

Molecular screening of Coloured South African breast cancer patients for the presence of *BRCA* mutations using high resolution melting analysis

by

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Hiermee erken ek met dank die finansiële hulp (beurs) wat ek van die Struwig-Germeshuysen Kankernavorsingstrust ontvang het, vir die voltooiing van my studies. Menings wat in die publikasie uitgespreek word of gevolgtrekkings waartoe gekom is, is die van die navorser alleen en strook nie noodwendig met dié van die SGKN-Trust nie.

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MOLECULAR SCREENING OF COLOURED SOUTH AFRICAN BREAST CANCER PATIENTS FOR THE PRESENCE OF *BRCA* MUTATIONS USING HIGH RESOLUTION MELTING ANALYSIS

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AM Grobler

Dedicated to my beloved friends and family

Your work is going to fill a large part of your life, and the only way to be truly satisfied is to do what you believe is great work. And the only way to do great work is to love what you do. If you haven't found it yet, keep looking. Don't settle. As with all matters of the heart, you'll know when you find it.

– Steve Jobs

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- The Creator, who gave me a glimpse of creation and filled me with wonder.

Summary

The populations of South Africa (SA) exhibit a rainbow of genetic diversity due to the high contribution of ancestral genetic admixture. The economic structure of this third world country has limited the exploration of this genetic diversity with regards to familial breast cancer (BC) testing. As the SA Coloured woman has a lifetime risk of 1 in 22 to develop BC, the main aim of the study involved targeting the highly penetrant genes *BRCA1* and *BRCA2* for comprehensive mutation analysis. This was done in order to determine the range of variants and mutations present within BC patients representing this group.

In order to perform such a comprehensive screen, High Resolution Melting Analysis (HRMA) was optimised and validated for use in conjunction with the protein truncation test (PTT), genotyping assays using real-time based PCR, single stranded conformational analysis (SSCP) and DNA Sanger sequencing to determine the presence of potential disease-causing mutations. A total of 229 Coloured BC patients were included based on a specific selection criteria. This criteria included being affected with BC and either having a positive family history of the disease, or an early age at onset (<45 years) or bilateral disease. All male BC patients were included.

Twelve different pathogenic or class 5 mutations were detected for a total of 33 patients. These mutations were identified using genotyping analysis, PTT and HRMA. These mutations were confirmed using Sanger sequencing. These mutations included all three the Afrikaner founder mutations, together with the Xhosa/Coloured mutation detected for the Xhosa and Coloured population residing in the Western Cape.

A total of 50 variants were identified using HRMA, ranging from single base changes to a 12bp deletion occurring within the coding region of *BRCA2*. The clinical significance of these variants were classified using computer-based analysis. Variants of unknown significance (VUS) were investigated using a multiple evidence-based approach in order to confirm their clinical status. These included using the BIC, ClinVar, the ENIGMA guidelines, and the 1000 Genomes project database. This was done in order to investigate whether the variant was

novel or allocated to a specific population cluster. The majority of the variants was class 1 polymorphisms, exhibiting normal variation. The portfolio of variants reflected 300 years of admixture between the Bantu-speaking Black African populations of the North Western Cape province, the European settlers and the slaves from the East as global, Eastern and African polymorphisms were observed.

Numerous new pathogenic mutations were identified, ranging from likely pathogenic (class 4) to class 5. Many of these mutations proved to be restricted to the southern tip of SA. Based on these results, recommendations can be made regarding the composition of targeted mutation panels for the diagnostic testing of SAC BC patients and their families.

Keywords: Coloured Population, South Africa, *BRCA1/2*, HRMA, mutation screening, computer-based analysis

Opsomming

Die bevolking van Suid-Afrika (SA) beskik oor 'n reënboog van genetiese diversiteit wat te wyte is aan die hoë bydrae van voorvaderlike genetiese vermenging. Die ekonomiese struktuur van hierdie derdewêreldse land het die ontginning van hierdie diversiteit met betrekking tot oorerflike borskankertoetsing verhinder. Die hoofdoelwit van die studie was om hierdie twee hoë-impak borskankergene te analiseer in Kleurlingpasiënte, aangesien hulle risiko vir die ontwikkeling van die siekte 1 uit elke 22 is. Hierdie studie is nodig om die tipe en verskeidenheid van mutasies te bepaal wat moontlik teenwoordig kan wees by Kleurling borskankerpatiënte.

Ten einde so 'n omvattende analise uit te voer, is die nuwe mutasie siftingstegniek, *High Resolution Melting Analysis* (HRMA), geoptimeer en gevalideer vir gebruik. In samewerking met die *Protein Truncation Test* (PTT), genotipering met behulp van qPCR, enkelstring konformasie-ontleding (SSCP) en DNA volgordebepaling kon hierdie ontledings gedoen word. 'n Totaal van 229 Kleurlingpasiënte is ingesluit op grond van spesifieke kriteria. Die kriteria is gebaseer op die teenwoordigheid van 'n positiewe familiegeskiedenis, of 'n vroeë ouderdom van diagnose (<45 jaar) of bilaterale aantasting. Alle geaffekteerde mans is ingesluit.

Twaalf verskillende siekte-veroorsakende of klas 5 mutasies is geïdentifiseer vir 'n totaal van 33 pasiënte. Hierdie mutasies is geïdentifiseer met behulp van genotipering, PTT en HRMA en is bevestig deur middel van DNA volgordebepaling. Al drie die Afrikaner stigtersmutasies sowel as die herhalende Kleurling- of Xhosamutasie is geïdentifiseer vir hierdie bevolking woonagtig in die Wes Kaap.

'n Totaal van 50 verskillende variante is geïdentifiseer deur gebruik te maak van HRMA. Hierdie variante het gewissel van enkelbasisveranderinge tot 'n 12 basis paar delesie teenwoordig in ekson 10 van *BRCA2*. Die kliniese belang van hierdie variante is bepaal deur gebruik te maak van rekenaaranalises. Variante waarvan die kliniese impak onseker was, is addisioneel ondersoek deur gebruik te maak van verskeie databasisse wat gebaseer was op konkrete bewyse. Hierdie databasisse het die volgende ingesluit, naamlik die BIC, ClinVar, die riglyne van

ENIGMA en die 1000 Genoomprojek. Hierdie addisionele studies was nodig om te bepaal of die variant nuut was of reeds in 'n spesifieke bevolkingsgroep voorgekom het. Die meerderheid van die variante was verteenwoordigend van gewone polimorfismes. Die versameling variante het 300 jaar se vermenging tussen die Bantoe-sprekende swart bevolkings van Afrika, die Europese setlaars en die slawe uit die Ooste versinnebeeld, aangesien die polimorfismes geïdentifiseer verteenwoordigend van die wêreld, die Ooste en Afrika was.

Verskeie nuwe siekte-veroorsakende mutasies is geïdentifiseer wat gevarieer het tussen klas 4 en klas 5. Baie van hierdie mutasies was beperk tot die suidelike gedeelte van Suider-Afrika. Aanbevelings vir die samestelling van spesifieke mutasiepanele vir die diagnostiese toetsing van SA Kleurlingpasiënte en hulle familieledede kan gemaak word, gebaseer op die resultate van hierdie studie.

Sleutelwoorde: Kleurlingbevolking, Suid-Afrika, *BRCA1/2*, HRMA, mutasieanalises, rekenaargebaseerde analises

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Abbreviations

aa	Amino Acid
ABRAXAS	Family with Sequence Similarity 175, Member A (OMIM 611143)
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
<i>BARD1</i>	BRCA1-Associated Ring Domain 1 (OMIM 601593)
BC	Breast cancer
BIC	Breast cancer Information Core
bp	Base pairs
<i>BRCA1</i>	Breast cancer susceptibility gene 1
<i>BRCA2</i>	Breast cancer susceptibility gene 2
BRCT	BRCA C Terminus
<i>BRIP1</i>	BRCA1- Interacting Protein 1 (OMIM 605882)
c	Coding DNA reference ID
ca	Cancer
C _p	Crossing point
Cys	Cysteine
ddNTP	Dideoxyribonucleotide triphosphate
del	Deletion
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ENIGMA	Evidence-based Network for the Interpretation of Germline Mutation Alleles
fs	Frame shift
g	Genomic nucleotide reference ID
Gln	Glutamine
GLOBOCAN	Global Burden of Cancer Study
Glu	Glutamic Acid

Gly	Glycine
GWAS	Genome-wide Association Study
HA	Heteroduplex Analysis
HCl	Hydrochloric Acid
HGVS	Human Genome Variation Society
His	Histidine
HR	Homologous Recombination
HRMA	High Resolution Melting Analysis
IARC	International Agency for Research on Cancer
Ile	Isoleucine
ins	Insertion
kb	Kilo base pairs
kDa	kilo Dalton
Leu	Leucine
Lys	Lysine
MAF	Global Minor Allelic Frequency
Met	Methionine
<i>MRE11A</i>	Meiotic Recombination 11, <i>S. Cerevisiae</i> , Homolog of, A (OMIM 600814)
mtDNA	Mitochondrial DNA
NaCl	Sodium Chloride
<i>NBN</i>	Nibrin (OMIM 602667)
NCBI	National Centre for Biotechnology Information
ng.μl ⁻¹	Nano gram per microliter
NHLS	National Health Laboratory Services
NLS	Nuclear Localization Signal
NRY	Non-recombining portion of the Y Chromosome
OMIM	Online Mendelian Inheritance In Man
OVC	Ovarian cancer
p	Protein reference sequence ID
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction

Phe	Phenylalanine
Pro	Proline
PTT	Protein Truncation Test
qPCR	quantitative Polymerase Chain Reaction
<i>RAD50</i>	RAD50, <i>S. Cerevisiae</i> Homolog of; RAD50 (OMIM 604040)
<i>RAD51C</i>	RAD51, <i>S. Cerevisiae</i> Homolog of, C; RAD51C (OMIM 602774)
<i>RAD51D</i>	RAD51, <i>S. Cerevisiae</i> Homolog of, D; RAD51D (OMIM 602954)
rs	Reference SNP ID number
SA	South Africa
SAC	South African Coloured
SDS	Sodium dodecyl sulphate
secs	seconds
Ser	Serine
SET	Sucrose-Tris-EDTA
SNP	Single Nucleotide Polymorphism
SSCP	Single Stranded Conformation Polymorphism
ssDNA	single stranded DNA
TAT	Turn-around-time
Thr	Threonine
T _m	Temperature of melting point
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
Tyr	Tyrosine
UFS	University of the Free State
v/v	Volume per volume ratio
Val	Valine
VUS	Variant of Unknown Significance
w/v	Weight per volume ration
WHO	World Health Organization
<i>XRCC2</i>	X-Ray Repair, Complementing Defective, in Chinese Hamster, 2; XRCC2 (OMIM 600375)

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

In the year 2016, the United Nations estimated the world to have a global living population of 7.4 billion people with approximately 82% residing in less developed regions. According to the World Health Organization (WHO) in 2015, the disease incidence and mortality rates in these regions are on the increase compared to more developed countries. Within these less developed regions, the disease incidence and mortality was the highest for cancer, at 135 in 100 000 and 89 in 100 000 respectively. Among all cancers, breast cancer (BC) had the highest incidence rate.

The completion of the Human Genome Project was one of the greatest feats of mankind and its exploration in history. An inward voyage of discovery gave mankind the ability to read our genetic blueprint and better understand inherited disease at the genetic level (National Human Genome Research Institute, 2015). Disease-causing mutations in the BC susceptibility gene 1 (*BRCA1*) and BC susceptibility gene 2 (*BRCA2*) increase the risk of developing BC by up to 80% (Claus *et al.*, 1996). The main challenges regarding screening for mutations in these genes are the long turnaround time (TAT) and a lack of population-specific diagnostic information (Feliubadaló *et al.*, 2013).

The objective for this study was to optimise High Resolution Melting Analysis (HRMA) as a more effective and higher throughput mutation screening technique in order to search for deleterious mutations within the familial BC genes *BRCA1* and *BRCA2* within the South African Coloured population (SAC). The study also hoped to provide insight into the landscape of naturally occurring variants within this genetically admixed population and identify possible familial-related mutations limited to the sequencing of the two BC genes.

1.2 CANCER BURDEN OF THE WORLD

In most economically developed countries, the majority of mortalities are due to the development of cancer (ca). This is a growing concern for the less developed countries such as South Africa (SA), as the ca burden will escalate due to the increase in the age of the average population. It is also in these less developed countries where roughly 82% of the world's population resides (United Nations, 2015). As the average life expectancy increases, abnormal lifestyle behaviours also increase. People tend to smoke more, follow poor diets, perform less physical activity in their day-to-day routine, and have their first child at a later average age in life (Lindsey *et al.*, 2015).

According to the World Health Organization (WHO), ca is a generic term that classifies a large group of diseases that can affect any part of the body. In 2012, the five most common ca types in men were lung, prostate, colorectal, stomach and liver cancer. For women, the top five ca types included ca of the breast, colorectal, lung, cervix and the stomach. According to the WHO, the five most common ca risk factors include a high body mass index, low fruit and vegetable intake, lack of activity, smoking and consumption of alcohol. Upon further investigation, the WHO states that the three main categories of external agents that contribute to ca development can be defined as physical carcinogens –for example ultraviolet and ionising radiation), chemical carcinogens – e.g. asbestos, components from smoking, food or water contamination and lastly biological carcinogens – e.g. infections from certain viruses, bacteria and parasites (World Health Organization, 2015).

The incidence and mortality of the five most common ca types among men and women worldwide are presented in Table 1.1. These statistics are based on worldwide GLOBOCAN estimates of ca incidences and mortalities presented by the International Agency for Research on Cancer (IARC) for 2012 (Ferlay *et al.*, 2015). From the data it can be concluded that the incidence rate of ca in more developed countries is higher than in less developed countries. For example, women develop breast cancer (BC) at almost double the rate (74.1) in developed countries compared to the latter (31.3) (Table 1.1). Interesting enough, the highest mortality rate is also due to BC, when compared to other ca types. For men, the highest incidence is seen for prostate ca, although more men pass away due to lung ca.

Table 1.1 Incidence and mortality rates and the cumulative probability of developing cancer by the age of 75 years, indicated for gender and cancer sites. The comparison is made between the more developed and less developed areas based on 2012 data (modified from World Health Organization, 2015). ASR indicates age-standardised rate per 100000.

	More developed Areas				Less Developed Areas			
	Incidence		Mortality		Incidence		Mortality	
	ASR	Cumulative risk, % (Aged birth to 74 years)	ASR	Cumulative risk, % (Aged birth to 74 years)	ASR	Cumulative risk, % (Aged birth to 74 years)	ASR	Cumulative risk, % (Aged birth to 74 years)
Females								
All cancers	240.6	23.3	86.2	9.0	135.8	13.4	79.8	8.1
Breast	74.1	8.0	14.9	1.6	31.3	3.3	11.5	1.2
Cervix	9.9	0.9	3.3	0.3	15.7	1.6	8.3	0.9
Colorectal	23.6	2.7	9.3	1.0	9.8	1.1	5.6	0.6
Lung	19.6	2.4	14.3	1.7	11.1	1.2	9.8	1.0
Stomach	6.7	0.8	4.2	0.4	7.8	0.9	6.5	0.7
Males								
All cancers	240.6	30.9	138.0	14.3	163.0	16.6	120.1	12.0
Colorectal	36.3	4.3	14.7	1.6	13.7	1.6	7.8	0.8
Liver	8.6	1.0	7.1	0.8	17.8	2.0	17.0	1.8
Lung	44.7	5.4	36.8	4.4	30.0	3.3	27.2	2.9
Prostate	69.5	8.8	10.0	0.8	14.5	1.7	6.6	0.6
Stomach	15.6	1.9	9.2	1.0	18.1	2.1	14.4	1.6

Table 1.2 lists the incidence and mortality rates of three subcontinents. The data for SA are represented by that of Southern Africa. The data indicate that for Africa, Southern Africa has the highest ca incidence and mortality rates, with Eastern Asia having the highest incidence and mortality rates for Asia (Table 1.2). For Europe the scenario seems quite different, with Western Europe having the highest incidence rates for ca, but not the highest mortality rate. The highest mortality rate is indicated for Central/Eastern Europe.

1.3 CANCER IN THE AFRICAN CONTINENT

Africa is a developing continent that currently lives in poverty and has a financially stricken health infrastructure compared to the more developed countries. It is normally expected that a disease would thrive in its population due to the limited access to doctors, proper diagnosis and treatment delay. This is however not the case as the ca incidence rates for Africa are lower overall compared to that of the developed world (Table 1.2). Valuable knowledge might be learned by studying Africa's gene pool.

Reliable data for Africa are hard to find due to the lack of infrastructure. Although the WHO has records for Africa and its diseases, gaps exist. From the data by IARC and GLOBOCAN 2012 in Table 1.2, it is evident that in Africa, Southern Africa has the highest incidence and mortality rates for ca. This could be due to more accurate data from SA as the country is broadly speaking more developed than the rest of its African neighbors. When the data of IARC and GLOBOCAN are scrutinised, it is interesting to note that the data obtained for SA are actually also intrinsically represented by the data for Central Africa, Europe and South Eastern Asia (Table 1.2), as the majority of the SA population groups (such as the Afrikaner, the various Black tribes and the SA Indian population) have their roots in these subcontinents. All these subcontinents are also more developed and have higher cancer incidence rates.

Table 1.2 Estimated ASR for incidence and mortality rates per 100 000 by world area, for 2012 (modified from World Health Organization, 2015).

	Incidence			Mortality		
	Male	Female	Overall	Male	Female	Overall
Eastern Africa	120.7	154.7	137.8	103.8	110.5	106.5
Central Africa	91.8	110.7	100.8	82.3	82.3	81.2
Northern Africa	133.5	127.7	129.7	99.9	75.7	86.8
Southern Africa	210.3	161.1	177.5	136.5	98.7	112.5
Western Africa	78.7	112.4	95.3	68.5	75.7	71.6
Eastern Asia	225.4	151.9	186.0	159.3	80.2	117.7
South-Central Asia	98.4	103.3	100.1	74.8	64.7	69.3
South-Eastern Asia	147.6	132.6	138.2	114.1	79.5	94.8
Central/Eastern Europe	260.0	193.5	216.1	173.4	91.6	123.4
Southern Europe	298.4	263.9	277.4	126.2	94.4	108.2
Southern Europe	297.6	220.4	253.6	137.9	78.9	105.2
Western Europe	343.7	263.7	298.7	131.3	83.6	105.0

1.4 CANCER INCIDENCE AND RISKS FOR SOUTH AFRICA

South Africa has a population of 54 million citizens (Statistics South Africa, 2015) which is divided mainly into four population groups, namely the Black African population (80.5%), the Coloured (8.8%) and White populations (8.3%), with 2.5% being Indian/Asian (Statistics South Africa, 2015). The genetic diversity is reflected in the 11 official languages for the country, which include the Black native languages (such as isiNdebele, isiXhosa and isiZulu), English (British ancestors) and Afrikaans (European descendants) (Patterson *et al.*, 2010).

The incidence of BC is increasing in sub-Saharan Africa, including Southern Africa where SA is located (Fregene & Newman, 2005; van der Merwe *et al.*, 2012). A summary of cancer incidence for SA is presented in Table 1.3. The three most common SA cancer types that women develop during their lifetime include BC, cervical ca and basal cell ca, with the highest incidence and risk being 26.94 in a 100 000 or 1 in 33 for CA. The three most common cancers SA men develop in their lifetime are firstly basal cell carcinoma, then prostate cancer and lastly squamous cell carcinoma, with the highest incidence and risk for basal cell carcinoma at 34.36 in 100 000 or 1 in 25 (Table 1.3).

When comparing the BC risk among SA women, according to data presented by Fregene and Newman (2005), it can be highlighted that Asian women have the highest risk for developing BC at 1 in 17 compared with the smallest chance for African women with a lifetime risk of 1 in 33. It is interesting to find that Coloured women have a risk of 1 in 22, which is closer to the risk of Asian and White women (1 in 18) than to the risk of their female African ancestor counterpart.

1.5 THE COLOURED POPULATION OF SOUTH AFRICA

The self-designated Coloured or Mixed Ancestry population, termed the South African Coloured (SAC) population (Quintana-Murci *et al.*, 2010), is genetically derived from various indigenous African populations (Khoi- and San-speaking or Bantu-speaking), slave labourers from West Africa, Indonesia, Madagascar, Java, India and Malaysia, as well as immigrants from Western Europe (Guidelines from the SASHG Committee for publication purposes compiled during 2013, Appendix A).

Table 1.3 Summary of the statistics of cancer types diagnosed histologically in women in SA during 2010 (National Cancer Registry, 2010).

Group	Site of Cancer	Percentage of all cancers	ASR	Cumulative risk, % (Aged birth to 74 years)	Life time Risk (Age 0-74)
South African	Breast	20.82	26.94	2.99	1 in 33
	Cervix	17.63	22.33	2.36	1 in 42
	BCC	13.40	17.40	1.93	1 in 52
Indian/Asian	Breast	39.57	51.15	5.81	1 in 17
	Cervix	8.01	10.32	0.99	1 in 101
	Primary Site Unknown	7.49	10.06	1.16	1 in 86
African	Cervix	28.01	26.19	2.80	1 in 36
	Breast	19.82	18.71	2.04	1 in 49
	Kaposi Sarcoma	6.69	4.91	0.40	1 in 252
Coloured	Breast	25.95	40.91	4.63	1 in 22
	BCC	13.15	22.77	2.49	1 in 40
	Cervix	12.11	17.76	1.88	1 in 53
White	BCC	33.89	84.44	8.86	1 in 11
	Breast	18.878	50.17	5.45	1 in 18
	SCC of Skin	11.42	25.22	2.58	1 in 39

ASR indicates age-standardised rate per 100 000. Rates are standardised in the World Standard Population.

BCC = Basal Cell Carcinoma and SCC = Squamous Cell Carcinoma

This group of people is unique to SA, with the majority (50.2%) residing in the Western Cape province, in the vicinity of Cape Town. In this dissertation, the term *Coloured* will therefore refer to this specific group of people that share the same complex history of ancestrally derived admixture.

In 1652 the Dutch East India Company established a trading and refreshment station in this area (Nurse *et al.*, 1985; Patterson *et al.*, 2010). Over generations social and demographic events fused these people into the SAC population that consists of the indigenous Khoi and San, various Bantu speaking populations, European settlers and slaves' descendants from Java, India, Mozambique and Madagascar (Mountain, 2003; Quintana-Murci *et al.*, 2010). The SAC population is therefore a highly admixed, but genetically unique ethnic group (Nurse *et al.*, 1985; Mountain, 2003). Genetic research involving this ethnic group is very limited. The first genome-based research involving this group commenced in 2009 (Quintana-Murci *et al.*, 2010).

Admixed populations have been the source of numerous challenges in clinical studies. The background of a mixed ancestry group causes difficulties trying to identify inherited diseases and their origins. Understanding the genetic structure of an admixed population will assist in the understanding of the evolution and impact on human disease.

In a study by Tishkoff and colleagues (2009), 1327 nuclear microsatellite and insertion/deletion markers in a large panel of African populations were compared. The study found that the SAC clustered in intermediate positions between African and non-African populations. In a second study by Patterson and colleagues (2010), genetic variation in 20 SAC individuals were compared to other worldwide populations by means of high-density genome-wide genotyping. They concluded that the SAC was the result of complex admixture involving the Bantu-speaking populations from SA as well as Europeans, South Asians and Indonesians.

These two studies conclude that the SAC population is admixed with the largest maternal genetic contribution coming from the Khoisan; secondly Indian, with the Bantu maternal lineage being the lowest. The paternal ancestry has almost equal contributions from the European and Khoisan ancestries. The second largest paternal ancestral contribution was Indian ancestry, with the South East Asia paternal ancestry in the third position. The ratios of paternal and maternal proportions are depicted in Figure 1.1 as described by Quintana-Murci and colleagues (2010).

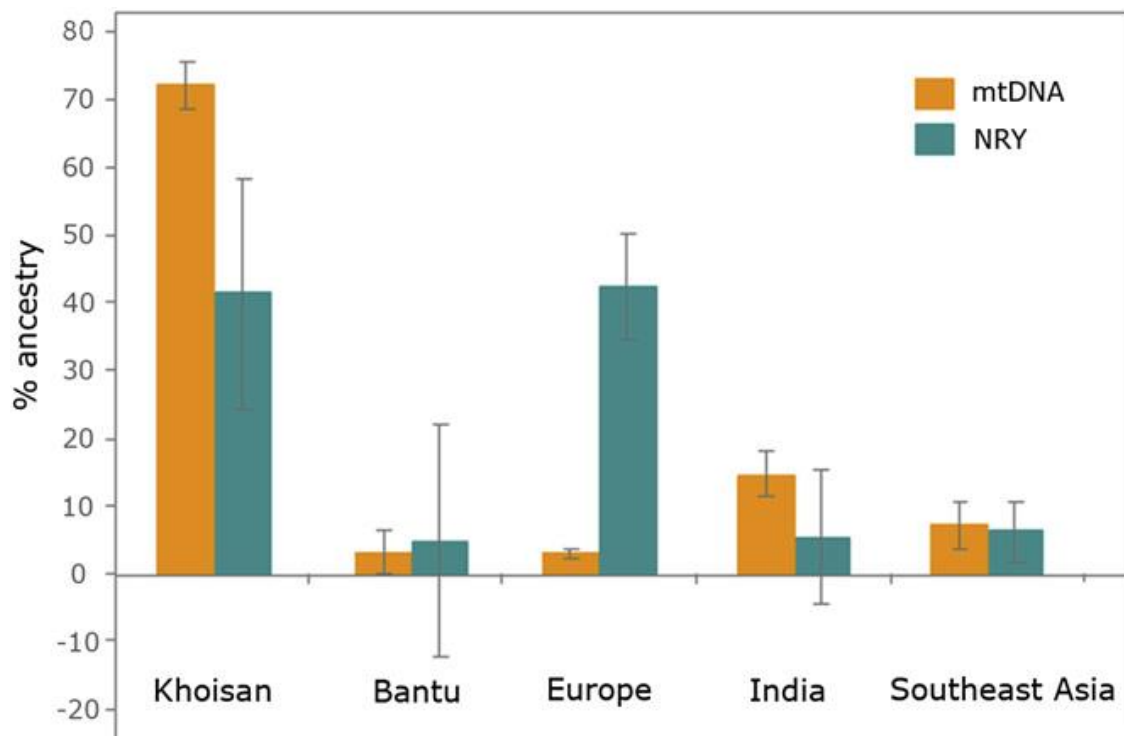


Figure 1.1 The mean diverse continental and within-continental admixture proportions in the SAC population for the five parental meta-population genetic contributors. The mean is indicated on the basis of maternally inherited mitochondrial DNA (mtDNA) and the non-recombining portion of the Y chromosome (NRY). Error bars indicate standard deviations (Source: Quintana-Murci *et al.*, 2010).

1.6 BREAST CANCER AND THE SUSCEPTIBILITY GENES

When breast cells undergo genetic damage that cause them to function abnormally and multiply to develop a malignant tumour, the disease is classified as BC (Lalloo & Evans, 2012). Numerous risk factors have been identified through the years that can lead to the development of BC. These factors include hormonal levels, reproductive and menstrual history, age, very little routine exercise, alcohol abuse, radiation, benign BC and obesity (Yang *et al.*, 2011). Although numerous risks have been identified, the clinical value is to understand the development of inherited BC.

Hereditary factors play a major role in the development of the disease. Studies have found that 10%-30% of BC cases are caused by hereditary factors with only 5%-10% of cases identified as having a strong inherited component. From these cases 4%-5% can be explained by mutations in high penetrant genes inherited in an autosomal dominant fashion (Newman *et al.*, 1988; Hall *et al.*, 1990). The evidence for these dominant inherited factors was found within the alleles on chromosome 17q12 and 13q12-13 where the *BRCA1* (Online Mendelian Inheritance In Man (OMIM 113705) and *BRCA2* (OMIM 600185) genes are localised (Miki *et al.*, 1994; Wooster *et al.*, 1995). Since their discovery the two genes have been linked to hereditary BC (Walsh *et al.*, 2010).

1.6.1 HIGH AND MODERATE PENETRANT BREAST CANCER GENES

The *BRCA1* gene encodes a protein that maintains genomic stability by acting as a tumour suppressor (Savage & Harkin, 2015). After the mRNA has been translated, it combines with various other tumour suppressor proteins, DNA damage sensors and signal transducers to form a large multi subunit complex, known as the *BRCA1*-associated genome surveillance complex (Wang *et al.*, 2000). Women who carry a *BRCA1* inherited mutation are predisposed to a high risk of breast and ovarian cancers at a younger age with a lifetime risk of 80% and 40% respectively (Welsch & King, 2001; Antoniou *et al.*, 2003).

BRCA2 also encodes a protein that forms part of the maintenance of genomic stability, but more specifically the homologous recombination (HR)

pathway. It assists in the repair of double stranded DNA breaks. Women who carry a *BRCA2* mutation have a lifetime risk of 26%-84% for developing BC and 20% for ovarian cancer while men who carry an inherited mutation have a lifetime risk to develop prostate (20%) and breast (6%) cancer (Easton, 1999; Chen *et al.*, 2006).

Since the dominant features of several cancer syndromes were identified, various other genes have also been found to be mutated in familial BC. These syndromes are listed in Table 1.4. These include *TP53* (OMIM 191170), *PTEN* (OMIM 601728), *STK11* (OMIM 602216) and *CDH1* (OMIM 192090). Other genes appear to have an increased risk for BC and OVC with moderate or intermediate penetrance due to being part of the BRCA complexes at certain points in different repair cycles. Each of these complexes leads to a specific cancer syndrome, yet in relation to BC patients, these genes have only been found in 0.1%-3% of BC cases (Apostolou & Fostira, 2013). The moderate penetrance genes, including the former, are listed in Table 1.4.

1.6.2 LOW PENETRANT BREAST CANCER GENES

Several BC susceptibility loci have been associated with a slightly increased or decreased risk for BC. These genetic modifiers can follow the polygenic model and can act synergistically with environmental or lifestyle factors to either increase or reduce BC risk. Together they account for a small fraction of familial BC cases (Apostolou & Fostira, 2013). The majority of these low-susceptibility loci have been identified through genome wide association studies (GWAS). From the Apostolou and Fostira study performed in 2013, only five single-nucleotide polymorphisms (SNPs) of the loci showed significant association specifically with BC. These include *MAP3K1* (OMIM 600982), *FGFR2* (OMIM 176943), *LSP1* (OMIM 153432), *TNRC19* (OMIM 602625) and *H19* (OMIM 616186).

1.7 THE HIGH IMPACT SUSCEPTIBILITY GENE *BRCA1*

The *BRCA1* gene spans a region of 80 kb of genomic DNA consisting of 24 exons. Exon 11 is the largest and codes for approximately 60% of the protein.

Table 1.4 Various high to moderate BC susceptibility genes involved in the development of the disease. Indicated are the syndrome, the gene or locus with its chromosomal location, the cancer types associated with the syndrome and the lifetime risk involved (copied from Apostolou & Fostira, 2013).

Syndrome	Gene or locus (Chromosomal location)	Neoplasm	Lifetime risk
Genes with high-penetrance mutations			
Hereditary breast/ovarian cancer syndromes	<i>BRCA1</i> (17q12–21)	Female breast, ovarian cancer	40–80%
	<i>BRCA2</i> (13q12-13)	Male and female breast, ovarian, prostate, and pancreatic cancer	20–85%
Li-Fraumeni syndrome	<i>TP53</i> (17p13.1)	Breast cancer, sarcomas, leukemia, brain tumours, adrenocortical carcinoma, lung cancers	56–90%
Cowden Syndrome	<i>PTEN</i> (10q23.3)	Breast, thyroid, endometrial cancer	25–50%
Peutz–Jeghers syndrome	<i>STK11</i> (19p13.3)	Breast, ovarian, cervical, uterine, testicular, small bowel, and colon carcinoma	32–54%
Hereditary gastric cancer	<i>CDH1</i> (16q22.1)	Hereditary diffuse gastric, lobular breast, colorectal cancer	60%
Genes with moderate-penetrance mutations			
ATM-related	<i>ATM</i> (11q22.3)	Breast and ovarian cancers	15–20%
CHEK2-related	<i>CHEK2</i> (22q12.1)	Breast, colorectal, ovarian, bladder cancers	25–37%
PALB2-related	<i>PALB2</i> (16p12.1)	Breast, pancreatic, ovarian cancer, male breast cancers	20–40%
Moderate risk breast/ovarian cancer	<i>BARD1</i> (2q34-q35), <i>BRIP1</i> (17q22–q24), <i>MRE11A</i> (11q21), <i>NBN</i> (8q21), <i>RAD50</i> (5q31), <i>RAD51C</i> (17q25.1), <i>XRCC2</i> (7q36.1), <i>RAD51D</i> (17q11), <i>ABRAXAS</i> (4q21.23)	Breast and ovarian cancers	variable

Only 22 exons are transcribed into a 7.8 kb mRNA strand, which encodes a protein chain consisting of 1863 amino acids (Miki *et al.*, 1994). The final protein has a molecular mass of 220 kDa (Chen *et al.*, 1995).

The protein is involved in homologous recombination (HR), cell cycle checkpoint regulation, transcription and apoptosis (Christou & Kyriacou, 2013). The N-terminus consists of a zinc finger RING binding motif, which indicates that the protein interacts with DNA or with proteins that have ubiquitin ligase activity (Freemont, 1993; Joazeiro & Weissman, 2000). *BRCA1* directs its mobilisation into the nucleus through two nuclear localisation signals (NLS), where it forms nuclear foci upon genotoxic stress (Scully *et al.*, 1996). The protein structure also includes a region between the NLS and the C terminal that has no known homology to any other protein. The domain however functions as a binding motif for various proteins that are collectively involved in DNA repair and cell cycle checkpoint control. Figure 1.2 illustrates two *BRCA1* C terminus (BRCT) motifs that assist in DNA repair and DNA damage response are present at the C terminal (Rodriguez & Songyang, 2008; Leung & Glover, 2011).

1.8 THE HIGH IMPACT SUSCEPTIBILITY GENE *BRCA2*

The *BRCA2* locus is smaller than the *BRCA1* locus, spanning a region of 70 kb of genomic DNA consisting of 27 exons, with exons 10 and 11 being the largest (Wooster *et al.*, 1995), but the gene transcript is larger than that transcribed for *BRCA1* at approximately 12 kb and encodes a much larger protein at 3418 amino acids. Similar to *BRCA1*, *BRCA2* shows no homology to any other proteins (Wooster *et al.*, 1995; Tavtigian *et al.*, 1996). The protein has eight conserved sequences termed BRC repeats (Fig.1.2) (Bork *et al.*, 1996). The function of these BRC repeats is to bind RAD51 (Fig. 1.2) (Wong *et al.*, 1997). Two NLS motifs are located within the C terminal for nuclear localisation of the *BRCA2* protein.

The mutations detected within these two genes will be presented according to the nomenclature recommendations of the Human Genome Variation Society (HGVS) (<http://varnomen.hgvs.org/recommendations/DNA/variant/substitution/>, version 15.11, accessed on 13 May 2016.) In the case of older mutations, the initial mutation's name will be listed in parentheses according to the BIC database,

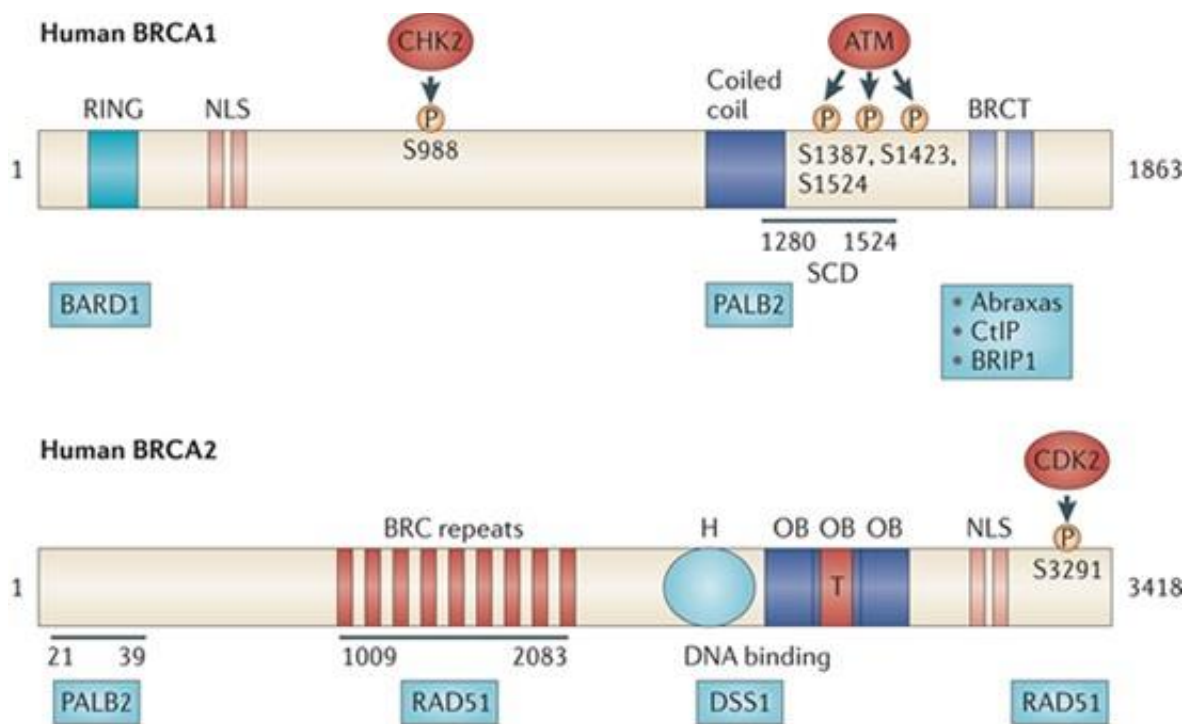


Figure 1.2 Illustration of the approximate regions of the most important motifs and functional domains of the *BRCA1* and *BRCA2* proteins. Protein binding domains as well as areas involved in phosphorylation are indicated. Various proteins bind to these two proteins that give rise to different and unique functionalities (adapted from Roy *et al.*, 2012).

where possible. *BRCA1* is numbered by GeneBank U14680 as the reference sequence, whereas GeneBank U43746 is used for *BRCA2*.

1.9 SOUTH AFRICAN FOUNDER MUTATIONS

Diagnostic testing for familial BC within SA has been available since 2005, although it was limited to specific mutations in certain population groups only. This was due to the identification of the first founder mutations within *BRCA1* and *BRCA2* in the Afrikaner population (van der Merwe & van Rensburg, 2009). Three mutations were found to be recurrent and represented more than 90% of the mutations detected within the Afrikaner population. Two of these mutations, *BRCA1* c.1374delC, p.Asp458Glu fsX17 (1493delC) and *BRCA1* c.2641G>T, p.Glu881X (p.E881X) were detected for the Afrikaner population. *BRCA2* c.7934delG, p.Arg2645Asn fsX3 (8162delG) was found in the Afrikaner population, but also occasionally within the SAC population from the Western Cape (van der Merwe & van Rensburg, 2009; van der Merwe *et al.*, 2012). Haplotype analysis for each of these mutations indicate a common ancestor that arose from a single mutational event more than 300 years ago (Reeves *et al.*, 2004; Van der Merwe & Van Rensburg, 2007).

The three Ashkenazi Jewish founder mutations were detected within the SA Jewish population. These three mutations are *BRCA1* c.66_67delAG, p.Leu22_Glu23LeuVal fs (185delAG), *BRCA1* 5263_5264insC, p.Ser1755?fs (5382insC) and *BRCA2* 5946_5946delT, p.Ser1982Arg fs (6174delT) (Van der Merwe *et al.*, 2012).

Only one recurrent mutation *BRCA2* c.5771_5774del, p.Ile1924Arg fsX38 (5999del4) was found among the Xhosa (Bantu-speaking) and SAC populations from the Western Cape region. Two other mutations, *BRCA1* c.1504_1508del, p.Leu502Ala fsX2 (1623del5) and *BRCA2* c.6447_6448dupTA, p.Lys2150Ile fsX19 (6676insTA) were also detected for the SAC population. The presence of these two mutations linked this group to Europe, but occurred in a small number of patients (n=2), (van der Merwe *et al.*, 2012). A single additional mutation was discovered which had not been previously reported to the Breast Cancer Information Core (BIC), namely *BRCA2* c.2826_2829del, p.Ile943Lys fsX16 (3054del4) (Van der Merwe *et al.*, 2012).

From the five parental ancestries presented in Figure 1.1, only the Afrikaner population with its European ancestry have been studied in SA. No literature is available on the *BRCA* status of the Khoisan population in Southern Africa. The SA Indian population is under investigation with a limited number of possible founder mutations present (Combrink HMVE, MMedSc dissertation, 2016). For Indonesia, one founder mutation has been identified that has to date not been detected within the SAC population. This mutation c.2699_2704delTAAATG, pGlu2183X in *BRCA2* (Purnomosari *et al.*, 2007) might be present due to its integration into the SAC population by slaves who were brought to SA.

1.10 MUTATION SCREENING TECHNIQUES

A variety of PCR-based techniques have been developed throughout the years for the detection of single nucleotide polymorphisms (SNP), small deletions or insertions and truncating codons. These techniques include single strand conformation polymorphism (SSCP) and heteroduplex analysis (HA), high resolution melting analysis (HRMA), protein truncation testing (PTT) and DNA sequencing. The selection of a mutation screening technique requires careful and thorough consideration due to variations in sensitivity, specificity and reproducibility. For diagnostic purposes, the technique should provide cost-effective accurate results within the minimal turnaround time (TAT). These criteria will determine the acceptance of a new mutation screening technique on a diagnostic platform.

1.10.1 COMBINED SINGLE STRAND CONFORMATIONAL POLYMORPHISM (SSCP) AND HETERODUPLEX ANALYSIS (HA)

The combination of SSCP and HA into a single technique is a screening method adapted from conventional polymerase chain reaction (PCR). The technique is widely utilised as a screening method for detecting variants within PCR amplicons. The method is based on the amplification of the targeted genomic sequence of interest using conventional PCR, whereafter it is denatured and the single stranded molecules separated by electrophoresis in a non-denaturing polyacrylamide gel (Orita *et al.*, 1989). These gels are visualised by silver staining.

The technique relies on the ability of small variants in a nucleotide sequence to alter the electrophoretic mobility of a single or double stranded molecule (Jordanova *et al.*, 1997). The sensitivity and resolution of the technique can be changed and are influenced by many parameters. These parameters include the size and GC content of the amplicon (Li *et al.*, 2003), the temperature at which electrophoresis takes place (Chen *et al.*, 1995) and the buffer composition (Kukita *et al.*, 1997). The technique entails the comparison of banding patterns between samples for the identification of possible alternating factors representing DNA changes. All the samples exhibiting band shifts are sequenced.

1.10.2 HIGH RESOLUTION MELTING ANALYSIS (HRMA)

HRMA is based on a combination of real-time PCR and PCR product melting analysis. The basic technique was first introduced in 1997 (Wittwer *et al.*, 1997), whereby DNA amplification during PCR was quantified using fluorescence dyes. The stability of the DNA duplexes are monitored by the release of these double stranded binding fluorescence dyes as the temperature is increased. Large differences between the PCR amplicons were easily distinguishable by melting temperature (T_m), but identification of the small single base changes were beyond the reach of the fluorescent melting analysis. The development of new revolutionary dyes years later resulted in the improvement of the melting resolution. This led to the development of a high resolution DNA melting method that could be used for either the genotyping of known variants or scanning for unknown variants (Wittwer *et al.*, 2003). All the samples exhibiting a different T_m will be sequenced to search for DNA changes in the targeted amplicon.

1.10.3 PROTEIN TRUNCATION TEST (PTT)

The protein truncation test which is also known as *in vitro* protein synthesis (Powell *et al.*, 1993; van der Luijt *et al.*, 1994) is a technique designed to detect mutations that lead to premature termination. The technique is ideal to screen larger genomic areas for the presence of truncating mutations. The technique is based on the addition of a translation primer to a genomic targeted sequence

during PCR amplification. After PCR, the generated amplicon is translated and the amino acid peptide size is visualised by separation using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The peptide separation is less influenced compared to SSCP, therefore size differences between the normal wild type and the truncated peptides are easily detectable. Once a truncation has been detected, the larger genomic region has to be screened using a set of smaller overlapping SSCP/HA or HRMA primers to determine the tentative position of the truncating DNA alteration. This specific fragment will then be sequenced to determine the precise position of the DNA alteration that resulted in the creation of a premature truncation.

1.10.4 DNA SANGER SEQUENCING

Sanger sequencing is the standard for obtaining the base composition of a targeted sequence. The method is PCR based with a single modification that entails the addition of chain-terminating dideoxynucleotides (ddNTP's) with fluorochromes, instead of the normal deoxynucleotides (dNTPs) to the PCR mixture (Sanger *et al.*, 1977). The ddNTPs each have different fluorochromes to distinguish between the four DNA bases. After the sequencing PCR, the targeted sequence will be amplified into single strands exhibiting various lengths. The sequencing product is separated by capillary electrophoresis where a laser excites each passing fluorochrome. The sequence of emission is captured and interpreted by the computer to produce a DNA code (Sanger *et al.*, 1977).

1.11 MUTATION ANALYSIS AND VARIANT CALLING

1.11.1 BREAST CANCER INFORMATION CORE (BIC)

The Breast Cancer Information Core is an online, open access mutation database specific for the reporting of mutations within the two familial BC genes (<https://research.nhgri.nih.gov/projects/bic/Member/index.shtml>, accessed 12 March 2016). The aim of the database is to facilitate in the detection and

characterisation of these mutations, as well as to provide technical support through detection protocols and primer sequences. Additionally for each mutation entered a link refers to literature reviews if available (Szabo *et al.*, 2000). A researcher has to apply for membership to the BIC to ensure that individuals using the database agree to guidelines regarding data entry and confidentiality. The BIC database consists of data derived from published literature as well as direct online entries by BC researchers worldwide. The data includes germline and somatic mutations (Szabo *et al.*, 2000). The BIC provides the BC research community with a central repository of mutations and polymorphisms that saves time when a certain mutation is detected for the first time in a specific population. For example, if the mutation is present in the BIC, no time need be spent on additional literature searches in order to determine the function of a variant of unknown significance (VUS) due to laboratory experimental limitations. Human functional assays are limited and costly.

1.11.2 THE 1000 GENOMES BROWSER

Variants and mutations in the BIC database are specifically captured for BC diagnostics. Some of these variants are population specific and do not necessarily cause disease. The pathogenic classification of a large number of the variants is incomplete and pending within the BIC database. In order to classify population variants, a global database is required to compare a variant across various geographical populations. For this reason, the 1000 Genomes Project was launched. The variants obtained through whole genome sequencing of a diverse sample group from distinct continental populations (The 1000 Genomes Project Consortium, 2015) were given a comprehensive description (<http://www.1000genomes.org/>, accessed 17 July 2016). A total of 2504 individual genomes from 26 populations have been sequenced and confirmed with multiple methods (Sudmant *et al.*, 2015). Whole genomes were acquired from five major continental ethnicities, which included 661 Africans, 347 Americans, 504 East Asians, 503 Europeans and 489 Southern Asians (Figure 1.3). In total the data produced over 88 million variants, which include 84.7 million SNPs, 3.6 million indels and 60 000 structural variants (The 1000 Genomes Project Consortium, 2015).

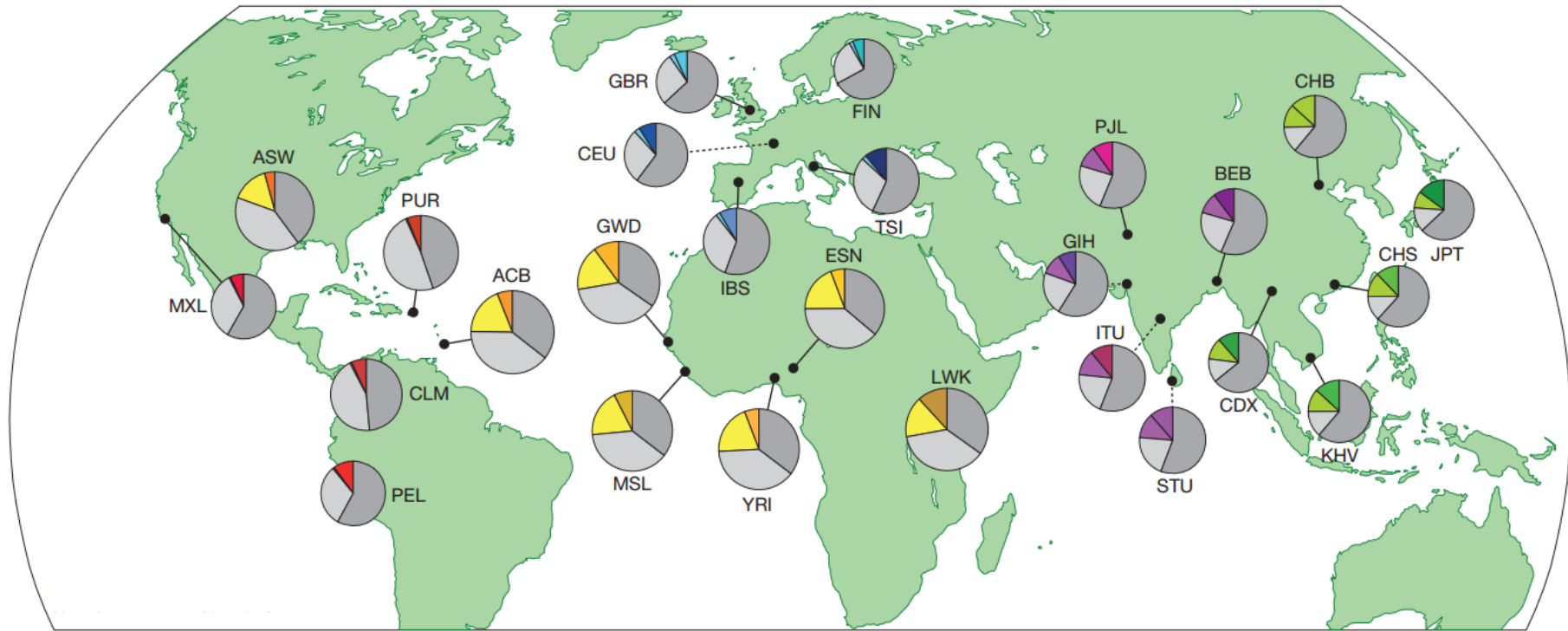


Figure 1.3 Illustration of the global geographical locations where individuals were sampled for whole genome sequencing in the 1000 Genomes Project (Adapted from the figure published by The 1000 Genomes Consortium, 2015). This study describes only sampling points from African origin as they are used for variant classification. African ethnicities included in the 1000 Genomes project are Americans of African Ancestry from SW USA (ASW), African Caribbeans in Barbados (ACB), Gambians in Western Divisions in the Gambia (GWD), Mende in Sierra Leone (MSL), Yoruba from Ibadan in Nigeria (YRI), Esan in Nigeria (ESN) and Luhya from Webuye in Kenya (LWK).

After excluding variants through population elimination, functional analysis could be performed on the remaining variants of unknown significance via *in silico* analysis.

1.11.3 IN SILICO ANALYSIS FOR VARIANTS OF UNKNOWN CLINICAL SIGNIFICANCE

Most genetic variants obtained from sequencing are in the form of SNPs. Some of these SNPs are found in the coding regions and result in an amino acid change within the protein product of the gene. These amino acid changes can affect the structure and function of the associated protein. To evaluate the clinical effect of these variants in patients, *in silico* analysis can be performed to predict the effect of the unknown variant. PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>, accessed 11 June 2016), is an automatic tool for these predictions (Adzhubei *et al.*, 2010). The prediction is based on sequence alignments, phylogenetic and structural features characterising the substitution (Adzhubei *et al.*, 2013). An additional software tool namely SIFT (<http://sift.jcvi.org/>, accessed 7 June 2016) can be used for predicting the function of a SNP. SIFT uses sequence homology through scores that are calculated using position specific scoring matrices with Dirichlet priors (Kumar *et al.*, 2009).

1.11.4 CLASSIFICATION OF MUTATIONS: EVIDENCE-BASED NETWORK FOR THE INTERPRETATION OF GERMLINE MUTANT ALLELES (ENIGMA)

ENIGMA is an international consortium of investigators focused on determining the clinical significance of sequence variants in the BC genes. This consortium compiled rules and guidelines to explain a 5 class system for the classification of variants (Appendix B, http://enigmaconsortium.org/documents/publications/ENIGMA_Rules_2015-03-26.pdf). These rules form a baseline for clinical classification to differentiate between high risk (pathogenic protein truncating variants) and low risk variants, to variants with no risk at all. At present, these guidelines are not intended for the evaluation and classification of variants with an intermediate level of risk (Spurdle *et al.*, 2012).

A class 1 variant (probability of being pathogenic <0.001) represents a variant that has a low clinical significance. Normally for these variants there is significant evidence against the variant being a dominant high-risk pathogenic variant. A class 1 variant may also be reported to occur in a large outbred control reference group at an allele frequency $\geq 1\%$. These variants will therefore have a minor allele frequency of $\geq 0.01\%$. A class 2 variant (probability of pathogenicity $0.001-0.049$) is likely not pathogenic and will have little clinical significance. There could also be evidence against the variant being a dominant high risk variant. A class 3 variant (probability of pathogenicity $0.05-0.949$) normally has insufficient evidence to be placed in classes 1, 2, 4 or 5. The term VUS (variant of unknown significance) is used to describe variants within class 3. Variants in class 4 (probability of pathogenicity $0.95-0.99$) will have strong evidence indicating the specific mutation as a likely dominant high-risk pathogenic variant. All the pathogenic mutations will represent class 5 (probability of pathogenicity >0.99). They will all affect the associated protein by the creation of a prematurely truncated peptide. For this class, there will be experimentally supported evidence that these mutations act as a dominant high risk pathogenic variant.

CHAPTER 2

THE IMPLEMENTATION OF HIGH RESOLUTION MELTING ANALYSIS AS A MUTATION SCREENING TECHNIQUE FOR THE FAMILIAL BREAST CANCER GENE *BRCA1*

2.1 INTRODUCTION

Familial breast cancer research has been the focus of the Molecular Genetics Laboratory of the Division Human Genetics for the past decade (1997-currently). The laboratory has been the referral centre for the African continent and even screened patients from the United Emirates during the early years. During this time, more than 1500 patients have been screened or genotyped.

Screening familial BC patients for mutations within *BRCA1* and *BRCA2* has posed many challenges during these initial years, mainly due to the limitations of techniques based on older technology. The methods and procedures were time consuming and included techniques such as single strand conformational polymorphisms (SSCP) and heteroduplex analysis (HA). The introduction of real-time PCR modernised the science industry. Although both conventional and real-time PCR are based on similar principles, the advantages of real-time PCR include the ability to identify amplified fragments during the PCR process, especially during the exponential phase compared to the plateau phase for standard PCR. There is no longer a need for post-PCR analysis, which in the case of *BRCA* screening, implies elimination of time-consuming gel electrophoresis (minimum of 16 hours), followed by silver-staining the following morning.

Many applications utilising real-time PCR analysis have since been developed. One of these include high resolution melt analysis (HRMA). This technique can be considered the next-generation application of amplicon melting analysis (Garritano *et al.*, 2009). It requires a real-time PCR detection system with

excellent thermal stability and sensitivity, and HRM-dedicated software. This combination, together with the advancement of saturating DNA-binding dyes has resulted in the development of this mutation screening technique. HRMA has the ability to detect small variations in nucleic acid sequences by the controlled melting of double-stranded PCR amplicons. This technique permits the discrimination of DNA sequences based on their composition, GC content, length or strand complementarity, resulting in an increase in sensitivity (Ngui *et al.*, 2012). Another advantage includes a decrease in contamination due to the entire process from amplification to analysis performed in a single tube.

In a gene scanning experiment, sample DNA is initially amplified via real-time PCR in the presence of a saturating DNA dye. Once completed, a melting curve is performed using high data acquisition rates, after which the data are finally analysed using gene scanning software (such as those present on the LightCycler® 480 II real time instrument from Roche Diagnostics, Applied Science, Germany). Analysis of the data commences with normalisation of the data (setting of the pre- and post-melt gliders) to ensure fluorescence signals of all samples are set to uniform and relative values. This is followed by temperature shifting, which comprises the shifting of the temperature axis to the point where the entire double-stranded DNA complement is completely denatured. Once the data have been normalised and the temperature shifted, a difference plot can be generated. This plot illustrates the differences in the melting curve shape by subtracting the curves from a reference curve (such as all the normal samples). This assists in the automatic clustering of the samples into groups depending on the shape of the melting curve (Application Manuals and Technical Guidelines: LightCycler® 480 Technical Note No. 1; <http://hrm.gene-quantification.info/>).

The aim of this part of the study was to optimise the use of HRMA and associated software as a mutation screening technique for *BRCA1*. Following optimisation, the technique is implemented and validated for use within the Molecular Genetics Laboratory by comparing results to those formerly obtained by combined SSCP/HA methods.

2.2 PATIENTS

2.2.1 SAMPLES USED FOR CONVENTIONAL PCR OPTIMISATION

Control DNA samples were obtained from three postgraduate students who gave blood voluntarily after signing informed consent. These individuals did not have a positive family history of breast and/or other cancer types, nor were they affected with the disease. These DNA samples were used for the initial optimisation of the conventional PCR reactions of the various *BRCA1* HRMA primer sets.

2.2.2 SAMPLES USED FOR THE VALIDATION OF HRMA

A cohort of 23 previously screened diagnostic BC patients using SSCP/HA was used for the validation of the new screening technique. These patients were diagnostically screened for *BRCA1* exons 2 – 10 and 12 – 24 using combined SSCP/HA as screening method. The patients exhibited different types of variants (single deletions, single base substitutions and small insertions) and represented four ethnic groups namely SA Blacks (n=6), Afrikaners (n=5), SACs (n=6) and SA Indians (n=6). The DNA of these patients was extracted on different occasions, using one of two DNA extraction methods. This included either the salting out method (Miller *et al.*, 1988) or the phenol:chloroform method (Sambrook *et al.*, 1989). The DNA was hydrated in T₁E (10 mM Tris-HCl pH 7.6, 0.1 mM EDTA). The entire cohort of samples (23 in total) was analysed in duplicate using HRMA. All band shifts were sequenced and the nucleotide differences documented. The HRMA results obtained were compared to that previously obtained using SSCP/HA in order to validate the optimised technique.

2.3 ETHICS

The proposed project was reviewed and approved by the Evaluation Committee of

the Faculty of Health Sciences, School of Medicine at the University of the Free State (UFS) in Bloemfontein. Once approved, ethics approval for the proposed research was obtained from the Research Ethics Committee of the Faculty of Health Sciences at the UFS (ETOVS 108/14 – Appendix C). Authorisation was also granted by the acting Business Manager of the National Health Laboratory Services and the Head of the Division of Human Genetics (Appendix D) for the use of the facilities and laboratory equipment.

2.4 METHODOLOGY

2.4.1 DNA EXTRACTION METHODS

Peripheral blood (10-20 ml) was received in ethylenediaminetetraacetic acid (EDTA) Vacutainer tubes. Two different DNA extraction methods were used for the familial BC patients within the laboratory during the period of the study. It included an adapted version of the phenol:chloroform method (Sambrook *et al.*, 1989) and the salting out procedure (Miller *et al.*, 1988). For both methods, the blood was transferred from the EDTA tubes into Nunc tubes and stored at – 20°C until the day of extraction.

2.4.1.1 PHENOL:CHLOROFORM METHOD

The frozen blood samples were removed from the freezer and slowly rolled on an orbital shaker until the samples were completely thawed. Once thawed, the red cells were ruptured using 45 ml cold lysis buffer [0.3 M sucrose, 10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) pH 7.8, 5 mM MgCl₂, 1% (v/v) t-octylphenoxypolyethoxyethanol (Trixton X-100)]. The ruptured cell suspension was placed on ice for 10 min before centrifugation (4 000 g) for 20 min at 15°C. The supernatant was discarded, after which the obtained pellet was cautiously washed and suspended in 1X SET buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl,

1 mM EDTA) containing $10 \mu\text{g}\cdot\mu\text{l}^{-1}$ proteinase K and 1% (w/v) sodium dodecyl sulphate (SDS). The solution was incubated overnight at 37°C .

The following day, equal volumes (5 ml) of phenol pH 8 (USB Corporation) and chloroform:isoamyl alcohol (24:1) were added to the solution and mixed thoroughly for 1 h on an orbital shaker. This step was followed by centrifugation (4 000 g) for 15 min at 15°C . After centrifugation, the supernatant was transferred to a new tube. An equal volume of chloroform isoamyl alcohol (24:1) was added, mixed thoroughly for 1h and centrifuged as described above. The DNA was precipitated from the supernatant using 2 volumes ice-cold 100% (v/v) ethanol and sodium acetate (pH 5.4) to a final concentration of 0.3 M. If the precipitated DNA was visible floating in the solution after 10 min, it was scooped from the solution, transferred to an Eppendorf tube and washed with 70% (v/v) ethanol for a minimum of 3 hours to overnight. Once the DNA was washed, it was briefly pelleted by centrifugation to separate the supernatant containing impurities from the purified DNA. The supernatant was discarded and the DNA pellet was air dried and dissolved in T₁E buffer. If no DNA was visible after precipitation, the tubes were incubated at -20°C overnight and centrifuged for 30 min (4 000 g) at 15°C the following morning to pellet the DNA. The pellet was washed with 70% (v/v) ethanol, air dried and dissolved in T₁E buffer.

2.4.1.2 SALTING OUT METHOD

The frozen blood samples were thawed by slowly rolling them on an orbital shaker. Once thawed, the cells were ruptured using the lysis buffer as described in 2.4.1.1. The mixture was placed on ice for 10 min before centrifugation (4 000 g) for 20 min at 15°C . The supernatant was discarded, after which the obtained pellet was washed and resuspended in 1 X SET buffer and incubated overnight as described in 2.4.1.1. A volume of 1.4 ml saturated NaCl (6 M) was added the following morning and the tubes shaken vigorously for 15 sec. The tubes were centrifuged (4 000 g) for 15 min (15°C). The tubes were removed from the centrifuge and for a second time vigorously shaken for 15 sec, and centrifuged as

before. After centrifugation, the supernatant was transferred to a new tube. The DNA was precipitated using 2 volumes ice-cold 100% (v/v) ethanol and handled according to the protocol described in 2.4.1.1.

2.4.2 DNA CONCENTRATION AND QUALITY

The concentration and purity of the extracted DNA was determined using spectrophotometry (NanoDrop® ND-100 Spectrophotometer v3.01, NanoDrop® Technologies Inc.) according to the manufacturer's instructions. The DNA concentration was expressed as $\text{ng}\cdot\mu\text{l}^{-1}$. The purity of the DNA was determined by measuring the absorbance wavelength ratios of three values, namely ($A_{260/280}$) and ($A_{230/260}$). The ratio of absorbance at $A_{230/260}$ was used to determine the presence of contaminants such as proteins. This ratio should ideally be between 2.0 and 2.2. The ratio of absorbance at $A_{260/280}$ was used to indicate the presence of contaminants such as phenol (absorbance should be 1.8-2.0). The DNA samples were initially diluted to $50 \text{ ng}\cdot\mu\text{l}^{-1}$ for use during the optimisation with the conventional polymerase chain reactions (PCR).

2.4.3 DNA DILUTION METHODS FOR HRMA

The students' DNA samples were diluted in a low salt buffer (T₁E) to eliminate the addition of possible contaminants such as too much salt or chemical factors that could influence the HRMA. The DNA aliquots were prepared in a meticulous manner in order to ensure equal DNA concentrations. In preparation for HRMA, the stock DNA samples were incubated for a minimum of 16h at 37°C to ensure a homogeneously diluted solution. The following day the samples were briefly vortexed and centrifuged before spectrophotometry. The DNA concentration of each sample was measured in triplicate using the NanoDrop® to obtain an average concentration throughout the dilution process. Once each stock concentration was known, a $150 \text{ ng}\cdot\mu\text{l}^{-1}$ aliquot was prepared. This new dilution was again incubated for at least 1h before being briefly vortexed, centrifuged and measured in triplicate. The

The concentration was meticulously adjusted by repeating this cycle until the average reading for the sample reached $150 \text{ ng}\cdot\mu\text{l}^{-1}$. This optimal dilution served as a HRMA stock dilution and was stored at -70°C for future use. The final PCR dilutions ($50 \text{ ng}\cdot\mu\text{l}^{-1}$) were prepared by adding DNA ($150 \text{ ng}\cdot\mu\text{l}^{-1}$ dilution) to T.4E in a ratio of 1:2. Dilutions were stored at -20°C until used.

2.4.4 BRCA1 HRMA PRIMER SETS

The sequences of 22 primer sets needed for HRMA of *BRCA1* (excluding exon 11 which is covered by PTT) were obtained from Van der Stoep *et al.* (2009) (Table 2.1). The primer sets covered all exons and exon-intron boundaries, except for exons 1 (which is non-coding) and 4, which was an error in the initial description of the gene (Fackenthal *et al.*, 2007). The primers were completely homologous to the *BRCA1* gene sequence, accession L78833, OMIM 113705, NM_007294.2 and NT_025965.11. An M13 primer tail was added to both the forward and the reverse primer sequences in order to sequence multiple exons simultaneously using a single universal primer. The primer lengths on average were 25 nucleotides long, excluding the length of the M13 tail. Amplification using these primer sets produced amplified products ranging from 217 to 348 bp (Table 2.1). The primer sets were manufactured and HPLC purified by Gibco® (Life Technologies, United Kingdom). Each set was diluted in T.4E to a final concentration of $20 \mu\text{M}$. Each primer dilution was aliquoted at a final concentration of $3\mu\text{M}$.

2.4.5 PCR OPTIMISATION FOR HRMA

2.4.5.1 OPTIMISING PRIMER ANNEALING TEMPERATURES

Amplification of the initial PCR product for HRMA of each primer set was optimised using a conventional PCR protocol. The PCR regime entailed one cycle at 95°C for 1 min, followed by 32 cycles at 94°C for 45 sec, the appropriate annealing temperature (ranged from 4°C below to 4°C above the optimal temperature as indicated by Van der Stoep *et al.*, 2009)

Table 2.1 Primer sequences for mutation screening of *BRCA1* using HRMA obtained from Van der Stoep *et al.* (2009). Indicated are the names of the primer pairs, the primer sequence including the M13 sequence (in bold), the proposed annealing temperatures with the number of cycles, the size of amplicon length, the GC content and the number of melting domains for each amplicon.

Primer name	Primer sequence 5' – 3'	T _a /cycles	Amplicon length	GC%	Melt domain count
BR1Ex2F	TG TAAAACGACGGCCAGTGAAGTTGTCATTTTATAAACCTTT	59/45	294	32	2
BR1Ex2R	CAG GAAACAGCTATGACCTGTGTCTTTTCTCCCTAGTATGT				
BR1Ex3F	TG TAAAACGACGGCCAGTTTGAGGCCTTATGTTGACTCAG	66/45	347	35	1
BR1Ex3R	CAG GAAACAGCTATGACCTGAAATGGAGTTGGATTTTTTCG				
BR1Ex5F	TG TAAAACGACGGCCAGTTTCATGGCTATTTGCCTTTTG	59/40	292	31	1
BR1Ex5R	CAG GAAACAGCTATGACC TGATGAATGGTTTTATAGGAACG				
BR1Ex6F	TG TAAAACGACGGCCAGTGGTTTTCTACTGTTGCTGCATCT	59/45	318	35	1
BR1Ex6R	CAG GAAACAGCTATGACCGAAAGTAATTGTGCAAACCTCCTG				
BR1Ex7F	TG TAAAACGACGGCCAGTGGGTTTCTCTTGGTTTCTTTGA	59/50	279	34	1
BR1Ex7R	CAG GAAACAGCTATGACCAGAAGAAGAAAACAAATGGTTT				
BR1Ex8F	TG TAAAACGACGGCCAGTTTCAGGAGGAAAAGCACAGAA	66/45	320	39	2
BR1Ex8R	CAG GAAACAGCTATGACCCACTTCCCAAAGCTGCCTAC				
BR1Ex9F	TG TAAAACGACGGCCAGTACCCTTTTAATTAAGAAAACCTTTAT	63/50	217	30	1
BR1Ex9R	CAG GAAACAGCTATGACCAAAGAGAGAAACATCAATCCT				
BR1Ex10F	TG TAAAACGACGGCCAGTTGGTCAGCTTTCTGTAATCGAA	59/50	318	39	1
BR1Ex10R	CAG GAAACAGCTATGACCAAGGTCCCAAATGGTCTTCA				
BR1Ex12F	TG TAAAACGACGGCCAGTCAGCAAGTTGCAGCGTT	63/45	251	44	1

BR1Ex12R	CAGGAAACAGCTATGACC ATACATACTACTGAATGCAAAGGAC				
BR1Ex13F	TGTA AAACGACGGCCAGTAAATGGAAAGCTTCTCAAAGTATT	63/45	341	41	2
BR1Ex13R	CAGGAAACAGCTATGACCC CTTACTCTTCAGAAGGAGAT				
BR1Ex14F	TGTA AAACGACGGCCAGTCTAACCTGAATTATCACTATC	63/45	348	34	1
BR1Ex14R	CAGGAAACAGCTATGACCG TGTATAAATGCCTGTATGCA				
BR1Ex15F	TGTA AAACGACGGCCAGTCTTTCACAATTGGTGCG	63/45	346	42	2
BR1Ex15R	CAGGAAACAGCTATGACCC CCAGAATATCTTTATGTAGGATTCA				
BR1Ex16AF	TGTA AAACGACGGCCAGTGACCAGAACTTTGTAATTC	59/45	296	46	2
BR1Ex16AR	CAGGAAACAGCTATGACCC CCAGCAGTATCAGTAGTAT				
BR1Ex16BF	TGTA AAACGACGGCCAGTAAAGTTCAGAATCTGCC	63/45	273	43	2
BR1Ex16R	CAGGAAACAGCTATGACCC ATAAACTCTTCCAGAATGTTG				
BR1Ex17F	TGTA AAACGACGGCCAGTACTAGTATTCTGAGCTGTGTGC	66/45	249	37	1
BR1Ex17R	CAGGAAACAGCTATGACCC CTCGCCTCATGTGGTT				
BR1Ex18F	TGTA AAACGACGGCCAGTCTTTAGCTTCTTAGGACAGCA	63/55	242	38	1
BR1Ex18R	CAGGAAACAGCTATGACCA AAATGCAATTCTGAGGTGTTA				
BR1Ex19F	TGTA AAACGACGGCCAGTTTGTGAATCGCTGACCTCTCT	66/45	247	37	1
BR1Ex19R	CAGGAAACAGCTATGACCG GTGCATTGATGGAAGGAAG				
BR1Ex20F	TGTA AAACGACGGCCAGTCTGGCCTGAATGCCTTAAAT	61/50	266	46	1
BR1Ex20R	CAGGAAACAGCTATGACCC AGAGTGGTGGGGTGAGATT				
BR1Ex21F	TGTA AAACGACGGCCAGTAGATTTTCTTCTCTCCATTCC	63/45	227	50	1
BR1Ex21R	CAGGAAACAGCTATGACCC CATCGTGGGATCTTGCTTA				
BR1Ex22F	TGTA AAACGACGGCCAGTCCCATTGAGAGGTCTTGCT	66/45	333	48	1
BR1Ex22R	CAGGAAACAGCTATGACCC GAGAAGACTTCTGAGGCTAC				
BR1Ex23F	TGTA AAACGACGGCCAGTATGAAGTGACAGTTCCAGTAG	63/45	225	50	1
BR1Ex23R	CAGGAAACAGCTATGACCG TGATAAACCAAACCCATGC				
BR1Ex24F	TGTA AAACGACGGCCAGTCCTAGTCCAGGAGAATGAATTGA	63/55	282	54	2
BR1Ex24R	CAGGAAACAGCTATGACCC TGGAAAGGCCACTTTGTAA				

for 1 min and 72°C for 45 sec, with a final elongation step at 72°C for 10 min. Each 50 µl PCR reaction contained 200 ng template DNA, 20 µM exon specific primers, 250 µM deoxyribonucleotide triphosphate, 100 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl and 1 U Taq DNA polymerase. The quality of the PCR products was visualised using electrophoresis. Electrophoresis was performed horizontally at 95 V in the presence of 0.5 µg.ml⁻¹ ethidium bromide (EtBr) and 1 X TBE (0.089 M Tris pH 8, 0.089 M boric acid, 2 mM EDTA) using a 2% (w/v) agarose gel. The products were sized against a 50 bp DNA ladder that acted as a sizing standard. Optimal annealing temperatures for each primer set were selected based on the intensity of the products across the annealing temperature ranges, the specificity of the amplicon and the correct size of the amplicon.

2.4.5.2 qPCR OPTIMISATION FOR HRMA

High Resolution Melting Analysis was to be performed on the LightCycler® 480 II real-time instrument. DNA was diluted to 25 ng.µl⁻¹ using the 150 µl⁻¹ dilution. The concentration of each sample was measured in triplicate. The PCR reaction was composed as recommended by the package insert of the LightScanner® Master Mix (BioFire Diagnostics, Inc., Salt lake City, Utah). Each 10 µl reaction contained 50 ng genomic DNA, 3 µM of each primer, 4 µl LightScanner® 2.5 X PCR Master mix, together with molecular grade dH₂O. Additional 25 mM MgCl₂ was available for further optimisation if required.

The amplification regime followed was as recommended by the package insert for the LightCycler® 480 High Resolution Melting Master (version July 2009). The regime consisted of a pre-incubation period (95°C for 10 min, with a ramp rate of 4.4/4.8°C/s). PCR cycling consisted of denaturation at 95°C for 10 sec (ramp rate of 4.4/4.8°C/s), annealing temperature as optimised for the specific primer set for 15 sec (ramp rate of 2.2/2.5°C/s) and extension for 72°C for 20 sec (ramp rate of 4.4/4.8°C/s). The amplified products for the HRMA PCR technique were initially separated using a 2% (w/v) agarose gel to confirm the quality and

specificity of the reaction (Sambrook *et al.*, 1989). Electrophoresis was performed horizontally at 95 V in the presence of 0.5 $\mu\text{g}\cdot\text{ml}^{-1}$ EtBr and 1X TBE.

2.4.5.3 OPTIMISATION OF HRMA AS A SCREENING TECHNIQUE

During HRMA, thermocycling amplification was directly followed by a high resolution melt, which consisted of a single cycle starting at 95°C for 1 min (ramp rate of 4.4°C/s). Afterwards, the temperature was gradually decreased to 40°C (where it was held for 1 min, ramp rate of 2.2°C) in order to allow amplicon renaturation and heteroduplex formation. Once renaturation occurred, the actual melting (T_m) was achieved by gradually increasing the temperature from 60-95°C (ramp rate of 0.03°C/s), with the instrument acquiring fluorescence data at a frequency of 25 readings per °C. The melt was completed by cooling for 10 sec at 40°C (ramp rate of 4.4°C/s). This HRMA regime was implemented as recommended by the package insert for the LightCycler® 480 High Resolution Melting Master (version July 2009).

2.4.6 SANGER SEQUENCING

All samples deviating from the baseline (cut-off beyond -2.5 to 2.5 for the relative signal difference) in the difference plots were bi-directionally sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States) to determine possible variants. The HRMA sample was purified using an enzymatic PCR clean-up (Illustra™ ExoProStar™ 1-step from GE Healthcare Life Sciences, United Kingdom) according to the manufacturer's instructions. It entailed adding 2 μl of the enzyme to 5 μl of the amplified HRMA product. The solution was briefly mixed by pipetting, after which it was incubated at 37°C for 15 min. This incubation was followed by a second incubation step at 80°C for 15 min to inactivate the enzyme. Each sequencing reaction contained 2 μl purified template, 1 μl BigDye® Ready Reaction terminator mix, 3.2 pmol of the respective primer and 2 μl BigDye® sequencing buffer. The sequencing regime was as

follows: one cycle at 96°C for 1 min, followed by 25 cycles at 96°C for 10 sec, 52°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% (v/v) ethanol, followed by a 30 min incubation period at room temperature in the dark. The products were centrifuged at 14 000 rpm for 30 min, after which the supernatant was aspirated, the pellet washed twice with 200 µl 70% (v/v) ethanol for a minimum of 1 hour and finally air dried in the dark.

Hi-Di™ formamide (Applied Biosystems, United States) (30 µl) was added to the dried products, after which it was denatured for 5 min at 96°C and the plates snap cooled in a cold block. The products were analysed using an ABI 3130 Genetic Analyzer (Applied Biosystems, US). The electropherograms were visually inspected using sequence analysis software (Chromas version 2.31, www.techneysium.com.au). The sequences were aligned to the reference sequences (NM_005905.3 for *BRCA1* and NM_00059.3 for *BRCA2*) with LALIGN (www.ch.embnet.org/software/LALIGN) and translation was performed using the ExPASy translate tool (<http://au.expasy.org/tools/dna.html>). Mutations were described according to the guidelines stipulated by the HGVS (accessed on 13 May 2016).

2.5 RESULTS AND DISCUSSION

2.5.1 DNA EXTRACTION METHODS

The DNA used for the optimisation and validation of HRMA as a mutation screening technique was extracted using either the phenol:chloroform or the salting out method. The phenol:chloroform method consists of hazardous chemicals such as phenol as its main agent for the isolation of DNA from cellular contents, whereas the salting out method isolates DNA by means of a non-hazardous substance, NaCl.

The best method for the extraction of DNA for HRMA experiments was proven by the Human Genetics Molecular Laboratory in Bloemfontein to be the salting out method (Combrink 2016, dissertation). Although the DNA extracted

using this method yielded lower fluorescence intensity, the technique delivered good quality DNA, had more consistent crossing point (Cp) values and clearer difference plots (Combrink 2016, dissertation).

The salting out method was selected as the method of choice for the familial BC samples, as it yielded a good amount of pure DNA and was safe to use within the laboratory. Upon comparison of samples previously extracted using both methods, the purity of the phenol:chloroform extracted DNA varied more between samples than when extracted using the salt (Combrink 2016, dissertation). The salt method normalised any possible salt contaminants that remained behind during the wash process.

2.5.2 OPTIMISATION OF DNA DILUTIONS

The DNA extracted from the three student samples using the salting out method, yielded high amounts of DNA. The DNA samples varied in concentration with none of the stock solutions being less than $1500 \text{ ng}\cdot\mu\text{l}^{-1}$. The high DNA concentration made the solution non-homogenous due to the densely coiled DNA, which took longer to dissolve completely. From this stock DNA, the first dilution was prepared at a concentration of $150 \text{ ng}\cdot\mu\text{l}^{-1}$ to ease the process of downstream dilutions. This dilution not only attempted to minimise differences in salt concentrations between samples, but also produced a more homogenous solution. The attempt to homogenise the dilutions proved to be critical as the undissolved DNA may contain impurities that can inhibit PCR reactions (Bio-Rad, 2016). In order to minimise differences between the samples, the dilutions were preferably made all on the same day using the same T₁E dilution buffer.

For the optimisation of the HRMA qPCR, each of the DNA dilutions was diluted down to $25 \text{ ng}\cdot\mu\text{l}^{-1}$ using two methods, namely dilution using the calculated method and the meticulous dilution method. The dilutions were prepared by adding DNA from the $150 \text{ ng}\cdot\mu\text{l}^{-1}$ into T₁E in a 1:5 ratio. The meticulous dilutions were incubated for a minimum of 1h and afterwards measured using the

NanoDrop®. These dilutions were adjusted if necessary, and the cycle repeated.

The importance of the critical dilution process is presented in Figure 2.1 A-B. From the first illustration, it is clear that scattering occurred around the C_p point and that there were inconsistencies amongst the samples at the exponential as well as the plateau phase of the qPCR reaction (Fig. 2.1 A). All these reactions were performed in duplicate. These samples were diluted from stock DNA using the normal calculation method. The experiment was repeated for the same fragment, using meticulously diluted DNA samples representing the same patients. From the second illustration, it is clear that all the samples exhibited the same C_p value and were tightly grouped during the exponential and plateau phases (Fig. 2.1 B). The data indicated that samples which were treated the same and had equal amounts of DNA, behaved similarly during qPCR. Grouped data as presented in Figure 2.1 B enabled the software to detect variation better, which in turn increased the sensitivity of the analysis compared to when major differences are present during the qPCR.

2.5.3 OPTIMISATION OF PRIMER ANNEALING TEMPERATURES

To test the primer specificity during PCR amplification of the various *BRCA1* amplicons, the annealing temperatures for each of the 22 primer pairs were initially used as proposed by Van der Stoep and co-workers (2009) (Table 2.1). The annealing temperatures of the primer sets proved to be very robust, as the published temperatures worked for the majority of the PCR products. The primer pairs for *BRCA1* were grouped into four main clusters (59°C, 61°C, 63°C and 66°C) which simplified the workflow within the laboratory during a comprehensive screen.

The majority of the products exhibited a single dark band when separated using horizontal electrophoresis (Fig. 2.2 A). To determine whether the size of the amplicons were correct, the PCR products were compared to a 50 bp DNA marker (Roche Diagnostics, Germany). One of the smallest (*BRCA1* exon 23, sized 225bp) and one of the largest (*BRCA1* exon 15, sized 346 bp) amplicons were confirmed to have the proposed fragment size (Fig. 2.2 B).

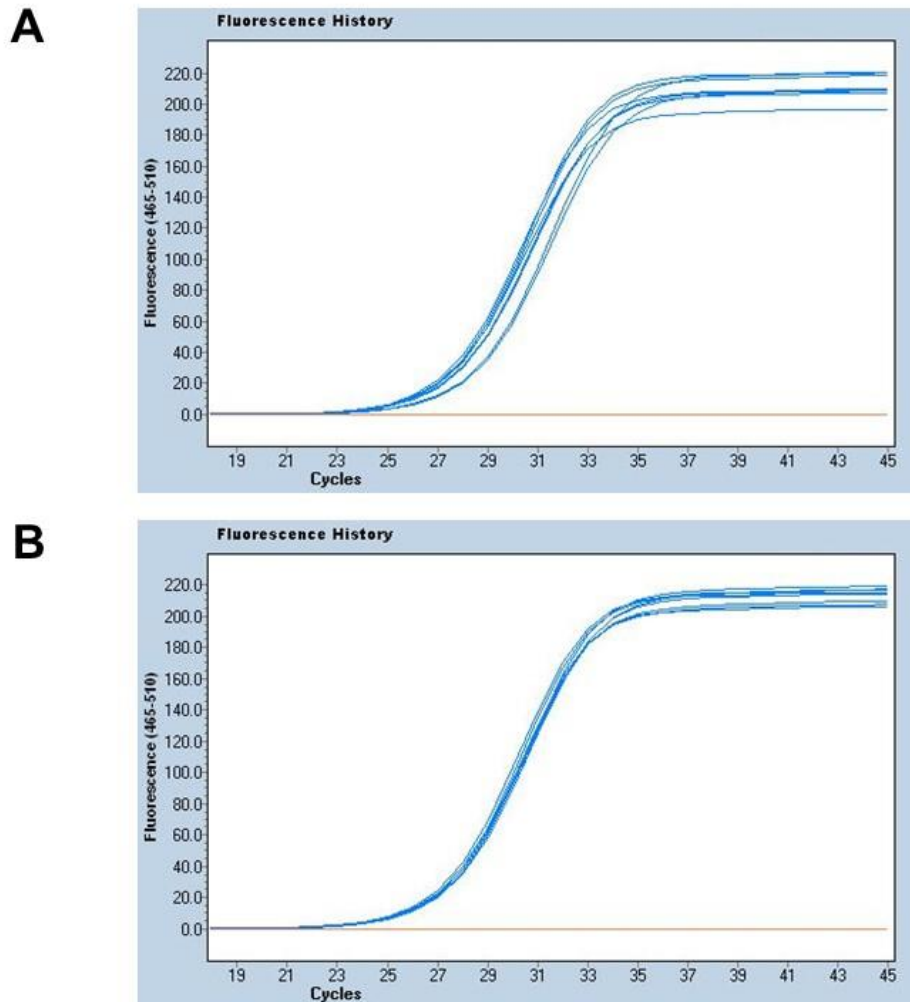


Figure 2.1 Effect of the two different DNA dilution methods on the shape of the qPCR for *BRCA1* exon 17. **A.** DNA diluted to $25 \text{ ng} \cdot \mu\text{l}^{-1}$ using a calculated method. **B.** DNA diluted to $25 \text{ ng} \cdot \mu\text{l}^{-1}$ using the meticulous method of DNA dilution.

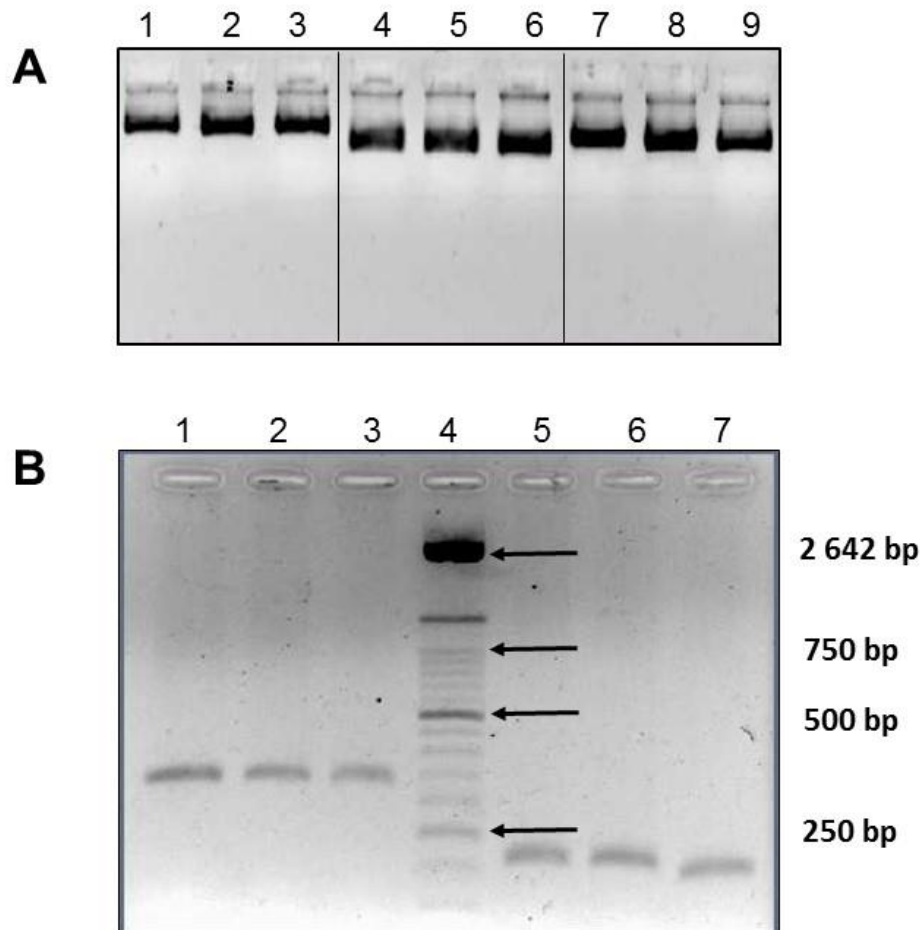


Figure 2.2 Confirmation of optimal annealing temperatures for PCR amplification of various primer sets representing *BRCA1*, using horizontal electrophoresis. Products were electrophoresed using a 2% agarose gel. **A.** Optimisation results for the PCR products obtained for exon 3 (lanes 1, 2 and 3), exon 9 (lanes 4, 5 and 6) and exon 20 (lanes 7, 8 and 9) of the three students. **B.** PCR products for exon 15 (sized 346 bp, lanes 1, 2 and 3) and exon 23 (sized 225 bp in lanes 5, 6 and 7) of the three students. The 50 bp molecular marker was loaded in lane 4.

2.5.4 OPTIMISATION OF qPCR FOR HRMA

The qPCR regime was performed as stipulated in the package insert of the LightCycler® 480 High Resolution Melting Master designed for the LightCycler® 480 II real time instrument. This regime did not prove to be optimal for all 22 exons representing *BRCA1*; therefore various experiments were performed in order to optimise HRMA for all the amplicons.

2.5.4.1 OPTIMISING DNA QUANTITY FOR qPCR

The effect of genomic DNA concentration on the qPCR amplification curve, Cp and total fluorescence was tested as the amount of total DNA indicated to be used in HRMA ranged from 100 pg to 100 ng per reaction (Application Guide for High Resolution Melt Analysis by Kapa Biosystems; Application Manuals and Technical Guidelines: LightCycler® 480 Technical Note No. 1; <http://hrm.gene-quantification.info/>). As Roche (according to the LightCycler® 480 High Resolution Melting Master, version July 2009) proposed an optimal final DNA concentration of 30-100ng, a total of 50 ng of DNA (at 25 ng.µl⁻¹) was used during the initial amplification of the various amplicons. This was an important aspect as many samples received from referral laboratories for a comprehensive screen have low DNA concentrations. By reducing the DNA concentration, it could ensure adequate amounts of template for the entire *BRCA* screen.

The DNA dilution for HRMA was lowered to 15ng.µl⁻¹, resulting in a final DNA amount of 30ng, which was at the lowest limit proposed by Roche. By lowering the DNA amount, it assured that salt and protein contamination remaining from the extraction process were at its lowest. By using T₁E as the dilution buffer, it provided adequate salt buffering that proved to be consistent throughout the samples. The reduction in the amount of DNA used as template resulted in the increase of the relative amplification fluorescence intensity by 20 units and the relative melting peak fluorescence by approximately 10 units (Fig. 2.3 A-B). It furthermore reduced the fluorescence starting baseline to some extent (Fig. 2.3 B – blue line was clearly visible before cycle 19) as less double stranded genomic

DNA was available for the melting dye to bind to. With more unbound melting dye available at this early stage of the qPCR, more dye could bind to the short double stranded amplicons which resulted in the increase in the relative amplification fluorescence (Fig. 2.3 B).

This effect gave the false impression that the C_p value decreased slightly. This was due to the reduction in background fluorescence that masked the fluorescence intensity of the short targeted amplicons. With the reduction in the initial amount of genomic DNA, amplification was quantified earlier in the qPCR in comparison to larger amounts of initial DNA. This was an early indication that the reduction in DNA concentration would also increase the sensitivity of mutation detection during melting analysis, as more unbound dye available for the amplicons would mean higher saturation and therefore increased sensitivity.

2.5.4.2 THE ADDITION OF $MgCl_2$

The addition of 1.0 mM $MgCl_2$ to the qPCR reactions had variable outcomes. Three specific areas of the qPCR were affected, namely an increase in the C_p value for some exons, a decrease in the relative signal intensity of the reaction and an increase in the melting temperature (T_m). For the majority of amplicons, the addition of $MgCl_2$ increased the C_p of the qPCR reaction. This was not the case for *BRCA1* exons 2, 5, and 6 presented in Figure 2.4. Here the C_p was not affected. However, the effect of additional $MgCl_2$ resulted in a reduction in the relative signal intensity of the qPCR (Fig. 2.4 A-C). The addition of $MgCl_2$ resulted in an increase in the T_m for all the amplicons (Fig. 2.4 Aii, Bii and Cii).

2.5.4.3 OPTIMISATION OF THE qPCR REGIME

The qPCR regime followed by the high resolution melt was utilized as proposed by the package insert for the LightCycler® 480 High Resolution Melting Master (version July 2009). The regime, which stipulated a total number of 45 qPCR cycles, seemed optimal for the majority of the *BRCA1* amplicons, although it was five more than that proposed by Van der Stoep and co-workers (2009).

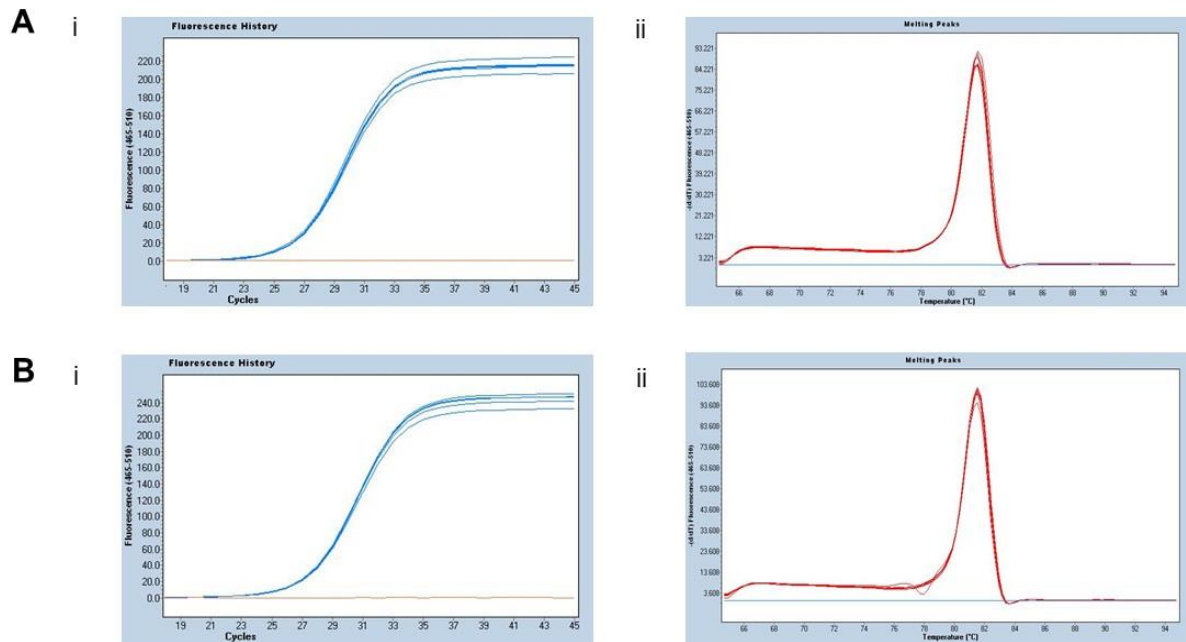


Figure 2.3 The effect of genomic DNA concentration on the qPCR amplification curve, Cp value and the total fluorescence detected for *BRCA1* exon 5. **A.** Amplification (i – amplification curve) and melting peak (ii) results for a qPCR reaction using 50ng of DNA. **B.** Amplification (i – amplification curve) and melting peak (ii) results for a qPCR reaction using 30 ng of DNA.

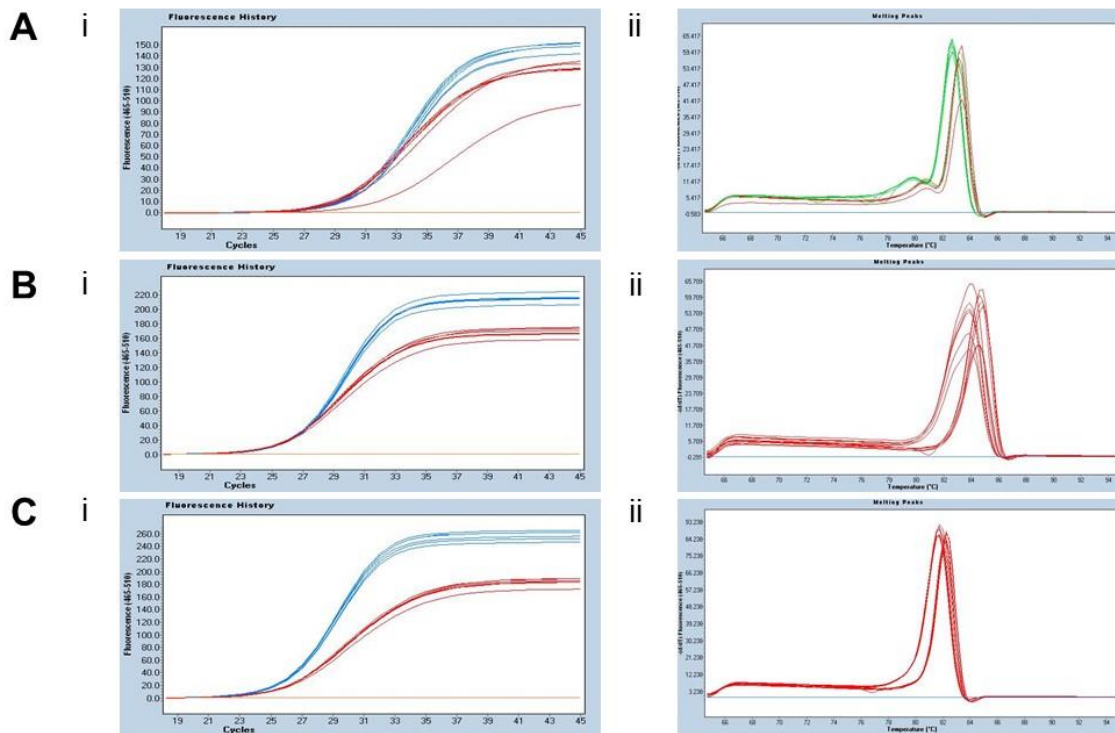


Figure 2.4 The effect of additional $MgCl_2$ on the amplification graphs (i) and the melting temperatures (ii) during qPCR optimisation. **A.** The amplification results for *BRCA1* exon 2, without (blue lines in figure i and green lines in figure ii) and in the presence of additional $MgCl_2$ (red lines in figures i and ii). **B.** The amplification results for *BRCA1* exon 5, without (blue lines in figure i and red lines in figure ii) and in the presence of additional $MgCl_2$ (red lines in figures i and ii). **C.** The amplification results for *BRCA1* exon 6, without (blue lines in figure i and red lines in figure ii) and in the presence of additional $MgCl_2$ (red lines in figures i and ii).

The extra five cycles gave ample opportunity for the amplification to reach a plateau before being terminated. Reactions that were terminated before they reached the plateau phase delivered inconsistent gene scanning results on the difference plots. All the primer sets, except for three, delivered amplification curves with a C_p between 25 and 30.

For exons 16B, 18 and 20, the C_p was above 30 cycles. In an attempt to increase the amplification rate for these exons, the annealing temperature was reduced from 63°C to 59°C. A reduction in annealing temperature is expected to decrease primer sensitivity but increase primer efficiency (Bio-Rad, 2016). For these three exons, the rate of amplification increased and the C_p shifted approximately three cycles earlier (≤ 30). This was accompanied by no loss in the specificity in the difference plots. Based on the evidence, the annealing temperature of these three exons were reduced to 59°C.

Some of the amplicons exhibited two melt domains in the normalised melt curve (Fig. 2.5 A). These were present for exons 2, 8, 13, 15, 16A, 16B and 24. In order to inspect these melt domains, melt peak analysis was performed for each of the exons (Fig. 2.5 B). In order to test whether the first smaller peak might be the presence of primer dimers, the primer concentration was reduced from 3 pmol to 2 pmol per reaction. The data obtained indicated that a reduction in primer concentration had no effect on the presence of the melt curve domains or the positions and intensity of the melt curve peaks (Fig. 2.5 B-C). The presence of these second peaks might be an indication of the GC content of amplicons or secondary structures which formed during amplification, as it was not due to non-specific binding of the primers during amplification, as the annealing temperatures had been optimised and checked for specificity.

Another aspect that was investigated was whether a reduction in the volume of LightScanner® Master mix used per reaction will influence amplification efficiency. By using less master mix, more reactions could be performed from a single vial which in return meant less reagent consumption per patient. This would make a comprehensive screen for the two familial BC genes more cost effective.

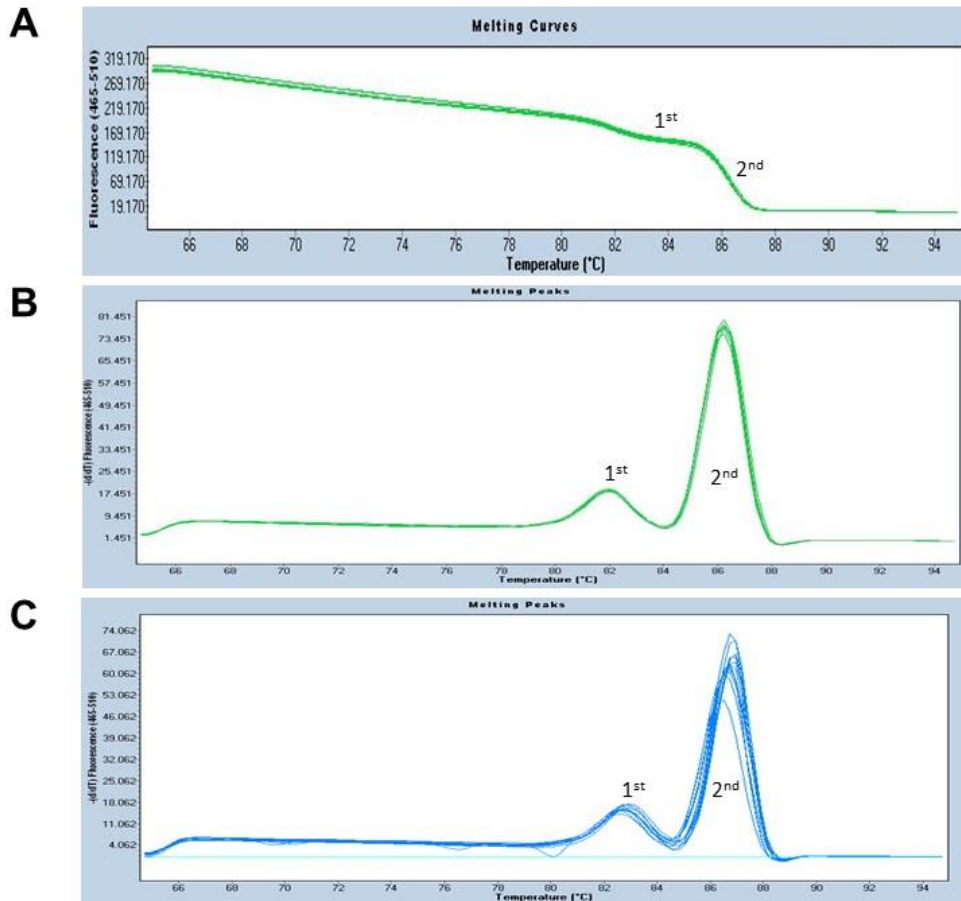


Figure 2.5 Effect of primer concentration on the presence of melt domains within *BRCA1* exon 16B. **A.** Melt curve for exon 16B indicating two melt domains in the presence of 3 pmol of each primer. **B.** Melt peaks for exon 16B, confirming the presence of two melt domains in the presence of 3 pmol of each primer. **C.** Melt peaks for exon 16B, confirming the presence of two melt domains in the presence of 2 pmol of each primer.

The volume of master mix used was reduced from 4 μ l to 3 μ l. Therefore the total reaction volume was reduced from 10 μ l to 9 μ l.

The data for this experiment is presented in Figure 2.6 (A-C). A variant detected within *BRCA1* exon 16 was used as positive control. Various DNA samples, including the positive control, were amplified for this exon using both the recommended (4 μ l) and the reduced volume (3 μ l) of master mix. The aim of the experiment was to determine whether the amplification rate and the sensitivity of the HRMA was affected by using less of the master mix. The amplification graphs indicated that there was no significant change in the qPCR slope or plateau rate amongst the samples after the amount of master mix was reduced. The reduced reactions compared well with regards to amplification rate against that in which the recommended amount was used (Fig. 2.6 A). It was expected that the reduction in the amount of master mix, therefore the high resolution melting dye, might influence the amplification and the detection of variation due to less dye being present.

It was initially hypothesised that if the ratio of the DNA to the dye was significantly higher, the background fluorescence of the unbound dye would overwhelm the amplicon relative intensity. This would not only provide a false reflection on the amplification efficiency and rate, but also of the C_p . The decrease from 4 μ l to 3 μ l had no difference in the amplification rate as both had an equal relative fluorescence intensity of 240 units (Fig. 2.6 A). Both compositions detected the presence of the variant equally well, although the shape of the melt peaks and the difference plot differed for the two groups (Fig. 2.6 A-B). The presence of the variant was indicated by the purple and green lines which were arranged on top of one another (Fig. 2.6 C). Based on the data presented, it was concluded that the laboratory could implement the use of less master mix (4 μ l to 3 μ l) in the reactions for HRMA, as the composition of the reaction was still suitable for the identification of variation in the DNA sequence of the amplicons screened.

Once the qPCR conditions were optimised, all the exons were thoroughly investigated step by step through the HRMA procedure. This was done in order to determine the expected parameters during amplification, normalisation, temperature shifting and the typical difference plot. The amplification curves, melt

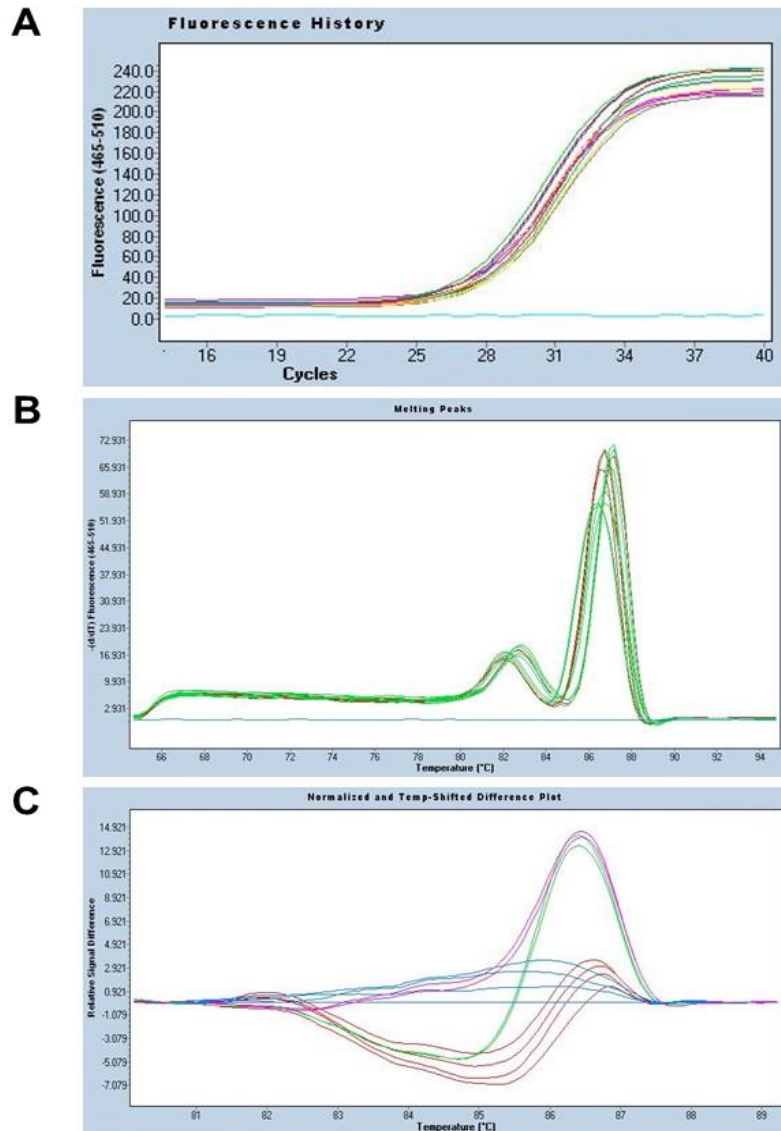


Figure 2.6 Determining the effect of master mix concentration on the amplification rate of qPCR and the sensitivity of HRMA utilising the *BRCA1* c.4837A>G variant present in *BRCA1* exon 16. The DNA of three students, together with the positive control, were used and each reaction was performed in duplicate. **A.** Amplification curves for the qPCR reactions for both the recommended 4 μ l and the reduced 3 μ l master mix reactions. **B.** Melt peaks for the recommended and the reduced master mix volumes. **C.** Difference plot for the experiment, with the samples with the recommended volume indicated in blue and the samples with the reduced volume indicated in red. Both sets of reactions accurately identified the positive control (indicated in green and purple).

curves and difference plots obtained from the three control students were used as negative reference samples for the predefined analysis settings for HRMA of *BRCA1* (Fig. 2.7 A-D). The melt curve was normalised by moving the pre- and post-melt gliders as close to the melt domain as possible (Fig. 2.7 B). For each exon's normalisation, the pre- and post-melt gliders were positioned with 1°C between the left and right glider bar. For the temperature shift the default setting was used at 5% correction, as recommended in the manual for the instrument (Fig. 2.7 C). From the data presented for the negative reference samples, the relative signal difference ranged from 0.85 to – 0.45 units. In the difference plot, any sample deviating more than 2.5 units from the negative baseline was highlighted to be sequenced (Fig. 2.7 D). This was used as base standard deviation for variant analysis during the validation study, and was applied for all amplicons analysed.

2.5.5 VALIDATION OF HRMA AS MUTATION SCREENING METHOD

In order to validate the use of HRMA as a screening method for the familial BC genes, a group of 23 familial BC patients were selected retrospectively. These patients were previously screened by the Universitas Diagnostic Laboratory in Bloemfontein using SSCP and HA as mutation screening techniques. Patients representing four different SA population groups (Black, Coloured, SA Indian and Afrikaner) were included to increase the genetic variation present, with the ultimate goal to determine the detection rate of HRMA of all possible variants. The majority of DNA samples were extracted using the salting out method, with only two archived samples previously extracted using the phenol:chloroform method. All the samples were meticulously diluted in T₁E to a final concentration of 15 ng.µl⁻¹. Each sample was amplified in duplicate for each exon to prevent detection errors due to inaccurate pipetting and to ensure accurate and consistent results per run. For HRMA, the duplicates of each sample had to group together in order for it to be called. In the case where one of the duplicates failed to amplify and the other was an outlier, the sample was sequenced.

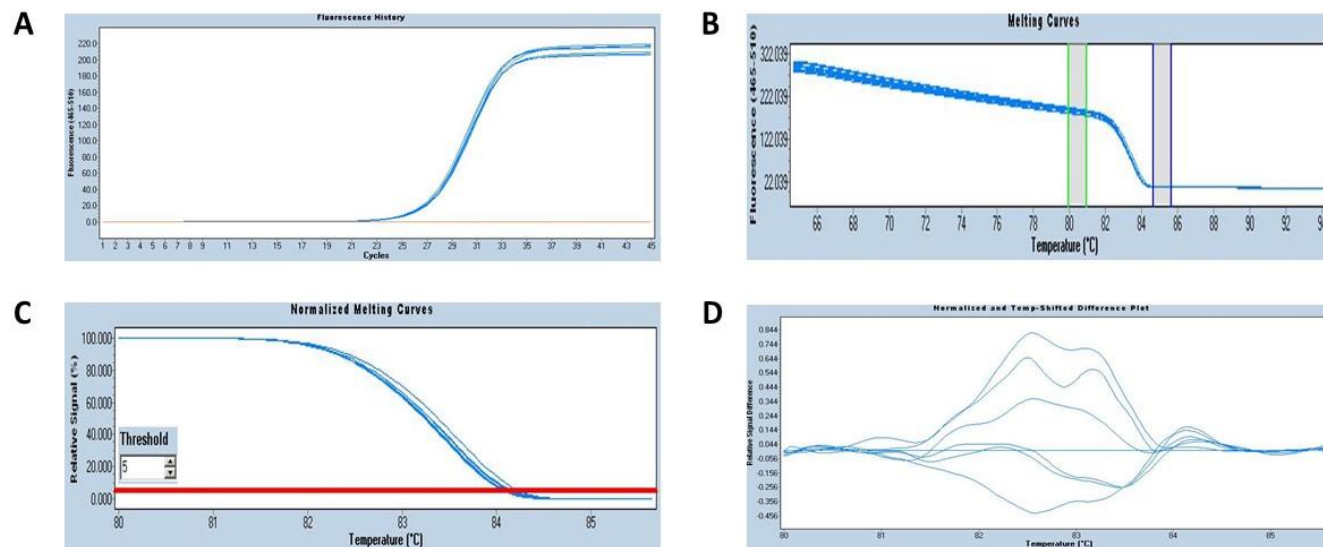


Figure 2.7 The amplification curves, melt curves and difference plots obtained for *BRCA1* exon 17 for the three control students who were used as negative reference samples for the predefined analysis settings for HRMA of *BRCA1*. **A.** Amplification curve. **B.** Normalising melt curve with the positioning of the pre- (indicated with green lines) and post- (indicated with blue lines) melt gliders. **C.** Normalised melting curve, based on a 5% threshold. **D.** Difference plot indicating no significant outliers (relative signal difference between 2.5 and -2.5 cut off).

BRCA1 exon 2 was the first exon screened using HRMA for the validation study (Fig. 2.8 A – E). From the amplification graph, a few samples amplified later and had a high C_p (Fig. 2.8 A). These samples melted slightly later during the post PCR melt step normalisation and temperature shifted curves, compared to the salt extracted samples (Fig. 2.8 B-C). For the normalisation, the pre-melt glider bars were placed at 77.42°C and 78.42°C (green lines) (Fig. 2.8 B). The post-melt gliders had to be placed at 84.21°C and 85.21°C to include these two samples that melted later (Fig. 2.8 B). The temperature shift excluded the last 5% of the data points which was captured when DNA might not be in single strand configuration. This ensured that only fluorescence data was analysed with an exponential decrease in fluorescence intensity. The 5% temperature shift included only amplicons during the melt phase and not genomic DNA in the process of melting, as genomic DNA requires a higher temperature to denature and might still release fluorescent dye (Fig. 2.8 C).

The final phase of HRMA was the analysis of the difference plot (Fig. 2.8 D). One clear distinguishable melt peak was observed in the difference plot for exon 2, reaching up to a relative difference signal of >12 (indicated in red) when compared to the samples grouped on the base line. This result was echoed by both reactions representing this sample, and was considered a valid result. The reactions representing the two other patient samples (highlighted by the purple ellipse) were also found to be outside the relative signal difference cut off (ranging from $->2.5$ to <2.5 , Fig. 2.8 D). These samples deviated from the base line by approximately 4 units. The three outliers were sequenced bi-directionally to investigate the reason for the deviation from the baseline. For the 12-unit outlier, an intronic variant *BRCA1* c.80+51G>A (rs180905862) was identified in the downstream area of exon 2. This intronic variant was previously identified by SSCP for this patient (Fig. 2.8 E). The electropherograms for the two samples in the purple ellipse did not indicate any changes occurring within the amplicon, and they were therefore labelled as false positives. These results corresponded to that previously found for these patients using SSCP/HA.

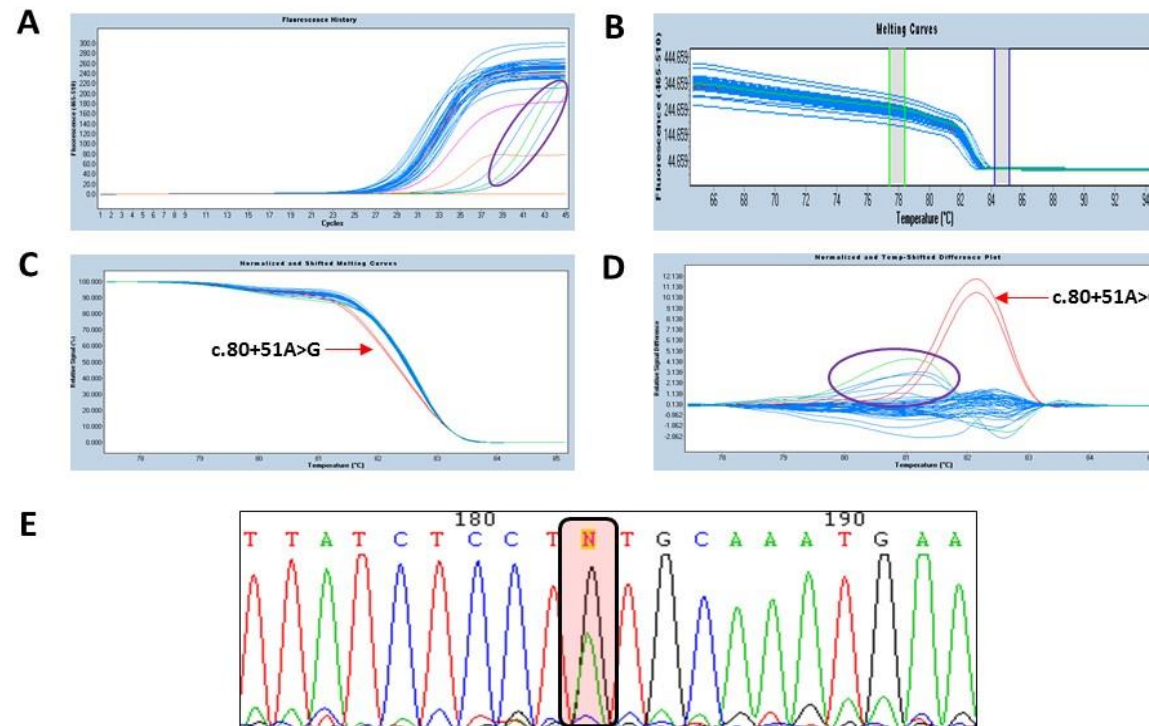


Figure 2.8 HRMA results for *BRCA1* exon 2 for 23 familial BC patients, of which one carried the heterozygous intronic *BRCA1* c.80+51A>G variant (rs180905862). **A.** The amplification graph, indicating two samples with a very late C_p .(enclosed in the purple ellipse). **B.** The normalising melt curve. **C.** Temperature shifted melt curve with 5% correction. **D.** Difference plot indicating the presence of the variant, together with two false positive samples (relative signal difference >2.5 and <-2.5 cut off indicated by the purple ellipse). **E.** Sequencing electropherogram indicating the presence of *BRCA1* c.80+51A>G variant.

Upon inspection, it was found that the curves representing the two false positive samples were extracted using the phenol:chloroform method. As these samples amplified later, they did not plateau with the rest of the samples over a period of 45 cycles (Fig. 2.8 A). This resulted in the samples displaying a relative difference signal of > 2.5 . Due to this value, these samples were incorrectly identified to be sequenced.

The late C_p of these samples could have been due to the presence of contaminants not washed out by the phenol and chloroform, or the low pH of the phenol. If the pH of the phenol and the chloroform was not high enough, it would not have been able to release histone proteins completely from the genomic DNA. In order to prevent this occurring for each of the fragments, the DNA of these two patients were measured using the NanoDrop[®] to ensure its concentration and to evaluate the quality parameters. The 230/260 and the 260/280 values indicated contamination at both wavelengths within the DNA, which resulted in the slow rate of amplification and the late C_p .

The stock DNA of these two patients were cleaned using the QIAamp[®] DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations (QIAamp DNA Micro Handbook, 3rd edition, December 2014). Once the DNA was cleaned using the QIAamp MiniElute[®] columns, it was re-measured and a new dilution prepared to a final concentration of $15 \text{ ng} \cdot \mu\text{l}^{-1}$. The cleaning of the genomic DNA and re-dilution resulted in these two patients mostly amplifying and grouping with the rest of the salt extracted samples during qPCR and the difference plots for *BRCA1* exon 3 (Fig. 2.9 A – F). The DNA of these two specimens were extracted approximately 15 years ago, and had been frozen/thawed many times before. This might have contributed to their unreliable performance during qPCR and HRMA. They occasionally had a later C_p for some of the amplicons, for which 5-10 additional cycles were added in order to provide enough time for these reactions to reach a plateau. The effect of samples that did not plateau was more dramatic on the difference plot, than the effect of the addition of more PCR cycles.

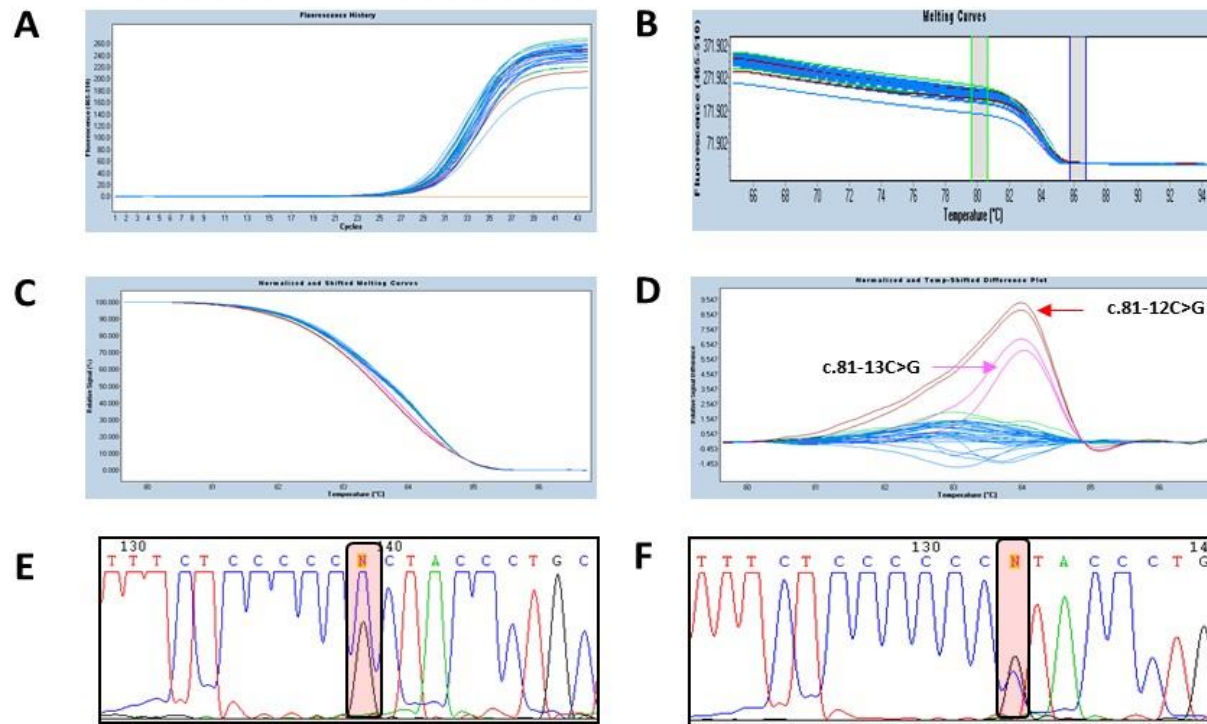


Figure 2.9 HRMA results for *BRCA1* exon 3 for 23 familial BC patients. **A.** The amplification graph. **B.** The normalising melt curve with pre- and post-melt glider positions. **C.** Temperature shifted melt curve with 5% correction. **D.** Difference plot indicating the presence of two variants, with relative signal differences >2.5, indicated by both reactions for each specimen. **E.** Sequencing electropherogram indicating the presence of *BRCA1* c.81-12C>G variant (rs80358055). **F.** Sequencing electropherogram indicating the presence of *BRCA1* c.81-13C>G variant (rs5638013).

For *BRCA1* exon 3 (Fig. 2.9 A-F) and all the other exons, the same set of rules was applied as for exon 2. For exon 3, all the samples had the same C_p (Fig. 2.9 A). On the difference plot, four clear distinguishable melt peaks were identified, representing two patients. The graphs had relative difference values of 6 and 9 respectively (Fig. 2.9 D). The rest of the samples were grouped on the baseline. The two patients representing the outliers were bi-directionally sequenced. Sequencing revealed the presence of two intronic variants in intron 2. These variants represented single base changes, one bp apart from one another (Fig. 2.9 E-F). They were the *BRCA1* c.81-12C>G variant (rs80358055) and *BRCA1* c.81-13C>G (rs5638013). Both variants were detected in a heterozygous state. The HRMA results corresponded with that previously detected using SSCP/HA for these two patients.

BRCA1 exon 16 was one of the exons where the number of qPCR cycles had to be increased in order for the two phenol:chloroform samples to reach a plateau (Fig. 2.10 A – E). Although the two irregular samples had high C_p values, the addition of cycles resulted in the exponential amplification to reach a plateau (Fig. 2.10 A). Due to these samples plateauing, they were included to the normalisation (Fig. 2.10 B – C), where two distinct melt domains were observed.

The effect of the positioning of the pre- and post-melt gliders on the difference plot were tested during this run, by normalising with and without both melt domains included. A 5% system default temperature shift correction was applied. For the first experiment, the pre- and post-melt gliders were positioned to include both the melt domains (Fig. 2.10 B) and were set 1°C apart (pre-melt represented by green lines and the post-melt by blue lines). Three melt groups were identified during the temperature shifted melt curve analysis (Fig. 2.10 D), which result was confirmed in the difference plot (Fig. 2.10 F). During the second experiment, the first melt domain was excluded during normalisation (Fig. 2.10 C). To accomplish this adjustment, the pre-melt glider criteria of 1°C had to be disregarded due to limited space. The two green lines were therefore less than a degree from each other (Fig. 2.10 C). Although the new positioning of the pre-melt gliders eliminated the first melt domain, there were still three melting groups present (Fig. 2.10 E). The difference plot produced from using only

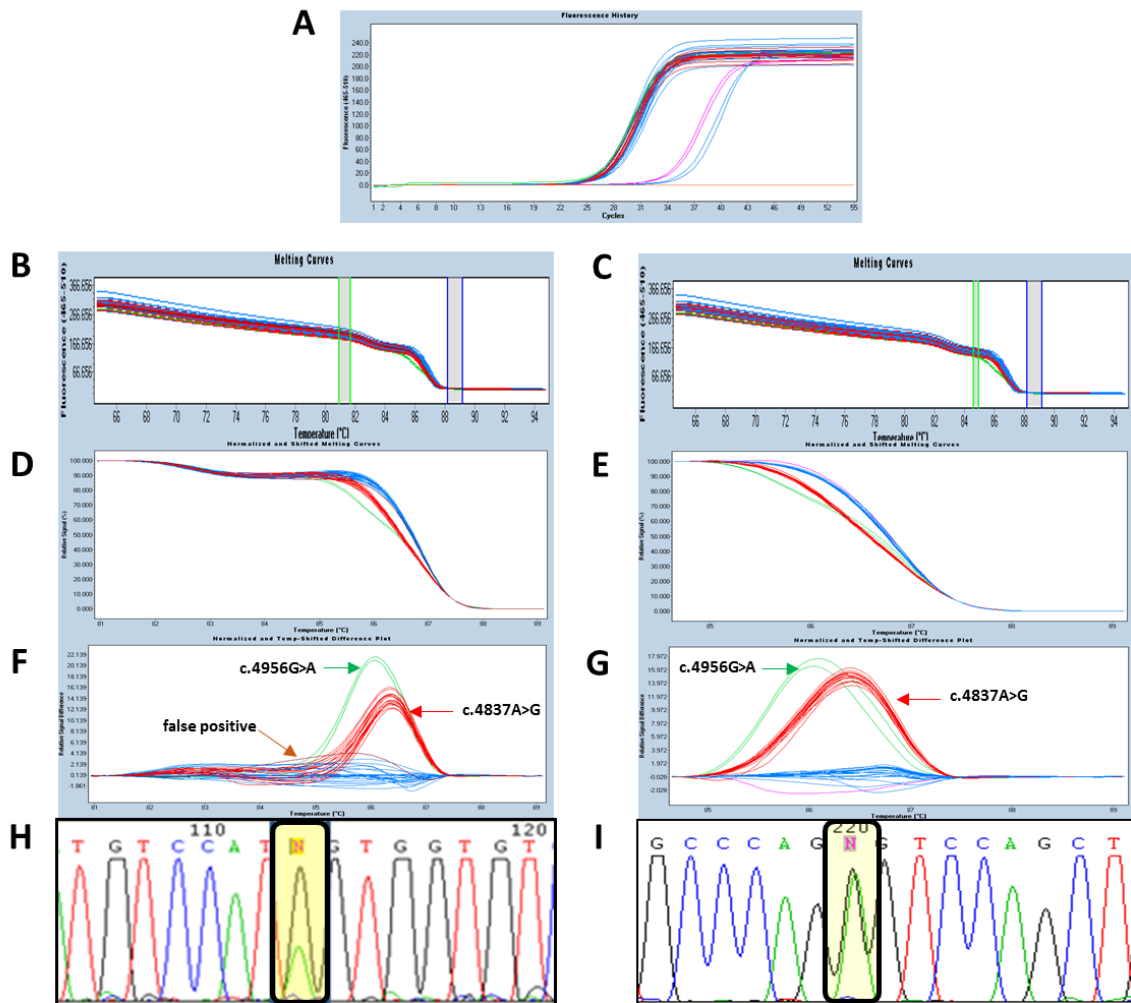


Figure 2.10 HRMA results for *BRCA1* exon 16B for 23 familial BC patients. **A.** Amplification graph. **B.** The melt curve including both melt domains with pre- and post-melt glider positions. **C.** The melt curve including only the second melt domain, with pre- and post-melt glider positions. **D.** Temperature shifted melt curve with 5% correction (including both melt domain). **E.** Temperature shifted melt curve with 5% correction (including only the second melt domain), with pre- and post-melt glider positions. **F.** Difference plot indicating the presence of potentially three variants, with relative signal differences >2.5. **G.** Difference plot indicating the presence of two variants, with relative signal differences >2.5, indicated by both reactions for each specimen. **H.** Sequencing electropherogram of *BRCA1* c.4956G>A variant (rs1799967). **I.** Sequencing electropherogram of the *BRCA1* c.4837A>G variant (rs1799966).

the second melt domain presented the identical groups, but the scattering on the base line was less and no false positives were observed in the negative curve group (Fig. 2.10 F). For the experiment that included both melt domains, a single patient (represented by two green lines) presented with a relative difference value of 22, with a cluster of patients at 14 (red lines) and a group clustering around the base line (blue lines). One sample was above the cut off value for the relative difference value (> 2.5 , indicated in one brown and one blue curve of similar shape, therefore conflicting results) as it had a value of 4 (Fig. 2.10 F). For the experiment that only included the last melt domain, the difference plot was clearer with less scattering around the base line and only two distinct outliers (the patient represented by the green lines and the group of patients indicated in red). The sample with a relative difference value of 4 now grouped with the rest on the base line, and was represented by two blue lines, therefore a conclusive result for this patient. From the data presented for the placement of the pre- and post-melt gliders, it can be concluded that for exon 16, only the second melt domain affected the difference plot (Fig. 2.10 D-E). Based on these results, only the sample indicated in green and a sample representing the red group were bi-directionally sequenced.

Sequencing results for the patient represented by the green lines indicated a single base change within the coding region of exon 16, namely *BRCA1* c.4956G>A (rs1799967). This variant was observed in a heterozygous state and resulted in the changing of a methionine (Met) to isoleucine (Ile) at amino acid (aa) position 1652 (Fig. 2.10 H). The sample representing the red group also exhibited a single base change at *BRCA1* c.4837A>G (rs1799966) and resulted in the changing of a serine (Ser) to a glycine (Gly) at aa position 1613 (Fig. 2.10 I). Both these variants have been detected within the SA population in both the heterozygous and homozygous state. They are benign polymorphisms with a minor allele frequency (MAF) of 0.0112 and 0.3558 respectively (National Centre for Biotechnology Information, 2016). The latter polymorphism has been detected for one of the samples extracted using the phenol:chloroform method. This confirmed the statement that by adding a few cycles to the qPCR regime, it will give non-optimal DNA HRMA reactions time to plateau,

to plateau, which could deliver an informative result for this type of sample.

The lessons learned during the initial optimisation using the student DNA samples were noted and incorporated into a working standard operating procedure, which was applied to the screening of the 23 BC patients for all 22 exons representing *BRCA1*. The results obtained for the validation are tabulated in Table 2.2. HRMA managed to successfully detect all the genetic differences previously documented for these patients using SSCP and HA. The data (each reaction performed in duplicate) indicated no discrepancies. This confirmed the successful optimisation of HRMA as a mutation screening technique within the Molecular Genetics Laboratory of the Division of Human Genetics. The main advantage of HRMA is the fact that no post-PCR analysis has to be performed by means of electrophoresis. All analyses are performed in a single tube, limiting contamination and potential sample swapping. All these factors contributed in a dramatic reduction in the hands-on and turnaround times for a comprehensive screen.

2.6 CONCLUSION

HRMA is a qPCR based molecular mutation 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). 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. The results in this chapter prove that the technique is very sensitive to DNA concentration in order to obtain equal amplification intensities. The factor that influenced the sensitivity the most and initially produced numerous false positives, was the presence of contaminants such as proteins, phenol and extra salts in the DNA. As HRMA is based on the melting of DNA in a solution and detecting differences with a light source, any contaminants that scatter or absorb light, increase or decrease the boiling temperature of the solution, increase or decrease the melting temperature of DNA will strongly influence the sensitivity and accuracy of the technique.

Table 2.2 Comparison of the genetic variation observed within *BRCA1*, for 23 BC patients obtained by two different mutation screening techniques (SSCP/HA and HRMA). Indicated are the exon number, the number of variants identified using SSCP/HA and HRMA, the designation of the variant, the associated reference SNP (rs number) and HGVS numbers.

Exon	SSCP/HA	HRMA	Nucleotide variant	rs number	HGVS
2	1	1	c.80+51G>A	rs180905862	g.41276080C>T
3	1	1	c.81-12C>G	rs80358055	g.41267808G>C
	1	1	c.81-13C>G	rs56328013	g.41267809G>C
5	1	1	c.212+23T>A	rs8176128	g.41258450A>T
6	1	1	c.213-54T>G	na	novel
8	1	1	c.442-34C>T	rs799923	g.41251931G>A
12	2	2	c.4115G>A, p.Cys1372Tyr	rs55848034	g.41243031C>T
13	11	11	c.4308T>C, p.Ser1436=	rs1060915	g.41234470A>G
16A	19	19	c.4837A>G, p.Ser1613Gly	rs1799966	g.41223094T>C
16B	19	19	c.4837A>G, p.Ser1613Gly	rs1799966	g.41223094T>C
	1	1	c.4956G>A, p.Met1652Ile	rs1799967	g.41222975C>T

For optimal HRMA, samples should be treated similarly during DNA extraction and time should be spent on the preparation of the DNA dilutions, using the same dilution buffer. Once the dilutions are optimally prepared, the technique delivers rapid, high quality and accurate mutation screening results.

Although it would be preferential to include DNA samples all extracted using the same method and chemicals in a run, this was not always possible as Bloemfontein is currently the referral centre for the comprehensive screening of patients for mutations in the familial BC genes. Various already extracted DNA samples have been received for HRMA screening. In order to include these samples into a single run, the quality and quantity should be rigorously checked. It was proven in this chapter that although samples have been extracted using different methods, they can still be included with others in the same run, once the differences in quality and elution buffers have been eliminated. For some of the exons, it may only entail an adjustment in the number of qPCR cycles. By increasing the number of cycles, these samples with a higher C_p values are given time to plateau, after which they can be normalised with the rest of the group.

To conclude, although SSCP/HA was more robust regarding DNA concentration differences and less sensitive to contaminants within the DNA, it is a very time consuming and laborious technique that requires hours of hands-on time during a pre- and post-PCR analysis. By using HRMA as mutation screening technique, there was a dramatic decrease in the turnaround time for the comprehensive screening of the familial BC genes, without compromising on the sensitivity and accuracy of the results.

CHAPTER 3

MOLECULAR SCREENING OF THE COLOURED POPULATION OF SOUTH AFRICA FOR MUTATIONS IN THE FAMILIAL BREAST CANCER GENES *BRCA1* AND *BRCA2*

3.1 INTRODUCTION

South Africa has a population of 54 million citizens, of which 8.8% (4.8 million) is represented by the South African Coloured (SAC) population. Of this number, approximately 52% is female (2.49 million) (Statistics South Africa, 2015). This population group is unique to SA, with the majority (50.2%, 2.4 million) residing in the Western Cape (Patterson *et al.*, 2010). The lifetime risk for Coloured women to develop BC is 1 in 22 (Fregene & Newman, 2005). Within this group this type of cancer is the most common and represents 25.95% (National Cancer Registry, 2010). The disease in this population has an ASR of 40.91 in 100 000. In 2010 alone, 394 new BC cases were reported among these women (National Cancer Registry, 2010).

This study attempted to investigate the landscape of genetic variants present for the SAC population within the familial BC genes *BRCA1* and *BRCA2*. Very few studies have searched for the presence of deleterious mutations in these genes specific to this unique group (Agenbag 2005; Van der Merwe *et al.*, 2012). By performing comprehensive screening, it will be possible to unravel the mystery of BC cancer development in this relatively young SA population. The aims of this study were to screen familial BC SAC patients for mutations within the two high-risk BC genes and to construct an appropriate diagnostic population directed mutation panel for use as a diagnostic tool in the future.

3.2 PATIENTS

3.2.1 FAMILIAL BREAST CANCER PATIENTS

A total of 229 self-identified Coloured patients were included in this study. The

selection criteria included being affected with BC and having one of the following: a positive family history (two or more affected family members), an early age at onset (<45 years) or bilateral disease. Male BC cases were also included, irrespective of the presence of a family history or an early age at onset. The request form which accompanied the patient's blood indicated the patient's demographics, clinical information, possible family pedigree and the patient's signature indicating consent (Appendix E).

3.2.2 ETHICAL ISSUES

As the majority of the self-identified BC patients were from the Western Cape, an application for the study of their patients and data was submitted to the Western Cape Provincial Government at Tygerberg Hospital (Appendix F). For the patients received from Inkosi Albert Luthuli Hospital in Durban, authorisation was obtained from the Provincial Government of Kwazulu-Natal (Appendix G).

All the patients were interviewed and counselled by genetic counsellors working in collaboration with the various Divisions of Human Genetics at the different referral hospitals. The patients were counselled according to the propagated protocol of the SA Genetic Counselling Association (Appendix H – <http://www.geneticcounselling.co.za/gcsa.php>, accessed 6 June 2016), which was specifically constructed for individuals or patients requesting information regarding the familial BC genes *BRCA1* and *BRCA2*. This protocol served as a guideline for genetic counsellors to structure the sessions according to a standardised format. This standardised format ensured that each patient counselled at the various referral centres received similar information regarding their risk, the influence of a disease-causing mutation and possible management options for the disease. After such a session, there was the opportunity to ask questions. Once the patient was sure he or she wanted to continue with testing, informed written consent was given by signing the informed consent section on the back of the request form. The blood was then drawn and sent to Bloemfontein for analysis. The Division of Human Genetics at the UFS had no direct contact with any of the patients participating in the project. Once the blood arrived, the sample was given a unique internal reference or CAM number to ensure confidentiality of the information.

3.3 METHODS

The molecular screening for the familial BC genes *BRCA1* and *BRCA2* was done in a tiered fashion. Once the DNA was extracted, genotyping for the most common mutations in SA was performed. If negative, the protein truncation test (PTT) was used to screen the largest exons of these two genes, namely *BRCA1* exon 11 and *BRCA2* exons 10 and 11. If a negative result was obtained, the remaining exons of both genes were screened using HRMA. If any indication of a DNA alteration was detected using any of these techniques, that section was sequenced using Sanger sequencing.

3.3.1 DNA EXTRACTION AND DILUTION PREPARATION

DNA was extracted using the salting out method as described in Chapter 2 (2.4.1.2). DNA quality and quantity were determined using the NanoDrop® (2.4.2). Stock DNA was diluted in T.E to $150 \text{ ng} \cdot \mu\text{l}^{-1}$ in an attempt to minimise variation in impurities and salt concentrations as described in Chapter 2 (2.4.3). All downward dilutions as required for each of the techniques were made in T.E. Dilutions for the various techniques were $50 \text{ ng} \cdot \mu\text{l}^{-1}$ for PTT and SSCP/HA, $15 \text{ ng} \cdot \mu\text{l}^{-1}$ for qPCR genotyping and HRMA. For HRMA, DNA was meticulously diluted as described in 2.4.3.

3.3.2 REAL-TIME GENOTYPING FOR SA FOUNDER MUTATIONS

Genotyping for the SA founder mutations (Van der Merwe *et al.*, 2012) were performed on the LightCycler® 480 II real-time instrument (Roche Diagnostics, Applied Science, Germany) using hybridisation probe technology for six of the mutations [*BRCA1* c.68_69delAG, p.Glu23ValfsX17 (185delAG); *BRCA1* c.1374delC, p.Asp458Glu fsX17, (1493delC); *BRCA1* c.2641G>T, p.Glu881X (2760G>T, E881X); *BRCA1* c.5266dupC, p.Gln1756ProfsX74 (5382insC); *BRCA2* c.7934delG, p.Arg2645AsnfsX3 (8162delG) and *BRCA2* c.5946delT, p.Ser1982

Table 3.1 Primers and probes used for the qPCR genotyping assays for the SA founder mutations. Indicated are the specific population the mutation was reported for, the mutation designation, the primer names with their 5' – 3' sequences, the amplicon length, the probe names and their specific sequences as designed by TIB MolBiol (Germany).

Population	Mutation name	Primer name	Primer sequence 5' – 3'	Amplicon length	Probe name	Probe Sequence 5' – 3'
Afrikaner	<i>BRCA1</i> 1493delC (c.1374delC)	1493delC S	ATGTATTGGACGTTCTAAATGAGGTA	179 bp	1493 [C]	TTCCCAAATATTTTGTCTTCAATATTAC--FL
		1493delC A	GCTTGCCTTCTCCGATAGG		1493delC Anchor	LC640-CTCTACTGATTTGGAGTGAACCTTTTCACTT--PH
	<i>BRCA1</i> E881X (c.2641G>T)	BRCA1 E881X S	GAATACATTCAAGTTTCAAAGCG	254 bp	881X [T]	GCAGAAGAGTAATGTGCAACAT--FL
		BRCA1 E881X A	TGGCATTATCAACTGGCTTATCT		881X Anchor	LC640-CTCTGCCCACTCTGGGTCCTTAAAG--PH
	<i>BRCA2</i> 8162delG (c.7934delG)	BRCA2 8162delG S	AGGGCTCTGTGTGACTCC	228 bp	8162delG [G]	TTGCTAATAGATGCCTAAGCCC-FL
		BRCA2 8162delG A	GAAACCTTAACCATACTGCCGT		8162delG Anchor	LC640-GAAAGGGTGCTTCTTCAACTAAAATACAGG--PH
Ashkenazi Jewish	<i>BRCA1</i> 185delAG (c.66_67delAG)	KA2 F	AAGTTGCATTTTATAAACCTT	258 bp	BRCA1delAG FL	TACAAAATGTCATTAATGCTATGCA--FL
		MY2 R	GTCTTTTCTTCCCTAGTATGT		BRCA1delAG LC	LC640-AAAATCTTAGAGTGTCCCATCTG--PH
	<i>BRCA1</i> 5382insC (c.5263_5264insC)	BRCA1in19S	TGTTTGGTTTCTTTCAGCATGA	153 bp	5382insCshort	GCAAGAGAATCCCCAGGAC--FL
		BRCA1in21A	AATACAGAGTGGTGGGGTGAGAT		Ex20 Anchor	LC640-AAGGTAAGCTCCCTCCCTCAAGTTGAC--PH
	<i>BRCA2</i> 6174delT (c.5946_5946delT)	BRCA2ex11 S	TCATCTGCAAATACTTGTGGGATT	168 bp	6174 [T]	CACAGCAAGTGGAAAATCTG--FL
		BRCA2ex11 A	GTGAGCTGGTCTGAATGTTCCG		6174delT Anchor	LC640-CCAGGTATCAGATGCTTCATTACAAAACGC--PH
Xhosa/Coloured	<i>BRCA2</i> 5999del4 (c.5771_5774delTTCA)	5999ex11 S mut.	AGATAATGATCAATGTAGCACGCAT	202 bp	SP 5999wt	TTTCTTCACTCTGAATGTCAGCAXIAAAACC--PH
		5999ex11 A	ACAAGTATTTGCAGATGAGACTGACT			

ArgfsX22 (6174delT)] and a simple probe assay for *BRCA2* c.5771_5774del, c.5771_5774del, p.Ile1924ArgfsX38 (5999del4). The primer and probe sequences are indicated in Table 3.1.

DNA was diluted down to 25 ng.µl⁻¹ from the initial dilution of 150 ng.µl⁻¹. Concentration was measured in triplicate. The PCR reaction was composed as recommended by the package insert of the LightCycler® 480 Genotyping Master Mix (Roche Diagnostics GmbH, Mannheim, Germany). Each 20 µl reaction contained 50 ng genomic DNA, 3 pmol of each primer (TIB MolBiol, Berlin, Germany), 2 pmol of each probe (TIB MolBiol), 4 µl LightCycler® 5 X Genotyping Master mix, together with 12.6 µl molecular grade H₂O.

The amplification regime followed was as recommended by the package insert for the LightCycler® 480 Genotyping Master (version February 2008). The PCR regime consisted of a pre-incubation period (95°C for 10 min, with a ramp rate of 4.4 °C/sec). PCR cycling entailed 45 cycles of: denaturation at 95°C for 10 sec (ramp rate of 4.4 °C/sec), annealing temperature at 50°C for 20 sec (ramp rate of 2.2 °C/sec) and extension at 72°C for 20 sec (ramp rate of 4.4 °C/sec).

Directly after amplification, a melt curve genotyping programme followed. The melt curve regime entailed a single cycle of amplicon denaturation at 95°C for 1 min, where after the temperature was rapidly decreased to 40°C (ramp rate of 4.4°C/sec) to allow amplicon renaturation and heteroduplex formation, where it was held for 1 min. After renaturation, the temperature was gradually increased to 95°C (ramp rate of 2.2°C/sec), where melting actually starts by gradually increasing the temperature from 40°C to 95°C at a ramp rate of 0.03°C/s and acquiring fluorescence data at a frequency of 5 readings per °C to determine the melting point (T_m) of the wild type and mutation.

Genotyping analysis was performed for both the hybridization and simple probe assays using a melting curve program subsequent to the qPCR. The LightCycler® 480 Genotyping Software grouped the samples with similar melting profiles together and identified each group as a genotype. To determine the various genotypes, the software analysed the shapes of all the melting curves and compared it to that of the confirmed positive control. With the comparison, each individual melting curve profile was called according to that standard. In runs where the positive control failed to amplify, the run was repeated in order to obtain a result.

3.3.3 PROTEIN TRUNCATION TEST (PTT)

The larger exons of *BRCA1* and *BRCA2* were screened for the presence of truncating mutations only, using PTT. Seven overlapping primers sets were used to amplify these exons, three for *BRCA1* exon 11 and four for *BRCA2* exon 11 (Table 3.2). A T7 promoter sequence was added to each forward primer, which acted as a translation initiation sequence. Each 25 μ l reaction consisted of 300 ng genomic DNA, 1 U Takara Ex *Taq* (Otsu, Japan), 250 μ M Takara dNTPs, 1.5 μ M $MgCl_2$, 20 pmol of each primer, 50 mM KCl and 100 mM Tris-HCl pH 7.2. The amplification regime was composed of 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, with a final extension at 72°C for 5 min.

In order to verify whether the conventional PCR produced the desired product, 8 μ l of each amplified product was separated on a 1.5% (w/v) agarose gel in 1 X TBE buffer. An *in vitro* transcription/translation reaction was performed for each amplified fragment, using the TnT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, Wisconsin, United States). The translation reactions contained 5 μ l of PCR product, 8 μ l TnT®T7 Quick Master Mix, 0.5 μ l PCR enhancer (Promega, Madison, Wisconsin, United States) and 0.6 μ l of 10mCi.ml⁻¹ L-[³⁵S] radio-active labelled methionine (PerkinElmer Inc., Boston, United States). The reactions were incubated at 30°C for 90 min. Translation was terminated by the addition of 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 ratio 0.2:100, followed by denaturation at 95°C for 5 min.

The translated peptides were separated using a 12% (w/v) SDS-PAGE running 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, Waltham, Massachusetts, United States). The peptide fragments were initially compressed (15 min at 120 V/cm⁻¹) where after they were separated for 90 min at 220 V/cm⁻¹ in a pre-cooled Tris/Glycine running buffer [25mM Tris, 192 mM glycine, 0.1% (v/v) SDS] (Bio-Rad Laboratories Inc., Hercules, California, United States). The gels were fixed in 10% (v/v) acetic acid, 65% (v/v) isopropanol for 15 min, rinsed with

Table 3.2 PTT primer sets used (obtained from the BIC) for the screening of exon 11 of *BRCA1* and *BRCA2*. Indicated are the primer names, the 5' – 3' sequence for both the forward and the reverse primers, with the T7 promoter highlighted in bold, the amplicon length and the associated peptide length.

Primer name	Primer sequence 5' – 3'	Amplicon Length	Peptide length
BR1EX11 Fragment 1F BR1EX11 Fragment 1R	CGCTAATACGACTCACTATAGGAACAGACCACCATGG CCTTGTGAATTTTCTGAGACGG CAGGAAACAGCTATGACATGAGTTGTAGGTTTCTGCTGT	1 344 bp	448 aa
BR1EX11 Fragment 2F BR1EX11 Fragment 2R	CGCTAATACGACTCACTATAGGAACAGACCACCATGG AACAATTCAAAAGCACCTAAAAAG CAGGAAACAGCTATGACAACCCCTAATCTAAGCATAGCATTTC	1 522 bp	507 aa
BR1EX11 Fragment 3F BR1EX11 Fragment 3R	CGCTAATACGACTCACTATAGGAACAGACCACCATGG CACCACTTTTTCCCATCAAGT CAGGAAACAGCTATGACATTATTTTCTTCCAAGCCCGTTCC	1 124 bp	375 aa
BR2EX11 Fragment AF BR2EX11 Fragment AR	CGCTAATACGACTCACTATAGGAACAGACCACCATGG TGCATTCTTCTGTGAAAAGAAGC GCACTTCAAATGTA CTCTCTG C	1 517 bp	506 aa
BR2EX11 Fragment BF BR2EX11 Fragment BR	CGCTAATACGACTCACTATAGGAACAGACCACCATGG TAAAGCAGCATATAAAAATGACTC GATCTTTTCATCACGTTCCG	1 673 bp	558 aa
BR2EX11 Fragment CF BR2EX11 Fragment CR	CGCTAATACGACTCACTATAGGAACAGACCACCATGG TTGATGGCAGTGATTCAAG TGCTACATTCATCATTATCTAGAGAG	1 739 bp	580 aa
BR2EX11 Fragment DF BR2EX11 Fragment DR	CGCTAATACGACTCACTATAGGAACAGACCACCATGG ATGACAAAAATCATCTCTCC AACTGACTACACAAAAATGGCTG	1 793 bp	598 aa

tap water and placed on the orbital shaker in Amplify (GE Healthcare, United Kingdom) for 15 min. The fixed gels were dried under vacuum (60°C) for 2h and exposed to X-ray film overnight. The X-ray film was developed by hand using Agfa G153 as the developer and Agfa G354 rapid fixer as the fixative (Agfa Healthcare, Morstel, Belgium). The dried film was captured digitally using the Bio-Rad Gel documentation system (Bio-Rad Laboratories Inc., Hercules, California, United States) and labelled accordingly.

3.3.4 HIGH RESOLUTION MELTING ANALYSIS (HRMA)

High Resolution Melting Analysis was performed as described in Chapter 2 for a total of 56 primer sets (*BRCA1* primer sets listed in Chapter 2, *BRCA2* primer sets listed in Table 3.3), which covered the splice site boundaries and exonic regions of both genes. Each amplicon per patient was performed in duplicate in order to eliminate pipetting errors. Both reactions had to deliver the same result in order for the amplicon to be scored.

3.3.5 COMBINED SSCP AND HETERODUPLEX ANALYSIS (SSCP/HA)

As PTT was used for the screening of large genomic sections (approximately 1.2 – 1.4 kb), conventional PCR based SSCP/HA was performed using smaller overlapping primer sets representing that specific PTT fragment in order to find the specific mutation in the case of a positive PTT result. The primer sets utilised were manufactured by Gibco (Gibco BRL Life Technologies, California, United States) and are listed in Table 3.4. PCR was performed using one cycle at 95°C for one min, followed by 32 cycles at 94°C for 45 sec, the optimal annealing temperature for the specific amplicon for 1 min and 72°C for 45 sec, with a final elongation step at 72°C for 10 min. Amplification was performed using the My Cycler from BioRad (Bio-Rad Laboratories Inc., Hercules, United States), utilising the gradient block. Each 50 µl PCR reaction contained 300 ng template DNA, 20 pmol exon specific primers, 250 µM deoxyribonucleotide triphosphate, 100 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl and 1 U *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany). A total of 10 µl loading buffer [95% (v/v)

Table 3.3 *BRCA2* primer sequences used for HRMA obtained from Van der Stoep and co-workers (personal communication). Each of the primer sets were amplified according to the settings optimized by Combrink (dissertation, 2016). Indicated are the names for all primers used, the primer sequence including a M13 sequence (in bold), the optimised annealing temperatures with the number of cycles and the amplicon length.

Primer name	Primer sequence 5' – 3'	T _a /cycles	Amplicon length
BREX2F	TGTAAAACGACGGCCAGT TTCCAGCGCTTCTGAGTTTT	61/40	264
BR2EX2R	CAGGAAACAGCTATGACCT GGGTTTTTAGCAAGCATTTTT		
BR2EX3.1F	CAGGAAACAGCTATGACCT CTTTAACTGTTCTGGGTCACAA	61/40	285
BR2EX3.1R	TGTAAAACGACGGCCAGT GAGATTGGTACAGCGGCA		
BR2EX3.2F	TGTAAAACGACGGCCAGT CAACAATTACGAACCAAACCTAT	61/40	209
BR2EX3.2R	CAGGAAACAGCTATGACCT GCCTAAATTCCTAGTTTGTAGT		
BR2EX4F	TGTAAAACGACGGCCAGT AAGAATGCAAATTTATAATCCAGAGT	61/40	285
BR2EX4R	CAGGAAACAGCTATGACCT TTCTACCAGGCTCTTAGCCA		
BR2EX5F	TGTAAAACGACGGCCAGT CCAGCAGCTGAAATTTGTGA	60/40	355
BR2EX5R	CAGGAAACAGCTATGAC CAAAAGGGGAAATTGTTAAGTTTTA		
BR2EX6F	TGTAAAACGACGGCCAGT AAAACCTTAACAATTTCCCTTTTT	60/40	192
BR2EX6R	CAGGAAACAGCTATGACCT GCCTGTATGAGGCAGAATG		
BR2EX7F	TGTAAAACGACGGCCAGT TCCTTAATGATCAGGGCATTTC	61/40	225

BR2EX7R	CAGGAAACAGCTATGACCT GACAATTATCAACCTCATCTGC		
BR2EX8F	TGTAAAACGACGGCCAGT TGTGCTTTTTGATGTCTGACAAA	60/40	308
BR2EX8R	CAGGAAACAGCTATGACCG GAGACAGCAGAGTTTCACAGGA		
BR2EX9F	TGTAAAACGACGGCCAGT TAAAGGGGGGACTACTACTATATGTGC	61/40	280
BR2EX9R	CAGGAAACAGCTATGACCG GAGATCACGGGTGACAGAGC		
BR2EX10.1F	TGTAAAACGACGGCCAGT TTCTATGAGAAAGGTTGTGTAGAATAAT	60/40	400
BR2EX10.1R	CAGGAAACAGCTATGACCG CTACATTTGAATCTAATGGATCAGTAT		
BR2EX10.2F	TGTAAAACGACGGCCAGT AAACCAAGTGAAAGAAAAATACTCATTGT	63/40	373
BR2EX10.2R	CAGGAAACAGCTATGACCA TCTCTCTTATTTACCACTGTTTCCTC		
BR2EX10.3F	TGTAAAACGACGGCCAGT GCCACGTATTTCTAGCCTACC	61/40	399
BR2EX10.3R	CAGGAAACAGCTATGACCG CCACGTATTTCTAGCCTACC		
BR2EX10.4F	TGTAAAACGACGGCCAGT GTCCAAATTTAATTGATAATGGAAGC	61/40	314
BR2EX10.4R	CAGGAAACAGCTATGACCC ACAGAAGGAATCGTCATCTA		
BR2EX12F	TGTAAAACGACGGCCAGT ATTTTTGTTAACATTTAAAGAGTCAATAC	60/40	281
BR2EX12R	CAGGAAACAGCTATGACCG GAGGTCAGAATATTATATACCATACCTA		
BR2EX13F	TGTAAAACGACGGCCAGT ACAGTAACATGGATATTCTCTTA	61/40	189
BR2EX13R	CAGGAAACAGCTATGACCA AAACGAGACTTTTCTCATACTG		
BR2EX14.1F	TGTAAAACGACGGCCAGT ATTCCTAAATATTTATATGTGTACTAGTCA	60/40	390
BR2EX14.1R	CAGGAAACAGCTATGACCT TACTATCATCAGAGCCATGTC		

BR2EX14.2F	TGTA AACGACGGCCAGTACAAGAAATGAAAAATGAGACACT	63/40	357
BR2EX14.2R	CAGGAAACAGCTATGACCGGGAAAACCATCAGGACATTAT		
BR2EX15F	TGTA AACGACGGCCAGTGCCAGGGGTTGTGCTTTTA	61/40	284
BR2EX15R	CAGGAAACAGCTATGACCCTCTGTCATAAAAGCCATCAG		
BR2EX16F	TGTA AACGACGGCCAGTTTTGGTAAATTCAGTTTTGGTTTG	61/40	379
BR2EX16R	CAGGAAACAGCTATGACCGCCA ACTTTTTAGTTTCGAGAGA		
BR2EX17F	TGTA AACGACGGCCAGTTTGAATTCAGTATCATCCTATGTGG	61/40	353
BR2EX17R	CAGGAAACAGCTATGACCGTGGGATGGCAACTGTCACT		
BR2EX18.1F	TGTA AACGACGGCCAGTTTTAACAGTGGAATTCTAGAGTCACA	61/40	284
BR2EX18.1R	CAGGAAACAGCTATGACCTCTAACTGGGCCTTAACAGCATA		
BR2EX18.2F	TGTA AACGACGGCCAGTTCTAGCAATAAACTAGTAGTGCAGATA	61/40	283
BR2EX18.2R	CAGGAAACAGCTATGACCAA CTCTAGAAATTTAACTGAATCAATG		
BR2EX19.1F	TGTA AACGACGGCCAGTATGAAA CTCTTATGATATCTGTAATAGAA	61/40	210
BR2EX19.1R	CAGGAAACAGCTATGACCATTACATCAACACAACCAACAT		
BR2EX19.2F	TGTA AACGACGGCCAGTCTCTGCCCTTATCATCGCTT	61/40	175
BR2EX19.2R	CAGGAAACAGCTATGACCGGCAAGAGACCGAACTCC		
BR2EX20F	TGTA AACGACGGCCAGTCCTGGCCTGATACAATTA ACT	60/40	276
BR2EX20R	CAGGAAACAGCTATGACCAGTCTCTAAGGACTTTGTTCTCA		
BR2EX21F	TGTA AACGACGGCCAGTTTTTAGTTGCTTTTGAATTTACAG	61/40	262

BR2EX21R	CAGGAAACAGCTATGACCTCCTGTGATGGCCAGAGAGT		
BR2EX22F	TGTAAAACGACGGCCAGTACATTAACCACACCCTTAAGAT	61/40	395
BR2EX22R	CAGGAAACAGCTATGACCTCATTTTGTTAGTAAGGTCATTTTT		
BR2EX23F	TGTAAAACGACGGCCAGTCAAACATTTAAATGATAATCACTTCTTCC	61/40	285
BR2EX23R	CAGGAAACAGCTATGACCGGAGATTCCATAAACTAACAAGC		
BR2EX24.1F	TGTAAAACGACGGCCAGTTTTATGGAATCTCCATATGTTGA	61/40	155
BR2EX24.1R	CAGGAAACAGCTATGACCCCTATTAGGTCCACCTCAG		
BR2EX24.2F	TGTAAAACGACGGCCAGTCAGCAAATTTTTAGATCCAGAC	63/40	174
BR2EX24.2R	CAGGAAACAGCTATGACCCCTGGTAGCTCCAACCTAATCAT		
BR2EX25.1F	TGTAAAACGACGGCCAGTTTCTTGCATCTTAAAATTCATCTAACAC	60/40	211
BR2EX25.1R	CAGGAAACAGCTATGACCCCTGATTTGGATTCTGGTCG		
BR2EX25.2F	TGTAAAACGACGGCCAGTAGGACATTATTAAGCCTCATATGTTAATTG	61/40	244
BR2EX25.2R	CAGGAAACAGCTATGACCGCTATTTCCCTTGATACTGGACTGT		
BR2EX26F	TGTAAAACGACGGCCAGTTGGGTTTGCAATTTATAAAGCAG	63/40	254
BR2EX26R	CAGGAAACAGCTATGACCCAGAATATACGATGGCCTCCA		
BR2EX27.1F	TGTAAAACGACGGCCAGTTTTCAATGAAAAGTTACTTTGATTTAGTT	61/40	400
BR2EX27.1R	CAGGAAACAGCTATGACCGTCATCTGAGGAGAATTCAGT		
BR2EX27.2F	TGTAAAACGACGGCCAGTTTGTGGCACCAATACGAA	61/40	397
BR2EX27.2R	CAGGAAACAGCTATGACCAACTGGAAAGGTTAAGCG		

Table 3.4 SSCP/HA primers utilised for investigating the genomic area represented by specific PTT regions for *BRCA1* and *BRCA2*. Indicated are the specific PTT fragment, the SSCP/HA primer pairs utilised, the 5' – 3' sequence of the primers, the size of the specific fragment and the annealing temperature.

PTT Fragment	SSCP Primer name	Primer sequence 5'-3'	Size of fragment	Annealing Temperature
<i>BRCA1</i> exon 11 Fragment 1	<i>BRCA1</i> Exon 11 Fragment DF <i>BRCA1</i> Exon 11 Fragment DR	GCCAAAGTAGCTGATGTATTGG CGCTTTAATTTATTTGTGAGGG	293 bp	58°C
	<i>BRCA1</i> Exon 11 Fragment EF <i>BRCA1</i> Exon 11 Fragment ER	CCCAACTTAAGCCATGTAAGTGG ATTCATCACTTGACCATTCTGC	228 bp	58°C
	<i>BRCA1</i> Exon 11 Fragment FF <i>BRCA1</i> Exon 11 Fragment FR	ATTTGGCAGTTCAAAGACTCC TTTAGGTGCTTTTGAATTGTGG	257 bp	58°C
<i>BRCA1</i> exon 11 Fragment 3	<i>BRCA1</i> Exon 11 Fragment PF <i>BRCA1</i> Exon 11 Fragment PR	AACTTAGAACAGCCTATGGGAA AACAAGTGTTGGAAGCAGGG	296 bp	58°C
	<i>BRCA1</i> Exon 11 Fragment QF <i>BRCA1</i> Exon 11 Fragment QR	AGGGGCCAAGAAATTAGAGTC CTTCCAATTCAGTCACTGTG	302 bp	58°C
	<i>BRCA1</i> Exon 11 Fragment RF <i>BRCA1</i> Exon 11 Fragment RR	GTAATATTGGCAAAGGCATCT TAAAATGTGCTCCCCAAAAGCA	360 bp	58°C

<i>BRCA2</i> exon 11 Fragment A	<i>BRCA2</i> Exon 11 Fragment EF <i>BRCA2</i> Exon 11 Fragment ER	TTCAAAAATAACTGTCAATCC GTTGCTTGTTTATCACCTGT	207 bp	54°C
	<i>BRCA2</i> Exon 11 Fragment FF <i>BRCA2</i> Exon 11 Fragment FR	AACCCATTTTCAAGAACTCTACCA CTGAAGCTACCTCCAAAACCTGTG	274 bp	54°C
	<i>BRCA2</i> Exon 11 Fragment GF <i>BRCA2</i> Exon 11 Fragment GR	ACAAATGGGCAGGACTCTTAGG CCTGCTTGAAAATAACATCTG	315 bp	54°C
<i>BRCA2</i> exon 11 Fragment D	<i>BRCA2</i> Exon 11 Fragment WF <i>BRCA2</i> Exon 11 Fragment WR	AAAGTAACGAACATTCAGACCA CTGGGTTTCTCTTATCAACACG	294 bp	54°C
	<i>BRCA2</i> Exon 11 Fragment XF <i>BRCA2</i> Exon 11 Fragment XR	AGTCTTCACTATTCACCTACG GTGAGACTTTGGTTCCTAAT	251 bp	54°C
	<i>BRCA2</i> Exon 11 Fragment YF <i>BRCA2</i> Exon 11 Fragment YR	TTCAACAAGACAAACAACAGT TGTCAGTTCATCATCTTCCATAAA	251 bp	54°C

formamide, 0.05% (w/v) xylene cyanol FF, 0.05% (w/v) bromophenol blue, 1 mM EDTA (pH 8.0)] were added post-PCR amplification. The reactions were denatured at 94°C for 5 min and snap-cooled on ice for 5 min until loaded.

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., Holliston, Massachusetts, United States). 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 16h at a constant voltage of 260-280 V.

The gels were visualised by silver staining. The staining commenced with the gel suspended in 1 M dithiothreitol (DTT) containing 0.5 M KOAc (pH 4.5) for 10 mins, followed by 30 sec in deionised water. This was followed by submerging the gel in 0.1% (w/v) silver nitrate solution for 10 min and rinsing for 1 min in deionised water. The gel was developed with 1.5% (w/v) NaCO₃, 0.155% (v/v) formaldehyde until the bands were visible (Bassam *et al.*, 1991; Pinar *et al.*, 1997). The reaction was stopped by gently agitating the gel in the presence of 0.01 M citric acid for 10 mins. The gel was rinsed in distilled water and digitally captured on the Bio-Rad Gel documentation system.

3.3.6 COMPUTER BASED ANALYSES

The analysis of variants of unknown significance (VUS) was conducted using a multiple evidence based approach in order to confirm their clinical status. All variants were initially checked for their existence using BIC and ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar/>, accessed 21 July 2016) databases. These databases were created for use by diagnostic laboratories. In BIC and ClinVar, variants were allocated a class (1-5) based on the ENIGMA guidelines for variant classification (Appendix A). Variants that were not found in these two databases were searched for in the 1000 Genomes project database to investigate whether the variant was novel or allocated to a specific population cluster. From the 1000 Genomes database, reference SNP numbers were also acquired if the variant had been reported and peer reviewed but not reported to the BIC database. Variant rate of occurrence was measured based on a minor allele frequency (MAF) score. If a variant was identified at a frequency greater than 0.01 and present in multiple

populations, the variant was accepted as benign (Appendix A). If a variant had a frequency less than 0.01, but was only found in a single population, the variant was accepted as likely benign (class 2).

Novel variants could not be classified as pathogenic if not reported to the 1000 Genomes database, since the database does not contain enough sub continental populations yet. For example, various variants in *BRCA1* and *BRCA2* were identified for Africa or more specifically for the Khoisan/Bushmen of Southern Africa. However, due to the database only containing the genome of a single individual representing this tribe, MAF scores carried no weight for evidential conclusions.

Post population data analysis for the VUS was grouped according to variant type. Variants were classified according to their main classes: nonsense, missense and synonymous variants. Nonsense variants led to terminating codons which are in most cases pathogenic. Missense variants were examined with computational functional analysis, using PolyPhen-2, SIFT and MutPred (<http://mutpred.mutdb.org/>, accessed 12 July 2016) to determine the variants' conservation across species and to assess possible protein structure disruption. Variants that delivered inconclusive results across these databases were then analysed with the synonymous VUS by means of splice effect predictions. Human Splicing Finder (<http://www.umd.be/HSF3/HSF.html>, accessed 17 June 2016) and Mutation Taster (<http://www.mutationtaster.org/>, accessed 3 July 2016) were used to determine splice defects. ProSite Domain (<http://prosite.expasy.org/>, accessed 24 July 2016) was used to investigate the peptide region where the mutation was located, in order to deduce a possible effect. Variants that showed possible pathogenicity were then searched across the literature for published functional assay confirmation.

3.4 RESULTS AND DISCUSSION

3.4.1 BREAST CANCER PATIENTS

A total of 229 patients were included in this study. These patients were all self-identified as Coloured. These patients were referred to Universitas Hospital from various NHLS regions distributed throughout SA. As SA is a third-world country,

medical care, treatment and in this case genetic testing for familial BC, are centralised in order to be more cost-effective. The majority (n=159, 69.4%) of patients were referrals from Tygerberg Hospital, with a further 28 (12.2%) received from Groote Schuur Hospital in the Western Cape. Together, 81.7% of the patient cohort represented the Coloured population of the Western Cape. The rest of the samples (18.3%) were received from Inkosi Albert Luthuli Hospital in Durban.

In the literature review presented in Chapter 1, it was stated that the majority (50.2%) of the SA Coloured population resides in the Western Cape. This makes the cohort a strong focus group narrowing the possibility of genetic diversity due to admixture of international ancestral gene pools. It narrowed the admixture of gene pools down to the first possible contributions from the Dutch East India Company during the establishment of its trading station in the now Western Cape province in 1652 (Nurse *et al.*, 1985; Patterson *et al.*, 2010). European settlers together with slaves from Java, the Indonesian islands and India were the first to establish themselves at Cape Point in SA (Nurse *et al.*, 1985; Mountain, 2003). This fact was confirmed by the genetic studies performed in 2009 (Tishkoff *et al.*, 2009) and 2010 (Quintana-Murci *et al.*, 2010). The SAC population has its biggest maternal contribution from the indigenous Khoisan, and second largest from Indian ancestry. Paternally, the biggest ancestral contribution was almost equally from the European and Khoisan, with secondly the Indian ancestry and third largest South East Asia (Quintana-Murci *et al.*, 2010).

Of the total number, 226 of the patients were female with only three being male. All these patients were affected with BC. The collection of clinical data was challenging and restricted due to incomplete request forms which often did not contain even the basic diagnostic information. As the laboratory in Bloemfontein did not have access to the patients, the clinical data set regarding the characteristics of the tumour and family details remained incomplete. From the 229 patients, 136 (59.4%) had a positive family history of BC, ranging from a single family member affected with BC (low risk) to more than three affected family members (high risk family). The familial status alone did not always strengthen the indications for comprehensive screening as the number of affected family members were not always indicated, nor their relationship with the index (first, second or third degree relatives). The majority of the patients represented a low risk family (ranging from

no family history to a single other family member being affected with the disease), with 22 indicating two affected family members (potentially medium risk). Twelve patients reported a strong family history of the disease, with three family members affected (potential high risk family). Only 11 of the patients reported an extensive family history of four or more family members affected with breast and other cancer types (high risk family). These families were affected by various ca types including colon, stomach and ovarian ca. The incompleteness of the clinical details made it difficult to select the most likely patients to carry a *BRCA* disease-causing mutation.

The age of the patients at request and age at diagnosis also varied widely. As for many the age at diagnosis was not indicated, the age at request was used to determine the various age groups requesting testing (Fig. 3.1). The majority of patients (32.2%) were in their 30's, with very few being in their 70's (3.8%). The data in the graph indicate three main periods during which the patients seemed to be concerned regarding their chances to be a *BRCA* pathogenic mutation carrier, namely in their early 30's, early 50's and mid 60's. It is hypothesised that the patients who requested testing during their early 30's might have been recently diagnosed with BC and wanted to determine their mutation status for the future, in order to do family planning. The cancers diagnosed at such an early stage might also be more aggressive, causing suspicion regarding the involvement of the *BRCA* genes. The patients in their early 50's might have been concerned regarding the use of hormone replacement therapies. By knowing their mutation status, their caregivers can make informed decisions regarding the optimal management options for their patients. It is hypothesised that the BC patients in their 60's might have requested genetic testing for the sake of their sons and daughters. It might be that they were the only remaining living individual affected for which mutation analysis could be performed, as the laboratory in Bloemfontein prefers testing of an affected family member first. As a mutation-positive status may be informative and life-changing for the extended related family members, determining this status could assist in the protection of the pre-symptomatic individuals representing their family.



Figure 3.1 Illustration of the age at which genetic testing for the familial BC genes had been requested by each of the 229 patients included in this cohort.

3.4.2 GENOTYPING FOR MOST COMMON SA MUTATIONS

All 229 SAC patients were screened for the presence of the seven most common mutations present within SA (Van der Merwe *et al.*, 2012). Three of these mutations represented the Afrikaner founder mutations [*BRCA1* c.1374delC, p.Asp458GlufsX17 (1493delC), *BRCA1* c.2641G>T, p.Glu881X (2760G>T, E881X) and *BRCA2* c.7934delG, p.Arg2645AsnfsX3 (8162delG)] (Reeves *et al.*, 2004), whereas another three were reported as founder mutations within the Ashkenazi Jewish population [*BRCA1*c.68_69delAG, p.Glu23ValfsX17 (185delAG); *BRCA1* c.5266dupC, p.Gln1756ProfsX74 (5382insC) and *BRCA2* c.5946delT, p.Ser1982ArgfsX22 (6174delT)]. Some of the Ashkenazi Jewish mutations are also known as European mutations, as they had been detected within populations representing that region. Due to the genetic association of the SAC population with that of Western Europe and the Afrikaner, all six of these mutations were tested.

The seventh mutation namely *BRCA2* c.5771_5774delTTCA, p.Ile1924ArgfsX38 is situated in exon 11 (5999del4) (Van der Merwe *et al.*, 2012). This mutation is specific to the Coloured and Xhosa populations from the Western Cape. A segregating haplotype between various mutation positive families indicated this mutation to be a founder or recurrent mutation.

Each genotyping assay was performed using a no template control (NTC) as well as a previously sequenced and confirmed positive control. The genotyping peaks of the unknown samples were compared to that of the known positive. If a melt peak was detected at the same temperature to that of the positive control, the patient was accepted as positive (Fig. 3.2). Of the 229 BC patients, 19 (8.3%) tested positive for the presence of either an Afrikaner founder or the Coloured/Xhosa mutation from the Western Cape. None of the Ashkenazi Jewish/European founder mutations were observed. All three the Afrikaner founder mutations were detected within this cohort, of which *BRCA2* c.7934delG (8162delG) was the most common.

3.4.2.1 *BRCA1* c.1374delC, p.Asp458GlufsX17 (1493delC)

The *BRCA1* c.1374delC, p.Asp458GlufsX17 mutation (1493delC - rs397508862, g.41246174delG) was first reported in various families in the Afrikaner population by Reeves *et al.* in 2004. This mutation was novel and previously restricted to SA. Haplotype analysis indicated a common ancestral event, as the families shared a common haplotype (Reeves *et al.*, 2004). Genealogical studies resulted in the identification of the founding couple by linking five unrelated mutation positive families. The founding couple was identified as Pieter Louw (born in 1667) who was married to Elisabeth Wendels (born in 1671). The couple was originally from the Netherlands (Van der Merwe & Van Rensburg, 2007). Based on these results, the mutation was declared an Afrikaner founder mutation.

This mutation was detected for a single SAC family (2/229, 0.87%), identified within two family members, namely the mother and daughter (diagnosed at age 53 and 34 respectively, Fig. 3.2). The single base deleted at position 1374 immediately resulted in a shift in the reading frame, from the position of the deletion to the end of the exon 11. This resulted in the premature truncation of the *BRCA1* protein at aa 474, leaving it incapable to function normally. The effects of this mutation have not been fully described as yet.

3.4.2.2 *BRCA1* c.2641G>T, p.Glu881X (E881X)

The *BRCA1* c.2641G>T, p.Glu881X (rs397508988, g.41244907C>A) mutation was reported for the first time in the Afrikaner population (Reeves *et al.*, 2004). This mutation was also detected using PTT and confirmed by Sanger sequencing. This mutation too has been proven to be an Afrikaner founder mutation, confirmed by a common haplotype segregating within eight BC families carrying the *BRCA1* c.2641G>T,p.Glu881X mutation. Genealogical studies successfully linked nine “current day” unrelated mutation positive families to identify a founding couple (Van der Merwe & Van Rensburg, 2007). They were Hercules des Prez (born 1645) from France, who was married to Cecilia d’Athis (born 1650 in France). The couple fled from France and represented the founders of the Du Preez family in SA.

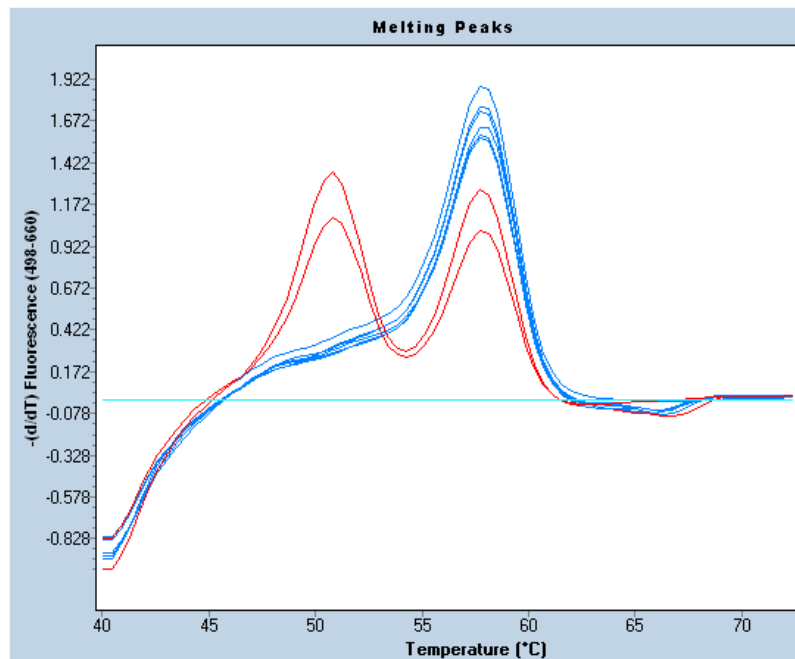


Figure 3.2 Genotyping for the *BRCA1* c.1374delC, p.Asp458GlufsX17 (1493delC) founder mutation using qPCR. The previously sequenced and confirmed positive control (represented by the red line) indicated two melt peaks, at approximately 51°C and 58°C indicative of a heterozygote. Genotyping of a Coloured patient (also indicated in a red line) displayed an identical pattern to that of the positive control (heterozygous for the single base deletion). All the other samples (indicated in blue lines) within this run were homozygous for the wild type (high peak at approximately 58°C).

This mutation is approximately 300 years old (Van der Merwe & Van Rensburg, 2007).

This Afrikaner founder mutation was detected only once within the Coloured cohort, in a patient 34 years of age (Fig. 3.3). Very little is known regarding her family history, as nothing was indicated on the request form. The mutation involved a single base substitution of a guanine to thymine at base 2641 within exon 11 of *BRCA1*. This single base change creates a nonsense terminating codon that normally produces a glutamic acid at residue 881. The full clinical implication of this truncation has not been explained. This mutation is novel and restricted to SA.

3.4.2.3 *BRCA2* c.7934_7934delG, p.Arg2645fsX2 (8162delG)

The *BRCA2* c.7934_7934delG, p.Arg2645fsX2 (8162delG, rs80359688, g.32936788delG) mutation was previously described by Agenbag (2005). She reported the presence of the mutation in three Afrikaner and a single SAC patient. In the current study, this mutation was detected for six patients (Fig. 3.4). Five of the patients had a moderate to extensive family history of BC. The age at onset was known for these patients and corresponded to that reported for *BRCA2* mutation carriers reported internationally. *BRCA2* mutation carriers tend to develop BC at a later stage in life than those who carry *BRCA1* mutations. This explains why BC caused by deleterious mutations in *BRCA2* cannot be easily separated from that of sporadic cases, partly due to the later onset in life.

The mutation is described as a deletion of a guanine at base 7934 in exon 17 of *BRCA2*. The mutation results in a frameshift which leads to a missense change from arginine to asparagine at residue 2645. The premature stop codon is created at residue 2647, which results in truncation of the protein.

3.4.2.4 *BRCA2* c.5771_5774delTTCA, p.Ile1924_Ala1925fsX38 (5999del4)

The *BRCA2* c.5771_5774delTTCA, p.Ile1924_Ala1925fsX38 (5999del4, rs80359535, g3214263_32914266delTTCA) mutation was first reported to the BIC

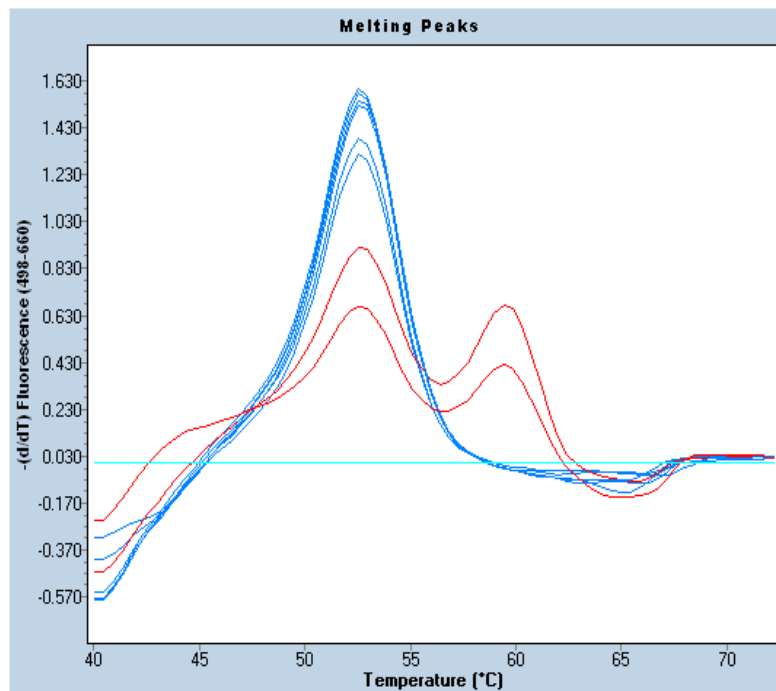


Figure 3.3 Genotyping for the *BRCA1* c.2641G>T, p.Glu881X (E881X) founder mutation using qPCR. The previously sequenced and confirmed positive control (represented by the red line) indicated two melt peaks, at approximately 52.5°C and 59°C indicative of a heterozygote. Genotyping of a Coloured patient (also indicated in a red line) displayed an identical pattern to that of the positive control (heterozygous for the single base deletion). All the other samples (indicated in blue lines) within this run were homozygous for the wild type (high peak at approximately 52.5°C).

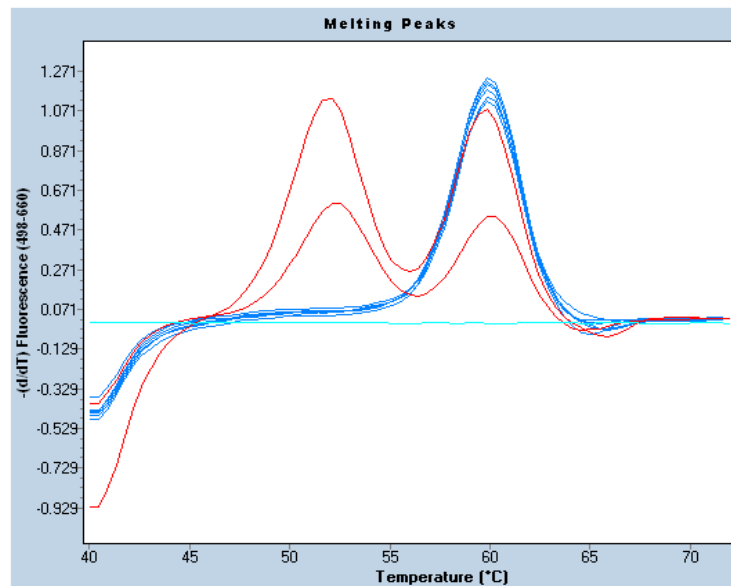


Figure 3.4 Genotyping for the *BRCA2* c.7934_7934delG, p.Arg2645fsX2 (8162delG) founder mutation using qPCR. The previously sequenced and confirmed positive control (represented by the red line) indicated two melt peaks, at approximately 52°C and 60°C indicative of a heterozygote. Genotyping of a Coloured patient (also indicated in a red line) displayed an identical pattern to that of the positive control (heterozygous for the single base deletion). All the other samples (indicated in blue lines) within this run were homozygous for the wild type (high peak at approximately 60°C).

database in 1997 by Ligtenberg and published by Ikeda and co-workers from Japan in 2002. Van der Merwe and associates described the mutation as a founder/recurrent mutation in non-Afrikaner BC patients of the Western Cape of SA in 2012 after detecting it with PTT. As this mutation was previously recorded for two patients in the Netherlands, Van der Merwe *et al.* (2012) initially speculated that the mutation was introduced into the SAC population via the admixture with Dutch settlers more than 300 years ago. This proved incorrect, as the Coloured patients did not share the same haplotype as the Dutch patients carrying the same mutation. From the haplotype data it was concluded that this mutation originated independently within the Coloured/Xhosa populations of the Western Cape many years before (Van der Merwe *et al.*, 2012). The mutation has not been detected within the Afrikaner population and has been limited to the Coloured/Xhosa population residing in the Western Cape. The mutation was detected in 10 of the 229 SAC patients (4.37%) (Fig. 3.5).

The mutation is classified by the deletion of four consecutive bases at nucleotide numbers 5771 to 5774, situated in exon 11. The mutation causes a frameshift resulting in a missense aa at residue 1924 (from isoleucine to arginine), with a premature stop codon following at residue 1962.

3.4.3 MUTATION SCREENING

3.4.3.1 Protein truncation test (PTT)

A total of 103 BC patients were retrospectively selected for a comprehensive screen using PTT and HRMA. The selection was based on the criteria provided in 3.2. These patients all reported a family history of BC, ranging from medium to high risk. Among the 103 patients, the average age at request was 44.4 years. Among these patients, 11 tested positive for the presence of a prematurely truncated peptide, indicative for the presence of a nonsense mutation or a deletion/duplication of a number of bases.

Two truncating mutations were observed within both *BRCA1* and *BRCA2* respectively. The *BRCA1* c.1504_1508delTTAAA, p.Leu502AlafsX2 mutation in exon 11 was previously known as 1623delTTAAA (rs80357888, g.41246040_

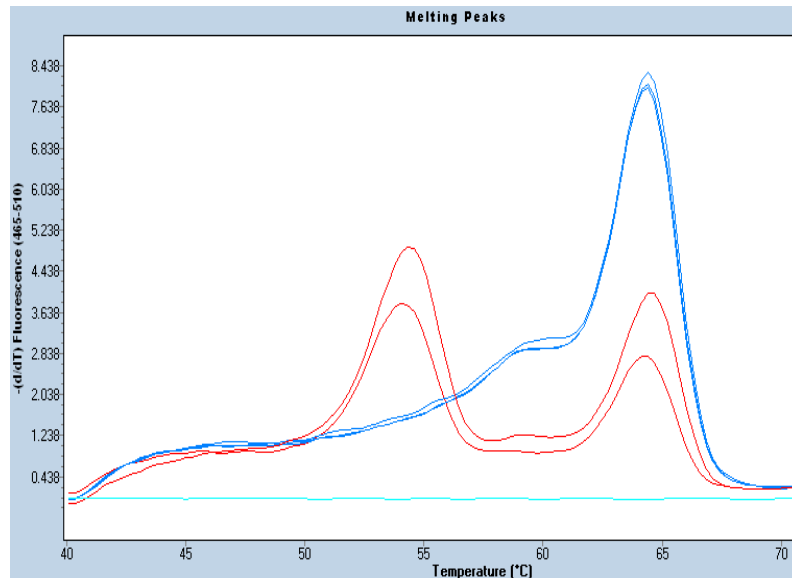


Figure 3.5 Genotyping for the *BRCA2* c.5771_5774delTTCA, p.Ile1924_Ala1925fsX38 (5999del4) recurrent mutation using qPCR. The previously sequenced and confirmed positive control (represented by the red line) indicated two melt peaks, at approximately 53°C and 62°C indicative of a heterozygote. Genotyping of a SAC patient (also indicated in a red line) displayed an identical pattern to that of the positive control (heterozygous for the four base deletion). All the other samples (indicated in blue lines) within this run were homozygous for the wild type (high peak at approximately 62°C).

41246044delTTAAA). This mutation had been reported 29 times before to the BIC with Myriad Diagnostic Laboratories being the largest contributor. The majority of entries were reported for Western Europeans, with the minorities being specifically indicated as German, Greek, Irish, Italian and Spanish. The mutation is characterised by the deletion of five bp at coding nucleotide 1504 to 1508 of *BRCA1* exon 11 (Fig. 3.6). The deletion altered the aa from a leucine to alanine. Although the mutation conserved the aliphatic nature of the specific aa at residue 502, the frameshift resulted in the formation of a termination codon at codon 504. The termination led to the early truncation of the *BRCA1* protein. The variant was detected in three female patients, CAM1677, CAM1703 and CAM2704, all referred from Tygerberg Hospital in the Western Cape. CAM1677 was affected with BC at the age of 71 and had three sisters and a daughter affected with BC, all diagnosed in their early 50's. For CAM1703, the available clinical data indicated that she was diagnosed with ductal BC at the age of 70. CAM2704 was the only patient of the three who had been diagnosed with infiltrating BC at an early age (dx 35). She had a sister (dx 30) and a grandmother affected with the disease.

A novel *BRCA1* mutation was identified, namely *BRCA1* c.3732_3733delTA, p.His1244GlnfsX9 (no rs number as yet). This mutation was identified for a single patient (CAM2747) (Fig. 3.7). The deletion of the two bp caused a change in the aa at codon 1244, from a histidine to an aspartic acid, with a premature termination codon following nine residues later, at position 1253. This mutation was novel and has not been reported before. Although the mutation function at DNA level has not been fully examined, the premature truncating effect on the protein will produce an incomplete and therefore ineffective *BRCA1* protein in the ecology of active repair mechanisms.

A novel mutation was also detected for *BRCA2*. The mutation entailed a four bp deletion at the beginning of exon 11, with a description of *BRCA2* c.2826_2829AATT, p.Ser942=fsX15 (3054del4) (no rs number as yet). The deletion did not disrupt codon 942 (Ser), but caused the termination of the protein 15 aa downstream (Fig. 3.8). The mutation was identified for two patients

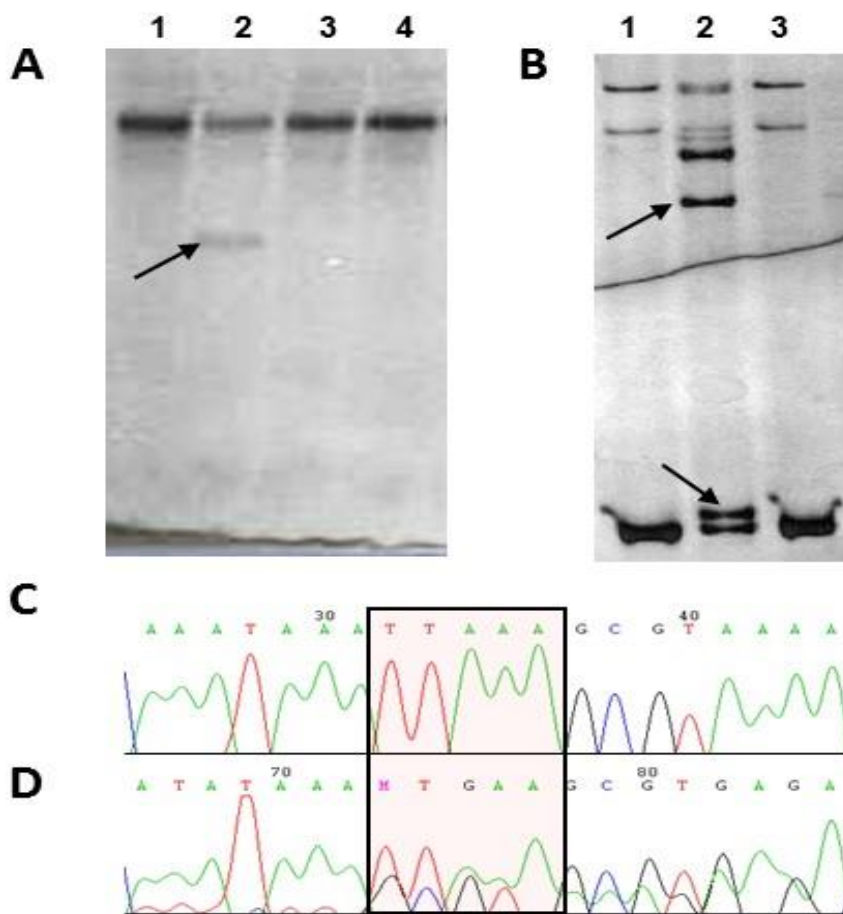


Figure 3.6 Identification and designation of *BRCA1* c.1504_1508delTTAAA, p.Leu502AlafsX2 (g.41246040_41246044delTTTAA). **A.** SDS page gel exhibiting *in vitro* transcription/translated peptides for *BRCA1* fragment 1. The patient with the truncated peptide (indicated by the arrow) is indicated in lane 2. **B.** The position of the mutation was determined by using smaller overlapping SSCP/HA primer sets. Indicated are the results for primer set E (lanes 1-3). The difference in SSCP/HA pattern is visible in lane 2, as indicated by the arrows. **C.** DNA sequencing results for CAM1608, indicating the wild type sequence. The bp involved in the mutation are indicated in the square box. **D.** Sequencing electropherogram for CAM1703, which clearly indicates the deletion of five bp, creating a shift in the reading frame.

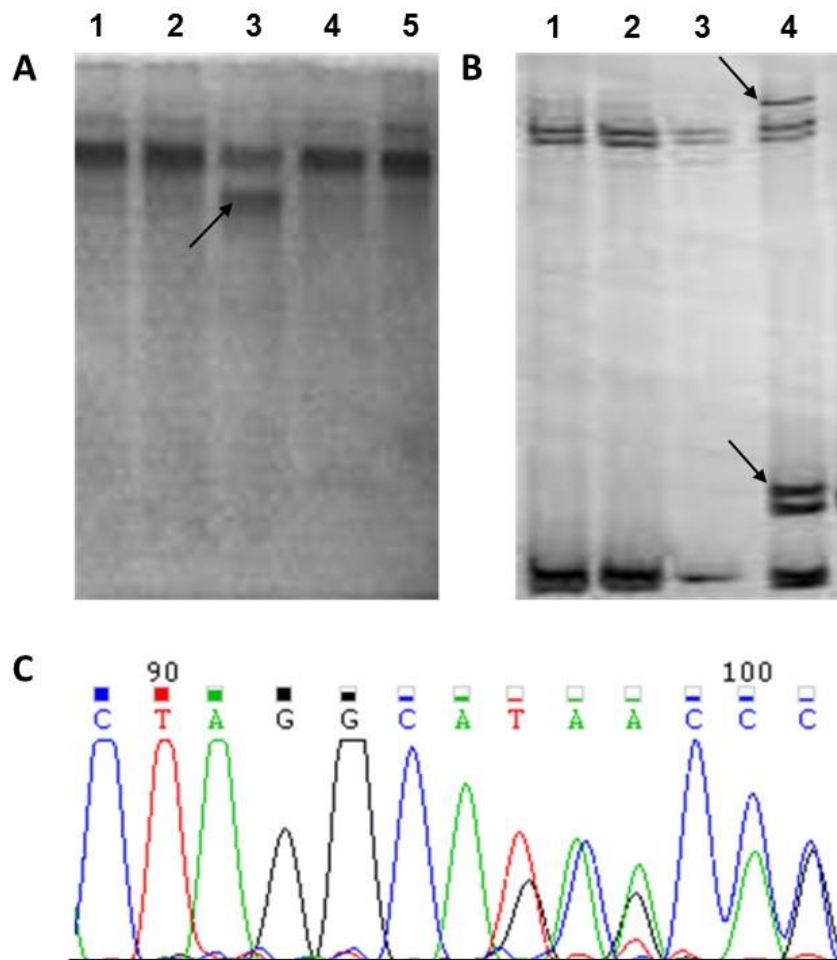


Figure 3.7 Identification and designation of *BRCA1* c.3732_3733delTA, p.His1244GlnfsX9 as a novel mutation. **A.** SDS page gel exhibiting *in vitro* transcription/translated peptides for *BRCA1* fragment 3. The patient with the truncated peptide (indicated by the arrow) is indicated in lane 3. **B.** The position of the mutation was determined by using smaller overlapping SSCP/HA primer sets. Indicated are the results for primer set Q (lanes 1-4). The difference in SSCP/HA pattern is visible in lane 4, as indicated by the arrows. **C.** DNA sequencing results for CAM2001, indicating the frameshifted sequence.

(CAM 1663 and CAM 1875). The first patient was diagnosed with aggressive stage IV disease at the young age of 27, who had an uncle and nephew affected with BC (Van der Merwe *et al.*, 2012). The second patient represented a female BC patient at the age of 49, who could not recollect the presence of a family history for the disease.

The second mutation detected for *BRCA2* using PTT was *BRCA2* c.6448_6449dupTA, p.Lys2150IlefsX18 (6676insTA) (rs397507858, g.32914939_32914940dupTA) (Fig. 3.9). The insertion of the two bp between nucleotides 6448 and 6449 resulted in a premature stop codon at residue 2168. The mutation was first reported by Meindl in 2002 in a study on *BRCA* variants in a German population, where the variant was reported in two patients affected with BC. In the current study, this mutation was observed for three SAC patients. The demographics for this mutation varied, as one patient had already been diagnosed at age 38 with stage IV disease, whereas another was only diagnosed at age 63. All these patients had a positive family history ranging from medium to high risk.

This mutation has been previously detected in the Coloured population by Agenbag (2005). The results were described in a MMedSc dissertation. Although the electropherogram indicating the two bp duplication looked identical to what has been found in the current study, the mutation was described differently. The author described the mutation as c.6449_6450insTA (6677insTA). The position of the insertion was described incorrectly as the insertion occurred at base c.6448_6449 (Fig. 3.9). Based on the results presented in 2005 and the current study, it is concluded that it is the same mutation occurring at c.6448_6449dupTA. Agenbag (2005) reported this mutation for three Coloured patients. When the data is combined, this mutation might represent the second recurrent mutation detected for the Coloured population from the Western Cape. Thus far, it has been detected within a total of six SAC BC patients and is currently specific to the Coloured population in SA.

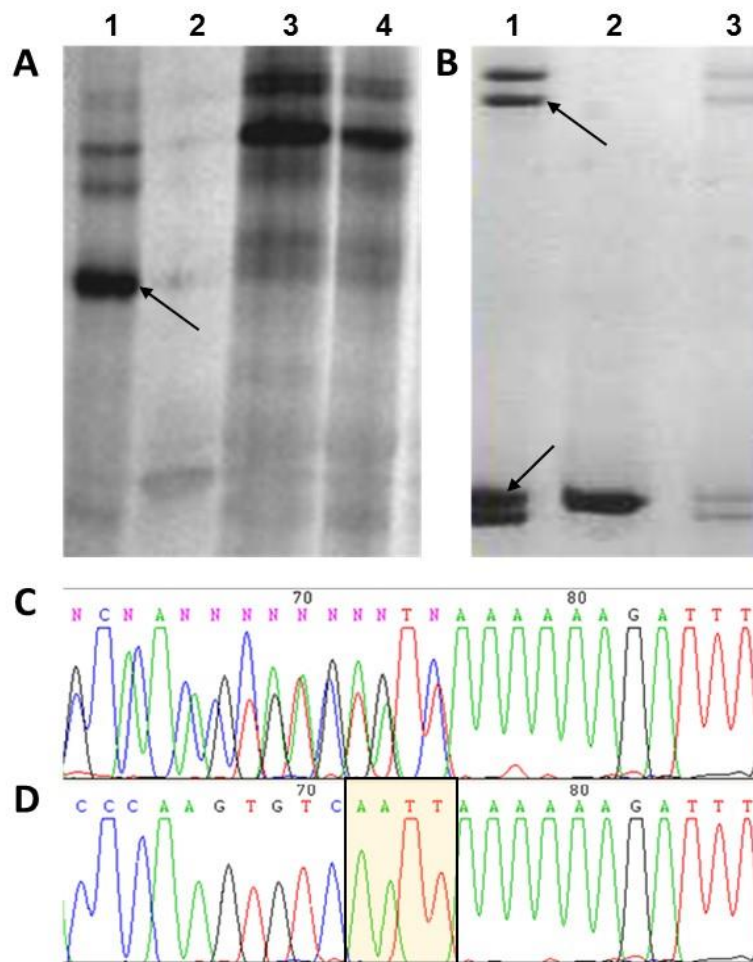


Figure 3.8 Identification and designation of *BRCA2* c.2826_2829AATT, p.Ser942=fsX15. **A.** SDS page gel exhibiting *in vitro* transcription/translated peptides for *BRCA2* fragment A. The patient with the truncated peptide (indicated by the arrow) is indicated in lane 1. **B.** The position of the mutation was determined by using smaller overlapping SSCP/HA primer sets. Indicated are the results for primer set *BRCA2* exon 11 SSCP fragment F (lanes 1-3). The difference in SSCP/HA pattern is visible in lanes 1 and 3, as indicated by the arrows. **C.** DNA sequencing results for CAM1875, indicating the shift in the reading frame as a result of a four bp deletion. **D.** Sequencing electropherogram for CAM2266 (wild type). The four bp involved in the mutation are highlighted by the square box.

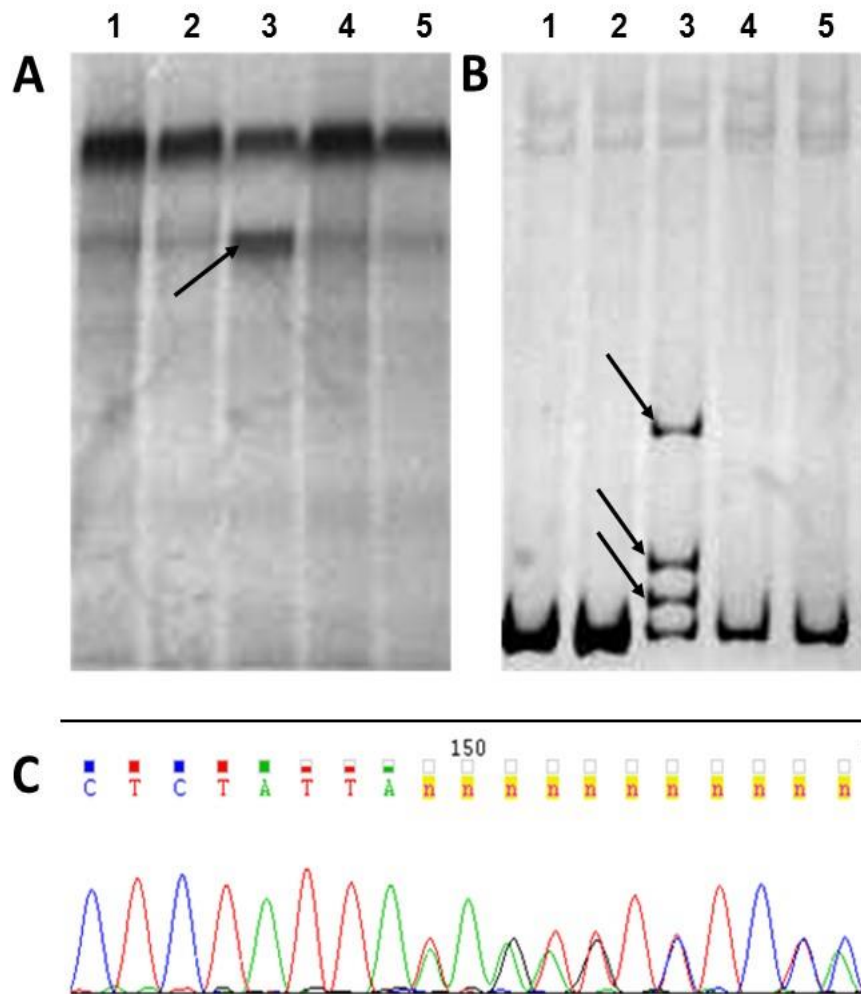


Figure 3.9 Identification and designation of *BRCA2* c.6448_6449dupTA, p.Lys2150IlefsX18. **A.** SDS page gel exhibiting *in vitro* transcription/translated peptides for *BRCA2* fragment D. The patient with the truncated peptide (indicated by the arrow) is indicated in lane 3. **B.** The position of the mutation was determined by using smaller overlapping SSCP/HA primer sets. Indicated are the results for primer set *BRCA2* exon 11 SSCP fragment X (lanes 1-5). The difference in SSCP/HA pattern is visible in lane 3, as indicated by the arrows. **C.** DNA sequencing results for CAM2664, indicating the shift in the reading frame as a result of a two bp duplication.

3.4.3.2 High resolution melting analysis (HRMA)

After truncation testing, the remaining exons of *BRCA1* and *BRCA2* were screened using HRMA to identify possible deleterious mutations present in any of the smaller exons. A total of 103 BC patients were screened using HRMA. The complete raw data set for the comprehensive screening of all 103 patients is included in Appendix I. The primers and PCR conditions for the screening of *BRCA2* were applied as optimised and researched by Combrink (2016) in a related Master's degree project on the Indian population of SA, which formed part of an umbrella study performed by Dr NC Van der Merwe at the Division of Human Genetics in Bloemfontein.

The number and the type of the variants detected varied amongst the patients. For some, no variants were detected whereas for others a total of eight were recorded. The intron splice site boundaries and exonic regions for *BRCA2* were more variable compared to *BRCA1*, as more variants were detected for *BRCA2* (total of 32) compared to the 18 in *BRCA1*.

Variant classification was performed according to that described in 3.3.6. It commenced with determining whether the variant had been detected before. The first database consulted was the BIC. If the variant had already been included in the BIC, the rs and g numbers would be available for future reference. In the majority of cases, the ENIGMA classification for that specific variant would be indicated. Variants not listed within the BIC database or without an ENIGMA pathogenic classification were searched for using the specific designation in the ClinVar database. ClinVar includes listings by several diagnostic companies that classify mutations on the basis of variant type, population frequencies, *in silico* functionality prediction and species-wide conservation analysis, familial pathogenicity segregation and lastly peer review publications. The entries into ClinVar are reviewed and evaluated by expert panels consisting of internationally acknowledged scientists, also involved in the Human Variome project. The recommendations for the various variants listed by ClinVar were considered as thoroughly curated and evaluated, and were therefore accepted as valid and current.

Variants not found in ClinVar were analysed by means of the population frequency in the 1000 Genomes database. The MAF gave an indication of the

frequency of the variant within the various genomes. Only rare variants qualified to be called a variant of unknown clinical significance (VUS). Allele frequencies for the closest relative population were obtained from the 1000 Genomes database. It was used to indicate if a variant was restricted to a unique single continental gene pool or whether the variant was present in multiple populations globally. If the variant had been identified globally at a frequency >0.01 or 1%, it was considered benign in most cases. If the variant had been observed in a single population at an allelic frequency of <0.01 or $<1\%$, it was considered to be likely benign. The chances of a general population risk increase could however not be ignored. Therefore, until proven non-pathogenic, variants that could possibly alter the silencing and enhancing elements at DNA level, cannot be accepted as benign or likely benign. All class 3 VUS variants (after population frequency comparison) were compared to clinical data obtained from the patient request form and literature. If no evidential literature was obtained, *in silico* analysis was performed to make a final functionality prediction. The sequencing data for all the variants detected are presented in Appendix J.

3.4.4 **BRCA1 VARIANTS DETECTED USING HRMA**

A total of 18 different variants were detected for *BRCA1* (Table 3.5). The majority of these variants (94%) were identified in a heterozygous form, with only *BRCA1* c.442-34C>T (rs799923) detected in both hetero- and homozygous states. Of the 21 exons screened with HRMA, only 13 (66.7%) exhibited variation. Exons 7, 10, 14, 19, 20, 21 and 23, and their intronic splice site boundaries proved to be conserved (Fig. 3.10).

The type of variants observed varied, ranging from intervening sequence changes to single base duplications (Table 3.5). Approximately half of the variants (52.6%, 10 of the 18) represented intronic variants, whereas nine (47.2%) were missense mutations, including two synonymous mutations. A single variant was identified in the 3' UTR region of *BRCA1*, together with two frameshift mutations (exon 2 and intron 12).

Various benign polymorphisms were observed, situated in both the coding and intervening regions. The class 1 SNPs situated within the exons were not limited to the SAC population, as they were detected within the 1000 Genome project at a frequency of $>0.01\%$ (Table 3.5). The majority of patients exhibited

Table 3.5 *BRCA1* variants identified in the SAC population. Indicated are the exon or region where situated, the designation of the variant, the associated protein change, the rs and g reference number, in how many patients the change had been detected, the variant type, how many times that specific variant had been entered into the BIC, the BIC and ClinVar classification, the minor allele frequency as detected within the 10000 Genome project, and the ENIGMA classification. Class 5 mutations are indicated in red, with likely pathogenic (class 4) indicated in orange, variants of unknown significance (class 3) in blue, likely benign (class 2) in green and benign polymorphisms (class 1) in yellow. The electropherograms utilised for the variant calling are indicated in Appendix J.

Exon / Region	Variants	Protein	rs number	Genomic reference number (NC_000017.10)	Recurrency (n = 103)	Variant type	# BIC Entries	BIC Classification	ClinVar Classification	1000 Genomes Minor allele frequencies			Proposed Classification based on all data evaluated
										Allele frequency for Africans	Highest Allele frequency of outbred population	AF Scoring	
2	c.66dupA	p.Glu23ArgfsX18	rs80357783	g.41276047_41276048insT	1	Frameshift	32	Class 5	Pathogenic SCV000282347.1	na	na	na	Class 5
3	c.110C>A	p.Thr37Lys	rs80356880	g.41267767G>T	1	Missense	1	Class 3	Pathogenic SCV000244295.1	na	na	na	Class 5
5	c.212+23T>A	Na	rs8176128	g.41258450A>T	1	IVS	na	na	Benign SCV000244801.1	0.0393	American 0.0014	African SNP	Class 1
8	Het c.442-34C>T Hom c.442-34C>T	Na	rs799923	g.41251931G>A	12 2	IVS	207	Class 1	Benign SCV000244771.1	na	European 0.1428	European SNP	Class 1
9	c.593+3G>A	Na	rs80358013	g.41249258C>T	2	IVS	1	Class 3	Likely Benign (Conflicting entries) SCV000209915.3	0.0008	na	African rare	Class 2
12	c.4113G>A	p.Gly1371=	rs147448807	g.41243033C>T	2	Synonymous	na	na	Likely Benign (Conflicting entries) SCV000252816.2	0.0053	American 0.0014	African SNP	Class 2
	c.4115G>A	p.Cys1372Tyr	rs55848034	g.41243031C>T	1	Missense	4	Class 3	Uncertain Significance SCV000076459.4	na	na	na	Class 2
	c.4185+12_4185 +13delGT	Na	rs273900723	g.41242948_41242949delAC	2	IVS	2	No But Pending	Likely Benign (Conflicting entries) SCV000145010.1	na	na	na	Class 4

13	c.4308T>C	p.Ser1436=	rs1060915	g.41234470A>G	29	Synonymous	251	Class 1	Benign SCV000244710.1	0.1595	Southern Asian 0.4968	Global SNP	Class 1
15	c.4675+32A>G	Na	rs762883588	g.41226316T>C	2	IVS	na	na	na	na	na	na	Class 3
16	c.4837A>G	p.Ser1613Gly	rs1799966	g.41223094T>C	38	Missense	248	No but Pending	Benign SCV000244369.1	0.3598	Southern Asian 0.4980	Global SNP	Class 1
	c.4956G>A	p.Met1652Ile	rs1799967	g.41222975C>T	1	Missense	62	Class 3	Benign SCV000244374.1	na	Southern Asian 0.0378	Asian SNP	Class 1
17	c.4987-20A>G	Na	rs80358035	g.41219732T>C	2	IVS	26	Class 3	Benign SCV000244376.1	0.0091	na	African rare	Class 2
18	c.5141T>G	p.Val1714Gly	rs80357243	g.41215902A>C	1	Missense	1	Class 3	Uncertain Significance SCV000184497.2	na	na	na	Class 4
22	c.5406+8T>C	Na	rs55946644	g.41201130A>G	3	IVS	16	Class 1	Benign SCV000244535.1	0.0159	American 0.0058	African SNP	Class 1
24	c.5468-10C>A	Na	rs8176316	g.41197829G>T	1	IVS	16	Class 1	Benign SCV000267721.1	0.0212	na	African SNP	Class 1
	c.5578C>G	p.His1860Gln	Novel	g.41197713G>C	1	Missense	na	Na	na	na	na	na	Class 4
3' UTR	c.5628C>G	Na	rs3092995	g.41197659G>C	3	3'UTR	7	Class 3	na	0.0552	American 0.0072	African SNP	Class 1

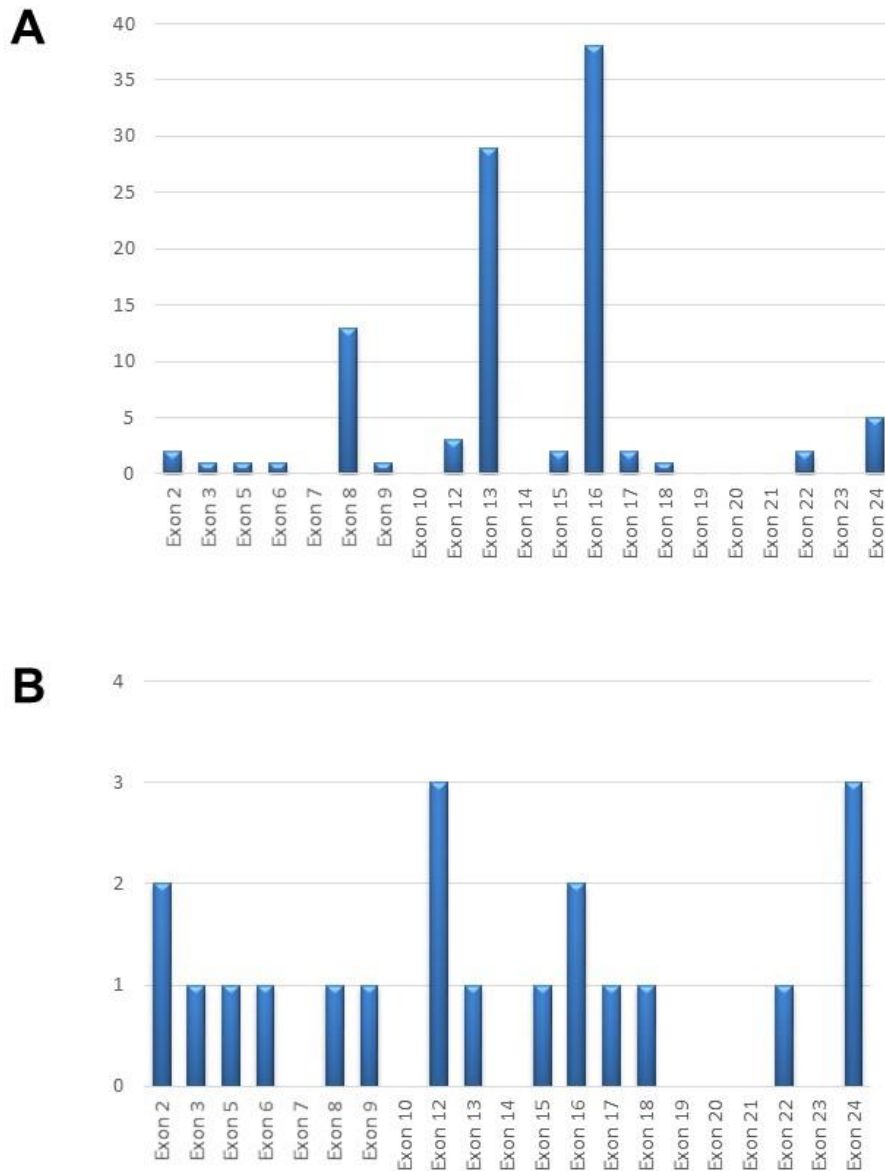


Figure 3.10 Demographics of the *BRCA1* variants identified for 103 BC patients representing the SAC population. **A.** Percentage of the patients who carried variants for each specific exon. **B.** The number of *BRCA1* variants observed per exon.

normal variation seen within these regions across the globe (Table 3.5). Out of all the exons exhibiting variants, only three had more than one specific variant, namely exons 12, 16 and 24 (Fig. 3.10). Within the SAC, exon 12 seemed to be the most variable, although the variants detected for this exon were not that common.

As so many of the variants detected for this cohort presented with class 1 and 2 likely benign polymorphisms for the two familial BC genes (ENIGMA class confirmed by either BIC and ClinVar or by ClinVar only), as listed in the results tables, they will not be discussed individually. Data regarding the number of patients it was detected for and other evidence such as entry numbers for each are presented in detail in Table 3.5. The focus for this section of the dissertation will therefore be on the results obtained for the variants for which conflicting results existed, or which fell into classes 3-5. Those variants for which additional information was gathered and tested – in order to clarify their specific role in BC carcinogenesis within this unique cohort –, will be discussed individually.

3.4.4.1 *BRCA1* frameshift mutations

Of the 18 variants detected, a single bp duplicated in exon 2 was recorded. - *BRCA1* c.66dupA, p.Glu23Argfs, rs80357783, g.41276047_41276048insT represented the duplication of a single base at nucleotide 66 in exon 2, which led to the formation of a premature termination codon 18 aa downstream at codon 40. The evidence for the identification and designation of rs80357783 is presented in Figure 3.11.

The designation of this mutation differed between the BIC and ClinVar, although the position of the duplication and the associated rs number was the same. This mutation is listed by the BIC as *BRCA1* c.66_67insA, p.Leu22_Glu23?fs (rs80357783, g.41276047_41276048insT), with the termination occurring at aa 40. ClinVar reports the mutation as *BRCA1* c.66dupA, p.Glu23Argfs (accession number SCV000282347.1, Table 3.5). As both databases refer to the identical mutation (insertion or duplication of a single adenine at position 66) and indicate the termination of the associated protein at aa 40,

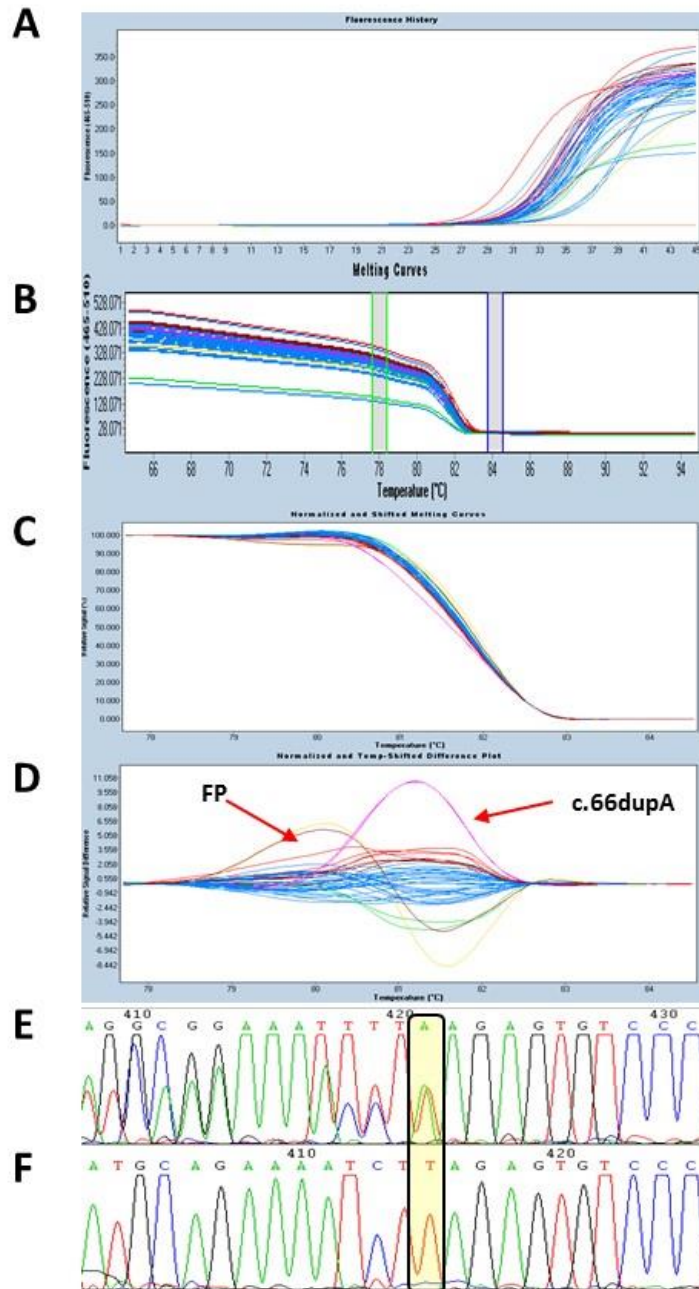


Figure 3.11 HRMA results for *BRCA1* exon 2 for 23 familial SAC patients, of which a single patient carried the *BRCA1* c.66dupA, p.Glu23Argfs mutation and another delivered false positive (FP) results. **A.** The qPCR curve indicating the yellow and brown reactions which failed to plateau. **B.** Positioning of the gliders for the HMRA melt. **C.** Temperature shifted melt curves of 5% correction. **D.** Difference plot indicating the presence of a variant, together with a false positive (brown and yellow lines). **E.** Sequencing electropherogram of the *BRCA1* c.66dupA, p.Glu23Argfs. **F.** Sequencing electropherogram of CAM2814 representing a sample negative for variation within the exon.

Table 3.6 Results presented for *in silico* analysis of all *BRCA1* VUS. Indicated are the variant location and type, the designation of the variant, the effect on the protein, the rs number, the summary of Mutation Taster and its prediction, the PolyPhen-2 score and prediction, SIFT score and prediction, the probability as per MutPred, the Human Splicing finder and the Prosite domain.

<i>BRCA1</i>	Variant Type	Variant	Protein	rs number	MutationTaster Summary	MutationTaster Prediction	PolyPhen-2 Score	PolyPhen-2 Prediction	SIFT score	SIFT Prediction	MutPred Probability of deleterious mutation scoring	Human Splicing Finder	Prosite Domain
Exon 3	Missense	c.110C>A	p.Thr37Lys	rs80356880	Known disease mutation (HGMD CM101974) Protein features might be affected Splice site changes	Disease causing	0.974	Probably Damaging	0	Damaging	0.936	Creation of an exonic ESS site	Zinc finger Ring
Exon 12	Missense	c.4115G>A	p.Cys1372Tyr	rs55848034	Protein features might be affected Splice site changes	Polymorphism	0	Benign	1	Tolerated	0.168	Creation of an exonic ESS site	Na
Intron 15	IVS	c.4675+32A>G	Na	rs762883588	Protein features might be affected Splice site changes	Polymorphism	Na	na	na	Na	na	Na	Na
Exon 18	Missense	c.5141T>G	p.Val1714Gly	rs80357243	Protein features might be affected Splice site changes	Disease causing	0.993	Probably Damaging	0	Damaging	0.683	Activation of an exonic cryptic donor site	BRCT domain
Exon 24	Missense	c.5578C>G	p.His1860Gln	novel	Amino acid changes	Polymorphism	0.023	Benign	0	Deleterious with low confidence	0.141	Alteration of an exonic ESE site	Na
3' UTR	Untranslated Region	c.*36C>G	Na	rs3092995	Splice cite changes	Polymorphism	Na	na	na	Na	na	Na	3'UTR

the ClinVar designation will be used for the purposes of this study.

The mutation has been reported 32 times to the BIC database and was classified as a class 5 deleterious mutation. ENIGMA classification was confirmed by ClinVar as pathogenic. The majority of the cases (43%) reported to the BIC were identified within the Caucasian Dutch, confirming the more than 300-year-old link between the SAC and the Dutch. Noël *et al.* (2010) described a 36-year-old female mutation carrier with invasive ductal carcinoma, which was diagnosed 13 years later at the age of 49 with low-grade adeno-squamous carcinoma, an extremely rare occurrence. The Coloured patient who tested positive for this mutation was affected with bilateral BC at the age of 42 and had two family members affected with the disease. The related family members have not as yet been tested for the presence of this high risk mutation in *BRCA1*.

3.4.4.2 *BRCA1* missense mutations

Nine different missense mutations have been identified, representing various exons throughout the gene. The clinical classification of these mutations varied from benign class 1 (*BRCA1* c.4308T>C, p.Ser1436= in exon 13 and c.4837A>G, p.Ser1613Gly in exon 16) to class 5 disease-causing variants (Table 3.5). The five VUS missense variants were selected for *in silico* mutation prediction software analysis using SIFT, PolyPhen-2, MutationTaster and MutPred online. It was used as described in 3.3.6. The results of the mutation simulations are presented in Table 3.6.

The second class 5 deleterious mutation detected using HRMA was *BRCA1* c.110C>A, p.Thr37Lys (rs80356880, g.41267767G>T). This missense mutation consisted of a single base change (C>A) within the critical RING finger domain (Meza *et al.*, 1999) of the gene, at nucleotide 110 (Appendix J). The base change resulted in the altering of a threonine into a lysine at codon 37 (Table 3.5). The aa substitution changed the peptide properties as the uncharged side chain of the threonine was substituted with an aa with a charged polar side chain.

The variant was initially described by the BIC database as a VUS, although the link to their CIRCOS data indicate it as class 5 (accessed on 18 July 2016). The classification as class 5 was confirmed by both ClinVar (reviewed by expert panel on 10 August 2015) and the *in silico* analysis presented in Table 3.6. This

type of substitution does not always have an effect at the specific location of the aa, but can influence the Van der Waal's radius of the surrounding amino acids and disrupt that specific location's stability and function. This could have played a role, as this mutation was situated in the critical binding area of *BRCA1* and *BARD1*. Confirmation of its class 5 classification is based on the findings of Sweet *et al.* (2010) and Lindor *et al.* (2012). They reported a disruption of the RING finger domain (between aa 24–65 of *BRCA1*) in the presence of this mutant.

The mutation has been reported only once before to the BIC (for an American patient) and was not detected in the 1000 Genomes project (Table 3.5). This rare mutation was detected for a single Coloured BC patient affected at the age of 57, who reported a positive family history of BC. Other family members have not as yet requested testing for the presence of this deleterious mutation in exon 3.

BRCA1 c.4115G>A, p.Cys1372Tyr (rs55848034, g.41243031C>T) was observed in exon 12 at position 4115 (Appendix J). The nucleotide substitution caused an aa change from a cysteine to a tyrosine. The substitution seemed conserved as the functional properties of the variant remained an uncharged polar amino acid although the length of the amino side chain differed. The variant has been reported four times within European and Jewish patients and is indicated by both the BIC and ClinVar as a class 3 VUS (Table 3.5). The allelic frequency for the minor allele is not indicated in the 1000 Genomes project, indicating the variant to be very rare (allele frequency <0.01). *In silico* predictions (Table 3.6) indicated the minor allele to represent a polymorphism, which might alter the exon splice silencer. In this cohort this variant was detected for a single BC patient diagnosed at a very young age (dx 29). The patient has a positive family history for BC with the grandmother's sister and a cousin being affected. Variant segregation in the family has not been examined as yet. Although this variant seems extremely rare, it is tentatively classified as likely benign (class 2) based on the *in silico* prediction results, which indicate that the change might be tolerated.

BRCA1 c.5141T>G, p.Val1714Gly (rs80357243) was reported only once to both the BIC and ClinVar (Appendix J). Due to its low occurrence, both databases have listed it as a VUS (Table 3.5). The variant encodes for a nonpolar aa residue in the *BRCT* binding region of *BRCA1* (at aa 1714). *In silico* analysis provided inconclusive results as two simulators predicted the variant to be damaging

(PolyPhen-2 and SIFT), whereas MutPred and Human Splicing Finder indicated the mutation as benign. PolyPhen-2 and SIFT predicted the variant not to be conserved and to disrupt the protein. The score for MutPred prediction was not high enough to establish a possible effect on the protein. Human Splicing Finder predicted the variant to activate a cryptic donor splice site which forms an extra or new 5' splice site within exon 18 (Table 3.6).

In order to conclude and postulate the classification of this *BRCA1* variant, its location was investigated. The C-terminal *BRCT* region (where the missense mutation is situated) of the protein is essential for the gene's participation in DNA repair, transcriptional regulation and tumour suppressor functions (Miki *et al.*, 1994; Friedman *et al.*, 1994). The domain stretches from aa 1646-1863 and contains two ~ 90-100 aa sequence repeats (Gayther *et al.*, 1996). The two repeats have similar structures and are packed together in a head-to-tail arrangement when interacting with other proteins in response to DNA damage (Williams *et al.*, 2001). Missense mutations such as *BRCA1* c.5141T>G, p.Val1714Gly could occur at the interface between the two repeats and thereby destabilise the structure. The manner by which the two *BRCT* repeats interact in *BRCA1* could regulate the cellular response to DNA damage. Based on these findings, the variant was tentatively classified as a class 4 likely pathogenic mutation until further evidence is available to discard the classification.

A novel missense mutation *BRCA1* c.5578C>G, p.His1860Gln (no rs as yet, g.41197713G>C) has been detected for a single Coloured patient, situated within exon 24 (Table 3.5). , and was present in a heterozygous state. As no information was available for this variant, it was included in the *in silico* analysis. These results proved to be inconclusive as the variant did not affect the function of the *BRCA1* protein (Table 3.6). PolyPhen 2 predicted the variant to be benign, whereas SIFT indicated it to be deleterious due to a charged polar class aa being replaced with an uncharged polar class aa which was not conserved among species. Splice site prediction simulated the alteration of an exon splice enhancer site (Table 3.6).

The BC patient with the variant was diagnosed at the age of 39, but did not have any recollection of a positive family history. Due to the variant's rare

occurrence and according to ENIGMA's guidelines, it is postulated that the variant be classified as a class 4 (likely pathogenic) mutation until a functional, splicing assay or disease segregation can be performed involving the variant.

Only a single missense mutation was located within the 3' UTR of the gene (*BRCA1* c.5628C>G, rs3092995, g.41197659G>C). This variant had been reported seven times before to the BIC, occurring mostly in Austrians and Ashkenazi-American patients (Table 3.5). The variant has been reported at an allelic frequency of 0.0552 in the African populations, and was detected in three BC patients. As the allelic frequency was >0.01, this SNP is classified as a benign class 1 variant.

3.4.4.3 *BRCA1* synonymous mutations

A single base change (G>A) detected in exon 12 at position 4113 resulted in no change of the aa at codon 1371 (Gly). This synonymous *BRCA1* c.4113G>A, p.Gly1371= (rs147448807) mutation (Appendix J) has not been reported before to the BIC, but was reported to ClinVar and included in the 1000 Genomes project (Table 3.5). This mutation has been previously reported for the American and African populations, although at a low frequency (Table 3.5). The variant was detected at an allelic frequency of 0.0053 out of 1322 chromosomes for the African population. As the SNP was detected at a very low frequency of 0.0014 on the East coast of America, it is hypothesised that the SNP could have been introduced into the African American population via slaves being transported out of Africa into America. In this study, the variant was detected in a heterozygous form within only two patients (2 of 206 chromosomes, 0.97%). Both these patients were from Kwa-Zulu Natal, and were affected with BC at ages 38 and 39 respectively. Although this missense mutation has not been detected before for other populations, it seems to represent an African SNP, as it was detected at a frequency of <0.01 in other populations. According to the ENIGMA rules, this variant occurring at a frequency less than 0.01 world-wide has to be classified as a class 2 (likely benign) SNP until proven otherwise.

3.4.4.4 *BRCA1* intervening sequencing variants

Various intervening sequence variants have been detected, ranging from novel detections, to rare changes to common previously detected SNPs. The majority of these variants appeared to be common for the African population and was classified as benign class 1 polymorphisms due to their high allelic frequencies within the 1000 Genome project (Table 3.5).

Only three of these intronic variants will be individually discussed, namely the variant detected in intron 5 (*BRCA1* c.212+23T>A, rs8176128), the change in intron 9 (*BRCA1* c.593+3G>A, rs80358013) and a two bp deletion in intron 12 (*BRCA1* c.4185+12_4185+13delGT, rs273900723, g.41242948_41242949delAC). The variant situated in intron 5 has not been previously reported to the BIC and was only detected once within this study. The variant has an allele frequency of 0.0393 for the African population, and has also been detected at a frequency of 0.0014 in the American populations (1000 Genomes database, Table 3.5). This indicates the variant to be possibly naturally occurring. This variant is therefore classified as a class 1 variant based on its high population frequency, until an mRNA study proves it to be disease-causing.

The intronic variant downstream of exon 9 is rare, as it has been reported only once to the BIC. The variant has been included in the ClinVar database, where it is indicated as possibly benign to likely benign (class 2). In the 1000 Genomes database the variant has been detected at a very low frequency of 0.0008 in the African population, confirming its uniqueness. The variant is located 3 bp from the 3' splice site. Utilising the Human Splicing Finder software, the results predicted the variant to have no effect on the 3' splice site. Based on these and the allelic frequency results available, the variant was tentatively classified as class 2 (likely benign).

The last variant (*BRCA1* c.4185+12_4185+13delGT, rs273900723, g.41242948_41242949delAC) represented the deletion of two bp in intron 12 (Table 3.5). The deletion was detected for two SAC BC patients. The frameshift variant had been reported twice before to the BIC, for a Caucasian patient and an African-American patient. Although the variant is indicated to have no clinical importance in BIC, no clinical classification is specified (accessed on 22 July

2016). The indication on ClinVar is conflicting, ranging from class 1 (benign) to class 2 (likely benign). This variant has not been observed within the 1000 Genomes database, therefore has no population frequency. As it has only been detected four times during >15 years of *BRCA* screening, it can be assumed that it is extremely rare. Using *in silico* prediction with the Human Splice Finder database, the confliction increased as the programme indicated that the variant could alter the intron/exon silencing site, thereby probably disrupting it (Table 3.6). In such a case according to the ENIGMA rules, the variant must remain classified as a class 3 (VUS) or possibly class 4 (likely pathogenic), until an mRNA study proves the variant to be either benign or disease-causing (Table 3.5). Another possibility could be to perform familial segregation studies using these two patients together with their family members. Pathogenicity could then be proven, should the variant segregate with the disease in each of the families.

Both patients carrying the variant were diagnosed at an early age (dx 39 and 38 respectively) and had a positive family history. One of the patients reported multiple cancer types within the family, with more than 10 extended family members affected with breast, colon, stomach and prostate cancer.

3.4.5 *BRCA2* VARIANTS DETECTED USING HRMA

A total of 32 variants were detected for *BRCA2*, with the majority (87.5%) being in a heterozygous state (Table 3.7). Of the 25 exons and associated splice sites screened, only 14 (56%) exons/regions exhibited variants (Fig. 3.12). Of all the variants detected, 13 (40.6%) were identified within an intervening sequence, with 16 (11 missense and 5 synonymous changes) in the coding region (34.4%) (Table 3.7). Only a single frameshift variant was detected, with another variant observed within the 5' UTR region of *BRCA2*.

HRMA revealed the presence of a single pathogenic mutation, 21 benign variants and 10 VUS. Only a single novel variant was detected (Table 3.7). The majority of patients had variants in exons 2, 8, 10, 14, 17 and 22. A quarter (26.6%) of the patients exhibited variants in exons 2 and 8, with another 32% showing variants in exons 10 and 14. The majority of patients (39.4%) contained a variant within exon 22 (Table 3.7).

Table 3.7 *BRCA2* variants identified in the SAC population. Indicated are the exon or region where situated, the designation of the variant, the associated protein change, the rs and g reference number, in how many patients the change has been detected, the variant type, how many times that specific variant has been entered into the BIC, the BIC and ClinVar classification, the minor allele frequency as detected within the 10000 Genome project and the ENIGMA classification. Class 5 mutations are indicated in red, with likely pathogenic (class 4) indicated in orange, variants of unknown significance (class 3) in blue, likely benign (class 2) in green and benign polymorphisms (class 1) in yellow. African allele frequencies indicated with an exponential a represented a single African individual from the Khoisan/Bushmen tribe. The electropherograms used for the variant calling are indicated in Appendix J.

Exon	Variants	Protein	rs Number	G number NC_000013.10	Recurrence (n=103)	Variant Type	BIC Entries	BIC Classification	ClinVar Classification	1000 Genomes Allele Frequencies			Proposed Classification based on all data evaluated
										Allele Frequency For Africans	Highest Allele Frequency of outbred population	AF Scoring	
2	c.-26G>A	na	rs1799943	g.32890572G>A	26	5'UTR	206	Class 1	Benign SCV000244917.1	0.0651	East Asian 0.3601	Global SNP	Class 1
3	c.68-7delT	na	rs276174878	g.32893207delT	2	IVS	4	Class 3	Uncertain Significance SCV000267840.1	na	na	na	Class 3
4	c.317-22C>T	na	rs81002794	g.32899191C>T	5	IVS	1	Class 3	Likely Benign SCV000072151.4	0.5 ^a	na	na	Class 1
	c.425+67A>C	na	rs11571610	g.32899388A>C	7	IVS	35	Class 1	Benign SCV000244962.1	0.0325	Southern Asian 0.1329	Global SNP	Class 1
5	c.426-89T>C	na	rs3783265	g.32900149T>C	2	IVS	36	Class 1	Benign SCV000244974.1	0.0325	Southern Asian 0.1329	Global SNP	Class 1
7	c.517-4C>G	na	rs81002804	g.32900632C>G	10	IVS	2	Class 3	Likely Benign (Conflicting entries) SCV000167325.4	0.5 ^a	na	na	Class 1
8	Het c.681+56C>T Hom c.681+56C>T	na	rs2126042	g.32903685C>T	24 1	IVS	Na	na	Benign SCV000244988.1	0.2746	American 0.2176	Global SNP	Class 1

10	c.865A>C	p.Asn289His	rs766173	g.32906480A>C	4	Missense	48	Class 3	Benign SCV000245001.1	0.0325	Eastern Asian 0.0962	Global SNP	Class 1
	c.891_902delAACAGT TG TAGA	p.Glu297AspfsX3119	novel	g.32906506_32906517	1	Frameshift	Na	na	na	na	Na	na	Class 3
	c.987G>A	p.Arg329=	rs561002197	g.32906602	1	Synonymous	Na	na	Likely Benign SCV000259525.1	na	Southern Asian 0.0010	Asian SNP	Class 3
	Het c.1114A>C Hom c.1114A>C	p.Asn372His	rs144848	g.32906729A>C	21 1	Missense	397	Class 1	Benign SCV000245002.1	0.0840	Southern Asian 0.3538	Global SNP	Class 1
	c.1365A>G	p.Ser455=	rs1801439	g.32906980A>G	5	Synonymous	43	Class 1	Benign SCV000245004.1	0.0325	Southern Asian 0.1329	Global SNP	Class 1
	c.1909+12delT	na	rs276174816	g.32907536delT	1	IVS	2	Class 3	na	na	Na	na	Class 3
12	c.6842-73T>A	na	rs11571673	g.32918622T>A	10	IVS	Na	na	na	0.0061	na	African SNP	Class 2
14	c.7017G>C	p.Lys2339Asn	rs45574331	g.32929007G>C	4	Missense	67	Class 1	Benign SCV000267805.1	0.0257	na	African SNP	Class 1
	c.7242A>G	p.Ser2414=	rs1799955	g.32929232A>G	24	Synonymous	183	Class 1	Benign SCB000245107.1	0.2073	Eastern Asian 0.3689	Global SNP	Class 1
	c.7319A>G	p.His2440Arg	rs4986860	g.32929309A>G	1	Missense	80	Class 1	Benign SCV000245110.1	0.0393	Na	African SNP	Class 1
	c.7435+53C>T	na	rs11147489	g.32929478C>T	1	IVS	38	na	Benign SCV000245110.1	0.0295	Eastern Asian 0.0952	Global SNP	Class 1
17	Hom c.7806-14T>C Het c.7806-14T>C	na	rs9534262	g.32936646T>C	6 13	IVS	329	Class 3	Benign SCV000245136.1	0.4160	Southern Asian 0.5327	Global SNP	Class 1
	c.7976+12G>A	na	rs81002827	g.32936842G>A	1	IVS	1	Class 3	Benign SCV000073375.4	na	na	na	Class 2
18	c.8092G>A	p.Ala2698Thr	rs80359052	g.32937431G>A	1	missense	1	Class 3	Likely Benign (Conflicting entries) SCV000108639.3	0.5 ^A	na	na	Class 1
	c.8010G>A	p.Ser2670=	rs146430937	g.32937349G>A	1	Synonymous	na	na	Likely Benign (Conflicting entries) SCV000253044.2	na	American 0.0014	American SNP	Class 2
	c.8149G>T	p.Ala2717Ser	rs28897747	g.32937488G>T	1	Missense	112	No But pending	Likely Benign (Conflicting entries) SCV000244479.1	Na	American 0.0029	Global SNP	Class1

19	c.8487+19A>G	na	rs11571743	g.32944713A>G	4	IVS	2	Class 1	Benign SCV000267846.1	0.0386	na	African SNP	Class 1
22	Hom c.8755-66T>C Het c.8755-66T>C	na	rs4942486	g.32953388T>C	8 22	IVS	319	Class 1	Benign SCV00024516.1	0.4887	Southern Asian 0.5317	Global SNP	Class 1
	c.8830A>T	p.Ile2944Phe	rs4987047	g.32953529A>T	6	Missense	116	Class 1	Benign SCV000073651.5	0.0325	American 0.0029	African SNP	Class 1
	c.8851G>A	p.Ala2951Thr	rs11571769	g.32953550G>A	1	Missense	43	Class 1	Benign SCV000073651.5	0.0008	American 0.0360	Global SNP	Class 1
27	c.9649-19G>A	na	rs11571830	g.32972280G>A	1	IVS	4	No But pending	Benign SCV000267831.1	na	Eastern Asian 0.0169	Asian SNP	Class 1
	c.9730G>A	p.Val3244Ile	rs11571831	g.32972380G>A	3	Missense	74	Class 1	Benign SCV000267832.1	0.0257	na	African SNP	Class 1
	c.9875C>T	p.Pro3292Leu	rs56121817	g.3297252C>T	2	Missense	7	Class 3	Likely Benign SCV000267833.1	0.0008	na	African SNP	Class 4
	c.10023C>T	p.Asp3341=	rs113507014	g.32972673C>T	2	Synonymous	na	na	Likely Benign SCV000214833.1	0.5 ^a	na	na	Class 2
	c.10234A>G	p.Ile3412Val	rs1801426	g.32972884A>G	6	Missense	114	Class 1	Benign SCV000154054.1	0.1188	Southern Asian 0.0605	Global SNP	Class 1

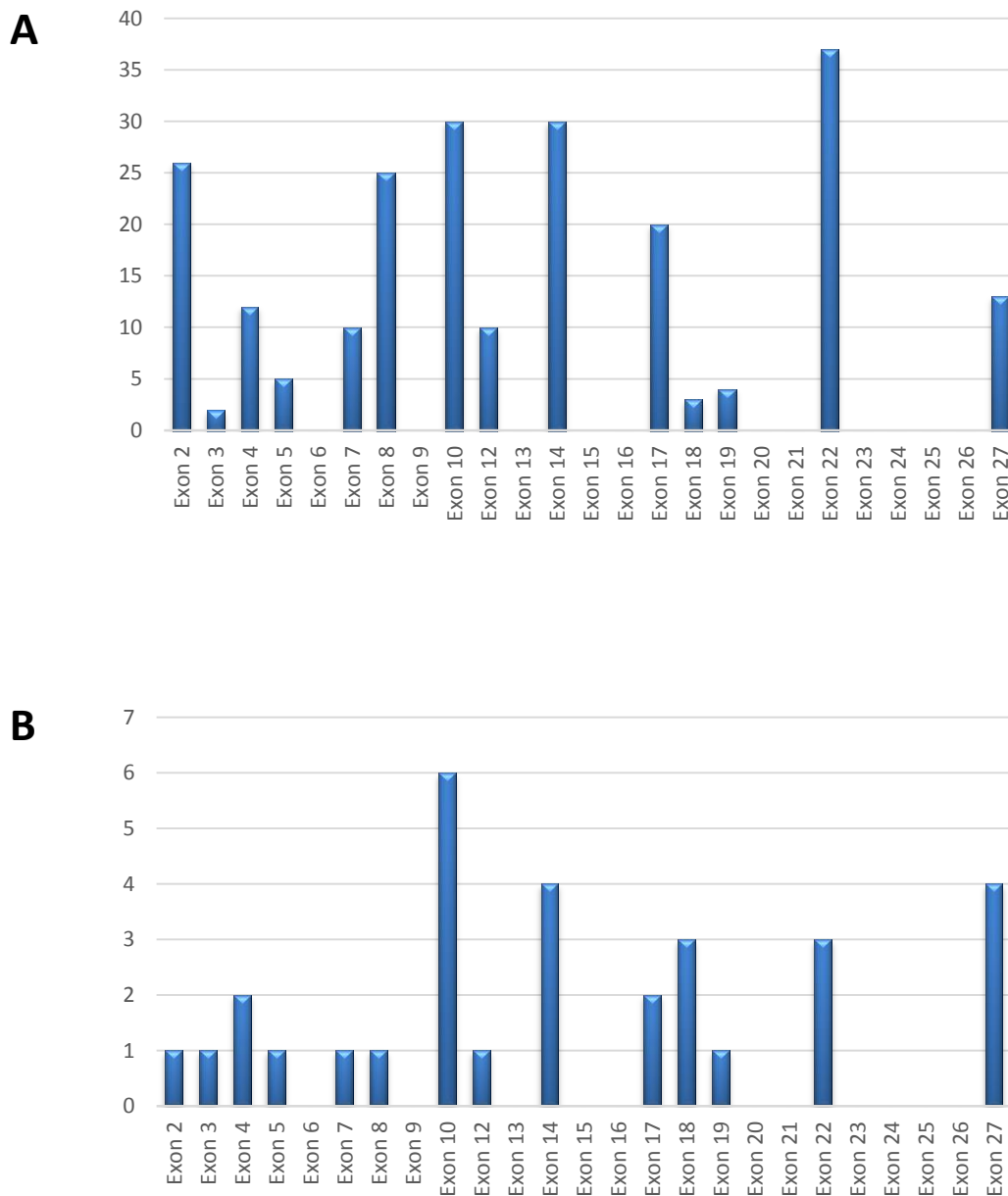


Figure 3.12 Demographics of the *BRCA2* variants identified for 103 BC patients representing the SAC population. **A.** Percentage of the patients that carried variants for each specific exon. **B.** The number of *BRCA2* variants observed per exon.

3.4.5.1 *BRCA2* in frame deletion

A deletion of 12 bp was detected within exon 10 for a single BC patient (CAM2411) (Fig. 3.13). This mutation *BRCA2* c.891_902delAACAGTTGTAGA, p.Glu297AspfsX3119 (no rs number as yet, g.32906506_32906517delAACAGTTGTAGA) was novel as it has not been previously reported before to any database. The deletion occurred in frame at nucleotide position 891-902 and did not result in the creation of a premature truncation, but the deletion of four aa at codon 298-301. It is postulated that this newly formed peptide will only be four residues shorter than the normal *BRCA2* protein. As the 12 bp deletion was novel, but did not result in the early termination of the protein, it was declared tentatively a VUS. The location of the four deleted aa did not fall within a critical known functional domain of the *BRCA2* protein [as tested by Prosite and Ensemble (www.ensembl.org/index.html)], and therefore might not have a dramatic effect. As the mutation was novel and extremely rare, it is postulated that this mutation represents a VUS. Additional information on the structural conformation is needed in order to classify its clinical significance.

A similar class 5 pathogenic deletion at exactly the same position has been observed and reported to the BIC four times before. This mutation involves the deletion of 9 bp instead of the 12 bp (c.891_902delAACAGTTGT), but has an insertion of 10 different bp afterwards. This mutation has been detected for BC patients from Western Europe. The SA mutation was detected in a single SAC BC patient (dx 38), who has a family history of cancer types associated with mutations in *BRCA2*.

3.4.5.2 *BRCA2* missense mutations

Various missense mutations had been observed (Table 3.7). Of these, only a few will be discussed individually as the majority represented normal benign class 1 polymorphisms confirmed by either both BC databases or ClinVar only. These were present throughout the gene, with four situated in exon 10.

Various missense mutations were detected that acted as benign polymorphisms within the populations representing the African continent. These include *BRCA2* c.7017G>C, p. Lys2339Asn (rs45574331), *BRCA2* c.7319A>G,

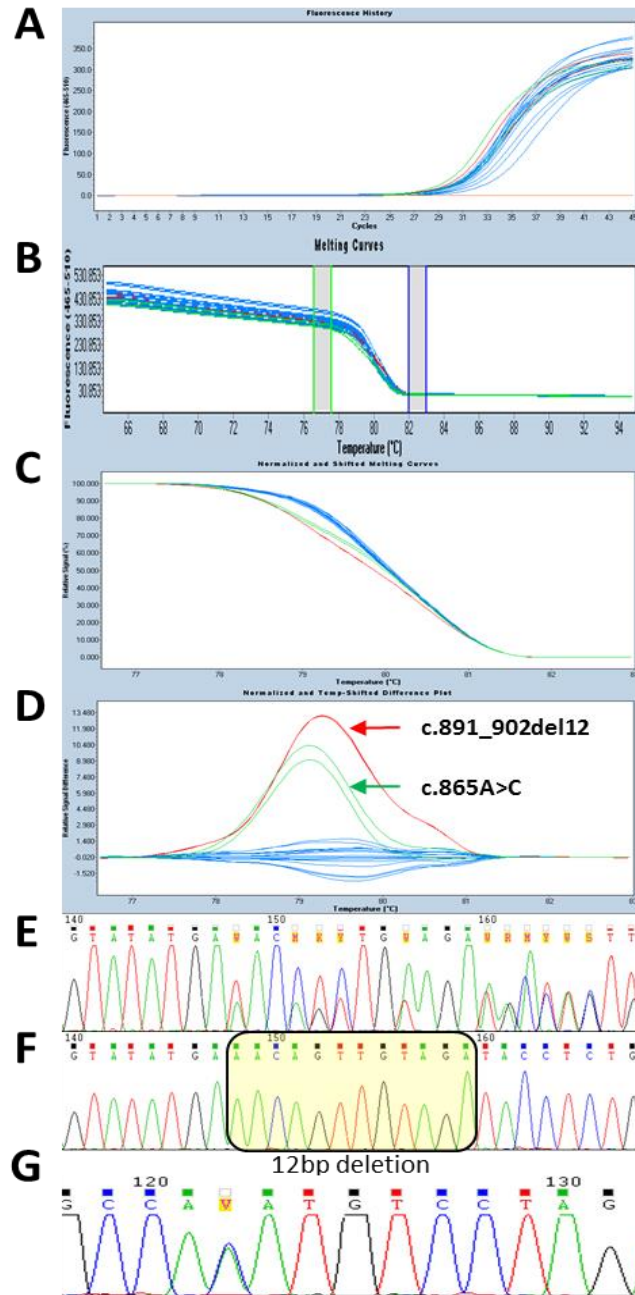


Figure 3.13 HRMA results for *BRCA2* exon 10 for 23 BC patients (performed in duplicate) with a single patient (CAM2411) carrying the *BRCA2* c.891_902del AACAGTTGTAGA, p.Glu297AspfsX3119 mutation and another presenting with a missense variant c.865A>C, p.Asn289His. **A.** Results for the qPCR curve. **B.** Positioning of the gliders for the HRMA melt. **C.** Temperature shifted melt curves with a 5% correction. **D.** Difference plot indicating the presence of the two variants (indicated in red and green arrows). **E.** Sequencing electropherogram for CAM2411, indicating a shift in the reading frame. **F.** Sequencing electropherogram of CAM2818, negative for the 12 bp deletion. **G.** Sequencing electropherogram for *BRCA2* c.865A>C, p.Asn289His present in CAM2023.

p.His2440Arg (rs4986860), *BRCA2* c.8830A>T, p.Ile2944Phe (rs4987047) and *BRCA2* c.9730G>A, p.Val3244Ile (rs11571831). Some of the other variants detected such as *BRCA2* c.865A>C, p.Asn289His (rs766173), *BRCA2* c.1114A>C, p.Asn372His (rs144848), *BRCA2* c.8149G>T, p.Ala2717Ser (rs28897747), *BRCA2* c.8851G>A, p.Ala2951Thr (rs11571769) and *BRCA2* c.10234A>G, p.Ile3412Val (rs1801426) indicated global variation as they have been described for multiple countries throughout the world, some also in a homozygous state (Table 3.7). As the BIC or ClinVar databases did not clarify the clinical importance of all the variants, some were selected for *in silico* mutation prediction using software analysis such as SIFT, PolyPhen-2, MutationTaster and MutPred online. The results of the mutation simulations are presented in Table 3.8.

A change (G>A) occurred at nucleotide 8092 (Appendix J) in exon 18 (*BRCA2* c.8092G>A, p.Ala2698Thr – rs80359052) and resulted in an aa change from an alanine to a threonine at residue 2698. The substitution replaced a nonpolar residue with an uncharged polar residue. This change has been reported once for an African individual to the BIC and was indicated as a VUS (Class 3). ClinVar indicated various reports, predicting it to be benign to likely benign. The allelic frequency for the variant as indicated by the 1000 Genomes project (indicated as a high 0.5) is actually a misrepresentation, as it was based on the variant being present in a single heterozygous individual from Africa representing the Khoisan/Bushman tribe (Table 3.7). The variant was furthermore only detected in one patient within the SAC population. Results of the *in silico* analysis (as presented in Table 3.8) conclusively indicated that the variant does not affect *BRCA2* protein function. Both PolyPhen-2 and SIFT predicted the variant to be conserved and tolerated. Splice site prediction by the Human Splicing Finder showed that the variant might have the possibility to alter an exonic splicing enhancer site. Therefore, according to the ENIGMA rules the variant is postulated to represent a class 1 benign natural occurring variant in African Khoisan/Bushman patients.

Another missense mutation was observed within exon 27, namely *BRCA2* c.9875G>A, p.Pro3292Leu (rs56121817) (Table 3.7). The nucleotide substitution resulted in a change of a proline to a leucine at residue 3292. This change semi-conserved the nonpolar side chains of the amino acid residues.

Table 3.8 Results presented for *in silico* analysis of all *BRCA2* VUS. Indicated are the variant location and type, the designation of the variant, the effect on the protein, the rs number, the summary of Mutation Taster and its prediction, the PolyPhen-2 score and prediction, SIFT score and prediction, the probability as per MutPred, the Human Splicing Finder and the Prosite domain.

BRCA2	Variant Type	Variants	Protein	rs number	MutationTaster Summary	MutationTaster Prediction	PolyPhen-2 score	PolyPhen-2 Prediction	SIFT score	SIFT Prediction	MutPred Probability of deleterious mutation scoring	Human Splicing Finder
Intron 2	IVS	c.68-7delT	na	rs276174878	Known disease mutation	Disease causing	na	Na	Na	na	na	Activation of an intronic cryptic acceptor site Alteration of the WT acceptor site
Exon 10	Synonymous	c.987G>A	p.Arg329=	rs561002197	Protein features might be affected Splice site changes	Disease causing	na	Na	Na	na	na	Creation of an exonic ESS site Alteration of an exonic ESE site
Intron 10	IVS	c.1909+12delT	Na	Rs276174816	Protein features might be affected Splice site changes	Poly-morphism	na	Na	Na	na	na	No effect
Intron 11	IVS	c.6842-73T>A	Na	Rs11571673	Protein features might be affected Splice site changes	Poly-morphism	na	Na	Na	na	na	Alteration of an intronic ESS site Creation of an intronic ESE site
Intron 17	IVS	c.7976+12G>A	na	rs81002827	Protein features might be affected Splice site changes	Poly-morphism	na	Na	Na	na	na	Alteration of an intronic ESS site Creation of an intronic ESE site
Exon 18	Missense	c.8092G>A	p.Ala2698Thr	rs80359052	Protein features might be affected Splice site changes	Poly-morphism	0.003	Benign	1	Tolerated	0.295	Alteration of an exonic ESE site
Exon 27	Missense	c.9875C>T	pPro3292Leu	rs56121817	Protein features might be affected Splice site changes	Disease causing	0.971	Probably Damaging	0.04	Deleterious	0.317	Creation of an exonic ESS site Alteration of an exonic ESE site

Although the variant has been reported seven times to the BIC for Western European individuals, it was indicated as a VUS. ClinVar classified the variant as benign to likely benign (Table 3.7). The allelic frequency for the variant in the 1000 Genomes project is indicated as 0.0008 for the African population, which is very rare. The variant was detected in two patients within the SAC population. *In silico* analysis was not conclusive. Among the protein function prediction software, the variant was predicted to be probably deleterious (class 4) or disease-causing (class 5). Splice site prediction by the Human Splicing Finder predicted the variant to alter an exonic splicing enhancer site and to create an exonic splice site. As no conclusive results were obtained for this variant regarding its clinical significance, a literature search was performed.

An article by Tram *et al.* (2013) assisted with the classification of this variant. They investigated various VUS missense mutations within the two genes regarding their ability to influence each of the gene's phosphorylation patterns. This included *BRCA2* c.9875G>A, p.Pro3292Leu. This variant was included in their search as *BRCA2* codon 3291 (Ser), the aa immediately upstream, was the best-characterised phosphorylation site for the *BRCA2* protein. This site was located at the carboxy-terminal region of the protein, which interacts with *RAD51* (Liu *et al.*, 2010). Research has proven that phosphorylation of the Ser at codon 3291 blocks the interaction of *BRCA2* with *RAD51* as a way to regulate the recombination activity (Au & Henderson, 2007). This phosphorylated residue at codon 3291 is surrounded by a kinase interaction or recognition motif that consists of 7-12 aa (Songyang *et al.*, 1994). Phosphorylation of the Ser at codon 3291 is therefore dependent on the surrounding sequence of the peptide that constitutes the kinase recognition motif in order for the *RAD51* protein to bind.

The aa at 3292 (Pro), next to the critical Ser, is therefore a very conserved residue. The presence of the missense mutation *BRCA2* c.9875G>A at nucleotide 9875 results in the changing of this conserved aa from a Pro to a Leu. According to Tram *et al.* (2013), this change leads to the abolishment of the kinase binding site at codon 3291 (Ser), thereby preventing the interaction between *BRCA2* and *RAD51*. This presented evidence that *BRCA2* c.9875G>A, p.Pro3292Leu is of high clinical significance, for it could impact on BC by negatively affecting the interaction between the two proteins.

Based on this evidence, this variant was classified as likely pathogenic (class 4, Table 3.7). Three other SA BC cases, representing other ethnicities, have been identified with this mutation. Each of these patients has an extensive family history of BC and other cancer types. An attempt will be made to perform segregation analysis within these families in order to determine whether the mutation segregates with the disease. With positive segregation results, it will be possible to classify this mutation as a class 5 disease-causing mutation.

3.4.5.3 *BRCA2* synonymous mutations

A single base change in exon 10 (*BRCA2* c.987G>A, p.Arg329=, rs561002197) represented a synonymous mutation at codon 987 as the nucleotide substitution did not affect the aa. ClinVar classified the variant as likely benign (Table 3.7). The variant was also observed in the 1000 Genomes project, although it was at a very low frequency for the South Asian population (frequency of 0.0010). In this cohort the variant was detected for a single individual. The Human Splice Site prediction software predicted the alteration of an exon splicing enhancer site and the possible creation of an exon splice site. As the variant occurred at a frequency less than 0.01, functional analysis is needed in order to confirm the variant to be disease-causing. For now, this variant was classified as potentially class 2.

Another synonymous variant (*BRCA2* c.8010G>A, p.Ser2670=, rs146430937) was detected in exon 18 (G>A). Although the variant has not been reported to the BIC, ClinVar classified the variant as likely benign and it was included in the 1000 Genomes project, previously reported for the American population gene pool at a frequency of 0.0014 (Table 3.7). According to ENIGMA, a synonymous variant occurring at a frequency less than 0.01 should be classified as a class 2 (likely benign) until it has been proven as disease-causing at mRNA level. This variant was only detected once within the cohort of 103 BC patients.

The last synonymous mutation was detected in exon 27, namely *BRCA2* c.10023C>T, p.Asp3341= (rs113507014). ClinVar classified the variant as likely benign and this variant was reported by the 1000 Genomes database at a frequency of 0.5 for an individual representing the Khoisan/Bushmen tribe of Africa. This could have resulted in the over-estimation of its frequency, therefore an incorrect classification as being common, when in fact the variant is rare. The

variant was detected twice within this study. It is therefore speculated that this variant might represent a natural polymorphism of African-Bushmen origin.

3.4.5.4 *BRCA2* intervening sequencing variants

Various intronic variants have been detected within the SAC population (Table 3.7), of which the majority was benign class 1 polymorphisms representing either the global or the African gene pool specifically. Some of these variants however, needed additional analyses to finalise their ENIGMA classification.

Two patients (CAM1254 and CAM1506) were identified who carried the *BRCA2* c.68-7delT (rs276174878, g.32893207delT) intronic variant (Appendix J). This variant has been reported to the BIC and ClinVar before and was indicated as a VUS (Table 3.7). It was absent in the 1000 Genomes project. *In silico* analysis indicated a potential pathogenic role for this variant (Table 3.8).

In order to resolve its clinical significance, a literature search was performed. Three research papers were obtained that studied the effect of aberrant splicing of exon 3 due to the presence of this and other variants (such as *BRCA2* c.68-7T>A, rs81002830) within a homo-thymine repeat at the same location. The authors performed functional assays by making use of splicing reporter hybrid minigene assays (Sanz *et al.*, 2010; Muller *et al.*, 2011; Théry *et al.*, 2011). By using this, they focused on the gene region carrying the variation and compared RNA splicing effects between the wild type and the variant under identical conditions (Sanz *et al.*, 2010). With these assays, they managed to obtain direct proof regarding the involvement of the variants at position c.68-7. Both intronic variants *BRCA2* c.68-7delT (rs276174878) and *BRCA2* c.68-7T>A (rs81002830) exhibited increased levels of the delta3-transcript, thereby enhancing the exclusion of exon 3 (Muller *et al.*, 2011; Théry *et al.*, 2011). The authors proposed that due to the allelic imbalance of the delta3-transcript, segregation studies are needed together with the evaluation of these variants' frequency in a control population to determine their precise effect. With more conclusive data, the classification of *BRCA2* c.68-7delT (rs276174878) could possible change from a VUS class 3 to a class 4 or 5.

Two intronic variants, the first in intron 3 (*BRCA2* c.317-22C>T, rs81002794) and the second in intron 6 (*BRCA2* c.517-4C>G, rs81002804) (Table 3.7) were detected in a heterozygous state. These variants were detected five and ten times respectively. Both these variants were reported previously to the BIC database (for an African patient) and the 1000 Genomes project (for the individual representing the Khoisan/Bushmen tribe). Only the c.317-22C>T variant effect could be predicted using the Human Splicing Finder software (Table 3.8). The results indicated that the variant could possibly create an intronic splicing enhancer site. Computer-based analysis indicated the second variant as benign, as the single base change four bp from the start of exon 7 was semi-conservative and seems to be tolerated (Human Splicing Finder). As these variants were restricted to the African population, it is proposed that they are naturally occurring variants within the Khoisan/Bushmen of Africa. Based on the evidence, they are both classified as class 1 benign variants.

The intronic variant detected in intron 10 (*BRCA2* c.1909+12delT, rs276174816) has been reported twice to the BIC as a VUS for German and Spanish individuals, but was totally absent from the 1000 Genomes project (Table 3.7). No additional analysis could be performed for this variant, as there was a conflict in the reference number and the variant register in the SNP database. This variant was only detected once in this study, and due to the lack of information, remains classified as a VUS class 3 (Appendix J).

Very little is known about a heterozygous variant present in intron 17 (*BRCA2* c.7976+12G>A). This variant has been reported twice to the BIC for an African individual. Clinvar reported the variant benign, but it was not detected in the 1000 Genomes project. This rare variant was only detected once within the SAC population, and is therefore very rare. The Human Splice Site prediction software predicted the alteration of an exon splicing site and the creation of an exon splice enhancer site (Table 3.8). Based on these observations the variant is tentatively classified as a class 2 polymorphism until more information can be obtained.

The last intronic variant to be discussed represents a relative common variant detected in intron 10. This single base change has been identified for 10 SAC BC patients. The variant is not included in the BIC database, although it has an allelic frequency of 0.0061 in African populations and 0.5 in the

Khoisan/Bushman individual screened in the 1000 Genomes project. As the allelic frequency was still relatively low indicating it to be a scarce variant, the variant was tentatively classified as a class 2 polymorphism.

3.5 CONCLUSIONS

A total cohort of 229 BC patients representing the SAC population was selected to be included in the study based on various selection criteria. These patients were screened for the presence of smaller genomic changes present within the coding and adjacent intronic areas of the familial BC genes *BRCA1* and *BRCA2*. A combination of mutation screening techniques was used, which included real-time (qPCR) based genotyping for known population specific mutations, PTT (radioactive screening of the largest genomic regions within the genes), DNA Sanger sequencing, SSCP/HA (to identify the precise location of the mutation detected by PTT) and HRMA.

A total of 50 variants were identified, ranging from single base changes situated within intronic regions to a 12 bp deletion within the coding region of *BRCA2* exon 10 (Fig. 3.14). The majority of the variants proved to be benign class 1 polymorphisms, exhibiting normal variation present within this unique population group. The benign variants detected were the result of 300 years of admixture between the Bantu-speaking Black African populations such as the Khoi- and San-speaking tribes of the North Western Cape province, the European settlers and the slaves from the East. The portfolio of variants reflected each of these different ethnicities (global, Eastern and African polymorphisms), combining all into a unique subset of genetic diversity.

Numerous pathogenic mutations were identified, ranging from class 4 to class 5. Many of these mutations proved to be restricted to the southern tip of SA, as it included the Afrikaner-specific founder mutations, the Xhosa/Coloured recurrent mutation and a few newly discovered Coloured specific mutations. Based on these results, recommendations can be made regarding the composition of targeted mutation panels for the diagnostic testing of these SAC BC patients and their families. It is interesting to note that to date, very few of the family members of these mutation-positive patients have requested pre-symptomatic

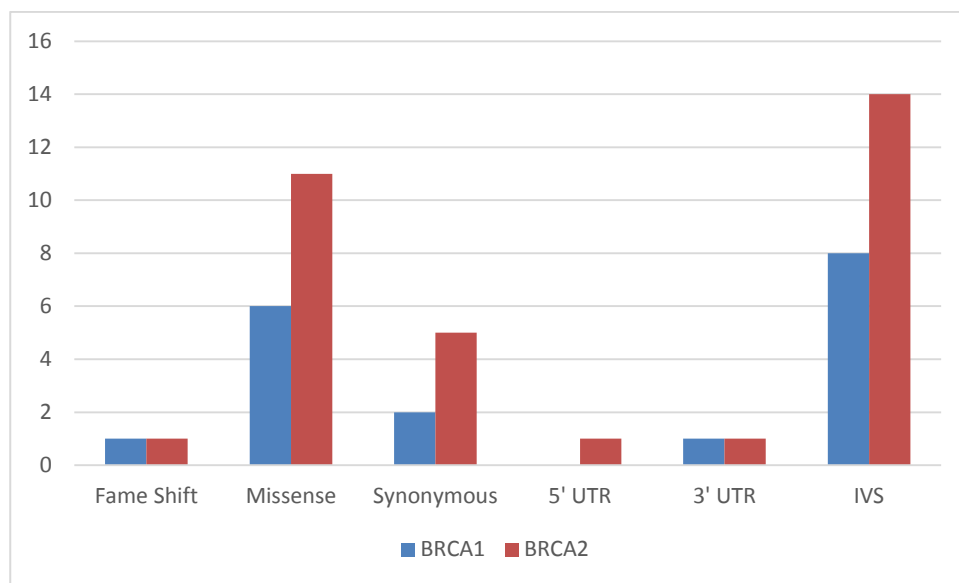


Figure 3.14. Number and types of variants determined for *BRCA1* and *BRCA2* for 103 BC patients representing the SAC population.

testing. Although both institutions have genetic counsellors on their operational platform explaining the implications of genetic testing to these families, the demand for testing remains low. It is therefore hypothesised that the uptake of genetic testing for familial BC in the SAC population is currently similar to that of the Black population (Moeti *et al.*, 2014). This might be due to various factors ranging from the inability to fully comprehend the information and benefits, a possibly low social and financial class, to religious convictions regarding human interference. Results such as these should be incorporated onto the diagnostic platforms in order to promote health care in SA and specifically to benefit this unique and enriching population group residing almost exclusively at the southernmost tip of the African continent.

CHAPTER 4

CONCLUSIONS

South Africa has been given the nickname “the rainbow nation” due to its mixture of genetically unique population groups. This uniqueness complicates genetic testing at a pathology level, as screening for deleterious mutations within the large familial BC genes can be an intricate task. With the optimisation of HRMA as a higher throughput mutation screening technique, the physical laboratory work will progress quicker. However, a bottleneck is expected to form, as classification of their clinical significance and interpretation of all the different variants is a time-consuming process.

This study attempted to investigate the landscape of genetic variants present within the familial BC genes *BRCA1* and *BRCA2* within the highly admixed SAC population. This attempt successfully identified 58 different variants (all inclusive). The comprehensive screen unravelled the mystery of BC development within the cohort to some extent as 14.4% of the patients tested positive for a deleterious high-risk mutation. The variants detected clearly reflect the various countries of origin of the SAC population, as a combination of typical southern African, southern Asian and European variants were observed (Quintana-Murci *et al.*, 2010).

Although the optimisation and implementation of HRMA reduced the turnaround times for performing a comprehensive screen, certain limitations within the screening process remained. One of these included the fact that the depth of mutation detection was not increased, as HRMA only detected smaller indels and SNP's and not larger rearrangements; these require to be screened additionally using multiplex ligation-dependent probe amplification (MLPA). Another limitation is based on the fact that PTT was used to screen the largest exons, which only identified mutations that resulted in the creation of a premature truncation; therefore frameshift and nonsense mutations. This technique does not have the ability to detect missense mutations, and therefore various variants present in *BRCA1* and *BRCA2* exons 11 might have been missed. Fortunately, the majority

of the missense variants detected within these regions are benign polymorphisms. According to the data reported for the BIC (accessed 29 July 2016), the total number of entries representing variants in *BRCA1* exon 11 was 6382. This number of entries represented 903 mutations described for this region. A small percentage (7.5%) of these was reported only once for a specific patient. The data for *BRCA2* looked similar, as a total of 6182 entries had been reported for exon 11, representing a total of 846 mutations. A small percentage (7.3%) of these was reported only once for a specific patient. The majority of these entries represented hundreds of missense mutations. The lists presented by the BIC were searched in an attempt to determine the percentage of deleterious missense mutations that this study could have missed by performing PTT for exon 11 instead of full sequencing for the region. No deleterious missense mutation could be found for either gene. What seemed therefore as a limitation can in fact now be interpreted as an advantage, as numerous missense mutations might have been detected for which currently no information exists regarding clinical classification. All the missense mutations listed for exon 11 of each gene are currently, with the exception of those already classified as benign class 1 variants, indicated as “clinical significance unknown”.

Another limitation of the study might reside in the fact that the cohort was not screened for the presence of larger duplications or deletions using MLPA. A parallel study under the umbrella project within the Division of Human Genetics in Bloemfontein focused on screening three SA population groups (including the SAC population) for the presence of these larger rearrangements. A total of 129 BC patients who tested negative for pathogenic *BRCA* mutations were screened using MLPA. The study included 29 Coloured patients. None were detected. The study of PJ Moeti concluded that this type of mutation did not contribute to the familial BC risk within the various SA population groups, therefore neither to the SAC population. Based on these results, it is speculated that not performing MLPA for the entire cohort would result only in a very low percentage (if any) of deleterious mutations that might have been missed.

In an attempt to improve the turnaround time and comprehensiveness of screening for familial BC, it would be ideal to advance to next generation sequencing (NGS). This new technology is able to combine HRMA, SSCP/HA, PTT and DNA sequencing all into one technique. Not only will it allow the

laboratory to screen for mutations within the high risk *BRCA* genes, it will also simplify the search for mutations present in other moderate familial BC genes such as *PALB2*. All these genes could then be screened and analysed together with even higher and greater depth of sensitivity within a single run. Screening these high to moderate BC genes may result in a higher percentage of familial BC patients with an inherited susceptibility being positively diagnosed, with immediate benefit to the extended related family.

CHAPTER 5

REFERENCES

5.1 REFERENCES

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5.2 ELECTRONIC RESOURCES

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CHAPTER 6

APPENDIXES

Appendix A

**Guidelines from the SASHG Committee for publication purposes regarding:
Nomenclature for South African populations**

Prepared by Lisa Roberts, Jacque Greenberg and Michele Ramsay, with input from Himla Soodyall and the current SASHG committee - June 2013

When publishing genetic or biomedical research studies involving patients, controls or population groups in South Africa, it is often relevant and necessary to identify the groups by their ethnic affiliations. The categories are usually used as proxies for genetic ancestry, but the ways in which individuals “self-identify” are influenced by many factors, including geographic origin, language and culture. As genetic and biomedical researchers, we take cognisance of the fact that these are labels of convenience that do not necessarily accurately reflect ancestry, nor do they define homogenous groups.

We recognise that preferred identifiers for South African ethnic groups vary between individuals, groups, nations and organisations. In citing ethnic labels, we therefore recommend the use of the names or categories that the people call themselves. Today, South Africans broadly consider themselves White, Black (black African), Coloured or Indian. Importantly too, these are also the categories used in the South African population census (also at www.statssa.gov).

Due to the international opinion that “Coloured” is a derogatory term, it is necessary to clarify that in the South African context, populations who self-identify as Coloured have a complex history of ancestrally derived admixture. These groups, also frequently termed “Mixed Ancestry” are ancestrally derived from admixtures of one or more of the indigenous African populations (Khoe- and San-speaking or Bantu-speaking), immigrants from Western Europe, or slave labourers from West Africa, Indonesia, Madagascar, Java, India and Malaysia. The term “Coloured” in South Africa is therefore a name that encompasses a wide range of people who are unique to this country.

As a guide, the SASHG committee therefore recommends, for publication purposes for genetic and biomedical studies, the use of the following nomenclature for South African populations: White, Black African, Coloured and Indian. This terminology should be accompanied by a brief description of the geographic origin and inclusion criteria of the participants of the specific research study.

If necessary, depending on the journal and/or the reviewers’ comments, it is acceptable to substitute the term “Mixed Ancestry” for “Coloured”, accompanied by a description of the cohort(s) under study.

Black African communities in South Africa also identify themselves by language: the Zulu people or amaZulu (who speak isiZulu), Xhosa or amaXhosa (isiXhosa), Sotho (seSotho), etc. and these affiliations should be used if the data are available and relevant to the study. Similarly, since San hunter-gatherers and Khoe pastoralists identify themselves with their community names (e.g. !Xun, †Khomani, //Ganaa, Hai//om, etc.) these affiliations should be used when available and relevant, however if they are grouped together the term Khoe-San is recommended.

We acknowledge that in the future there may no longer be a need for such “labels” but at present, particularly for genetic research purposes, this information is pertinent and relevant.

Appendix B


SCHEME FOR AUTOSOMAL DOMINANT AND X-LINKED MENDELIAN DISEASES

CLASS	AMERY CLASSIFICATION	CATEGORY	CRITERIA	Exceptions (New Baseline Class)
3	Pathogenic Mutation	A 1 needed	Confirmed de novo alteration in the setting of a new disease (appropriate phenotype) in the family Alterations resulting in premature truncation (e.g. reading frame shift, nonsense) Other ACMG-defined mutation (i.e. initiation codon or gross deletion) Strong segregation with disease (LOD >3 + >10 meioses) Functionally validated splicing mutation Significant disease association in appropriately sized case-control study(ies) Detected in individual satisfying established diagnostic criteria for classic disease without a clear mutation	Confirmed de novo alteration in a novel gene with possible disease implications (A) Likely de novo alteration (i.e. assembly not confirmed) with known disease association (A) Confirmed de novo alteration in the setting of a discordant phenotype (B) Truncation in close proximity to 5' terminal 5246 gene specific LOD has not been established in mechanism of pathogenicity (e.g. MPOV) (B) In-frame gross deletion of a single exon not in a known protein functional domain (A), initiation codon that is not well conserved (A) In-frame skipping a single exon not in a known protein functional domain (A)
		B 4 needed	Start nucleotide of exon Good segregation with disease (LOD 1.5-3 + 5-9 meioses) Deficient protein function in appropriate functional assay(s) Well-characterized mutation at same position Other strong data supporting pathogenic classification	
4	Variant, Likely Pathogenic	3 needed	Alterations at the terminal three/nucleolar sites (1-3, 2) without other strong (B-level) evidence supporting pathogenicity Rarely in general population databases (dbSNP, ESP, 1000 Genomes)	Dependent on disease penetrance and inheritance pattern.
		C 4 needed	In silico models in agreement (deleterious) and/or completely conserved position in appropriate species Moderate segregation with disease (at least 3 informative meioses) for rare diseases. Other data supporting pathogenic classification	In-silico splicing predictions not used as independent line of evidence for last nucleotide of exon.
3	VUS		3 of B 2 of B and at least 1 of C 1 of B and at least 2 of C	
	Variant, Likely Benign	D 1 needed	Insufficient or conflicting evidence Gross Duplications without Strong Evidence for Pathogenic or Benign Least protein function observed in appropriate functional assay(s) Isogenic alteration with no splicing impact by RT-PCR analysis or other splicing assay Other strong data supporting benign classification	
		E 2 needed	Co-occurrence with mutation in same gene (disease unknown) Co-occurrence with a mutation in another gene that clearly explains a proband's phenotype Subpopulation frequency in support of benign classification (in silico models in agreement (benign)) Does not segregate with disease in family study (genes with incomplete penetrance) No disease association in small case-control study Other data supporting benign classification	Genes without a defined, severe allelic phenotype (B)
1	Benign	F 1 needed	General population or subpopulation frequency is too high to be a pathogenic mutation based on disease/phenotype prevalence and penetrance Does not segregate with disease in family study (genes with complete penetrance) Internal frequency is too high to be a pathogenic mutation based on disease/phenotype prevalence and penetrance Seen in homo with a mutation or in homozygous state in individual without severe disease for that gene No disease association in appropriately sized case-control study(ies) 1 of D and at least 2 of E 2 or more of D 3 of E with conflicting data	Genes without a defined, severe allelic phenotype (B)

The variant classification scheme is not intended for the interpretation of alterations considered epigenetic, factors including genetic modifiers, multifactorial disease, or low-risk disease association alleles and may be limited in the interpretation of alterations conformed by incomplete penetrance, variable expressivity, phenocopies, trisomic or oligogenic inheritance, or skewed X-inactivation.

Appendix C

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[REDACTED]

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2014-09-22

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DR N VAN DER MERWE
DIVISION OF HUMAN GENETICS
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UFS


Dear Dr van der Merwe

ECUFS NR 108/2014
MR J OOSTHUIZEN

DIVISION OF HUMAN GENETICS
PROJECT TITLE: MOLECULAR SCREENING OF SOUTH AFRICAN COLOURED BREAST
CANCER PATIENTS FOR THE PRESENCE OF BRCA MUTATIONS USING HIGH RESOLUTION
MELTING ANALYSIS

1. You are hereby kindly informed that at the meeting on 16 September 2014 the Ethics Committee approved the above project after all conditions have been met when the following was submitted:
 - **Signed permission letter from the Tygerberg Hospital**
2. You are hereby kindly informed that the Ethics Committee is concerned about this study being the same as the study with the project title: *"Molecular screening of the South African Indian population for BRCA1 and BRCA2 using High resolution melting analysis"*. Kindly note that the studies may be the same but the write up for the M.Med studies should not be the same word for word.
3. 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.
4. Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.
5. The Committee must be informed of any serious adverse event and/or termination of the study.
6. 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).

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Appendix D



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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.

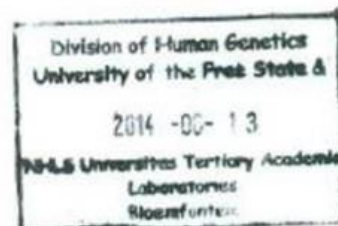
Yours sincerely

Dr NC vd Merwe PhD
Principal investigator

E-mail: gnmgncy.MD@mail.uovs.ac.za

On behalf of: Pakiso Moeti
HMVE Combrink
J Oosthuizen

Approved:



Appendix E

CONSENT FOR ADDITIONAL DNA STUDIES

1. I, _____, request that an attempt be made using genetic material to assess the probability that: I (name: _____) might have inherited disease-causing mutation in the gene for: **FAMILIAL BREAST CANCER.**
2. I understand that the genetic material for analysis is to be obtained from: blood cells.
3. I acknowledge that a portion of the sample will be stored indefinitely for later use for:
 - (a) possible re-analysis for the same disorder
 - (b) 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.
5. I authorise my doctor(s) to provide relevant clinical details in order for the research to be published.
6. I have been informed that:
 - (a) I will not receive any remuneration for my involvement in this study.
 - (b) I will not endure additional financial costs due to the tests being performed.
 - (c) The risks and benefits associated with genetic analysis 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) 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 _____
of person giving consent

Date: _____
Capacity _____

Patient signature: _____

Witnessed consent: _____

Appendix F



Tygerberg Hospital

REFERENCE: Research Projects
ENQUIRIES: Dr G G Marinus
TELEPHONE: 021 938-6267

ETHICS NO: ECUF 108/2014

Molecular screening of South African coloured breast cancer patients for the presence of BRCA mutations using high resolution melting analysis

Dear Van der Merwe

PERMISSION TO CONDUCT YOUR RESEARCH AT TYGERBERG HOSPITAL

In accordance with the Provincial Research Policy and Tygerberg Hospital Notice No 40/2009, permission is hereby granted for you to conduct the above-mentioned research here at Tygerberg Hospital.

A handwritten signature in black ink, appearing to be 'D Erasmus', written over a circular stamp or mark.

**DR D ERASMUS
CHIEF EXECUTIVE OFFICER**

Date: 22 August 2014

Appendix G



health

Department:
Health
PROVINCE OF KWAZULU-NATAL

INKOSI ALBERT LUTHULI CENTRAL HOSPITAL
Ethekewini Health District
Office of the Chief Executive Officer
800 Vusi Mzimela (Bellair) Road, Mayville, 4091
Private Bag X03, Mayville, 4058
Tel: 031 - 240 1034
Fax: 031 - 240 1005
Email: Gugu.Duma@ialch.co.za
www.kznhealth.gov.za

Enquiries: Dr S.T. Mtshali

Date: 15 August 2014

Ms Gugu Khumalo
c/o Department of Health KwaZulu-Natal

Email: gugu.khumalo@kznhealth.gov.za


RESEARCH PROPOSAL

A research proposal has been submitted to me by another centre requesting that data of IALCH patients be used for the study "MOLECULAR SCREENING OF THE SOUTH AFRICAN INDIAN POPULATION FOR BRCA1 AND BRCA2 .

The protocol has been forwarded to the Research Committee in Pietermaritzburg for clearance and the research has been approved.

I wish to inform you that I consent to the request for samples of IALCH patients to be used for the research in question.

Yours sincerely,



Dr S.T. Mtshali
Chief Executive Officer

uMnyango Wezempilo. Departement van Gesondheid
FIGHTING DISEASE, FIGHTING POVERTY, GIVING HOPE

Appendix H

**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 I

HRMA Full Screens raw data *BRCA1* exons 2 - 15

CAM nr.	Patient Number	BRCA1 : 2	BRCA1 : 3	BRCA1 : 5	BRCA1 : 6	BRCA1 : 7	BRCA1 : 8	BRCA1 : 9	BRCA1 : 10	BRCA1 : 12	BRCA1 : 13	BRCA1 : 14	BRCA1 : 15
1009	1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1187	2	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	✓	✓	✓
1236	3	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	✓	✓	✓
1254	4	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1410	5	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C,p.Ser1436= rs1060915, g.41234470A>G	✓	✓
1481	6	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436= rs1060915 g.41234470A>G	✓	✓
1482	7	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1485	8	IVS-58delTGT	T37K	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	✓	✓	✓
1497	9	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1506	10	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C,p.Ser1436= Rs1060915 g.41234470A>G	✓	✓
1595	11	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

1643	12	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	✓	✓	✓
1663	13	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1666	14	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436= rs1060915 g.41234470A>G	✓	✓
1679	15	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C, p.Ser1436= rs1060915,g.41234470A>G	✓	✓
1701	16	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	IVS-58delT	✓	✓	✓	✓	c.4485-64 C>G
1710	17	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1715	18	✓	✓	✓	✓	✓	✓	IVS-58delT	✓	✓	✓	✓	✓
1762	19	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1784	20	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436= rs1060915 g.41234470A>G	✓	✓
1785	21	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1799	22	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1808	23	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C, p.Ser1436= rs1060915, g.41234470A>G	✓	✓
1860	24	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1861	25	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1871	26	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436= rs1060915 g.41234470A>G	✓	✓
1872	27	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1889	28	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	✓	✓	✓
1890	29	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1892	30	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436= rs1060915 g.41234470A>G	✓	✓
1918	31	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

1923	32	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436=rs1060915 g.41234470A>G	✓	✓
1927	33	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	Het c.4308T>C p.Ser1436=rs1060915 g.41234470A>G	✓	✓
1940	34	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1998	35	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2017	36	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2023	37	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436=rs1060915 g.41234470A>G	✓	✓
2027	38	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2129	39	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2137	40	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C, p.Ser1436=rs1060915, g.41234470A>G	✓	✓
2220	41	✓	✓	✓	Leu85Ile	✓	✓	c.548-58delT	✓	✓	✓	✓	✓
2221	42	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2223	43	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2232	44	✓	✓	✓	✓	✓	✓	c.548-58delT	✓	✓	✓	✓	✓
2248	45	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436=rs1060915 g.41234470A>G	✓	✓
2257	46	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2267	47	✓	✓	c.212+66 A>G	✓	✓	✓	✓	✓	✓	✓	✓	c.4675+32A>G
2271	48	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4675+32A>G
2277	50	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2287	51	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2304	52	✓	✓	✓	c.301+75C>G	✓	✓	✓	✓	✓	✓	✓	✓
2318	53	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4485-64 C>G
2320	54	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

2330	55	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2332	56	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2341	57	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	c.4308T>C, p.Ser1436= rs1060915, g.41234470A>G	✓	✓
2342	58	✓	✓	✓	✓	✓	✓	c.548-58delT	✓	✓	✓	✓	✓
2349	59	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2353	60	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2356	61	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2358	62	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C, p.Ser1436= rs1060915, g.41234470A>G	✓	✓
2361	63	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436= rs1060915 g.41234470A>G	✓	✓
2381	64	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436= rs1060915 g.41234470A>G	✓	✓
2389	65	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2398	66	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C,p.Ser1436= rs1060915, g.41234470A>G	✓	✓
2411	67	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C, p.Ser1436= rs1060915, g.41234470A>G	✓	✓
2414	68	✓	✓	✓	✓	✓	✓	✓	✓	c.4115G>A, p.Cys1372Ty r Het	✓	✓	✓
2451	69	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2494	70	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2503	71	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2507	72	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2524	73	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	✓	✓	✓
2530	74	✓	✓	✓	✓	✓	c.442-34C>T,hom Rs799923	✓	✓	✓	c.4308T>C,p.Ser1436= rs1060915,	✓	✓

							g.41251931G>A					g.41234470A>G		
2531	75	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2533	76	✓	✓	c.212+23 T>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2562	77	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2578	78	c.66_67dupAp.Leu22 _Glu23fs	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C, p.Ser1436= rs1060915, g.41234470A>G	✓	✓
2579	79	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2580	80	✓	✓	✓	✓	✓	Het c.442-34C>T, Rs799923 g.41251931G>A	✓	✓	✓	✓	✓	✓	✓
2604	81	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2606	82	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2614	83	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2635	84	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2703	85	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2704	86	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2706	87	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2729	88	✓	✓	c.212+66 A>G	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2747	89	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2755	90	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2761	91	✓	✓	✓	✓	✓	✓	✓	✓	c.4113G>A, p.Gly1371= c.4185+12_4185- 13delGT	✓	✓	✓	✓
2767	92	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2804	93	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2811	94	✓	✓	✓	✓	✓	c.442-34C>T,het Rs799923 g.41251931G>A	✓	✓	✓	✓	Het c.4308T>C p.Ser1436= rs1060915 g.41234470A>G	✓	✓
2816	95	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

2848	96	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C, p.Ser1436=rs1060915, g.41234470A>G	✓	✓
2860	97	✓	✓	✓	✓	✓	c.442-34C>T,hom Rs799923 g.41251931G>A	✓	✓	✓	✓	✓	✓
2877	98	✓	✓	✓	✓	✓	✓	✓	✓	c.4113G>A, p.Gly1371=Het c.4185+12_418 5+13delGT Het	✓	✓	✓
2882	99	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.4308T>C p.Ser1436=rs1060915 g.41234470A>G	✓	✓
2883	100	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2921	101	✓	✓	✓	✓	✓	c.302-41T>C	✓	✓	✓	✓	✓	✓
2943	102	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2983	103	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2987	104	✓	✓	c.212+66A>G	✓	✓	✓	✓	✓	✓	c.4308T>C,p.Ser1436=Rs1060915 g.41234470A>G	✓	✓
3007	105	✓	✓	c.212+66A>G	✓	✓	✓	c.593+3G>A Rs80358013 g.41249258C>T	✓	✓	✓	✓	✓
3012	106	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C,p.Ser1436=Rs1060915 g.41234470A>G	✓	✓
3018	107	✓	✓	✓	✓	✓	c.442-34C>T Rs799923	✓	✓	✓	✓	✓	✓
3019	108	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3027	109	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C,p.Ser1436=Rs1060915 g.41234470A>G	✓	✓
3033	110	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3034	111	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3038	112	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

3039	113	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
3040	114	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.4308T>C.p.Ser1436= Rs1060915 g.41234470A>G	✓	✓
3056	115	✓	✓	✓	✓	✓	✓	c.593+3G>A Rs80358013 g.41249258C>T	✓	✓	✓	✓	✓	

Full Screens raw data BRCA1 – exons 16 to 24

CAM nr.	Patient Number	BRCA1 : 16.1	BRCA1 : 16.2	BRCA1 : 17	BRCA1 : 18	BRCA1 : 19	BRCA1 : 20	BRCA1 : 21	BRCA1 : 22	BRCA1 : 23	BRCA1 : 24	BRCA1 : PTT frag 1	BRCA1 : PTT frag 2	BRCA1 : PTT frag 3
1009	1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1187	2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1236	3	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1254	4	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1410	5	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1481	6	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1482	7	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1485	8	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1497	9	Het c.4837A>G p. Ser1613Gly	Het c.4837A>G p. Ser1613Gly	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

		rs1799966 g.41223094T>C	rs1799966 g.41223094T>C											
1506	10	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1595	11	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1643	12	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1663	13	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1666	14	✓	✓	Het c.4987- 20A>G, rs80358035, g.41219732T >C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1679	15	✓	✓	✓	Het c.5075- 53C>T, rs8176258, g.41216021 G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓
1701	16	✓	✓	✓	IVS18+73 G>C	✓	✓	✓	✓	IVS22+78C >A	✓	H1860Q	✓	✓
1710	17	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1715	18	✓	✓	✓	IVS18+73 G>C	✓	✓	✓	✓	✓	✓	✓	✓	✓
1762	19	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1784	20	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1785	21	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1799	22	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1808	23	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1860	24	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1861	25	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1871	26	Het c.4837A>G p. Ser1613Gly	Het c.4837A>G p. Ser1613Gly	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

		rs1799966 g.41223094T>C	rs1799966 g.41223094T>C											
1872	27	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1889	28	✓	✓	Het c.4987- 20A>G, rs80358035, g.41219732T >C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1890	29	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1892	30	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1918	31	✓	✓	✓	c.5141T> G, p.Val1714 Gly	✓	✓	✓	✓	✓	✓	✓	✓	✓
1923	32	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1927	33	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	Het c.5406+8T>C Rs55946644 g.41201130A> G	✓	Het c.5628C>G g.41197659G>C	✓	✓	✓
1940	34	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1998	35	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2017	36	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2023	37	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2027	38	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2129	39	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2137	40	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2220	41	Het c.4837A>G p. Ser1613Gly	Het c.4837A>G p. Ser1613Gly	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

		rs1799966 g.41223094T>C	rs1799966 g.41223094T>C											
2221	42	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2223	43	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2232	44	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2248	45	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2257	46	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2267	47	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2271	48	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2277	50	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2287	51	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Pos	✓
2304	52	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2318	53	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2320	54	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2330	55	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2332	56	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2341	57	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2342	58	✓	✓	✓	✓	✓	✓	✓	✓	✓	IVS23-10C>A	✓	✓	✓
2349	59	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR
2353	60	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2356	61	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2358	62	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2361	63	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

2381	64	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
2389	65	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR
2398	66	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2411	67	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2414	68	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2451	69	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	Het c.5406+8T>C Rs55946644 g.41201130A> G	✓	c.5628C>G g.41197659G>C	NR	NR	NR	NR
2494	70	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2503	71	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR	NR
2507	72	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2524	73	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR
2530	74	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	c.4956G>A, p.Met1652Ile, rs1799967 Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR
2531	75	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2533	76	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	3' UTR c.5628 C>G	✓	✓	✓
2562	77	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2578	78	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2579	79	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

2580	80	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2604	81	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2606	82	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2614	83	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2635	84	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2703	85	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2704	86	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Pos	✓	✓
2706	87	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2729	88	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2747	89	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Pos
2755	90	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2761	91	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2767	92	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2804	93	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2811	94	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2817	95	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2848	96	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	Het c.4837A>G p. Ser1613Gly rs1799966 g.41223094T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2860	97	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2877	98	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2882	99	Het c.4837A>G p. Ser1613Gly	Het c.4837A>G p. Ser1613Gly	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

		rs1799966 g.41223094T>C	rs1799966 g.41223094T>C											
2883	100	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2921	101	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2943	102	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2983	103	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2987	104	✓	✓	✓	✓	✓	✓	✓	c.5406+8T>C Rs55946644	✓	✓	✓	✓	✓
3007	105	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	c.4837A>G,p.S1613G rs1799966,g.41223094T C	✓	✓	✓	✓	✓	c.5406+8T>C Rs55946644	✓	✓	✓	✓	✓
3012	106	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3018	107	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3019	108	✓	✓	✓	✓	c.5175A>G,p.Glu1725= ClinVar - rare (benign - likely benign)	✓	✓	✓	✓	✓	✓	✓	✓
3027	109	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	c.4837A>G,p.S1613G rs1799966,g.41223094T C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3033	110	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3034	111	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	c.4837A>G,p.S1613G rs1799966,g.41223094T C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3038	112	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3039	113	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3040	114	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3056	115	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	c.4837A>G,p.S1613G rs1799966,g.41223094T >C	✓	✓	✓	✓	✓	c.5406+8T>C Rs55946644	✓	✓	✓	✓	✓

HRMA Full Screens raw data BRCA2 exons 2 – 10

CAM nr.	Patient number	BRCA2 : 2	BRCA2 : 3.1	BRCA2 : 3.2	BRCA2 : 4	BRCA2 : 5	BRCA2 : 6	BRCA2 : 7	BRCA2 : 8	BRCA2 : 9	BRCA2 : 10.1	BRCA2 : 10.2	BRCA2 : 10.3	BRCA2 : 10.4
1009	1	✓	✓	✓	✓	✓	✓	c.517-4C>G, rs81002804, g.32900632C >G	✓	✓	✓	✓	✓	✓
1187	2	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
1236	3	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	Het c.425+67A>C rs11571610 g.32899388A> C	✓	✓	✓	✓	✓	✓	✓	✓	✓
1254	4	Het c.-26G>A rs1799943 g.32890572G>A	c.68-7delT g.32893207d elT	✓	✓	✓	✓	c.517-4C>G, rs81002804, g.32900632C >G	✓	✓	✓	✓	✓	✓
1410	5	✓	✓	✓	✓	✓	✓	✓	c.681+56C>T	✓	✓	✓	✓	✓
1481	6	✓	✓	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓	✓
1482	7	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1485	8	✓	✓	✓	Het c.317- 22C>T rs81002794 g. 32899191C>T	✓	✓	c.517-4C>G, rs81002804, g.32900632C >G	✓	✓	✓	✓	✓	✓
1497	9	✓	✓	✓	✓	✓	✓	✓	IVS8+56C>T	✓	✓	✓	✓	✓

1506	10	✓	c.68-7delT g.32893207d eIT	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
1595	11		Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1643	12	✓		✓	✓	Het c.425+67A>C rs11571610 g.32899388A> C	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	Het c.1365A>G p.Ser455= rs1801439 g.32906980A >G	✓
1663	13	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1666	14	✓	✓	✓	✓		Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓	✓	✓	✓
1679	15	✓	✓	✓	✓			✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
1701	16	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1710	17	✓	✓	✓	✓	Het c.317- 22C>T rs81002794 g. 32899191C>T	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓
1715	18	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1762	19	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
1784	20	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042	✓	✓	✓	✓	c.1114A>C rs144848 Het	✓	✓

1785	21	✓	✓	✓	✓	✓	✓	c.517-4C>G, rs81002804, g.32900632C >G	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓
1799	22	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1808	23	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1860	24	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042	✓	✓	✓	✓
1861	25	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓
1871	26	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1872	27	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1889	28	✓	✓	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓
1890	29	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1892	30	✓	✓	✓	✓	✓	✓	✓	Hom c.681+56C>T rs2126042	✓	✓	✓	✓
1918	31	✓	✓	✓	✓	✓	✓	c.517-4C>G, rs81002804, g.32900632C >G	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓
1923	32	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓

1927	33	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1940	34	✓	✓	✓	Het c.425+67A>C rs11571610 g.32899388A> C	✓	✓	✓	✓	✓	✓	✓	✓	✓
1998	35	✓	✓	✓	✓	✓	✓	✓	c.681+56C>T	✓	✓	✓	✓	✓
2017	36	✓	✓	✓	✓	✓	✓	c.517-4C>G, rs81002804, g.32900632C >G	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓	✓
2023	37	✓	✓	✓	Het c.425+67A>C rs11571610 g.32899388A> C	Het c.426- 89T>C rs3783265 g.32900149T >C	✓	✓	✓	✓	Het c.865A>C p.Asn289His rs766173 g. 32906480A> C	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C, Het c.1365A>G p.Ser455= rs1801439 g.32906980A >G	Het c.1365A>G p.Ser455= rs1801439 g.32906980A >G	✓
2027	38	✓	✓	✓	✓	✓	✓	✓	IVS8+56C>T	✓	✓	✓	✓	✓
2129	39	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	Het c.425+67A>C rs11571610 g.32899388A> C	✓	✓	✓	✓	✓	Het c.865A>C p.Asn289His rs766173 g. 32906480A> C	Het c.1365A>G p.Ser455= rs1801439 g.32906980A >G	Het c.1365A>G p.Ser455= rs1801439 g.32906980A >G	✓
2137	40	✓	✓	✓	Het c.317- 22C>T rs81002794 g. 32899191C>T	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓	✓
2220	41	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2221	42	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2223	43	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2232	44	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2248	45	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

2257	46	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2267	47	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2271	48	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2277	50	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1909+12delTT, g.32907536_329 07537delTT
2287	51	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2304	52	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2318	53	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2320	54	✓	✓	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓	✓
2330	55	✓	✓	✓	✓	✓	✓	c.517-4C>G, rs81002804, g.32900632C >G	✓	✓	✓	✓	✓	✓
2332	56	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2341	57	✓	✓	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓	✓
2342	58	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2349	59	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
2353	60	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓

2356	61	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓
2358	62	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
2361	63	✓	✓	✓	✓	✓	✓	✓	IVS8+56C>T	✓	✓	✓	✓
2381	64	✓	✓	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	c.987G>A, p.Arg329=, g.32906602, rs561002197	✓	✓
2389	65	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2398	66	✓	✓	✓	✓	✓	✓	✓	Het c.317- 22C>T rs81002794 g. 32899191C>T	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓
2411	67	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.891_902de IAACAGTTG TAGA, p.Glu297_30 1Asp?fs, g.32906506_ 32906517	✓	✓
2414	68	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2451	69	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2494	70	c.-26 G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2503	71	✓	✓	✓	✓	✓	✓	✓	c.517-4C>G, rs81002804, g.32900632C>G	✓	✓	✓	✓
2507	72	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

2524	73	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2530	74	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Homo c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
2531	75	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
2533	76	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2562	77	✓	✓	✓	✓	✓	✓	✓	c.681+56C>T	✓	✓	✓	✓	✓
2578	78	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1365A>G p.Ser455= rs1801439 g.32906980A >G	Het c.1365A>G p.Ser455= rs1801439 g.32906980A >G	✓
2579	79	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2580	80	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2604	81	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2606	82	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2614	83	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
2635	84	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2703	85	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2704	86	✓	✓	✓	✓	✓	✓	✓	c.681+56C>T	✓	✓	✓	✓	✓

2706	87	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2729	88	✓	✓	✓	✓	✓	✓	c.517-4C>G, rs81002804, g.32900632C >G	✓	✓	✓	✓	✓	✓
2747	89	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2755	90	✓	✓	✓	c.425+67A>C g.32899388A> C	✓	✓	✓	✓	✓	Rs766173 c.865A>C, p.Asn289His	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	Het c.1365A>G p.Ser455= rs1801439 g.32906980A >G	✓
2761	91	✓	✓	✓	c.425+67A>C g.32899388A> C	c.426-89T>C rs3783265 g.32900149T>C	✓	✓	IVS8+56 C>T	✓	Rs766173 c.865A>C, p.Asn289His	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
2767	92	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
2804	93	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2811	94	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	Het c.681+56C>T rs2126042 g.32903685C >T	✓	✓	✓	✓	✓
2816	95	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2848	96	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2860	97	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C p.Asn372His rs144848 g.32906729A >C	✓	✓
2877	98	✓	✓	✓	c.317-22C>T rs81002794	✓	✓	✓	✓	✓	✓	✓	✓	✓

2882	99	Het c.-26G>A rs1799943 g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	c.1114A>C rs144848 Het	✓	✓
2883	100	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2921	101	✓	✓	✓	✓	✓	✓	c.681+56C>T	✓	✓	✓	✓	✓	✓
2943	102	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2983	103	✓	✓	✓	✓	✓	✓	c.681+56C>T	✓	✓	✓	✓	✓	✓
2987	104	c.-11C>T g.32890587C>T c.-26G>A g.32890572G>A	✓	✓	✓	✓	✓	c.681+56C>T	✓	✓	✓	✓	✓	✓
3007	105	✓	✓	✓	✓	✓	✓	c.681+56C>T	✓	✓	c.1114A>C,p.N372 H Rs144848	✓	✓	✓
3012	106	✓	✓	✓	✓	✓	✓	Rs81002804 c.517-4C>G ClinVar – conflicting Het	c.681+56C>T Hom	✓	✓	✓	✓	✓
3018	107	c.-26G>A g.32890572G>A	✓	✓	✓	✓	✓	✓	c.681+56C>T Hom	✓	✓	✓	✓	✓
3019	108	✓	✓	✓	✓	✓	✓	✓	✓	✓	1011C>T,p.Asn33 7= Rs41293473 g.32906626C> T	Het c.1114A>C,p.N372 H Rs144848	✓	✓
3027	109	✓	✓	✓	✓	✓	✓	Rs81002804 c.517-4C>G ClinVar – conflicting Het	✓	✓	Het c.1114A>C,p.N372 H Rs144848	✓	✓	✓
3033	110	c.-26G>A g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3034	111	✓	✓	✓	✓	✓	✓	Rs81002804 c.517-4C>G ClinVar – conflicting Hom c.517-19C>T	✓	✓	✓	✓	✓	✓

								Rs 11571623 g.32900617C> T Het						
3038	112	c.-26G>A g.32890572G>A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3039	113	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.1114A>C,p.N372 H Rs144848	✓	✓
3040	114	✓	✓	✓	✓	✓	✓	c.681+56C>T Het	✓	✓	✓	✓	✓	✓
3056	115	✓	✓	✓	✓	✓	✓	c.681+56C>T Hom	✓	✓	✓	✓	✓	✓

HRMA Full Screens raw data *BRCA2* exons 12 – 21

CAM nr.	Sample nr.	BRCA2 : 12	BRCA2 : 13	BRCA2 : 14.1	BRCA2 : 14.2	BRCA2 : 15	BRCA2 : 16	BRCA2 : 17	BRCA2 : 18.1	BRCA2 : 18.2	BRCA2 : 19.1	BRCA2 : 19.2	BRCA2 : 20	BRCA2 : 21
1009	1	Het c.6842-73T>A, g.32918622T>A	✓	✓	✓	✓	✓	✓	c.8092G>A	✓	✓	✓	✓	✓
1187	2	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	✓	✓	✓	✓	✓	✓	✓
1236	3	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	✓	✓	✓	✓	✓	✓	✓

1254	4	Het c.6842-73T>A, g.32918622T>A	✓	Het c.7242A>G p.Ser2414=rs1799955 g.32929232A>G	Het c.7242A>G p.Ser2414=rs1799955 g.32929232A>G	✓	✓	Homo c.7806-14T>C rs9534262 g.32936646T>C	✓	✓	✓	✓	✓
1410	5	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1481	6	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.8487+19A>G, g.32944713A>G, rs11571743	✓	✓
1482	7	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1485	8	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1497	9	✓	✓	Het c.7242A>G p.Ser2414=rs1799955 g.32929232A>G	Het c.7242A>G p.Ser2414=rs1799955 g.32929232A>G	✓	✓	✓	✓	✓	✓	✓	✓
1506	10	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1595	11	✓	✓	Het c.7242A>G p.Ser2414=rs1799955 g.32929232A>G	Het c.7242A>G p.Ser2414=rs1799955 g.32929232A>G	✓	✓	✓	✓	✓	✓	✓	✓
1643	12	Het c.6842-73T>A, g.32918622T>A	✓	✓	✓	✓	✓	Het c.7806-14T>C rs9534262 g.32936646T>C	✓	✓	✓	✓	✓
1663	13	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1666	14	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.8487+19A>G, g.32944713A>G, rs11571743	✓	✓
1679	15	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1701	16	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1710	17	✓	✓	Het c.7242A>G p.Ser2414=rs1799955	Het c.7242A>G p.Ser2414=rs1799955	✓	✓	✓	✓	✓	Het c.8487+19A>G, g.32944713A	✓	✓

				g.32929232A >G	g.32929232A >G							>G, rs11571743		
1715	18	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1762	19	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	✓	✓	✓	✓	✓	✓	✓
1784	20	✓	✓	✓	✓	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓
1785	21	Het c.6842- 73T>A, g.32918622T >A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1799	22	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1808	23	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1860	24	✓	✓	c.7017G>C rs45574331 Het	c.7017G>C rs45574331 Het	✓	✓	✓	✓	✓	✓	✓	✓	✓
1861	25	✓	✓	Het c.7017G>C p.LYS2339As n rs45574331 g.32929007G >C Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7017G>C p.LYS2339As n rs45574331 g.32929007G >C Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	Homo c.7806-14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓
1871	26	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓
1872	27	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1889	28	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Het c.8487+19A> G, g.32944713A >G, rs11571743	✓	✓

1890	29	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓
1892	30	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1918	31	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1923	32	✓	✓	c.7242A>G, p.Ser2414 Het	c.7242A>G, p.Ser2414 Het	✓	✓	✓	✓	✓	✓	✓	✓
1927	33	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓
1940	34	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
1998	35	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2017	36		✓	Het c.6842- 73T>A, g.32918622T >A	✓	✓	✓	✓	✓	✓	✓	✓	✓
2023	37	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2027	38	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2129	39	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G Het c.7435+53C>T rs11147489 g.32929478C >T	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G Het c.7435+53C>T rs11147489 g.32929478C >T	✓	✓	Homo c.7806-14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓
2137	40	✓	✓	✓	✓	✓	✓	Homo c.7806-14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓
2220	41	✓	✓	✓	✓	✓	✓	IVS16-14T>C	✓	✓	✓	✓	✓
2221	42	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2223	43	✓	✓	✓	✓	✓	✓	IVS16-14T>C	✓	✓	✓	✓	✓

2232	44	✓	✓	✓	✓	✓	✓	8162	✓	✓	✓	✓	✓	✓
2248	45	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2257	46	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2267	47	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2271	48	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2277	50	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓
2287	51	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2304	52	Het c.6842- 73T>A, g.32918622T >A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2318	53	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2320	54	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2330	55	Het c.6842- 73T>A, g.32918622T >A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2332	56	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2341	57	✓	✓	✓	✓	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓
2342	58	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2349	59	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓
2353	60	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2356	61	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	✓	✓	✓	✓	✓	✓	✓

2358	62	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2361	63	✓	✓	✓	✓	✓	✓	c.8010G>A, p.Ser2670	✓	✓	✓	✓	✓	✓
2381	64	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2389	65	✓	✓	✓	✓	✓	✓	Het c.8149G>T p.Ala2717Ser rs28897747 g.32937488G >T	✓	✓	✓	✓	✓	✓
2398	66	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2411	67	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2414	68	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2451	69	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2494	70	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2503	71	Het c.6842- 73T>A, g.32918622T >A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2507	72	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2524	73	✓	✓	c.7242A>G, p.Ser2414 Het	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2530	74	✓	✓	✓	✓	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓
2531	75	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2533	76	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2562	77	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2578	78	✓	✓	✓	✓	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓
2579	79	✓	✓	Het c.7242A>G p.Ser2414= rs1799955	Het c.7242A>G p.Ser2414= rs1799955	✓	✓	✓	✓	✓	✓	✓	✓	✓

				g.32929232A >G	g.32929232A >G										
2580	80	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2604	81	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2606	82	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2614	83	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2635	84														
2703	85	✓	✓	c.7242A>G,p.Ser 2414= rs1799955, g.32929232A>G	c.7242A>G,p.Ser 2414= rs1799955, g.32929232A>G	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2704	86	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2706	87	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2729	88	Het c.6842- 73T>A, g.32918622T >A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2747	89	✓	✓	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	Het c.7242A>G p.Ser2414= rs1799955 g.32929232A >G	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2755	90	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2761	91	✓	✓	c.7242A>G,p.Ser 2414= rs1799955, g.32929232A>G	c.7242A>G,p.Ser 2414= rs1799955, g.32929232A>G	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2767	92	✓	✓	✓	✓	✓	✓	Het c.7806- 14T>C rs9534262 g.32936646T >C	✓	✓	✓	✓	✓	✓	✓
2804	93	✓	✓	c.7242A>G,p.Ser 2414= rs1799955, g.32929232A>G	c.7242A>G,p.Ser 2414= rs1799955, g.32929232A>G	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2811	94	✓	✓	Het c.7242A>G p.Ser2414= rs1799955	Het c.7242A>G p.Ser2414= rs1799955	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

				g.32929232A >G	g.32929232A>G									
3019	108	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3027	109	c.6842-73T>A, g.32918622T>A	✓	✓	✓	✓	✓	✓	c.8092G>A, pAla2698Thr, rs80359052, g.32937431G >A	✓	✓	✓	✓	✓
3033	110	✓	✓	c. 7242A>G, p.Ser2414= Rs1799955 g.32929232A >G	c. 7242A>G, p.Ser2414= Rs1799955 g.32929232A>G	✓	✓	✓	✓	✓	✓	✓	✓	✓
3034	111	✓	✓	✓	✓	✓	✓	✓	c.8092G>A, pAla2698Thr, rs80359052, g.32937431G >A	✓	✓	✓	✓	✓
3038	112	✓	✓	c. 7242A>G, p.Ser2414= Rs1799955 g.32929232A >G	c. 7242A>G, p.Ser2414= Rs1799955 g.32929232A>G	✓	✓	✓	✓	✓	✓	✓	✓	✓
3039	113	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3040	114	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
3056	115	✓	✓	✓	c.7397C>T, p.A2466V rs169547,g.32 929387C>T	✓	✓	✓	✓	✓	✓	✓	✓	✓

HRMA Full Screens raw data *BRCA2* exons 22 – 27

CAM nr.	Sample nr.	BRCA2 : 22	BRCA2 : 23	BRCA2 : 24.1	BRCA2 : 24.2	BRCA2 : 25.1	BRCA2 : 25.2	BRCA2 : 26	BRCA2 : 27.1	BRCA2 : 27.2	BRCA2 :PTT A	BRCA2 :PTT B	BRCA2 :PTT C	BRCA2 :PTT D	BRCA2 :PTT E
1009	1	c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1187	2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1236	3	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1254	4	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1410	5	c.8755-66T>C, Rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1481	6	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1482	7	c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1485	8	c.8755-66T>C, Rs4942486 g.32953388T> C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1497	9	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1506	10	c.8830A>T,p.L29 44F rs4987047, Class 1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1595	11	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1643	12	c.8755-66T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM

		rs4942486 g.32953388T>C													
1663	13	Het c.8830A>T, p.Ile2944Phe, g.32953529A>T, rs4987047	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1666	14	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1679	15	c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1701	16	c.8755-66T>C, Rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1710	17	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1715	18	c.8755-66T>C, Rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1762	19	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1784	20	c.8755-66T>C rs4942486 Het	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1785	21	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	6676insTA	✓	HRM
1799	22	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1808	23	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1860	24	c.8755-66T>C rs4942486 Het	✓	✓	✓	✓	✓	✓	Het c.9730G>A p.Val3244Ile rs11571831	✓	✓	✓	✓	✓	HRM
1861	25	✓	✓	✓	✓	✓	✓	✓	Het c.9875C>T, p.Pro3292Leu, rs56121817, g.3297252C >T, Het c.9730G>A p.Val3244Ile rs11571831	✓	✓	✓	✓	HRM	
1871	26	c.8755-66T>C rs4942486 Het	✓	✓	✓	✓	✓	✓	✓	Het c.10023C>T p.Asp3341=	✓	✓	✓	✓	HRM

										Rs113507014					
1872	27	✓	✓	✓	✓	✓	✓	✓	c.9649-19G>A, g.32972280G>A, rs11571830	✓	✓	✓	✓	✓	HRM
1889	28	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1890	29	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1892	30	c.8755-66T>C rs4942486 Hom	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1918	31	Het c.8830A>T, p.Ile2944Phe, g.32953529A>T, rs4987047	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1923	32	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1927	33	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1940	34	c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
1998	35	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2017	36	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2023	37	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Pos	✓	✓	HRM
2027	38	✓	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR	NR	HRM
2129	39	Homo c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2137	40	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2220	41	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2221	42	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2223	43	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2232	44	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2248	45										✓	✓	✓	✓	HRM

2257	46		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2267	47		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2271	48	Het c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2277	50		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2287	51														✓	
2304	52		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2318	53	c.8755-66T>C, Rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2320	54	Homo c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2330	55		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2332	56	IVS21-66 T>C hetero	✓	✓				✓	✓	✓	✓	✓	✓	✓	✓	
2341	57		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2342	58	c.8755-66T>C, Rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2349	59		✓	✓	✓	✓	✓	✓	✓		Het c.10234 p.Ile3412 rs180142 g.32972884 A>G	NR	NR	NR	NR	HRM
2353	60	Het c.8830A>T, p.Ile2944Phe, g.32953529A>T , rs4987047	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2356	61	Homo c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	

2358	62	Het c.8830A>T, p.Ile2944Phe, rs4987047, g.32953529A>T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2361	63	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2381	64	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2389	65	✓	✓	✓	✓	✓	✓	✓	✓	Het c.10234 p.Ile3412 rs180142 g.32972884 A>G	NR	NR	NR	NR	HRM
2398	66	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2411	67	✓	✓	✓	✓	✓	✓	✓	✓	Het c.10234 p.Ile3412 rs180142 g.32972884 A>G	✓	✓	✓	✓	HRM
2416	68										✓	✓	✓	✓	✓
2451	69	✓	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR	NR	HRM
2494	70	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2503	71	✓	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR	NR	HRM
2507	72										✓	✓	✓	✓	✓
2524	73	c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	NR	NR	NR	NR	HRM
2530	74	Het c.8755- 66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	Het c.10234 p.Ile3412 rs180142 g.32972884 A>G	NR	NR	NR	NR	HRM
2531	75	c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2533	76	c.8755-66	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM

		T>C het													
2562	77	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2578	78	c.8830A>T p.I2944P	✓	✓	✓	✓	✓	✓	✓	Het c.10234 p.Ile3412V rs180142 g.32972884 A>G	✓	✓	✓	✓	HRM
2579	79	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2580	80	c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2604	81	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2606	82														✓
2614	83	✓	✓	✓	✓	✓	✓	✓	✓	Het c.10234 p.Ile3412V rs180142 g.32972884 A>G	✓	✓	✓	✓	HRM
2635	84											✓	✓	✓	✓
2703	85	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2704	86	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2706	87											✓	✓	✓	✓
2729	88	S2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2747	89	c.8830A>T, p.Ile2944Phe	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2755	90	c.8755-66T>C rs4942486 c.8851G>A p.Ala2951Thr rs11571769	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM
2761	91	c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM

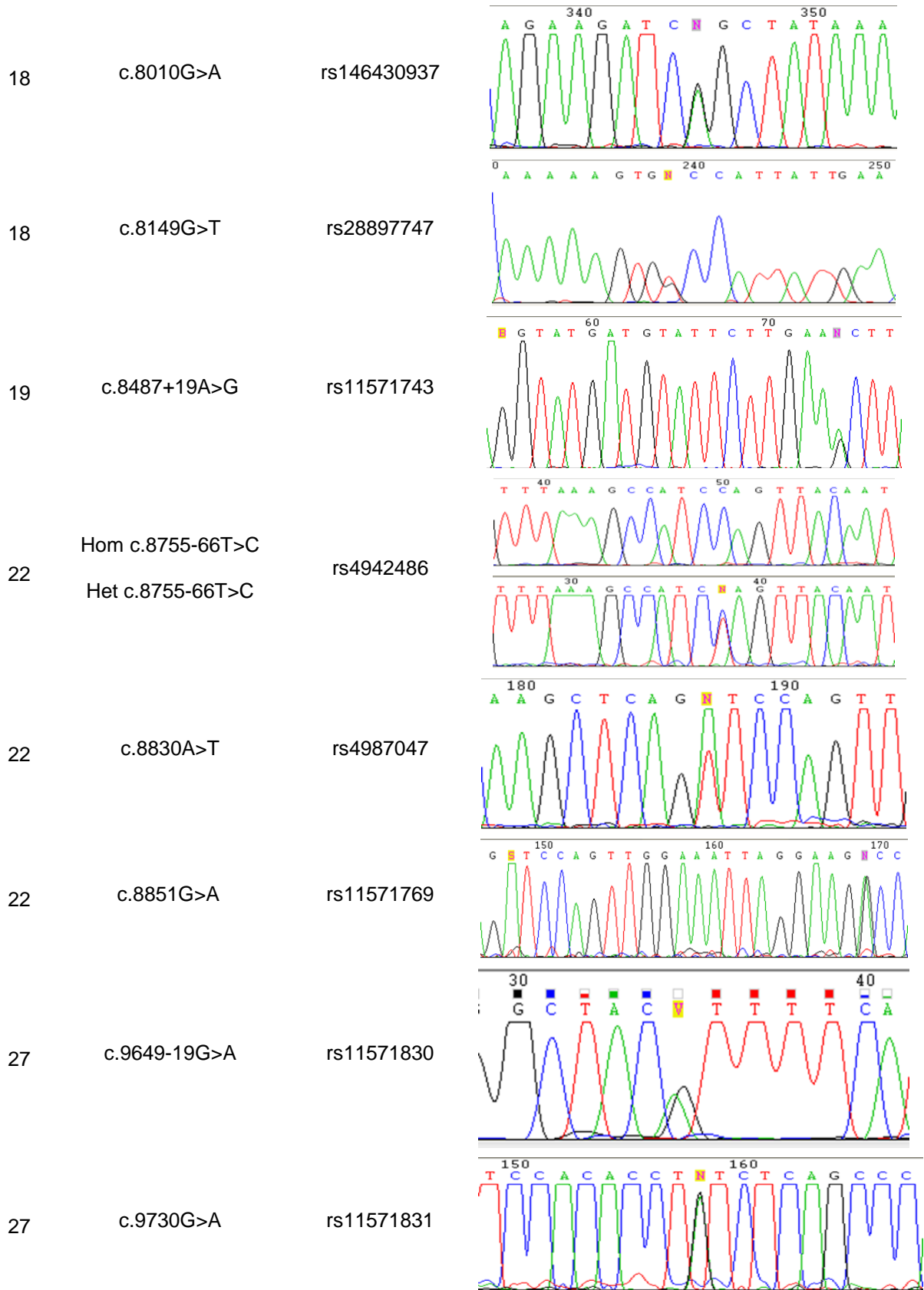
2767	92	Het c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2804	93	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2811	94	Homo c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2816	95											✓	✓	✓	✓	
2848	96	Homo c.8755-66T>C rs4942486 g.32953388T>C	✓	✓	✓	✓	✓	✓	c.9875C>T, pPro3292Le u, rs56121817, g.3297252C >T	✓	✓	✓	✓	✓	HRM	
2860	97	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2877	98	✓	✓	✓	✓	✓	✓	✓	Het c.9730G>A p.Val3244Ile rs11571831	✓	✓	✓	✓	✓	HRM	
2882	99	c.8755-66T>C rs4942486 Hom	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2883	100											✓	✓	✓	✓	
2921	101	c.8755-75T>C het (also homo) (likely benign - Class 2)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
2943	102	✓	✓	✓	✓	✓	✓	✓	Rs11571831 c.9730G>A,p.Val3 244Ile	c.10023C>T,p.A 5P3344= NOVEL	✓	✓	✓	✓	HRM	
2983	103	c.8755-66T>C Rs4942486 (het)	✓	✓	✓	✓	✓	✓	✓	✓	c.10234A>G,p.Ile 3412Val CAM2766 - rs1801426	✓	✓	✓	✓	HRM
2987	104	c.8755-66T>C Rs4942486 (het)	✓	✓	✓	✓	✓	✓	✓	✓	c.10234A>G,p.Ile 3412Val CAM2766 - rs1801426	✓	✓	✓	✓	HRM
3007	105	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	

3012	106	c.8755-66T>C Rs4942486 (het)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
3018	107	✓	✓	✓	✓	✓	✓	✓	✓	✓	Pos	✓	✓	✓	HRM	
3019	108	c.8755-66T>C Rs4942486 (hom)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
3027	109	c.8755-66T>C Rs4942486 (hom)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
3033	110	c.8755-66T>C Rs4942486 (het)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
3034	111	c.8755-66T>C Rs4942486 (hom)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
3038	112	c.8755-66T>C Rs4942486 (het)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
3039	113	c.8755-66T>C Rs4942486 (hom)	✓	✓	✓	✓	✓	✓	✓	✓	c.10234A>G,p.Ile 3412Val CAM2766 – rs1801426	✓	✓	✓	✓	HRM
3040	114	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	
3056	115	c.8755-66T>C Rs4942486 (het)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	HRM	

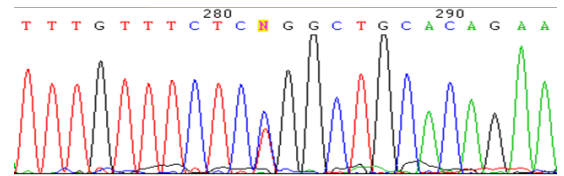
Appendix J

BRCA1 variants

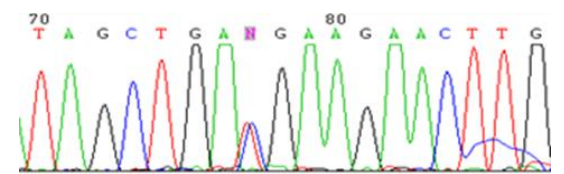
Exon Region	Variants	rs Number	Electropherograph
2	c.66dupA	rs80357783	
3	c.110C>A	rs80356880	
5	c.212+23T>A	rs8176128	
8	Het c.442-34C>T Hom c.442-34C>T	rs799923	
9	c.593+3G>A	rs80358013	
12	c.4113G>A	rs147448807	
12	c.4115G>A	rs55848034	



27 c.9875C>T rs56121817



27 c.10023C>T rs113507014



27 c.10234A>G rs1801426

