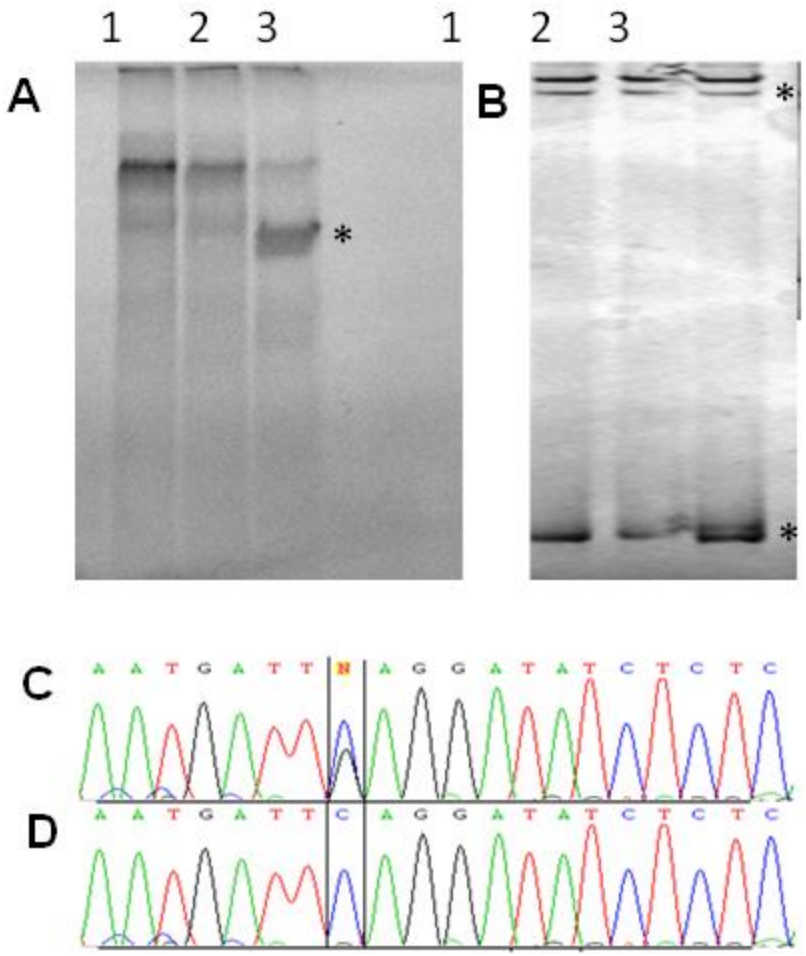

Figure 4.9 Identification and designation of *BRCA2* c.5279C>G,p.Ser1760Ter (g.32339634C>G). **A**. SDS page gel exhibiting *in-vitro* transcription/translated peptides for *BRCA2* fragment D. Loaded in lane 1 is CAM2481, with CAM2513 in lane 2, CAM2695 in lane 3. **B**. The position of the mutation was determined by using smaller overlapping SSCP/HA primer sets. Indicated are the results for primer set S (lanes 1 - 3). The difference in SSCP/HA pattern is visible in lane 3, as indicated by the asterisk. **C**. DNA sequencing results for CAM2695, indicating the nonsense mutation (C/G) as indicated. **D**. DNA sequencing results for CAM2481, which represented a normal control (C/C).

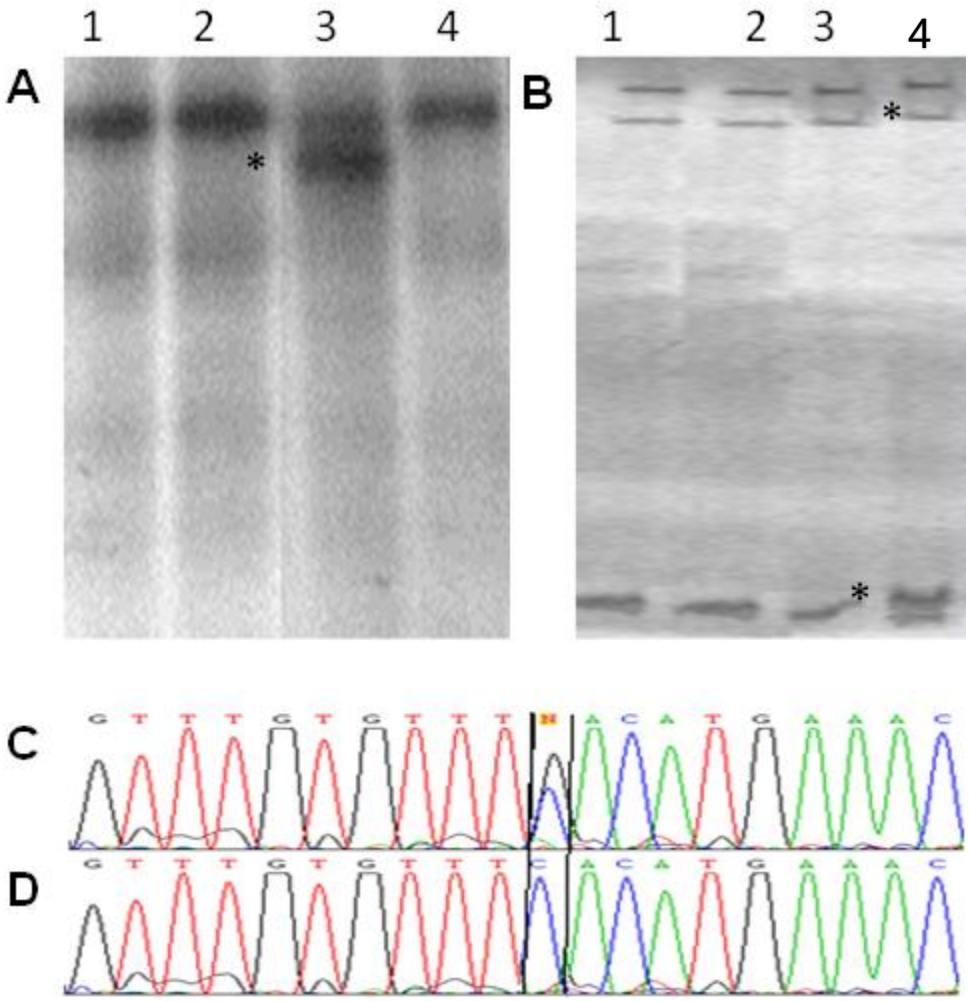

Figure 4.10 Identification and designation of *BRCA2* 5563C>G, p.Ser1855Ter (g.32914127G>A). **A**. SDS page gel exhibiting *in-vitro* transcription/translated peptides for *BRCA2* fragment D. Loaded in lane 1 is CAM2338, with CAM2339 in lane 2, CAM2359 in lane 3 and CAM2360 In lane 4. **B**. The position of the mutation was determined by using smaller overlapping SSCP/HA primer sets. Indicated are the results for primer set T (lanes 1 - 4). The difference in SSCP/HA pattern is visible in lane 4, as indicated by the asterisk. **C**. DNA sequencing results for CAM2359, indicating the nonsense mutation (C/G). **D**. DNA sequencing results for CAM2339, which represented a normal control (C/C).

An additional missense polymorphism, called *BRCA2* c.865A>C p.Asn289His (g.32332343A>C), was detected within two individuals (CAM2335 and CAM2651) in this study.

One deletion, called *BRCA2* c.9435_9436delGT, p.Val3145_Phe3146=fs (g.32394867_32394868delGT), was detected within one individual in this cohort (CAM2601) for *BRCA2* exon 25. This variant is classified as a frameshift mutation caused by the deletion of a GT. Figure 4.11 A - E shows the qPCR and temperature shift respectively for this mutation.

Table 4.7 is a summary of the genetic differences that were detected within *BRCA2* exons. Included in this table is the designation according to the BIC, the AA change, the rs number, and the Human Genome Variation (HGVS) number, as well as the clinical significance for each genetic difference found within the coding region of *BRCA2.*

4.3.1.1 Intronic variation observed within *BRCA2*

Nine different *BRCA2* intronic variants were observed within this study group. The most common intronic polymorphism that was detected was found within the intron of exon 22 and has the designation of *BRCA2* c.8755-66T>C (g.32953388T>C). This polymorphism was detected within four individuals (CAM2108, CAM2113, CAM2331 and CAM2752). Two other upstream variants were detected prior to the coding regions of *BRCA2* exon 2 and exon 22 namely *BRCA2* c.-26G>A (g.32890572G>A) and *BRCA2* c.8755-75T>C respectively. The *BRCA2* c.-26 G>A polymorphism was detected within four individuals (CAM2113, CAM2481, CAM2651 and CAM2335) and the non-coding polymorphism *BRCA2* c.8755-75T>C within one individual (CAM2481).

The rest of the non-coding variants within *BRCA2* were found downstream from the coding regions of exons 2, 4, 8, 14 and 21. The non-coding variant *BRCA2* c.425+67A>C (g.32899388A>C) was found within two individuals (CAM2651 and CAM2335). This polymorphism is found 67 bp downstream from the coding region of *BRCA2* exon 4.

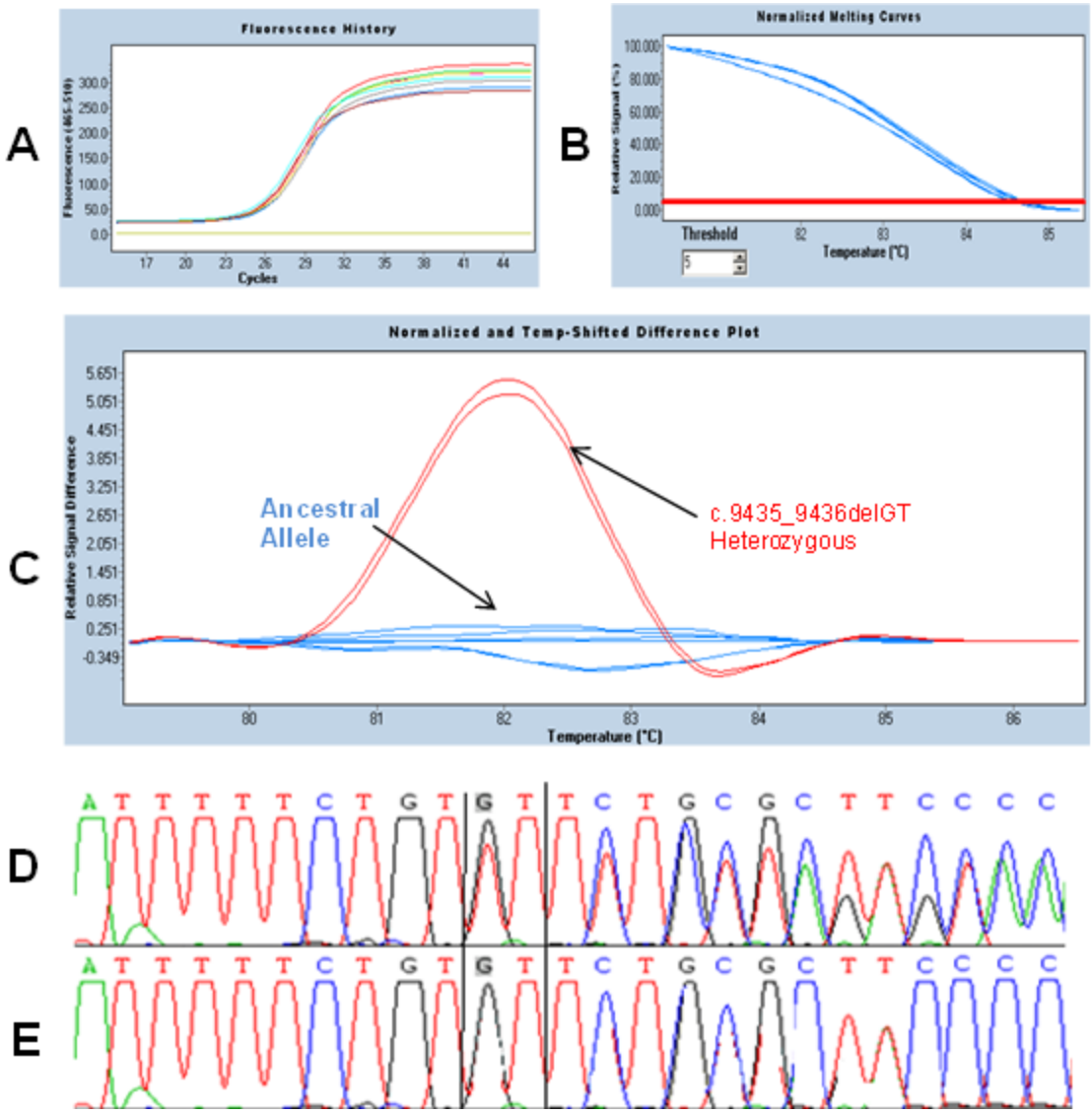

Figure 4.11 Results for the frameshift mutation *BRCA2* c.9435_9436delGT, p.Val3145_Phe3146=fs (g.32394867_32394868delGT). **A**. The qPCR amplification curve. **B**. Normalisation of the amplification curves. **C**. Difference plot indicating the two genotypes with a relative signal difference less than 5.6 units. **D**. DNA sequence indicating the presence of a heterozygote (delGT/GT). **E**. DNA sequence indicating the ancestral allele (GT/GT).

Table 4.7 A summary of all the coding variation detected within *BRCA2.* Indicated are the *BRCA2* exon, BIC designation, AA designation, how many patients had the variation (n x/50) in the study, the HGVS nucleotide number, rsid and the clinical significance of the genetic difference.

Exon	BIC designation	Protein designation	n x/50	HVGS	rsid	Clinically significant
10	c.865A>C	p.Asn289His	2	g.32332343A>C	rs766173	UV
11 E	c.5279C>G	p.Ser1760Ter	1	g.32339634C>G	rs80358751	Yes
11 E	5563C>G	p.Ser1855Ter	1	g.32339913G>A	rs587782694	Yes
14	c.7242A>G	p.Ser2414Ser,	3	g.32355095A>G	rs1799955	No
25	c.9435_9436delGT	Val3145_Phe3146=fs	1	g.32394867_32394868delGT	rs80359763	Yes

The splice site mutation c.8754+1G>A (g.32376792G>A) is located one bp from the coding region of *BRCA2* exon 21 (Figure 4.12 A - E). This splice site mutation was detected within three individuals (CAM2291, CAM2465 and CAM2715). Table 4.8 is a summary of all non-coding *BRCA2* variants that were detected using HRMA. The raw identified sequence data for *BRCA1* and *BRCA2* may be found in Appendix E and Appendix F. All the information was submitted for review to the dsSNP as well as BIC.

4.4 Discussion

4.4.1 SA Indian Families

Familial BC is a disease that is characterized by the presence of multiple affected family members present within the immediate and extended family. The majority of the index cases in this study had a family history for BC, with these families exhibiting more than two affected family members (Table 4.1).

The presence of such a family history for BC increased the likelihood of these families carrying a deleterious *BRCA* mutation (Ford *et al*., 1998; Nilsson *et al*., 2014). The average age of diagnosis of the affected patients enrolled in this study was 43. Within these families there were also cases of OVC, lung cancer, stomach cancer, kidney cancer and leukaemia.

4.4.2 Mutational Profile

In terms of the Indian population of SA, up until now there has been an absence of information with regard to familial BC for this population. This state of affairs complicated diagnostic testing at pathology institutions, as testing for the most common SA *BRCA* mutations was found to be ineffective. This was expected as this diagnostic screen was based on data obtained for the Afrikaner, the Ashkenazi Jewish and the Xhosa and Coloured populations. It was therefore not applicable to the SA Indian population.

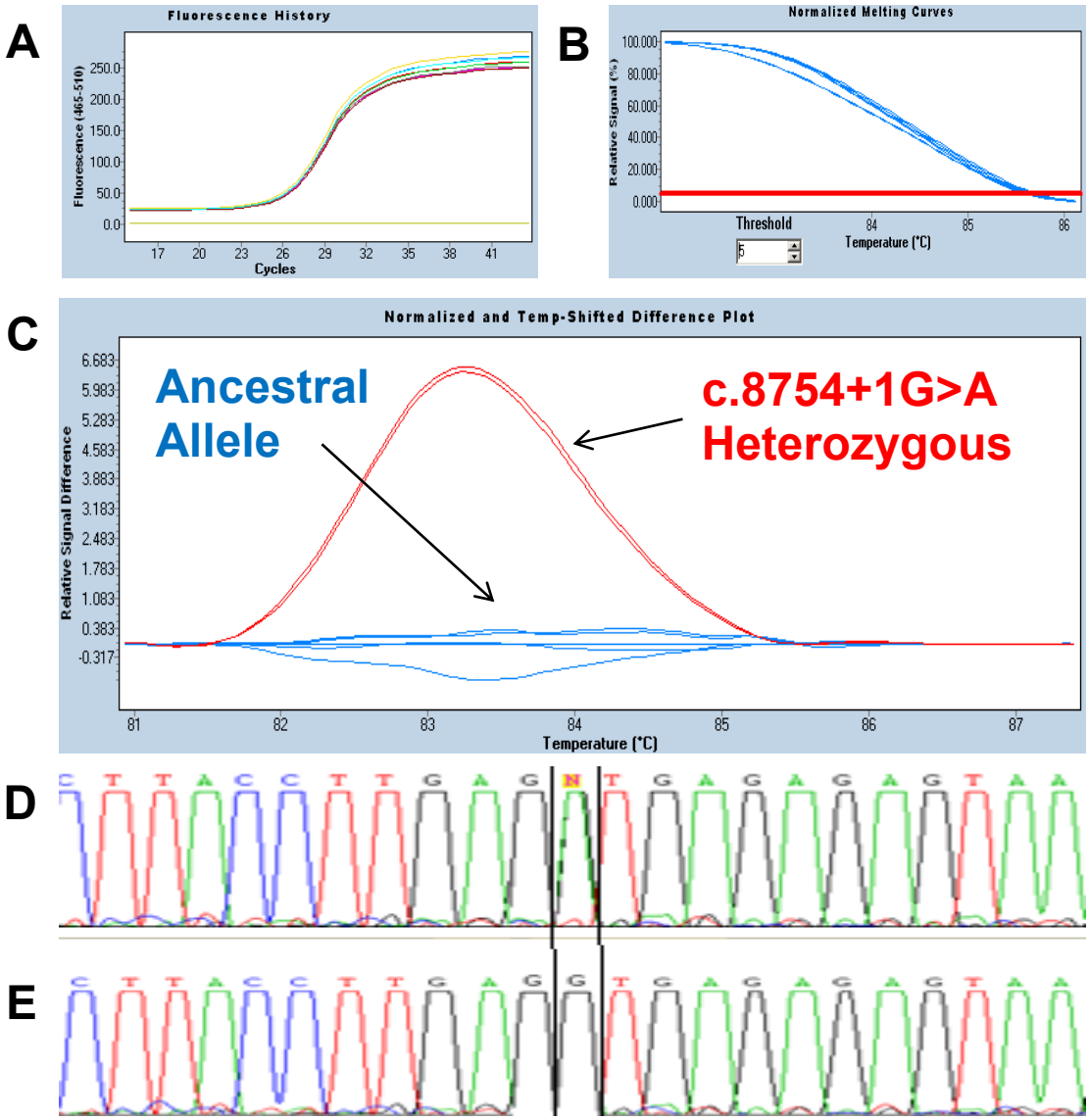

Figure 4.12 Results for the splice site mutation *BRCA2* c.8754+1G>A (g.32376792G>A). **A**. The qPCR amplification curve. **B**. Normalisation of the amplification curves. **C**. Difference plot indicating the two genotypes with a relative signal less than 6.6 units. **D**. DNA sequence indicating the presence of a heterozygote (G/A). **E**. DNA sequence indicating the ancestral allele (G/G).

Table 4.8 A summary of all the intronic variation detected within *BRCA2.* This table indicated the intron, BIC designation, IVS Designation, how many patients in the study had the difference (n x/50), HVGS nucleotide number, rs number and the clinical significance of each genetic difference.

Intron	BIC designation	IVS Designation	n x/50	HVGS	rsid	Clinically significant
2	c.-26 G>A	c.-26 G>A	3	g.32890572G>A	NA	No
I-2	c.67+58A>G	IVS2+58A>G	1	NA	NA	UV
I-4	c.425+67A>C	IVS4+67A>C	2	g.32899388A>C	NA	UV
I-8	c.681+56C>T	IVS8+56C>T	1	g.32329548C>T	rs2126042	No
I-21	c.8755-66T>C	IVS21-66T>C	4	g.32953388T>C	rs4942486	No
I-21	c.8755-75T>C	IVS21-75T>C	1	NA	NA	UV
I-21	c.8754+1G>A	IVS21+1G>A	3	g.32376792G>A	rs397508006	Yes

UV: Unclassified Variant

NA: Not applicable

4.4.2.1 Molecular Screening of *BRCA1*

Two truncating mutations were detected for *BRCA1* exon 11 (CAM2360 and CAM2695). As this technique only detected the presence of base changes that have an effect on the translated peptide, it was not influenced by the multiple missense mutations present within the exon. Both these mutations were classified according to the ENIGMA guidelines that state that any nonsense mutation or any other mutation that causes a shift in the reading frame upstream of amino acid 1 855 of the BRCA1 protein shall be regarded as pathogenic. As both these mutations occurred at respectively amino acid 454 and amino acid 1 198, both variants were officially classified as pathogenic mutations.

The ENIGMA classification was verified by the BIC, which also indicated that both these mutations were class 5 pathogenic variants. As *BRCA1* exon 11 forms part of the functional regulation of the BRCA1 protein, it contributed in homologous repair. In a study by Raponi *et al.* (2014) deleterious mutations within *BRCA1* exon 11 not only inhibited the function of the BRCA1 protein complex, but also directly affected the binding of Tra2beta and hnRNP L, two regulatory proteins responsible for *BRCA1* expression. Both patients that tested positive for these mutations had either a moderate or high risk for BC based on the number of affected family members. In both cases, direct family members (siblings, mother and aunt) had BC. Interestingly, CAM2251 was diagnosed with BC at the age of 81. This late age at onset just further showed that there are various factors involved in the onset of the disease.

All the remaining exons of the gene were screened using HRMA (exons 2 - 10 and 12 - 24). This mutation screening technique revealed the presence of multiple variants, detected for both the coding and non-coding regions. Mutation screening using HRMA successfully identified other pathogenic mutations in the cohort, of which the Ashkenazi Jewish founder mutation *BRCA1* p.Leu22_Glu23LeuValfs (*BRCA1* 185delAG, g.41276047_41276048delCT) detected in exon 2, was the first and was detected within a single patient (CAM2770). This mutation is a class 5 pathogenic variant logged in the dbSNP database as rs80357713 and was previously detected within the Indian population of mainland India (Sharma *et al.,* 2014; Singh *et al.,* 2015). Couch (2002) suggested that British colonisation and trade unions between

the Netherlands and Middle East were the events that introduced this mutation into the Indian population from the Ashkenazi Jewish population. The patient that was affected was diagnosed at age 33. This patient had two family members that tested positive for OVC. Supporting evidence showed that a *BRCA1* mutation increased an individual's risk of OVC by 20 - 40% (Bolton *et al*., 2012).

The fourth pathogenic mutation *BRCA1* c.191G>A, p.Cys64Tyr (g.43106477C>T, rs55851803) was located within exon 5 (CAM2699). This mutation fell within the zinc-finger domain, an essential part of the BRCA1 protein that facilitates binding with the BARD1 protein (Gomez and Hergovich, 2014). Although the age of onset for the patient was 48 years of age, this individual had a moderate risk with two sisters also affected with BC diagnosed at ages 40 and 45 respectively.

The fifth pathogenic mutation was detected within the coding region of exon 22. This mutation involved a deletion of two base pairs, with the insertion of an A (*BRCA1* c.5365_5366delGCinsA, p.Ala1789_Ile1790LeuTrpfs), detected for a single patient (CAM2261). According to the ENIGMA classification guidelines, this mutation is regarded as pathogenic and was novel. CAM2261 had a moderate risk for BC because there were two family members affected with BC.

The most frequent coding variant detected was the synonymous *BRCA1* c.4308T>C,p.Ser1436Ser mutation (g.41234470A>G, rs1060915). This variant in exon 13 was present in 14 of the 50 BC patients, in both the heterozygous (T/C) and homozygous (C/C) alleles (Figure 4.3). The synonymous variant had an MAF score of C = 0.3363/1684 and was reported repeatedly in the BIC with additional data that supported this variant to be benign (Riahi *et al.,* 2014).

The second most common variant was *BRCA1* c.4837A>G,pSer1613Gly (g.41223094T>C, rs1799966) (Table 4.5). This missense mutation was present in nine individuals, in both heterozygous (A/G) and homozygous (G/G) alleles and was included in the BIC and dbSNP database as a benign allele with an MAF score of G = 0.3558/1782.

The study brought about the identification of rare variants present within the coding region of the gene. These included *BRCA1* c.591C>T,p.Cys197Cys (g.41249263G>A, rs1799965) in exon 9, *BRCA1* c.4535G>T,p.Ser1512lle (g.43074471C>A, rs1800744) in exon 15 and *BRCA1* c.4812A>G,p.Glu1604Glu (g.41223119T>C, rs28897693) in exon 16.

The coding variant in exon 9, was listed by the BIC as a synonymous variant with unknown clinical significance. According to the dbSNP database, the variant allele had an MAF score of A = 0.0004/2. A study involving this variant was conducted by Dosil *et al.* (2010) to evaluate the role this unclassified variant had on RNA-splicing. In the study, they tested to see whether this specific variant caused overexpression of this exon or influenced splicing from within the exon itself. From their data, they concluded that this variant acted as a non-pathogenic polymorphism.

The missense polymorphism found in exon 15 was only observed for a single individual and changed the amino acid from a serine to an isoleucine. The presence of the variant allele had an MAF score of A = 0.0006/3. Several authors have investigated a potential association between this variant and the potential to cause disease. They have published data that indicate that its contribution towards the function on the BRCA protein complex is significantly low (Abkevich *et al*., 2004; Phelan *et al.,* 2005; Osorio *et al*., 2007).

The rare synonymous mutation detected within exon 16, namely *BRCA1* c.4812A>G,p.Glu1604Glu (g.41223119T>C, rs28897693) present in a single carrier. This variant had an MAF score for the variant allele of C = 0.0014/7 and was classified by the BIC as a UV. According to information on NCBI, this rare variant was likely benign. As there was no amino acid change this variant did not influence the polypeptide chain, thus it was most likely to not influence the function of the polypeptide.

The last group of variants that was detected for *BRCA1* were the intronic variants. These ranged from Class 1 benign polymorphisms to UV. The most common intronic variant was *BRCA1* c.442-43C>T (g.41251931C>T, rs799923) in intron 7. This

specific variant had an MAF score of A = 0.0986/494, with supporting evidence by Marth *et al*. (1999) that confirmed that this variant was a benign polymorphism.

The intronic deletion *BRCA1* c.548-58_548-58delT (g.32329548C>T, rs2126042) did not cause a frameshift in the reading frame of the adjacent exon and did not actively influence the splice site or reading frame of the adjacent coding region of exon 8. This polymorphism was classified by the BIC as not clinically significant. With an MAF score of T/T = 0.3349/1677 and supporting evidence, this polymorphism was classified as a benign allele that did not contribute towards an increased risk of familial BC (Russo *et al*., 2007).

Seven UVs within the intronic region of *BRCA1* were detected in this study. The non-coding variant *BRCA1* c.-19-115 T>C (g.41276247A>G, rs3765640) was found within one individual. According to the dbSNP (NCBI), this UV had an MAF score of C = 0.3536/1771 showing it was not classified as a rare variant (MAF<0.01). Furthermore, this variant was 115 bp from the coding region of exon 2 and did not influence the translation of the amino acid sequence in the coding region. This allele was detected in both its heterozygous and homozygous forms. Various authors have shown that the *BRCA1* gene was involved in multiple mechanisms that were vital for foetal development and when the *BRCA1* mutation carriers from both the maternal and the paternal cell line pass both mutations causing a homozygous mutant allele, the results were fatal (Gowen *et al.,* 1996; Hakem *et al*., 1998; Chandler *et al*., 2001). This UV was therefore considered as being non-pathogenic because of its frequency and mode of inheritance.

The *BRCA1* c.4485-64C>G (g.41226602G>C, rs-Unknown) variant was detected within one individual and described by the BIC as an UV. Currently no information regarding the clinical significance of this variant is available. As the variant lies 64 bp from the start of exon 15, it was therefore not considered to be within any of the critical regions according to the ENIGMA and may be regarded as benign.

The *BRCA1* c.302-41T>C (g.41256319A>G, rs-Unknown) non-coding variant was detected within two patients and is found within intron 6 with an MAF score of G = 0.0038/19. This relatively high frequency was above the threshold for the criteria

according to the ENIGMA clinical significance guidelines to be considered rare. This factor removed the clinical significance status from a class 5, 4 or 3 variant to a low or of no clinical significance status.

The following UVs found within the intronic regions of *BRCA1* do not fit the classification criteria as designated by ENIGMA and did not pose a threat of being pathogenic for the carriers of these variants. They include the non-coding variant *BRCA1* c.5152+28T>A (g.41215907A>T, rs-Unknown) found 28 bp downstream from exon 17, the *BRCA1* c.5152+73G>A (g.41215818A>G, rs Unknown) variant detected in intron 18, the *BRCA1* c.547+44A>G (g.41251788T>C, rs-Unknown) variant located in intron 8 and *BRCA1* c.5075-13T>G (g.41215981A>C, rs-Unknown) variant found in intron 17.

4.4.2.2 Molecular Screening of *BRCA2*

Two truncation mutations were detected for *BRCA2* exon 11. The first mutation detected was a nonsense mutation namely *BRCA2* c.5279C>G,p.Ser1760Ter (g.32339634C>G, rs80358751). This mutation was classified as a pathogenic class 5 variant (BIC). The second nonsense mutation *BRCA2* c.5563C>G,p.Ser1855Ter (g.32339913G>A, rs587782694) was considered to affect the function of the BRCA2 protein and cause a premature truncation in the polypeptide chain. According to the ENIGMA guidelines, any other mutation that caused a shift in the reading frame upstream of amino acid 3 327 of the BRCA2 protein was regarded as pathogenic and all variation downstream from that position was classified as benign. As these mutations occurred at amino acid 1 760 and 1 855 respectively, both mutations were classified as pathogenic as they fell within the BCR repeat region that was responsible for the binding of RAD51 and PALB2 to the BRCA1 protein. These mutations have previously been shown to not only inhibit the function of the BRCA2 protein, but also incapacitated the binding mediated by RAD51 and PALB2 (Nieborowska-Skorska *et al.,* 2014).

Another two pathogenic mutations were detected within *BRCA2* namely the splice site mutation *BRCA2* c.8754+1G>A (g.32376792G>A, rs397508006) and the coding

mutation *BRCA2* c.9435_9436delGT, p.Val3145_Phe3146=fs (g.32394867_32394868delGT). The first pathogenic mutation was found 1 bp downstream from exon 21. This mutation caused exon trapping that resulted in the activation of a cryptic site 46 base pairs from the 3' end of exon 21 (Hansen *et al.,* 2008). This activation led to a premature stop codon resulting in a faulty BRCA2 protein by truncating the size of the protein product. The second deleterious frameshift mutation *BRCA2* c.9435_9436delGT was classified according to the BIC as a class 5 pathogenic frameshift mutation. This mutation was novel and no supporting literature was available. The remaining variation observed for *BRCA2* were either benign or UV.

The synonymous polymorphism *BRCA2* c.7242A>G,p.Ser2414Ser (g.32355095A>G, rs1799955) was found within exon 14. The MAF score for this variant was G = 0.2326/1165. A study showed that this polymorphism was not pathogenic and did not influence the function of the BRCA protein complex (Hadjisavvas *et al.,* 2003).

The coding variant *BRCA2* c.865A>C,p.Asn289His (g.32332343A>C, rs766173) is an UV according to the BIC. The MAF score for this variant was C = 0.0737/369 and it was not classified as rare. There was conflicting evidence regarding the pathogenic status of this variant, however, according to the NCBI it was likely to be benign and was classified as not clinically significant (Freedman *et al*., 2004; Tommasi *et al*., 2008).

There were three benign intronic variants detected for *BRCA2*. *BRCA2* c.8755-66T>C (g.32953388T>C, rs4942486) was detected within four individuals with an MAF score of C = 0.4884/2446. The BIC classified this variant as not clinically significant as it was proven to not alter the function of the BRCA2 protein complex (Ozcelik *et al.,* 2012). The *BRCA2* c.-26 G>A (g.32890572G>A, rs-Unknown) variant was detected within four individuals. According to the BIC, these polymorphisms did not clinically contribute towards the pathology, familial BC.

Two individuals in this study had exhibited *BRCA2* c.425+67A>C (g.32899388A>C, rs-Unknown). According to the BIC and UMD-*BRCA2* mutations database, this

polymorphism had no clinical significance and did not contribute to familial BC as it did not influence the expression or translation of the *BRCA2* gene.

Three intronic UVs were detected within *BRCA2.* The first was *BRCA2* c.67+58A>G (Unknown HGVS, rs-Unknown) located 58 bp from the coding region of exon 2. As this variant was more than 6 bp from the coding region and no evidence regarding the influence of this variant was available, this variant could not be classified as a high risk for the pathology. The second UV, known as *BRCA2* c.681+56C>T (g.32329548C>T, rs2126042), was identified within one patient. According to the dbSNP, it had an MAF score of T = 0.1859/931 and was detected in both the heterozygous and homozygous forms. In a study, an *in silico* analysis of this variant predicted that it is most likely benign (Wagner *et al.,* 1999). The final UV, *BRCA2* c.8755-75T>C (Unknown HGVS, rs-Unknown), was detected once and according to the ENIGMA classification did not fall within the criteria of being pathogenic based on the position of the variant. No further supporting literature could be obtained for this UV.

Chapter 5

5.1 Conclusion

Familial BC testing at this facility was previously based on the use of older technology. These included techniques such as SSCP/HA (Hayashi, 1991). Conventional SSCP/HA was costly and labour intensive. The optimised HRMA worked well as a molecular screening technique for *BRCA1* exons 2 - 10 and 12 – 24, as well as *BRCA2* exons 2 - 10 and 12 - 27. The molecular screening technique, PTT, worked well at identifying truncated proteins within the *BRCA1* exon 11 and *BRCA2* exon 11. When SSCP/HA and the newly optimised HRMA results were compared there were no inconsistencies in the nucleotide detection of these techniques. The setup time of HRMA was considerably faster and less labour intensive compared to SSCP/HA. HRMA had the same sensitivity to detect genetic differences as compared to SSCP/HA. There were however false positive results when using HRMA. Thus, any result on HRMA that indicates an outlier, has to be sequenced. It is for this reason that HRMA cannot be used as a genotyping technique, only a screening technique.

The Division of Human Genetics has been involved in familial BC research for more than a decade. Their work resulted in the identification of founder mutations within the Afrikaner (Reeves *et al*., 2004) and Coloured/Xhosa populations from the Western Cape (van der Merwe *et al*., 2012). The Division has since become the testing facility for Indian patients from KwaZulu-Natal. This endeavour was complicated because no information was available regarding the mutation profile for *BRCA1* and *BRCA2* for this unique population group.

The Indian population of SA displayed a high rate of genetic variability observed within *BRCA1/2* and this research opened the possibility of further investigations. A total of nine pathogenic mutations and 25 non-pathogenic variants were identified within the cohort. Furthermore, 45% of the pathogenic mutations were found within *BRCA1*/2 exon 11, respectively. Three patients presented with the same disease causing mutation that was found within the splice site of *BRCA2* exon 21.

The patients that presented with the mutations identified to be clinically significant, were genetically counselled and testing was offered to relatives of the affected individuals. These patients had the option of preventative measures that would reduce their risk factor significantly. The patients that were high-risk individuals and who did not present with a pathogenic *BRCA1/2* mutation had the option to explore other alternatives.

To conclude, mutation detection is vital within medical research as it is fundamental to disease identification and associated diagnoses. Currently, samples from individuals are sent to international laboratories that either have the facilities or mutation panels to appropriately test them. The familial BC research that was conducted, opens doors for more specific mutation panels to be designed within SA for this population. This research will contribute to improved diagnostics and disease management for familial BC in SA within the Indian population.

Chapter 6

6.1 References

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Appendix A

Ethical approval

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS UV

[redacted]

Research Division
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E-mail address: EthicsFHS@ufs.ac.za

Ms J du Plessis/gn

2014-11-12

REC Reference nr 230408-011
IRB nr 00006240

MR HMVE COMBRINK
DIVISION OF HUMAN GENETICS
FACULTY OF HEALTH SCIENCES
UFS

Dear Mr Combrink

ECUFS NR 107/2014
PROJECT TITLE: MOLECULAR SCREENING OF THE SOUTH AFRICAN INDIAN POPULATION FOR BRCA1 AND BRCA2 USING HIGH RESOLUTION MELTING ANALYSIS.

1. You are hereby kindly informed that at the meeting on 04 November 2014 the Ethics Committee approved the above project after all conditions were met when the following was submitted:

   - ***Revised Information Leaflet and satisfying answers to the reviewer's questions***

2. Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition (2006); the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.

3. Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

4. The Committee must be informed of any serious adverse event and/or termination of the study.

5. All relevant documents e.g. signed permission letters from the authorities, institutions; changes to the protocol, questionnaires etc. have to be submitted to the Ethics Committee before the study may be conducted (if applicable).

6. A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.

Appendix B

Permission obtained from Prof M Theron: Head of the Division of Human Genetics and Acting Business Manager

National Health Laboratory Service
Human Genetics \ Mensgenetika

Tel: +27 51 4053047
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Practice/Praktyk no:5200296

Block C
Faculty of Health Sciences
University of the Free State
Bloemfontein

P.O. Box 339 (G11)
UFS
Bloemfontein 9300

Prof M Theron
Head of Department of Human Genetics
National Health Laboratory Services
Faculty of Health Sciences, UFS
BLOEMFONTEIN

13 June 2014

Dear Prof M Theron

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

I am in the process of expanding our current research project on familial breast cancer involving the genes *BRCA1* and *BRCA2*. We aim to screen young breast cancer patients (especially of Indian, Mixed Ancestry or African decent) or patients with a positive family history (any ethnic group). Blood samples from patients will be collected for DNA extraction. The project involves screening these patients for disease-causing mutations present in these genes, with the hope to establish a more informative diagnostic mutation screening protocol for each of the population groups within South Africa. It is currently limited to the Afrikaner population.

I would therefore like to ask your permission to use the space and equipment of the Division of Human Genetics (Molecular Laboratory) for this project. We hope to present the data at the local Faculty Forum as well as on other national congresses, depending on patient numbers and the results obtained. This study will be ongoing and will commence in April/May of 2014, after ethic approval has been obtained.

Your prompt reply will be appreciated.

Yours sincerely

Dr NC vd Merwe PhD
Principal investigator
E-mail: gnmgncv.MD@mail.uovs.ac.za
On behalf of: Pakiso Moeti
HMVE Combrink
J Oosthuizen

Approved :

Division of Human Genetics
University of the Free State &
2014 -06- 13
NHLS Universitas Tertiary Academic
Laboratories
Bloemfontein

Appendix C

SA Genetic counselling protocol for familial breast cancer: used by all South African counsellors as compiled on their discipline specific website

http://www.geneticcounselling.co.za

Can breast cancer be inherited?

- Breast cancer is common with an average lifetime **risk of breast cancer of 1 in 10 women** .
- The cause is mainly **sporadic** (random/ as a once off) influenced by environment and lifestyle.
- In a small number of cases (5-10%) breast cancer is **inherited** (passed from one generation to the next).
- Individuals from these families have an **increased chance of developing cancer in their lifetime**.
- Knowing your risk of hereditary breast cancer is **important for your future health** as it allows access to healthcare options to reduce the impact of cancer in your life.

What is hereditary breast cancer?

- Hereditary breast cancer is an **inherited** condition.
- When present your risk of developing breast cancer and other related cancers at a young age is greater than the average person.

What is the role of genetics in hereditary breast cancer?

- Genes carry information that defines how our bodies work, look and develop.

- These are inherited from both parents and hence each person has two copies of each gene.
- Faults (mutations) in certain genes can lead to an increased risk for cancer.
- Hereditary breast cancer has been associated with mutations in one of two genes, ***BRCA1*** or ***BRCA2***.
- The role of these genes is to prevent the development of cancer.
- Mutations in these genes are inherited in an **autosomal dominant** manner.
- Meaning the risk for hereditary breast cancer is 50/50 and this is the same for both males and females.
- If you have a BRCA1 or BRCA2 mutation you have a:
    - 50 to 80% lifetime risk of developing breast cancer.
    - 60% of developing a second primary breast cancer.
    - 20% to 60% risk of developing ovarian cancer.

When should you be concerned about hereditary breast cancer?

- A *BRCA1* **or** *BRCA2* **mutation** has been identified in another family member.
- You have a **personal history** of **breast cancer or any other associated cancer** such as ovarian, melanoma, prostate cancer.
- You have a **family history** of **breast cancer and/or other associated cancers** such as ovarian, melanoma, prostate cancer.
- You have a family history of cancer **diagnosed at an early age** such as younger than 50 years for breast cancer.
- You have a family history of unusual or **rare cancers**.
- **Multiple primary (first) cancers** in the same individual – same or different organs.
- **Bilateral (both sides) cancers** in paired organs e.g. breasts, eyes.
- Family history of **male breast cancer**.
- You are from an **ethnic group** known to be at risk for hereditary breast cancer such as Ashkenazi Jewish or Afrikaans.

How will genetic counselling help you if you are concerned about a hereditary breast cancer?

- You will be given information on the **role of genetics** in the development of hereditary breast cancer.
- A cancer risk model will be used to **assess your risk for hereditary breast cancer** in your family and used to define the risk for you and your family.
- Your results will be used to develop a **personalised plan** for screening, management and genetic testing.
- The benefits, risks and limitations of these health options will be discussed in an emotionally supportive environment to allow for **optimal decision-making** for the future.

What happens during a genetic counselling session?

During genetic counselling the genetic counsellor will:

- Obtain a **family** and **personal medical history**.
- Draw up a **family tree**.
- Use the medical information and family tree to **assess the risk** of a hereditary cancer syndrome.
- Discuss the **cause, inheritance, your risk of a hereditary breast cancer** and the likelihood of cancer in you and other of your family members.
- Discuss **genetic testing** (if appropriate), **preventative and screening options**, including the benefits, risks and limitations.

What are the recommended screening options if you have/ are at risk for hereditary breast cancer?

- Monthly **breast self examinations** from early adulthood.
- Six monthly to annual **clinical breast examinations**.
- Annual **mammography or sonography or MRI** from 40 years of age (or 5 years earlier than youngest age of diagnosis).

Please note that these are guidelines and may differ between specialists. The types of healthcare options/interventions and their timing will be defined by the treating specialist.

Is there genetic testing available for *BRCA1* and *BRCA2* genes?

- Limited genetic testing of the *BRCA1* and *BRCA2* genes is available in South Africa but more comprehensive testing is available overseas.
- Genetic testing is ideally first performed on a family member that has had a diagnosis of cancer.
- If a mutation is identified in either the *BRCA1* or *BRCA2* genes then testing can be offered to other relatives.

Please note that most medical aids do not provide funding for the cost of genetic tests.

What if no gene mutation is found in the *BRCA1* or *BRCA2* gene?

- If no mutation is identified but your family was assessed as being at high risk for hereditary breast cancer, then you would continue with recommended screening options as for individuals where a mutation was found.

How does knowing your risk help you?

- **Preventative and management options** are based on your family history and/or *BRCA1* and *BRCA2* gene test result.
- If your family is at high risk for a hereditary breast cancer accessing to preventative treatment and more intensive screening methods could substantially **reduce the risk of developing cancer**.
- Your risk for hereditary breast cancer can be used to **define the risk for other family members**.

Need more information about hereditary breast cancer?

Please Contact Us should you need more information about hereditary breast cancer, explore your risk for hereditary breast cancer or want to make an appointment to determine whether your family is at risk for hereditary breast cancer.

Appendix D

DNA consent form to be used by Inkosi Albert Luthuli Hospital in Durban

CONSENT FOR DNA ANALYSIS AND STORAGE

1. I, _____ , request that an attempt be made using genetic material to assess the probability that: I / my child (name:_____) / my unborn child (DELETE WHERE NOT APPLICABLE) might have inherited a disease-causing mutation in the gene for:_____
2. I understand that the genetic material for analysis is to be obtained from: blood cells / skin sample/ other tissue (specify _____) (DELETE WHERE NOT APPLICABLE) :
3. I request that **no** portion of the sample be stored for later use. ☐ **(**MARK IF APPLICABLE)

   or

   I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):

   (a) possible re-analysis for the same disorder

   (b) analysis (for the same disorder) for the benefit of members of my immediate family

   (c) research purposes (for any disorder), subject to the approval of the Institution's Ethics Committee, provided that any information from such research will remain confidential and anonymous.
4. The results of the analysis carried out on this sample of stored biological material will be made known to me, via my doctor, in accordance with the relevant protocol, if and when available. In addition, I authorise that these results may be made known to: (DELETE WHERE NOT APPLICABLE) :

   other doctors involved in my / my child's care

   the following family members: _____

   other persons: _____
5. I authorise / do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the _____ at _____.
6. 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.
7. 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.
8. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY: _____**
9. **Address:**

Tel. no.:

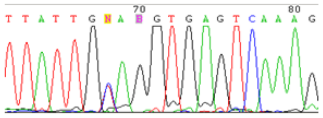
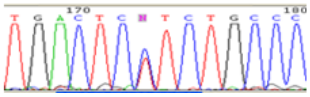
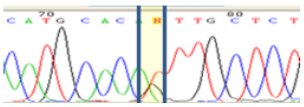
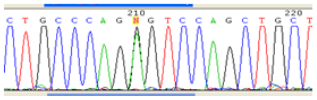
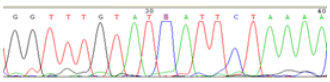
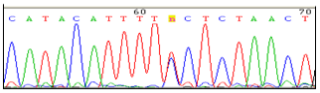
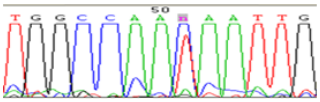
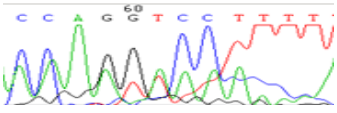
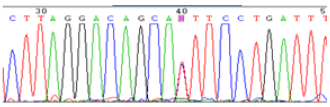
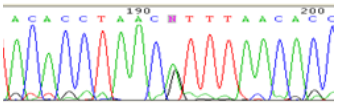
Signature _____ Date: _____
of person giving consent Capacity _____

Patient /guardian signature: _____ **Witnessed consent:_____**

NOTE - PLEASE INSERT A FAMILY PEDIGREE DRAWING ON THE REVERSE OF THIS FORM

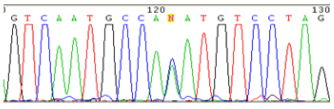
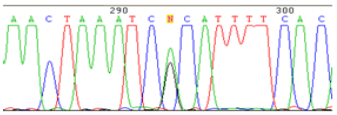
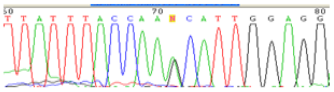
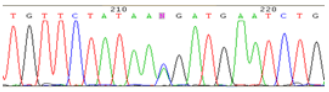
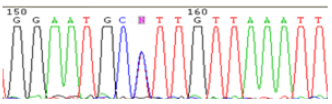
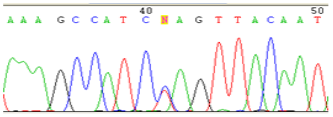
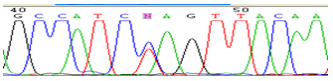
Appendix E

Raw sequence data for *BRCA1*

c.591C>T	**p.Cys197Cys**	
c.4308T>C	**p.Ser1436Ser**	
c.4535G>T	**p.Ser1512Ile**	
c.4837A>G	**p.Ser1613Gly**	
c.-19-115T>C	**IVS1-115T>C**	
c.302-41T>C	**IVS6-41T>C**	
c.442- 34C>T	**IVS7-34T>C**	
c.548-58_548-58delT	**IVS8-58delT**	
c.5153 - 73T>C	**IVS18-73T>C**	
c.5152+73G>A	**IVS18+73G>A**	

Appendix F

Raw sequence data for *BRCA2*

c.865A>C	**p.Asn289His**	
c.7242A>G	**p.Ser2414Ser,**	
c.-26 G>A	**c.-26 G>A**	
c.425+67A>C	**IVS4+67A>C**	
c.681+56C>T	**IVS8+56C>T**	
c.8755-66T>C	**IVS21-66T>C**	
c.8755-75T>C	**IVS21-75T>C**	

For the complete raw sequencing, including the identification of all the raw data not indicated contact Dr NC van der Merwe at vanderMerweNC@ufs.ac.za

Appendix G

Freeware licence agreement for the use of Patric J. Lynch illustration of the breast.

Description	English: Breast normal anatomy, cross-section, now thought to be incorrect[1] with numbered legend arrows Español: Sección representativa normal de la anatomía del pecho con las flechas numeradas para una leyenda Deutsch: Normaler Anatomiequerschnitt der Brustes mit nummerierten Pfeilen Français : Vue en coupe anatomique et schématique d'un sein avec flèches numérotées pour une légende Polski: Schemat przekroju sutka (gruczołu mlecznego) kobiety w przekroju strzałkowym. Opis wg. legendy Italiano: Rappresentazione anatomica di sezione sagittale di mammella umana. Frecce numerate legate a legenda.
Date	3 September 2007
Source	Patrick J. Lynch, medical illustrator
Author	Original author: Patrick J. Lynch. Reworked by Morgoth666 to add numbered legend arrows.
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Other versions	Originally from Image:Breast anatomy normal.jpg