MammaPrint risk score distribution in breast cancer patients with BRCA1/2 mutations.

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Declaration

I, Lwando Mampunye, declare that the content of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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Dedication

To my parents Nokwanda Mampunye & Mkuseli Jonathan Mampunye

Table of content

Abstract

Background: Tumour characteristics such as estrogen receptor, progesterone receptor and human epidermal growth factor receptor-2 status are routinely assessed using immunohistochemistry in all newly-diagnosed breast cancer patients. These biomarkers form part of the selection criteria used to determine the appropriateness of transcriptional gene profiling using MammaPrint, a 70-gene assay with level 1A evidence for chemotherapy selection in patients with early-stage breast carcinoma. This MammaPrint pre-screen algorithm does not include screening for pathogenic germline variants underlying differences in tumour pathology, increasingly considered to predict *BRCA1/2*-related cancer and response to PARP inhibitors that target the DNA repair pathway.

Aim: The aim of this study was to determine whether one or more of the eight most common pathogenetic *BRCA1/2* variants previously identified in the South African population are predictive of a MammaPrint high risk score. This investigation was prompted by detection of the *BRCA2* c.7934delG founder/recurrent mutation in tumour DNA of a female patient diagnosed with bladder cancer four years after receiving a low-risk MammaPrint result. Next generation sequencing of her tumour DNA furthermore revealed genetic variation affecting cytochrome P450 2D6 (CYP2D6) enzyme activity associated with resistance to Tamoxifen, previously used by this patient together with anti-depressants that may inhibit enzyme function.

Methods: A database query was performed to identify early-stage breast cancer patients referred for the MammaPrint test followed by *CYP2D6* genotyping using the same pathology-supported genetic testing platform. A rapid point-of-care DNA assay was used to screen 50 DNA samples for eight *BRCA1/2* founder/recurrent mutations: *BRCA1* c.68_69delAG, c.1374delC, c.2641G>T, c.5266dupC and *BRCA2* c.5771_5774delTTCA, c.5946delT, c.6448_6449insTA and c.7934delG.

Results: The pathogenic *BRCA2* c.7934delG variant was confirmed in the germline DNA of the index case with bladder cancer and was the only pathogenic variant detected in 10.2% of the study population (5/49, 1 sample failed). Two breast cancer patients with this pathogenic variant had a low-risk MammaPrint profile (2/25, 8.3%), while three patients with the same *BRCA2* variant had a high-risk profile (3/24, 12%) for breast cancer metastasis. None of the other seven *BRCA1/2* founder/recurrent mutations were detected in the study cohort. Patients with the *BRCA2* c.7934delG founder/recurrent mutation was diagnosed at a significantly younger age than those without this pathogenic variant (p=0.02). Intermediate (36%) and poor metabolizer (2%) status based on *CYP2D6**4 genotype was detected in 18 of the 50 patients included in the study. Three of these patients also had the *BRCA2* c.7934delG founder/recurrent mutation, one with a low-risk (index case) and two with a high-risk MammaPrint recurrence risk profile.

Conclusions: Our findings support use of the MammaPrint pre-screen algorithm to identify a subgroup of early-stage hormone receptor-positive breast cancer patients who may benefit from pharmaco-

diagnostic *BRCA* screening as part of the MammaPrint service. Use of laboratory-based technologies can take several days or weeks from sample collection to report generation, posing a unique opportunity for rapid *BRCA1/2* testing during the genomic counselling session. Delivery of a positive test result generated at the point-of-care, or a negative result requiring extended genome sequencing, are important considerations in clinical settings where loss to follow-up or access to gene-based cancer treatment remain a problem.

List of Figures

List of Tables

List of Abbreviations

ASCO - American Society of Clinical Oncology

FISH - fluorescence *in situ* hybridization

AS - Allred score

BMI – Body mass index

BOADICEA - Breast and ovarian analysis of disease incidence and carrier estimation algorithm

BSE - Breast self-examination

CAP - College of American Pathologists

CBE - clinical breast examination

CGH - comparative genomic hybridization

CVD - cardiovascular disease

CYP2D6 - cytochrome P450 D6

DISH - bright-field double

EGFR - epidermal growth factor receptor

EPA - Exome pre-screen algorithm

ER – Estrogen receptor

FDA - Food and Drug Administration

FH – Familial hypercholestemia

FFPE – formalin-fixed paraffin embedded

HBOC - hereditary breast-ovarian cancer

HREC - Health and Research Ethics Review Committee

HER2 - human epidermal growth factor receptor-2

HRT - hormone replacement therapy

IHC - immunohistochemistry

IS – Intensity score

ISH - *In situ* hybridization

LGC – Laboratory Goverment Chemist Limited

MGS - Multigene signature

MINDACT - Microarray In Node Negative Disease may Avoid Chemotherapy

MPA - MammaPrint microarray pre-screen algorithm

MRI - Magnetic resonance imaging

NCCN - National Comprehensive Cancer Network

NCDs - Non-communicable diseases

NGS - next-generation sequencing

NOS - carcinoma of no special type

PCC - Probe check control

pCR – pathologic complete response

POC – point-of-care

POCT – point-of-care technology

PR – Progesterone receptor

PRS - polygenetic risk score

PS - positive staining cells

PSGT - Pathology-supported genetic testing

RT - Radiation treatment

RT-qPCR - real-time polymerase chain reaction

SERM - selective estrogen receptor modulator

SNPs - single nucleotide polymorphisms

VUS – Variant of uncertain clinical significance

WES - whole exome sequencing

WGS - whole genome sequencing

Chapter 1

Literature Review

1.1. Introduction

The risk of death from non-communicable diseases (NCDs) such as cancer or cardiovascular disease (CVD) increases with advancing age in both men and women. CVD is the leading cause of death in low- and middle-income countries, while in some higher income countries cancer deaths are twice as high compared to CVD (Bowry et al. 2015). Cancer is a complex disease involving multiple genes acting in combination with lifestyle and other environmental factors that may result in malignant transformation of cells. Some forms of cancer are associated with a family history of the disease, but in most instances no definitive pattern of inheritance is observed. This complicates dissection of genetic and environmental contributing factors and limits preventative strategies based on the causal pathways underlying cancer subtypes (Sas-Korczyriska et al. 2017).

Approximately 1 in every 6 deaths are caused by cancer (Ferlay et al. 2019), with breast cancer recognised as the most common histologically diagnosed cancer in women. The incidence of breast cancer is steadily increasing in African women as they adopt a more westernised lifestyle, accompanied by altered reproductive cultures including delayed childbearing and smaller families, forgoing of breastfeeding, and diet changes that may lead to weight gain (Adeloye et al. 2018). The increase in breast cancer incidence is reflective of the increased global incidence from 1.7 million in 2005 to 2.4 million reported cases in 2015 (Fitzmaurice et al. 2017). More than 8000 new breast cancer cases are reported in Africa every year, making up approximately 20% of all cancers (Brinton et al. 2014). These findings have established breast cancer as a leading cause of cancer among women globally, and South Africa is no exception.

Poor survival and higher mortality rates reported in Africa are generally ascribed to late-stage presentation and a delay in diagnosis, which may partly be due to poor socio-economic status of the patient as well as sub-optimal health care systems (Espina et al. 2017). From studies conducted in Sub-Saharan Africa more advanced breast disease is seen in patients living in rural areas compared to those in the urban centres (Elgaili et al. 2010; Kantelhardt et al. 2014). The stage as well as the age at which the diagnosis of breast cancer is made, vary between the different population groups living in South Africa, this could possibly be partly due to personal financial or psychosocial reasons (Friedman et al. 2006). Geographic location has a significant effect on accessibility to medical centres (Vorobiof et al. 2001). Lack of infrastructure and resources in rural areas and long-distances from centres of excellence where routine screening mammography are available contribute to the increased mortality in rural areas. Fear of dying from cancer or refusal of recommended medical treatment methods due to cultural beliefs are all factors affecting overall survival (Dickens et al. 2014). Conversely, where patients do agree to undergo therapy, the costs related to follow-up visits may be unsustainable for patients who have to use public transport services.

Due to the above-mentioned factors, breast cancer patients often decide not to continue with their treatment (Goudge et al. 2009). In addition to the financial burden, concerns about recurrence risk and therapy-induced co-morbidities are major stress-related factors associated with a diagnosis of cancer. Chemotherapy, hormonal therapy and radiation therapy, as well as some of the new targeted therapies such as poly ADP ribose polymerase (PARP) inhibitors targeting *BRCA1/2* gene defects, can result in severe clinical complications. Chemotherapy overtreatment in early-stage breast cancer is of particular concern and raised an intense debate about the most appropriate way to identify patients at a sufficiently high risk of relapse to justify aggressive treatment (Michiels et al. 2016, Esserman et al. 2017, Tsai et al. 2018). Laboratory methods developed for this purpose include a 70-gene profile called MammaPrint (van't Veer et al. 2002), a genomic decision-making tool with level 1A evidence for differentiating a subgroup of early-stage breast cancer patients with a low risk for distant metastasis from those experiencing metastasis in the first year after surgery (Cardoso et al. 2016). The magnitude of chemotherapy benefit found to be the highest in the first five years after diagnosis needs to be counterbalanced by the associated therapy-induced risks, including premature menopause, cardiac damage, cognitive dysfunction and leukaemia (Azim et al. 2011; Ramalho et al. 2017).

Concerns about medication side effects such as bone loss associated with aromatase inhibitors and Tamoxifen resistance caused by variation in drug metabolizing enzymes (Baatjes et al. 2017, 2019; van der Merwe et al. 2012a, 2017), supports the application of germline genetic testing in conjunction with tumour gene profiling of hormone receptor-positive breast cancer using MammaPrint. Severe therapy-induced hypertriglyceridemia due to the estrogenic effect of Tamoxifen on lipid metabolism may, for example, result in life-threatening pancreatitis (Singh et al. 2016). Deep vein thrombosis is also a common complication of malignancy, prolonged post-surgical immobility, and the effect of chemotherapy and endocrine treatment on the blood clotting cascade. PARP inhibitors recently approved by the Food and Drug Administration (FDA) for treatment of breast, ovarian and pancreatic cancer in patients with pathogenic *BRCA1/2* variants, has furthermore been linked to severe deficiency of folate required as a co-factor in the DNA methylation process (Shammo et al. 2019). This finding highlighted the need for research to elucidate the mechanism of association between *BRCA1/2* gene defects and the folate-homocysteine pathway also implicated in CVD.

Breast cancer patients referred for diagnostic *BRCA1/2* testing based on standard referral guidelines (Schoeman et al. 2013) may consider CVD multi-gene testing (Kotze and Thiart 2003; Kotze et al. 2003) as part of the pre-screen algorithm developed for whole exome sequencing (WES) in genetically uncharacterised cases (van der Merwe et al. 2017). In this context, pathology-supported genetic testing (PSGT) of disease pathways shared by breast cancer and other NCDs (Kotze et al. 2013, 2015) provides a framework for combining diagnostic, prognostic and pharmacogenetic testing on the same platform (Figure 1). This approach, first applied in the differential diagnosis of patients with familial

hypercholesterolaemia (FH) and less severe forms of dyslipidaemia (Kotze and van Rensburg 2012; Marais et al. 2019) are increasingly used to detect common risk factors shared between breast carcinoma and associated comorbidities (van der Merwe and Kotze 2018). Both FH and breast cancer have strong familial risk implications in South Africa due to founder effects and the major genes underlying these conditions can be screened for simultaneously in eligible patients using WES.

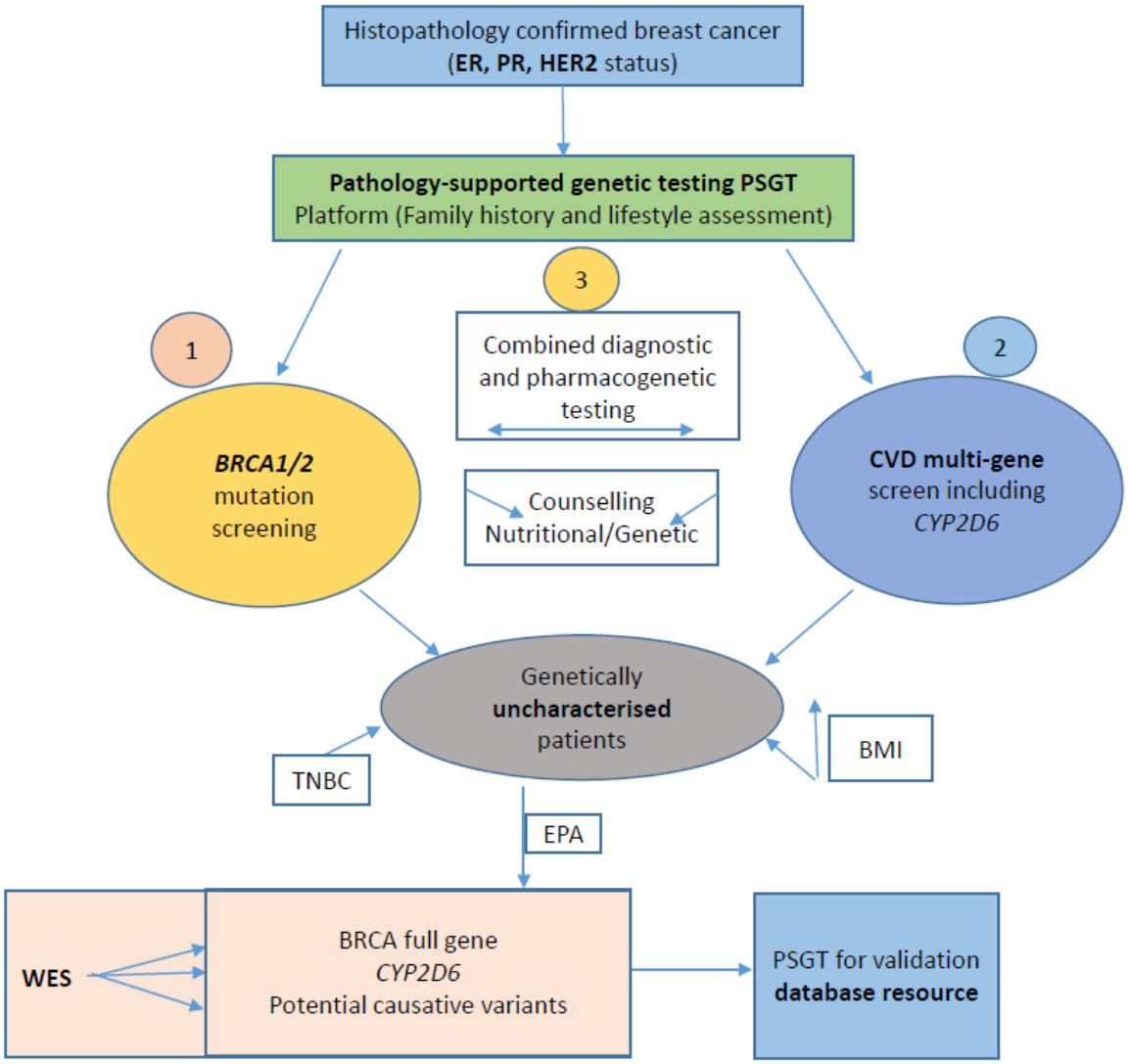

Figure 1: A pathology-supported genetic testing (PSGT) framework for differential diagnosis of inherited and lifestyle-related breast cancer using a pharmaco-diagnostic exome pre-screen algorithm (EPA). BRCA1/2 founder/recurrent mutation testing is combined with assessment of common cardiovascular disease (CVD) risk factors to facilitate selection of genetically uncharacterised patients for whole exome sequencing (WES): 1) diagnostic BRCA1/2 testing is based on standard referral guidelines including early-onset breast cancer (<40 years), triple-negative breast cancer (TNBC), bilateral breast cancer, additional ovarian cancer, one 1st degree relative with breast or ovarian cancer, > 2nd degree affected relatives and male breast cancer in a relative (Schoeman et al. 2013); 2) Biochemical genetic testing using a CVD multi-gene assay is combined with CYP2D6 pharmacogenetics based on co-morbidities identified in patients treated with PARP inhibitors or hormone-, chemo- or immunotherapies; 3) genomic counselling is recommended based on treatment-induced comorbidities, drug failure and/or familial risk to determine the appropriateness of WES in genetically uncharacterized breast cancer patients [Reproduced with permission from van der Merwe and Kotze 2018].

Genomic counselling is advisable in breast cancer patients with treatment-induced comorbidities, drug failure and/or familial risk to determine the appropriateness of WES or other next generation sequencing (NGS) technologies. The PSGT approach enables identification of high-risk patients eligible for WES, which in turn, enables extended analysis of disease susceptibility or drug metabolizing pathways implicated in genetically uncharacterised patients, using stored DNA sequence data that may in future become clinically relevant. CYP2D6 poor or intermediate metabolizers with pathogenic *BRCA1/2* variants will not gain the full benefit from Tamoxifen treatment and are therefore at higher risk of recurrence (Newman et al. 2008). This finding is of particular relevance in the South African population due to a founder effect responsible for the increased *BRCA1/2* mutation frequency across ethnic groups (Reeves et al. 2004; van der Merwe and van Rensburg 2009; van der Merwe et al. 2012b), especially in hormone receptor-positive breast cancer patients using antidepressants that may inhibit CYP2D6 enzyme activity (van der Merwe et al. 2012a). The eight most common *BRCA1/2* founder/recurrent mutations were recently incorporated into a rapid point-of-care (POC) assay (https://gtr.ukri.org/projects?ref=103993) applied for the first time in the present research study. The clinical impact of POC technology enabling rapid clinical decision-making at or near the site of care continues to grow with the shift from reactive to preventative medicine (Wang and Kricka 2018).

1.2. Modifiable and non-modifiable risk factors associated with breast cancer

Based on epidemiological studies, there are multiple risk factors associated with development of breast cancer. Some are non-modifiable meaning that these risk factors cannot be altered by the individual (e.g. inherited genetic variants), while others are modifiable (e.g. BMI) and can be controlled to influence the risk of breast cancer. Modifiable risk factors are lifestyle and environmentally orientated and there is sufficient evidence that obesity is significantly associated with development of breast cancer, especially in postmenopausal individuals (Neuhouser et al. 2015). Protani et al. (2010) found that among breast cancer survivors, obese women experienced poorer survival than those who were non-obese; however, clinical outcomes did not improve with weight loss after diagnosis (Protani et al. 2010). Alcohol consumption increases the risk of developing breast cancer (Khan et al. 2010) as well as recurrence risk in postmenopausal and obese women (Ettinger et al. 2016).

The risk of using hormone replacement therapy (HRT) in breast cancer development is complex and multifactorial (Hou et al. 2013). While the use of oral contraceptives may reduce the risk of colorectal and endometrial cancers, it increases the risk of breast cancer (Gierisch et al. 2013), as does a high BMI, also notable in premenopausal women (Cecchini et al. 2013). Pregnancy before the age of 30 and breastfeeding lowers breast cancer risk, while nulliparity increases the risk (Shapiro et al. 1971). Breastfeeding may be protective due to delayed return of regular ovulatory cycles postnatally, with an estimated risk reduction of 4.3% for every 12 months a woman is nursing (Shapiro et al. 1971).

Moderate exercise has been shown to infer a decreased risk of about 2% for development of breast cancer, while more regular activity is associated with a 5% reduction in risk (Wu et al. 2013). These findings may explain a proportion of the variation in the risk of developing breast cancer.

The major non-modifiable risk factors are being female as male breast cancer accounts for less than 1% occurrence worldwide (Yalaza et al. 2016), and ageing given the fact that most breast cancers are diagnosed after the age of 50 years (DeSantis et al. 2014). Menarche before the age of 12 and menopause after the age of 55 extends the time breast tissue is exposed to hormonal influence and therefore raises the risk. A family history of breast cancer, especially when diagnosed in first-degree or multiple relatives, increases risk, as does a personal history of atypical hyperplasia or carcinoma *in situ* of the breast. Radiation treatment (RT) to the chest area for other malignancies before the age of 30, especially if the women are left with intact ovarian function for ≥ 20 years post-RT, may increase the risk of breast cancer by up to 5 times (Cooke et al. 2013). Breast density is an independent risk factor, with the added disadvantage of not being able to differentiate a mass within the dense breast tissue, resulting in false-negative mammography (Boyd et al. 2011).

A family history of cancer that may be caused by pathogenic gene variants in high penetrance genes such as *BRCA1/2* predisposes women to hereditary breast-ovarian cancer (HBOC). *BRCA1/2* and other high-moderate risk genes such as *ATM*, *CDH1*, *CHEK2*, *PTEN*, *STK11* and *TP53* accounts for approximately 25% of familial breast cancer and less than 10% of all breast cancers (Apostolou and Fostira 2013). Genetically predisposed individuals have a 10 to 20 times greater risk of developing breast cancer than those without pathogenic variants that may underlie diverse tumour molecular pathologies (Nagel et al. 2012), compared to women in the general population who carry a risk for breast and ovarian cancer of 12% and 1.3% respectively (Chen and Parmigiani 2007). Inherited pathogenic *BRCA1* variants afford a lifetime risk of approximately 40% for ovarian and 80% for breast cancer, where the latter are generally found to be triple-negative breast cancers with early age of onset. Similarly, women with highly penetrant *BRCA2* variants are confronted with a lifetime risk of approximately 20% for ovarian and up to 85% for breast cancer, where these tumours are usually hormone receptor-positive with features similar to non-BRCA associated breast cancer (Apostolou and Fostira 2013). Although relatively rare, breast cancer can also affect men with a risk of approximately 6% in cases with pathogenic *BRCA1/2* variants (Evans et al. 2010).

With advancing NGS technologies, the mutational effects of other high penetrance cancer susceptibility genes were further substantiated, such as *TP53* associated with Li-Fraumeni syndrome, *PTEN* mutations in patients with Cowden syndrome and *STK11* associated with Peutz-Jeghers syndrome. Besides their association with developing hereditary breast cancer, these genes confer differing lifetime risks of developing these syndromes or cancer in individuals with pathogenic gene variants (Table 1).

Table 1: Estimated lifetime risk of developing breast cancer associated with pathogenic variants in high penetrance genes with established clinical guidelines.

Gene	The lifetime risk of developing breast cancer
BRCA1	40–80%
BRCA2	20–85%
TP53	56–90%
PTEN	25–50%
CDH1	60%
STK11	32–54%

Moderate penetrant variants are also increasingly detected in genes such as *PALB2, CHEK2*, *BRIP1* and *ATM* in families with breast cancer, accounting for approximately 3% of familial breast cancers (Nagel et al. 2012, Hollestelle et al. 2010, Goldgar et al. 2011). Population studies indicates that breast cancer susceptibility gene variants predominantly found in Polish breast cancer patients are *PALB2, BRIP1, MRE11 and ATM* (DeSantis et al. 2013, Podralska et al. 2018). These genes are normally responsible for maintaining genomic stability (Podralska et al. 2018). Notably, pathogenic germline variants in the *CDH1* gene are associated with hereditary lobular breast carcinoma, but not (ductal) carcinoma of no special type (NOS) (Shrader et al. 2011). Moderate or intermediate-penetrance genes associated with hereditary breast cancer confer variable lifetime risks of developing breast cancer (Figueiredo et al. 2019) (Table 2). Intermediate/moderate penetrant genes present a relative risk from 1.5 to 5, for low-penetrant genes the risk is less than 2, while high penetrant genes are associated with a relative cancer risk of more than 5 (Apostolou and Fostira 2013).

Table 2: Estimated lifetime risk of developing breast cancer associated with pathogenic variants in moderate penetrance genes.

Gene	Lifetime risk
PALB2	20–40%
CHEK2	25–37%
BRIP1	Variable
ATM	15–20%

Low-penetrance risk variants can follow a multifactorial inheritance pattern influenced by lifestyle and other environmental factors. These include single nucleotide polymorphisms (SNPs) in the *H19, TNRC19, MAP3K1, LSPL1, CAS*P8 and *FGFR2* genes found to be significantly associated with breast cancer development (Li et al. 2011, Zheng et al. 2009, Cox et al. 2007 and Milne et al. 2010). While the clinical utility of low-penetrance variants in breast cancer is controversial, their use in a polygenetic risk score (PRS) may identify a subgroup of familial breast cancer cases not explained by a single gene defect. However, the clinical utility of polygenic risk scores will differ between population groups and disease subtypes such as ER-positive and -negative breast cancer. According to a study performed by Mavaddat et al. (2019), stratification of female patients according to clinical risk factors associated with

breast cancer together with the use of a PRS may facilitate improved breast cancer management. The study supports previous findings and suggested that a PRS might improve preventative screening programs (Mavaddat, et al. 2010). Evidence was also provided for incorporating tumour pathology into risk models to facilitate distinction between carriers of pathogenic and benign variants or functional SNPs underlying breast cancer subtypes amenable to precautionary intervention. Breast cancer risk stratification, taking modifiable and non-modifiable factors such as SNPs into account, may identify subsets of a population at greater risk who may benefit from a risk-reducing approach by altering modifiable factors (Maas et al. 2016). While these risk identifying and reducing approaches are important, findings from a study conducted in a rural context in South Africa reported that 69% of the participants had not heard of breast cancer before, only 5.3% ever had any kind of breast cancer screening performed, while holding the belief that they carried no significant risk factors for developing breast cancer (Ramathuba et al. 2015). These authors put forward recommendations of an educational intervention to enhance knowledge about breast cancer, the associated risk factors and symptoms as well as encouraging basic screening methods, such as breast self-examination (BSE).

1.3. Breast cancer screening

One of the most cost-effective screening methods to detect early breast cancer is to conduct a BSE, performed by assessing each breast for asymmetry or nipple discharges as well as feeling the breast to check for lumps or any thickened or swollen areas. Despite the ease of this self-administered test, women have to have some insight into breast health and cancer awareness to conduct a BSE and studies on the African continent report low percentages of women conducting these tests (Abay et al. 2018, Suh et al. 2012). At a tertiary hospital in the Western Cape province of South Africa, 55% of interviewed women indicated that they regularly practised BSE (Moodley et al. 2018).

The effectiveness of a BSE is debatable as the advantages in terms of reducing mortality has not been established (Loh et al. 2013). Most health professionals advise women to do regular BSE to familiarise them with their normal breast anatomy. The 2016 National Comprehensive Cancer Network (NCCN) guidelines endorse yearly clinical breast examination (CBE) for women of average risk after 40 years of age (Loh et al. 2013). Due to South Africa being a resource-poor region, a national mammography-screening program is not currently in place and is mainly opportunistic or limited to symptomatic or high-risk patients in the public sector. The goal of breast imaging is to detect early malignancies and differentiate these from non-malignant breast disease.

1.3.1. Mammography

The first randomized control trials which compared periodic mammography screening with clinical examination confirmed a reduced mortality of roughly one third in the experimental group (Shapiro et al. 1971). The 2016 NCCN guidelines endorsed yearly mammography for women of average risk to

commence from the age of 40 years, with annual screens at 25 years of age for women who are identified at higher risk for developing cancer (Ettinger et al. 2016). However routine mammography can lead to needless stress and false-positive results, leading to unnecessary surgery with no reduction in mortality (Shapiro et al. 1971, Gøtzsche et al. 2013). There is a growing awareness of a larger population of women for whom mammography has decreased sensitivity, notable in the younger age groups where it is most commonly due to dense breasts (Gøtzsche et al. 2013). For this reason, these patients are screened using alternate technologies such as magnetic resonance imaging (MRI) and automated whole breast ultrasound (Kelly et al. 2010). Ultrasound is performed on pregnant women, patients younger than 30 years of age or for those who are lactating (Kelly et al. 2010). The increased detection of breast cancer by ultrasound following screening mammography suggests the added advantage of this technique for women with an average risk as well as high-risk patients with dense breasts (Berg et al. 2008, Chae et al. 2013). MRI can be used for evaluation of inconclusive findings or where mammographic evaluation is limited, for example in patients with breast implants. Furthermore, MRI is useful for monitoring neoadjuvant chemotherapy treatment response as well as identifying the presence of minimal residual disease post lumpectomy. According to Lee et al. (2010), screening using both MRI and mammography can decrease the rate of mortality in breast cancer patients. Reidl et al. (2015) reported that MRI is effective in the detection of familial breast cancer irrespective of the patient's age or breast density. Furthermore, there is no added value in using mammography and ultrasound in patients who are screened using MRI (Kelly et al. 2010). MRI is the preferred screening method for cancer detection in healthy individuals with pathogenic *BRCA1/2* variants (Buchanan et al. 2018).

1.3.2. Genetic counselling for risk evaluation

Further investigations into breast cancer risk would require the services of genetic counsellors who are trained to identify patients with a strong family history of cancer who may benefit most from genetic testing in a family context. Genetic services in South Africa, especially in rural areas, do not meet the genetic needs of the local population (Kromberg et al. 2012), where the responsibilities of counsellors are often carried out by other healthcare professionals who lack the formal specialized training (Gøtzsche et al. 2013). Inherited *BRCA1* and *BRCA2* mutations are identified respectively in about 52% and 32% of families where four or more members have breast cancer, while somatic mutations in these genes are rarely detected in patients presenting with sporadic breast cancer (De Silva et al. 2019). However, a negative family history or late-onset disease may eliminate a patient from testing and should not be the only criteria used for mutational analysis of *BRCA1/2*. A study determining the prevalence of these mutations in sporadic breast/ovarian cancer patients showed a relatively high mutation detection rate (42.9%), including the identification of a de novo pathogenic *BRCA1* variant in a patient older than 50 years of age (De Leeneer et al. 2012).

Research on the genetics of breast cancer in South Africa was initially focused on detection of pathogenic variants related to a founder effect, resulting in an increased frequency of certain *BRCA1/2* mutations in Afrikaners of European descent (Reeves et al. 2004). To date, at least eight pathogenic *BRCA1/2* founder/recurrent mutations have been identified in the South African population across ethnic groups (Table 3) (Reeves et al. 2004, Sluiter and van Rensburg 2011, van der Merwe et al. 2012b, Francies et al. 2015). The *BRCA1* c.68_69delAG variant shares the same haplotype among Ashkenazi and Iraqi Jews characteristic of a founder effect but arose independently in several other population groups due to recurrent mutational events (Bar-Sade et al. 1998).

Table 3: BRCA1/2 founder/recurrent mutations associated with hereditary breast cancer in the South African population, with variants shown in brackets provided in the bank identifier code (BIC) format.

Gene	**Region**	**dbSNP ID Number**	**Nucleotide Change** (HGVS Nomenclature Bold)	**Protein Change**	**Population**
BRCA1 NM_007300.4	Exon 2	rs80357914	**c.68_69delAG** [c.185delAG]	p.Glu23Valfs	European
	Exon 11	rs397508862	**c.1374delC** [c.1493delC]	p.Asp458Glufs	Afrikaner
		rs397508988	**c.2641G>T** [c.2760G>T]	p.Glu881Ter	Afrikaner
	Exon 20	rs80357906	**c.5266dupC** [c.5382insC]	p.Gln1756Profs	European
BRCA2 NM_000059.3	Exon 11	rs80359535	**c.5771_5774delTTCA** [c.5999delTTCA]	p.Ile1924Argfs	Xhosa, Mixed Ancestry
		rs80359550	**c.5946delT** [c.6174delT]	p.Ser1982Argfs	European
		rs397507858	**c.6448_6449insTA**	p.Lys2150Asnfs	Mixed Ancestry
	Exon 17	rs80359688	**c.7934delG** [c.8162delG]	p.Arg2645Asnfs	Afrikaner, Mixed Ancestry

Breast cancer patients have an approximately 17% increased risk of developing a second cancer (Molina-Montes et al. 2015). *BRCA1/2* mutation carriers are more prone to multiple malignancies which may appear as either synchronous or metachronous. The former is defined as two or more primary malignancies coexisting at the time of diagnoses or develop within six-months period, and the latter as

developing years after resection of the first primary cancer (Kim and Song 2015). In breast cancer patients, it is important to distinguish whether the contralateral or other lesion is metastatic or a second primary tumour. This distinction is not always clear (Kromberg et al. 2012). Chaudary et al. (1984) proposed criteria for the diagnosis of a second primary breast cancer in 1984 as follows: (i) there must be an *in situ* change in the contralateral tumour, (ii) the tumour in the second breast is histologically different from the cancer in the first breast, (iii) the degree of histological differentiation of the tumour in the second breast is distinctly greater than that of the lesion in the first breast, (iv) there is no evidence of local, regional, or distant metastases from the cancer in the ipsilateral breast (ipsilateral:tumor recurrence on the opposite breast). Despite recently developed novel methods such as cDNA microarray-based comparative genomic hybridization (CGH) that could be applied to assist in distinguishing a second primary cancer from the metastatic lesion, Chaudary's criteria remains the most widely accepted to date.

Additional features which could possibly influence the risk of a second cancer include age and lifestyle factors, such as body weight and alcohol intake, as well as subsequent tumour genetic alternations or the toxic effects of radiation and chemotherapy delivered during the course of the primary treatment (Sas-Korczyńska et al. 2017). Patients with primary malignant tumours should be meticulously followed up and counselling could provide risk assessment with suggested prevention strategies. All secondary tumours should be evaluated using histopathology and genetically profiled as the cancer genome often expresses differently in the metastasis, requiring different and targeted therapies (Mehdi et al. 2010).

1.4. Interventions relevant to the detection of pathogenic *BRCA1/2* variants

Patients who test positive for a pathogenic *BRCA1/2* variant are faced with risk-reducing options which have a significant impact on their psychosocial wellbeing as well as the quality of life of their family members (Jeffers et al. 2014). Women may feel less feminine after risk-reducing surgical interventions have been performed, although it results in reduced anxiety about cancer risk (Gopie et al. 2013). In high-risk individuals with pathogenic gene variants, risk-reduction surgery reduces breast cancer risk by 85% to 100% and breast cancer mortality rate by 81% to 100%, compared to patients without surgery. In *BRCA1/2* mutation carriers, prophylactic oophorectomy may reduce the risk of ovarian, fallopian tube or peritoneal cancer by 80%, as well as significantly decrease mortality (Finch et al. 2014).

Although risk reduction and therapeutic options for patients with advanced disease are well-established, optimal management of early-stage breast cancer patients with pathogenic *BRCA1/2* variants remain unclear. Sporadic breast cancer may require different adjuvant chemotherapy, even when pathological features are similar to *BRCA1/2* associated breast cancer. The lack of benefit or response of hormone

receptor-positive breast cancer to adjuvant chemotherapy can be predicted by using multigene genomic assays such as the 70-gene (MammaPrint) or 21 gene (Oncotype DX) expression profiles that have the ability to generate a prognostic recurrence score (Cardoso et al. 2016; Sparano et al. 2019). Less favourable intrinsic factors are associated with pathogenic germline *BRCA1/2* variants detected in patient referrals with hormone receptor-positive breast cancer (Shah et al. 2016). The use of NGS on tumour DNA extracted from cancer biopsies (solid or liquid) have promising capabilities of identifying the cause for underlying differences (Kanagal-Shamanna et al. 2014). Use of only 10 nanogram of DNA to perform NGS mutational profiling on cell blocks or fine needle aspiration cytological smears were shown to have high sensitivity to detect clinically relevant variants in the *APC, ATM, CDKN2A, CTNNB1, FGFR2, FLT3, KDR, KIT, KRAS, MLH1, NRAS, PIK3CA, SMAD4, STK11* and *TP53* genes than traditional platforms with high sample requirements.

1.5. Diagnosis and treatment of breast cancer subtypes

Routine pathology testing is performed for all patients presenting with suspected breast cancer. This may initially be performed using fine-needle aspiration cytology, followed by a tissue biopsy often performed at mammography. Tissue biopsies are placed in fixatives and sent to a consulting histopathologist who confirms the diagnoses and type of breast cancer, as well as reporting on commonly used prognostic markers, including ER and PR, HER-2 and Ki-67 (Senkus, et al. 2015).

The sex steroidal hormones are produced by the ovaries, and estrogen plays a significant role in the growth, development and differentiation of normal breast tissue as well as a stimulatory role in the development and progression of breast cancer. ER and PR are nuclear hormone receptors that act as transcription factors in breast epithelial cells. When attaching to their respective receptors, ER/PR stimulate the formation of proteins within the cell, which in turn influence the growth and function of breast cells (Tafe et al. 2014). ER is overexpressed in about 70% of breast cancers and is evaluated microscopically in tumour samples using IHC staining. When the cells are found to express either ER or PR by a positive immunostaining reaction, the diagnoses of hormone receptor-positive breast cancer is made where either or both of these hormones are regarded as the key drivers of the malignancy. These cancers respond to the accompanying hormonal signals which enhace cellular proliferation and tumour growth. Hormonal or endocrine therapy is offered to these patients in the form of aromatase inhibitors or Tamoxifen, a selective estrogen receptor modulator (SERM). Depending on the risk profile, some patients are offered a combination of Tamoxifen and ovarian suppression, if they are premenopausal. However, in postmenopausal patients and when there is loss of ovarian function, aromatase inhibitors are prescribed as first-line endocrine therapy, which block the action of the enzyme aromatase in turning androgen into estrogen, effectively making less estrogen available (Awan and Esfahani et al. 2018). SERMs are drugs such as Tamoxifen and Raloxifene which act as antagonists in breast tissue, meaning that they interfere with the ERα transcriptional activity by blocking the physiological action of estradiol

and displacing it from ER, thereby mitigating the proliferative action of the hormone on the cancer cells. With impeded mitogenic activity, cells are unable to divide resulting in decreased tumour size as well as reducing the risk of cancer recurrence and distant metastasis (Keen and Davidson. 2003). Interestingly, Lui et al. (2014) have shown that Tamoxifen may induce apoptosis in ER-negative breast cancer cell lines via a novel mechanism of CIP2A-dependent p-Akt inhibition, which may partly explain why some ER-negative tumours respond to Tamoxifen treatment (Osborne. 1998). This finding suggests that Tamoxifen may induce other pathways related to anti-tumour activity.

Aromatase inhibitors can affect the action of aromatase in fat, liver, and muscles, but cannot block the amount of aromatase present in ovaries. For this reason, the ovaries must be inactive for the therapy to be most effective (Słopień and Męczekalski 2016). Both aromatase inhibitors and Tamoxifen reduce estrogen levels, which impede cancer cell growth and can be used in the metastatic as well as the adjuvant setting. This treatment provides considerable benefit by reducing breast cancer recurrence and improving associated mortality in patients with endocrine responsive breast cancer (Kadakia and Snyder 2016). However, some breast cancers do not express any hormone receptors, may only express one of the two receptors or vary in the levels of expression from a strong to a weak immunostaining reaction. ER-positive tumours generally do not respond as well to chemotherapy as ER-negative breast cancer.

Determining HER2 tumour status is an equally important prognostic and predictive factor. HER2 is a member of the epidermal growth factor receptors and a membrane tyrosine kinase involved in cellular growth and overexpressed in approximately 20- 30% of breast cancers. Malignancies which are found to be HER2 positive tend to be fast-growing, aggressive tumours with a high tumour grade. The HER2 protein is expressed on the cell surface of normal breast epithelial cells and binds to growth factors which in turn stimulates cell division. Breast cancer tumour cells with a positive HER2 status have abnormally high levels of HER2 protein expression on their surface, resulting in increased proliferation with anti-apoptotic signals, which in turn drives tumour development and disease progression. Importantly, HER2 positive breast cancer responds to and can be treated with a targeted monoclonal antibody Trastuzumab (Herceptin), which binds to the HER2 receptor and reduces cellular replication and angiogenesis, and induces cell cycle arrest with an anti-tumour immune-response (Fiszman and Jasnis. 2011, Ross, et al. 2009). This has been shown to be effective in the adjuvant (Smith at el. 2007) as well as neo-adjuvant settings by shrinking and down staging breast cancer tumours (Goldhirsch. 2013).

1.5.1. Routine laboratory assessment of hormone and HER2 receptor status

As nuclear receptors, the ER and PR are commonly assessed using the Allred system of scorning, which is only performed on the invasive malignant tumour cells as ER/PR positive staining may be evident in the surrounding normal breast tissue as well. This system uses two scores based on the proportion of

positive staining cells (PS) in addition to the intensity at which they have stained (IS) (Qureshi and Pervez 2010). Proportion score classifies the percentage of the stained cells into six groups, where a score of zero indicates no staining and a score of 5 indicates that at least 67 to 100% of the cells are positive for hormone receptors (Table 4).

Table 3: Allred scoring system based on the proportion of estrogen receptor (ER-positive stained cells.

Score	Percentage of stained cells
0	ER-negative
1	<1% cells are ER positive
2	1-10% of cells are ER-positive
3	1-33% of cells are ER-positive
4	34-66% of cells are ER-positive
5	67-100% of cells are ER-positive

Score 0: no positive cells, score 1: < 1% of cells are positive, score 2: 1%-10% of cells are positive, score 3: 11-33% of cells are positive, score 4: 34-66% of cells are positive and score 5: 67-100% of cells are positive.

The intensity score (IS) categorises the staining intensity into classes, which are negative (0), weak (1), intermediate (2) or strong (3) (Qureshi and Pervez 2010). The final Allred score (AS) is calculated by adding the proportion score and intensity score, where 0 – 1 is regarded as negative and 2 – 8 as positive. A breast cancer tumour is regarded as hormone receptor-positive with any positive staining result for either ER, PR or both receptors. The potential benefit of hormonal therapy is reflected in increasing Allred scores (Table 5).

Table 4: Allred intensive scoring method for potential benefit from hormonal therapy.

Allred score	Effect of hormone therapy
0-1	No effect
2-3	Small (20%) chance of benefit
4-6	Moderate (50%) chance of benefit
7-8	Good (75%) chance of benefit

To determine HER2 tumour status, breast cancer tissue is microscopically examined and scored from 0 to 3 depending on the intensity of cell membrane IHC staining, where a score of zero or 1+ confirms a HER2-negative status. An immunostaining score result of 3+ is regarded as a positive HER2 tumour status while that of 2+ is deemed equivocal and needs to be further tested by *in situ* hybridization (ISH) to confirm HER2 gene amplification. Although fluorescence *in situ* hybridization (FISH) is the most

commonly used technique, other technologies include chromogenic or silver-enhanced ISH that are equally acceptable methods employed for confirming HER2 status. Chromogenic ISH uses a peroxidase enzyme labelled probe for visualisation while silver-enhanced ISH incorporates a silver-based detection on the same system. Advantages of the latter is that standard bright field microscopy can be used thereby avoiding the problem experienced with fading of fluorescent dye in the tissue samples. FISH is a cytogenetic procedure that uses fluorescent probes to identify targeted DNA sequences in formalin-fixed paraffin embedded (FFPE) tissue sections. The technique uses single-colour for the assessment of HER2 gene copy number per nucleus or a dual-colour assessment where chromosome 17 centromere and HER2 probes are differently labelled making the calculation of the HER2/chromosomal 17 ratio possible. Patients with ISH HER2/centromeric probe 17 (CEP17) ratio $\geq$ 2.0 or with a HER2 copy number > 6.0 qualify for anti-HER2 therapy. When HER2 ISH results are reported as equivocal, a reflex test should be requested and analysed on the same sample.

The American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) first issued guidelines on ISH HER2 testing in breast cancers in 2007 and has since added revisions in 2013 and 2018. These revisions are aimed at incorporating new knowledge to make the diagnosis of HER2 more definitive, resolving clinical dilemmas that impact otreatment decision making, as well as an attempt to standardise laboratory testing procedures and reporting.

1.5.2. Surrogate molecular subtyping

The four major intrinsic/molecular breast cancer subtypes, luminal A, luminal B, HER2-enriched and basal-type, were originally described based on genomic microarray analysis (Perou et al. 2000). Following this discovery, less expensive IHC tests were shown to provide a reasonable approximation of these subtypes, based on expression of ER, PR and HER2. This pathology-only approach translates into categorising invasive breast cancer into luminal, HER2-positive, and triple-negative subtypes. These categories may be better approximated by incorporating proliferating index (Ki-67) immunostaining to determine the percentage of malignant cells that stain positive for this nuclear proliferative marker. Cancers expressing high levels of Ki67 tend to correlate with poor clinical outcome. When combined with results obtained from cytokeratin 5 and epidermal growth factor receptor (EGFR) markers, luminal tumours are stratified into luminal A and B subtypes, while triple-negative tumours are separated from the basal-like subtype (Tang and Tse 2016). IHC molecular classification is limited by lack of standardisation in terminology, use of biomarkers included for analysis and agreement on cut-off values for each IHC biomarker.

Hormone and HER2 receptors as well as Ki67 assessment of FFPE breast cancer tumour specimens may be challenging to perform and standardise, while automated platforms such as the GeneXpert, using the Xpert Breast Cancer STRAT4 assay, may provide a less labour intensive method for assessment of these markers as well as faster turnaround time. This semi-quantitative real-time

polymerase chain reaction (RT-qPCR) based testing platform is used in research as well as clinical settings. The Xpert Breast Cancer STRAT4 assay enables qualitative measurements for ER, PR, HER2 and Ki 67 mRNA from FFPE specimens and has been shown to correlate well with central IHC/FISH tumour testing (Wu et al. 2018). The samples need to be prepared by an experienced anatomical pathologist to ensure suitable FFPE material for assessing the mRNA levels of ESR1, PGR, ERBB2, and MKi67. The reference gene, CYFIP1, serves to verify specimen adequacy and is used to normalise mRNA expression levels of the four genes analysed simultaneously in the kit. Breast cancer FFPE tissue samples are prepared as tissue sections on glass slides and treated with a lysis kit prior to use. Once the tissue lysate has been prepared, an aliquot is placed into the appropriate sample chamber in the assay cartridge and placed into the GeneXpert instrument for processing. A probe check control (PCC) is used to verify reagent rehydration, real-time PCR tube-filling in the cartridge, probe integrity and dye stability. In total, the assay utilises six fluorescent channels for target or control detection in a distinct channel with its own detection cut-off parameters for target detection. The GeneXpert instrument consists of a barcode scanner and a personal computer with preloaded software for performing the test and viewing the results.

The purpose of molecular or IHC sub-typing is to identify the likely outcomes for each patient. Breast cancer patients with luminal type tumours are ER/PR-positive, respond well to endocrine therapy and have a favourable prognosis (Parise et al. 2014). Endocrine therapy is generally prescribed for luminal-type tumours, where pre-menopausal women are offered Tamoxifen and post-menopausal breast cancer patients are usually advised to use aromatase inhibitors. Breast cancer patients with luminal B tumours are more often diagnosed at a younger age than those with luminal A tumours and tend to have a poorer prognosis. Basal-type breast cancers are usually triple-negative tumours (ER-, PR-, and HER2-) and are mostly detected in younger women. These tumours display an aggressive immunophenotype and have a poorer prognosis when compared to the luminal A and B subtypes. A subgroup of HER2 positive tumours is reclassified by genomic testing as HER2-enriched, with a tendency to be lymph node-positive at diagnosis. On a DNA level, HER2-enriched tumours demonstrate the highest number of mutations expressed in the genome, with 73% and 39% of these tumours found to be PIK3CA and TP53 genetically altered, individually. Although 68% of HER2-positive tumours show HER2 overexpression, identification of HER2-enriched subtypes among HER2-negative breast cancer may occur when more genes are analysed beyond the limitations of single-gene IHC (Roberts et al. 2013). HER2-positive disease is caused by the amplification the HER2 gene which leads to uncontrollable cell growth and has a higher recurrence rate than HER2-negative tumours (Slamon et al. 2011). They show poor tumour grading and are diagnosed at a younger age compared to the luminal A and B subtypes. Anti-HER2 treatments such as Herceptin are more effective in breast cancer patients with the HER2-enriched compared with luminal B HER2-positive tumours (Ross et al. 2009, Myburgh et al. 2016).

1.5.3. Molecular subtyping using microarray analysis

Breast cancers are classified as luminal on histopathology when found to be ER+/HER2-negative. However when using the 80-gene BluePrint assay for molecular subtyping, a subset of these tumours can be reclassified as ER+/basal, high genomic recurrence risk tumours (Whitworth et al. 2017). Once reclassified, these ER+/basal cancers show a significantly higher pathologic complete response (pCR) compared to ER+/luminal A or B molecularly classified tumours, while displaying a similar pCR to ER-negative/basal patients in the neoadjuvant setting (Groenendijk et al. 2019). Reasons for this reclassification may be due to borderline ER positivity on routine assessment, or the inability of IHC or mRNA to separate functional ER from non-functional ER as many ERα variants are present in human tissue displaying varying degrees of activity. For this reason, genomic risk assessment using the 70-gene assay or the 21-gene assay will not identify this group of clinically relevant patients, which can be classified by addition of the molecular subtyping 80-gene signature, BluePrint.

The value of combining MammaPrint with BluePrint was demonstrated in South African patients with early-stage breast cancer based on the following findings of Grant et al. (2019): 1) Neither IHC nor single-gene genomic mRNA reporting of ER/PR status can replace the combined use of these two tests for molecular subtyping; 2) Reliable distinction between luminal A and B type tumours is not possible using IHC or single-gene ER/PR/HER2 genomic mRNA assessment; 3) IHC combined with microarray gene profiling enables the identification of endocrine treatment resistant ER/PR-positive tumours lacking ERα function (basal-like), despite positive expression at the protein and single-gene RNA level. Several studies reported that the 21-gene breast cancer recurrence score has value in ER+/HER2 negative breast cancers, but may contribute similar information to that received from more cost-effective histopathological reports and surrogate molecular subtyping tools, including novel nomograms which use the commonly reported clinicopathologic variables (Orucevic et al. 2017).

Determination of ER, PR and HER2 status forms part of the MammaPrint pre-screen algorithm (MPA) developed as a cost-saving strategy for microarray gene profiling in South Africa (Grant et al. 2013), in a similar way that eligibility for *BRCA1/2* screening may be based on age of onset and family history (Schoeman et al. 2013). Subsequent studies performed in the UK and USA (Slade et al. 2016; Sun et al. 2019) demonstrated a cost-effective implementation of inherited cancer screening in unselected breast cancer patients when *BRCA1/2* testing is performed as an integral part of oncology practice. This finding raised the possibility that use of the pathology-supported genetic testing (PSGT) platform (Figure 1) to incorporate germline genetic testing for patients referred for the MammaPrint test may improve clinical management in a subgroup of high-risk patients that may not otherwise be considered for *BRCA1/2* gene screening.

1.6. Pathology-supported genetic testing

Irrespective of whether a patient presents with a risk of cancer development or with a nodule suspicious of breast cancer, the clinician is required to perform a clinical assessment and interpret these findings in the context of the personal and family history. Unsuitable or irrelevant testing needs to be avoided and only that which is deemed necessary and able to add preventive, diagnostic, prognostic or therapeutic value should be conducted after this initial assessment. If breast cancer is suspected, a tissue biopsy is required for histopathological confirmation, and by combining the results of the clinicopathological parameters a cancer management strategy is developed for the patient. Although the pathology report may not always allow a clear clinical decision on the use of adjuvant chemotherapy, it provides the basis for identification of the need for tumour genomic testing. Inappropriate use of the MammaPrint test can be avoided by using the PSGT approach described by Grant et al. (2013, 2019). By combining recurrence risk assessment using MammaPrint with molecular subtyping using BluePrint, additional information not provided through routine pathology testing and germline genetic testing is obtained on which to base anti-cancer therapy (Figure 2). Tumour gene expression profiling and NGS is used to determine the underlying breast cancer biology, which in turn assists decisions regarding chemotherapy use and more accurately suggest use of targeted agents such as Herceptin aimed at interrupting the active pathways within the tumour genome. Germline DNA testing may furthermore improve clinical outcome by identifying potential therapy-related risks associated with genetic variation in drug metabolizing enzymes such as CYP2D6, which is associated with Tamoxifen resistance. In Tamoxifen-treated patients who have experienced relapse, metastatic or metachronous malignancies, despite being classified with low-risk tumours by genomic profiling, *CYP2D6* genotyping or concomitant medication affecting enzyme activity may provide an explanation for poor clinical outcome. Patients referred for genomic tests such as MammaPrint, BluePrint or OncoType DX may also benefit from *BRCA1/2* testing regardless of the lack of family history, as such cases could be offered alternate or additional targeted therapies such as PARP inhibitors. Clinicians should be encouraged to use the opportunity to advise screening for other family members at risk of developing breast cancer or other *BRCA* related malignancies.

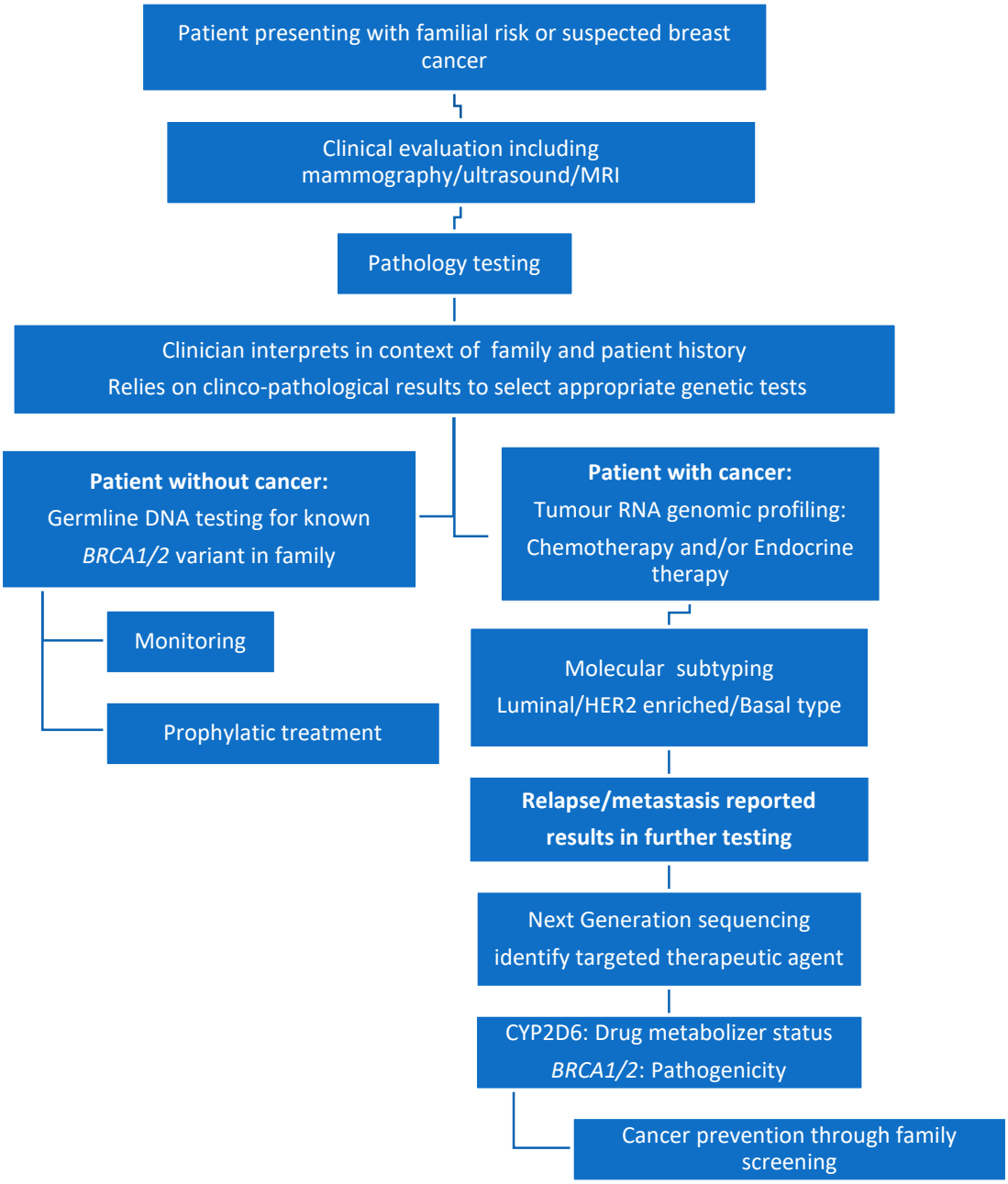

Figure 2: Schematic diagram illustrating the concept of pathology-supported genetic testing (PSGT) where routine clinical and pathology reports are evaluated to determine the appropriateness of breast cancer genetic/genomic testing. The PSGT approach incorporates multiple assay platforms to facilitate improved quality assurance on which to base treatment decisions. Clinical observations and family history identify the need for germline testing of individuals at risk of developing breast cancer, therapeutic risk or not gaining optimal benefit from certain drugs used in the treatment of endocrine positive breast cancer. In cases of therapeutic failure, BRCA1/2 testing may reveal pathogenic variants in cases who could be offered alternative therapies to improve outcomes or risk reduction of developing metachronous malignancies. Genetic screening of family members is advised to prevent them from developing BRCA1/2 related cancers.

Use and improvement of the PSGT platform has been a focus of breast cancer research since the 70-gene MammaPrint microarray test became available in South Africa, following FDA approval in 2007. Development of local referral criteria led to reimbursement of the MammaPrint by medical schemes in South Africa (Grant et al. 2013). An important requirement was to establish a sustainable breast cancer genomics database (accessible to registered users at www.gknowmix.org) for monitoring of clinical outcome and ongoing comparative effectiveness studies (Grant et al. 2019).

Traditional clinicopathological factors assessed in all newly diagnosed breast cancer patients, such as age, number of positive axillary nodes, tumour size, grade, ER, PR, HER2 and Ki67 status, are important for prognostic reasons and hold important therapeutic implications. However, patients with similar clinicopathological characteristics may experience remarkably different disease outcomes, indicating the inability of these standard indicators to fully explain the biological complexity of or accurately reflect the heterogeneity of breast cancer. In this context, genomic technology has advanced our understanding of genetic variation in tumour gene expression and driver mutations.

The use of adjuvant systemic therapy has resulted in a steady decrease in breast cancer mortality. Neoadjuvant chemotherapy may be administered before surgery to shrink the tumour *in situ*, affording more surgical options. Adjuvant chemotherapy is administered after breast cancer surgery and is intended to prevent recurrence of the disease, particularly distant recurrence (Rampurwala et al. 2014). Chemotherapy may also be suggested when patients with invasive breast cancer have unfavourable prognostic factors, or if there is spread to nearby lymph nodes. Although the use of these chemotherapeutic treatments has demonstrated improved survival, not all patients benefit from it.

1.7. MammaPrint

Many patients with early-stage breast cancer suffer side effects of chemotherapy without optimal benefit, while increasing the economic burden on health care. MammaPrint allows accurate distinction between patients at low and high risk of developing distant metastases and could identify those patients most likely to benefit from adjuvant therapy (van de Vijver et al. 2002, Buyse et al. 2006). Breast cancer patients classified as high risk for recurrence was shown to have a less than 50% chance of survival after 10 years and less than 44% chance to be metastasis-free after 10 years without adjuvant treatment. In comparison, patients classified as low risk of recurrence had a 97% chance of survival after 10 years and 87% chance to be metastasis-free after 10 years without adjuvant treatment. Br[i]east cancer patients with a poor prognosis profile based on the 70-gene MammaPrint assay were shown to benefit from administration of chemotherapy (Straver et al. 2010). Although several multi-gene assays are commercially available for assessment of recurrence risk in early-stage breast cancer patients, MammaPrint is the only test to date that have both been cleared by the FDA and awarded level 1A clinical utility evidence for chemotherapy benefit in early-stage breast cancer patients with up to three nodes involved. The FDA does not evaluate tests for clinical utility, only for analytical and clinical validity which is based on the prognostic value in the case of MammaPrint (Slodkowska et al. 2009).

Several retrospective studies have shown that MammaPrint can predict that patients with a low-risk gene profile can safely avoid chemotherapy. Mook et al. (2010) explored the use of the 70-gene assay to predict clinical outcomes in breast cancer patients with lymph node invasion. The study showed that the 70-gene assay is expressively superior to histological grading, ER status prediction, and to lymph

node invasion. The authors recommended the use of MammaPrint in patients with node-positive breast cancer. Whitworth et al. (2017) compared MammaPrint/BluePrint intrinsic subtyping with clinical IHC/FISH subtyping in clinical luminal breast cancer patients to predict treatment sensitivity. This study revealed that there was a pathological complete response for clinical luminal patients to neoadjuvant chemotherapy, with reclassification of approximately 20% patients by the BluePrint test to the basal subtype type.

The MINDACT (Microarray In Node negative Disease may Avoid Chemotherapy) trial was a prospective, randomised, phase III controlled clinical trial, designed in 2005 to assess the clinical utility of the MammaPrint genomic assay. The trial demonstrated that the patients who were considered high risk based on traditional and clinical-pathological features, but have a low-risk genomic signature and did not receive adjuvant chemotherapy, had a 5-year distant metastatic free survival rate of at least 92% or more (Cardoso et al. 2016). It was concluded that chemotherapy may not be required in about 46% of women diagnosed as clinically high-risk, early-stage breast cancer. The results of the MINDACT trial provides the highest level of evidence to support the use of MammaPrint as an accurate breast cancer assay in a subset of clinically high-risk patients.

Conventional histopathological subtyping of breast cancer tumours using IHC and FISH has limited reproducibility and accuracy and cannot determine intrinsic molecular subtypes accurately. The BluePrint molecular subtyping profile determines the mRNA levels of 80 genes that best discriminate between luminal A and B breast cancer subtypes, HER2 enriched and basal-type. Each of these genetically determined intrinsic subtypes has marked differences in long-term outcome and response to chemotherapy. The BluePrint assay was validated using four independent validation cohorts including 784 patients studied and offers a further stratification of the luminal subgroup into types A and B when used in combination with MammaPrint, which is critical in determining the need for chemotherapy (Whitworth et al. 2014). Patients with the luminal A molecular subtype can avoid chemotherapy while patients with a MammaPrint high-risk profile are equivalent to luminal B and may be offered chemotherapy (Whitworth et al. 2014). HER2-enriched is one of the four main molecular subtypes of breast cancer that can further be stratified by the 80-gene BluePrint profile as a valuable addition to the 70-gene MammaPrint profile (Whitworth et al. 2014). Although several prognostic and predictive tests have been approved for clinical use, their translation into routine clinical practice is not straight forward and requires processing and interpretation at specialised facilities.

1.8. Limitations to predict tumour response to any specific anticancer agent

Many pathology tests used in clinical practice have limited ability to predict tumour response to a specific anticancer agent. With improved genomic microarray or NGS analyses, it is possible to more accurately predict potential benefit of therapy targeted at the individual tumour biology. Biomarkers

can be used to assist in clinical decision making by identifying the most appropriate therapy targeted to a particular cancer. In cases where the application of traditional clinicopathological risk factors or genomic profiling tools such as MammaPrint reflected a low risk for breast cancer recurrence, and patients did not receive chemotherapy, it may also help to explain drug resistance and why a small number of breast cancer patients relapse. In order to effectively treat metastatic tumours based on molecular alterations which may not have been present in the primary tumour, tumour DNA could be sequenced for identification of actionable gene targets (Bombonati et al. 2011). The aim is to identify specific mutational events expressed in metastatic breast cancers in an attempt to match and personalise treatment plans. Biomarkers can be used to assist in clinical decision making for selection of the most appropriate therapy targeted to a particular cancer.

Several NGS applications with varying degrees of evidence have become commercially available over recent years. One of these tests offered to South African patients called OncoDEEP, combines NGS with IHC tailored to a specific cancer in order to identify treatment targets at both the protein and tumour DNA level. An international study published by Laes et al. (2018) on the performance of this integrated approach includes South African data from breast and other solid cancers. A combined genomic assessment allows for the complete profiling of the tumour and tailors the matching treatment to target-specific immunotherapy and chemotherapy. However, the clinical utility of NGS results reported to the ordering clinician and the extent to which different genomic applications compare with each other remains largely unknown. A key issue in clinical oncology practice is the ability to accurately interpret pharmacogenomic reports in order to distinguish clinically meaningful results from those that are not actionable. Treating clinicians have time constraints and require concise reporting of clinically relevant results that may be considered by some as an elusive target due to the ever-changing cancer genomics landscape.

In a study by Weiss et al. (2015) using the Foundation One (F1) and Paradigm Cancer Diagnostic (PCDx) tests on FFPE tumour tissue of 21 cancer patients, genomic data generated by different laboratories using the same samples were compared. The F1 test returned information on chromosomal abnormalities, somatic mutations, insertion and deletion polymorphisms, and DNA copy number variants at approximately 250 times coverage, while PCDx generated similar data at a much higher (5,000x) coverage, in addition to mRNA expression levels. Differences in turnaround time (TAT) was noted as one of the most important considerations for patients with progressing metastatic cancer; the longer the delay in initiating treatment the shorter the window of opportunity to change the disease course. A higher percentage of clinically relevant actionable targets were reported with the PCDx assay using the Ion PGM sequencer in relation to commercially available drug or clinical trial drugs, with some discrepancies noted. KRAS and ERBB2 variants were missed by PCDx as the gene regions spanning these specific alterations were not included in the platform at the time of the study. However, the resulting categorization for PCDx was the same as F1 since none of the mRNA targets indicated

available commercially or clinical trial drugs at the time of the study. Conversely, elevation of TOP2A mRNA confirmed clinical actionability through expression studies. These contrasting findings raised questions about variants detected in tumour DNA when there are no indication of protein expression of the relevant biomarkers, which could be misleading. Addition of an expression measurement to NGS platforms is therefore important to identify clinically relevant treatment targets. Indeed, after the results of Weiss et al. (2015) had been published, both the KRAS and ERBB2 variants were added to the PCDx platform in addition to several actionable IHC biomarkers, including AR, ER, HER2, MET, MGMT, PTEN, PR, TOP1, TYMS, PD-L1, and PD-1. In this comparative study, clinically relevant actionable targets were identified in 47%–67% of diverse cancer types at a total charge of US $4,800 for PCDx to medical insurance, versus US $5,800 for the F1 test per sample. To improve cost-effectiveness, the following selection criteria were defined as appropriate for tumour NGS at the time: 1) stage 4 solid tumor with 2) progression on at least one line of standard therapy, or 3) no standard of care available for the type of cancer diagnosed.

The pathology of breast cancer may not only affect the decision about tumour DNA or RNA analysis, but also germline genetic testing. ER/PR status was an important consideration for development of a clinical pipeline used to identify the target population most likely to benefit from aromatase inhibitors or tamoxifen pharmacogenetics in South African breast cancer patients (Baatjes et al. 2017). Targeting genetic/genomic testing to individuals with a high chance for a positive result improves the likelihood of demonstrating cost-effectiveness and overall clinical utility of new molecular technologies.

1.9. From tumour to germline genetics

The clinical utility of the MammaPrint microarray for assessment of metastatic potential (Cardoso et al. 2016), and single-gene *BRCA1/2* mutation testing of tumour or germline DNA as a treatment target for PARP inhibitors (Dziadkowiec et al. 2016) or to assess risk for familial breast cancer (Møller et al. 2014), is well established. However, the use of NGS on tumour DNA extracted from breast biopsies (solid or liquid) for targeted therapies requires further research (Cummings et al. 2016). The clinical utility of *CYP2D6* genotyping for determination of Tamoxifen resistance is, for example, less clear than the pharmacogenetic effect on response to anti-depressants, which led to an implementation study in South Africa (van der Merwe et al. 2012a). This translational research performed in breast cancer patients considered for concomitant treatment with Tamoxifen and antidepressants that may inhibit CYP2D6 enzyme function, increased the awareness of clinicians about the potential benefits of multi-gene testing to facilitate prevention of cumulative risk (Baatjes et al. 2017).

Detection of bilateral breast cancer or multiple primary tumours in a patient increases the probability of hereditary disease. NGS systems capable of analysing massive amounts of sequencing data at the same time are increasingly used to screen breast cancer patients for causative gene variants and

pharmacogenomic markers. The differences between NGS platforms lie mainly in the technical details of the sequencing reactions and can be categorized into four groups, namely pyrosequencing (Qiagen Pyromark Q24 - Pyrosequencing analysis system), sequencing by synthesis, or by ligation (Illumina HiSeq 2000 sequencing machine), and ion semiconductor sequencing (Ion Torrent Personal Genome Machine) (Van Dijk et al. 2014).

Third-generation nanopore sequencing technologies such as MinION use a portable device for analysis of long-range DNA and RNA fragments in real-time. The MinION sequencing device is capable of reading more than 100Kb and has been used in field laboratory work to analyse samples as small as viruses or as large as the whole human genome (Jain et al. 2016). Nanopore long reads simplify the assembly and repetitive regions, also improving the speed of species identification in experiments. The MinION is the world first POCket-sized device that offers ultralong read length sequencing in real-time at a relatively low cost.

A study performed by Liau et al. (2019) showed that nanopore sequencing offers a high throughput method for detection of both known and new pharmacogenetic variants, including duplicated alleles. Due to the long reads generated by nanopore sequencing, accurate *CYP2D6* haplotyping can be achieved without the need for statistical phasing, as also reported previously (Ammar et al. 2015). This third-generation sequencing technology was also used to accurately categorise in-frame and out-of-frame splicing events after sequencing of whole *BRCA1* mRNA transcript sequencing (de Jong et al. 2017). Massive parallel sequencing for identification of *BRCA1/2* variants are usually performed on NGS platforms such as the llumina MiSeq and Ion Torrent, followed by Sanger sequencing considered the ‘gold standard’ for confirmation of *BRCA1/2* or other variants (Toland et al. 2018). Further studies are needed to determine whether MinION nanopore sequencing can replace multiplex ligation-dependent probe amplification (MLPA) as the method of choice for identification or confirmation of large rearrangements including duplications and deletions in *BRCA1/2* or other cancer-related genes.

1.9.1. BRCA1/2 testing at the point-of-care

The two major breast cancer susceptibility genes, *BRCA1* and *BRCA2*, help protect against the development of tumours by acting as tumour suppressors that regulate cell division and repair DNA damage that can lead to uncontrolled cell growth. A pathogenic *BRCA1/2* variant can affect the ability of the affected genes to perform functions such as DNA repair and recombination. With recent development of the ParaDNA POC System using HyBeacon probes to detect the eight most common *BRCA1/2* founder/recurrent mutations (https://gtr.ukri.org/projects?ref=103993) previously described in South Africa (Reeves et al. 2004; van der Merwe and van Rensburg 2009; van der Merwe et al. 2012b), rapid DNA testing can now be performed as a stand-alone test or incorporated into the PSGT algorithm.

HyBeacon probes can process samples at a rapid pace using extracted DNA or crude saliva, swab, urine and blood samples (Howard et al. 2011). Within approximately 60 minutes, the POC diagnostic tool performs PCR amplification and analysis of the sample, while not taking more than 15 minutes to set up the instrument (Blackman et al. 2015). A summarised result is generated automatically and visualised via an attached laptop. Successful application of POC testing in a clinical setting was first demonstrated for genotyping of *CYP2C9*2, CYP2C9*3* and *VKORC1* related to differences in the ability to metabolise Warfarin (Howard et al. 2011). This pharmacogenetics POC assay using HyBeacon probes produced excellent and accurate results for dosage monitoring for patients on anticoagulant therapy. In a subsequent implementation study of genotype-guided dosing of the oral anticoagulant, successful integration of POC genetics was demonstrated in a clinical setting in the UK (Jorgensen et al. 2019). A similar process is required in South Africa for implementation of *BRCA1/2* founder/recurrent mutation testing, as explored in breast cancer patients referred for the MammaPrint test or NGS of tumour DNA in the present study.

Compared to other genomic assays such as Oncotype DX (Shah et al. 2016), the MammaPrint test has not previously been studied in *BRCA1/2* mutation carriers or cytochrome P450 2D6 (*CYP2D6*) intermediate and poor metabolizers (Ross et al. 2008). Cytochrome P450 is a superfamily of enzymes expressed in the liver and some areas of the central nervous system, with CYP2D6 identified as one of the most significant enzymes responsible for the metabolism of medication in the human body (Samer et al. 2013). Van der Merwe et al. (2012a) highlighted the fact that *CYP2D6* genotyping may be of particular relevance for prevention of cumulative risk in *BRCA2* breast cancer patients who receive Tamoxifen, or antidepressants known to inhibit enzyme function (Newman et al. 2008). However, it is uncertain whether *BRCA1/2* mutation screening and *CYP2D6* genotyping could add value to the PSGT approach when applied in the MammaPrint service in South Africa (Grant et al. 2013). This research question was addressed in the present study based on the knowledge that an integrated analysis of germline and tumour genetics may facilitate the identification of signal pathways and genetic alterations underlying biological changes with different treatment requirements (Kalia 2015).

Use of a single platform that brings together fragmented pathology and genetic data facilitates improved quality assurance on which to base treatment decisions (Figure 3). The relatively long turn-around time (2-3 weeks) of complex genomic tests performed on genetic material extracted from tumour biopsies (RNA/DNA) creates an opportunity for evaluation of rapid POC germline DNA assays that could add value during the waiting period from sample collection to report generation. When used as a first-line screening assay during a genomic counselling session, *BRCA1/2* and/or *CYP2D6* pharmacogenetic testing may provide a cost-effective entry level for implementation of personalised medicine.

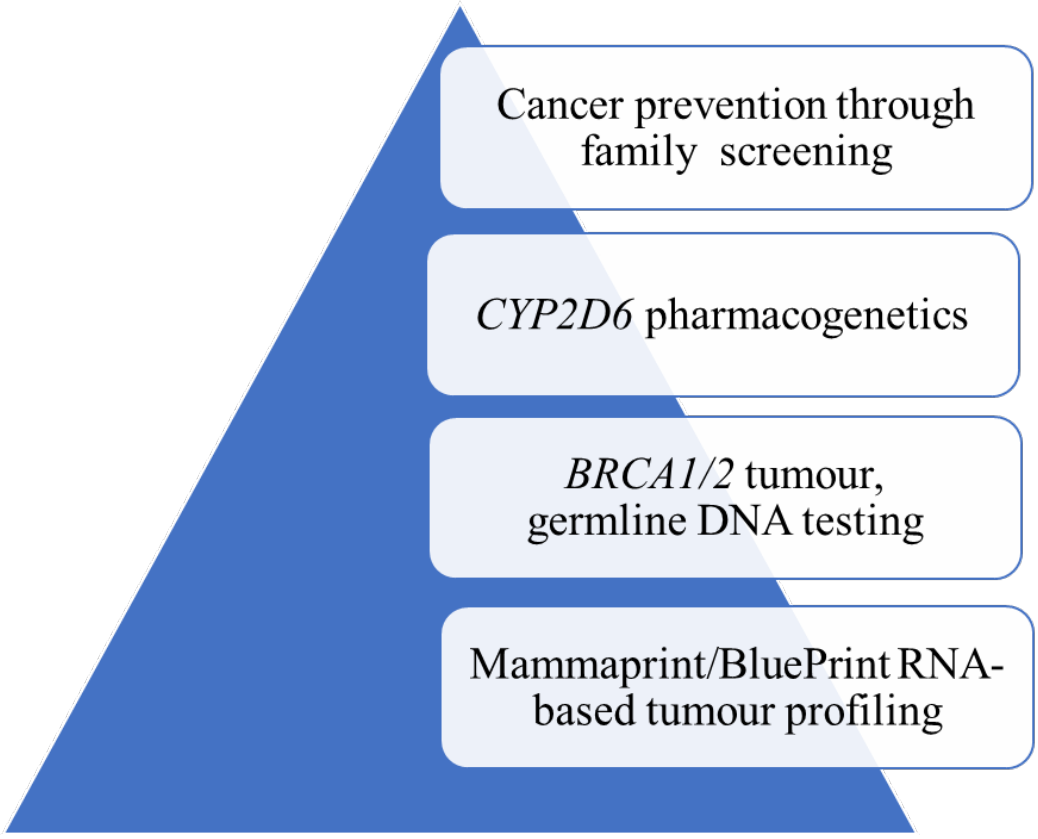

Figure 3: Pathology-supported genetic testing strategy incorporating multiple assay platforms to facilitate improved quality assurance on which to base treatment decisions.

Chapter 2

Rationale & Aims

2.1. Purpose

The purpose of this study is to translate research into clinical practice using an integrated service and research approach. This involves the ongoing development and improvement of a central genomics database generated at the interface between the laboratory and clinical practice. Pathology assessments and results obtained with different assays performed on genetic material extracted from breast tumours and blood or saliva samples of the same patients, evolved into a pathology-supported genetic testing (PSGT) platform for comparative effectiveness studies. Follow-up studies are performed in collaboration with participating clinicians to identify patients who developed secondary cancer or relapsed. This may be due to genetic variation in genes underlying cancer susceptibility or drug resistance, and/or non-compliance to anti-cancer therapy due to medication side effects. The well-being of breast cancer patients relying on the MammaPrint test to determine the need for chemotherapy, and *CYP2D6* genotyping for assessment of Tamoxifen resistance, was an important consideration in choosing point-of-care technology (POCT) as the method of choice for *BRCA1/2* founder/recurrent mutation testing, following test development and analytical validation at the Laboratory of Government Chemist Limited (LGC) in the UK.

2.2. Study aim

The primary aim of this study was to compare the frequency of *BRCA1/2* founder/recurrent mutations between early-stage breast cancer patients with a MammaPrint low- versus high-risk profile. The secondary aim was to evaluate the clinical outcome (cancer recurrence) of breast cancer patients with a pathogenic *BRCA1/2* variant in relation to *CYP2D6*4* intermediate- or poor metabolizer status.

2.3. Rationale

Currently, the application of PSGT to address different aspects of the same disease is limited to patients with private health care insurance. This problem is of particular relevance to the RNA-based 70-gene expression profile (MammaPrint®) with level 1A evidence for safe avoidance of chemotherapy in early-stage breast cancer patients (Cardoso et al. 2016). Although this microarray test is one of the most expensive genomic assays available in South Africa, reimbursement as part of oncology benefits by most medical schemes is based on reduced chemotherapy expenditure and improved well-being of patients who are spared the side effects of treatment. Recurrence risk assessment using the 70 genes analysed to obtain the MammaPrint high/low score based on RNA extracted from formalin fixed paraffin embedded (FFPE) tumour tissue, is usually performed in conjunction with functional tumour subtyping including 80 genes (BluePrint®) on the same microarray. Combined use of these two microarray assays outperforms approximation of tumour molecular subtypes using standard immunohistochemistry (IHC) (Whitworth et al. 2017).

IHC assessment of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) status forms part of the MammaPrint/Blueprint microarray pre-screen algorithm (MPA) developed as a cost-saving strategy in South Africa (Grant et al. 2013, 2019; Myburgh et al. 2016, Pohl et al. 2016). The finding that "biology matters, and it is more than just expression of ER" (Groenendijk et al. 2019: 5), warrants further study to determine the appropriateness of adding germline DNA testing to microarray analysis of tumour DNA/RNA. The central genomics database developed during implementation of the MammaPrint test in South Africa provides a valuable resource for this purpose, beyond a single research objective (Figure 4).

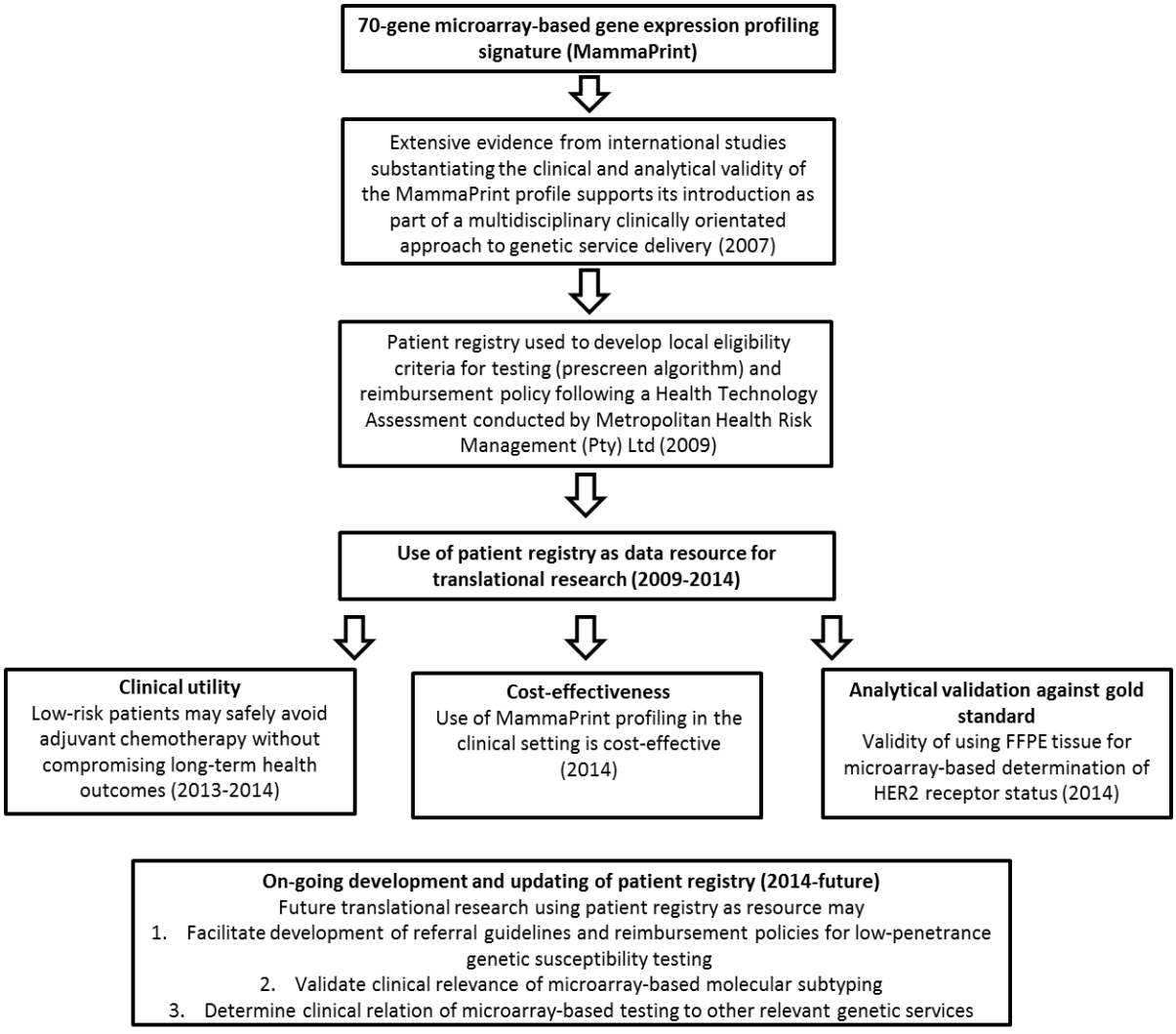

Figure 4: Introducing the MammaPrint test into the South African healthcare system by establishing a patient database at the interface between the laboratory and the clinic. A systematic approach was undertaken by using a pathology-supported genetic testing (PSGT) strategy to complement current testing procedures and establish clinical utility of gene profiling in early-stage breast cancer (Reproduced with permission from KA Grant PhD thesis, 2015)

The value of PSGT to bring together different test applications on the same platform was first demonstrated in a female breast cancer patient diagnosed with bilateral hormone receptor-positive breast cancer in 2008. She remains disease-free to date, despite omission of chemotherapy based on a low-risk MammaPrint profile for both tumours; which were classified by histopathology as invasive ductal/of no special type and lobular carcinomas (Grant et al. 2013). Additionally, Tamoxifen treatment was terminated in 2009 due to side effects unrelated to cytochrome P450 2D6 (*CYP2D6*) pharmacogenetics (van der Merwe et al. 2017). Pathway-based whole exome sequencing (WES) using the PSGT framework (Figure 1) proved to be a valuable tool to help distinguish between inherited and lifestyle-related breast cancer, or a combination of both in this family. The daughter of the above-mentioned patient with bilateral breast cancer was not referred for MammaPrint when aggressive HER2-positive breast carcinoma of no special type was diagnosed in 2010, as chemotherapy and anti-HER2 treatment was clearly indicated in this patient with early-onset breast cancer (<30 years). Full gene *BRCA1/2* DNA screening was negative in both the mother and daughter based on high-coverage germline NGS followed by WES. In 2018, the daughter developed liver metastasis, which was flagged on the PSGT platform when next generation sequencing (NGS) on tumour DNA was requested by her oncologist for identification of a gene-targeted therapy. When the NGS results of 75 cancer-related genes using the method described by Laes et al. (2018) became available, these were compared to the WES data obtained from her germline DNA three years earlier. This helped to identify a sequencing error in her tumour DNA and prevention of inappropriate treatment. This patient is currently in remission as IHC-based gene expression data, also provided in the NGS report, led to effective treatment of the liver metastasis.

Clinical monitoring over more than a decade in the above-mentioned family confirmed the value of a sustainable cancer genomics database with patient information collected at the protein, RNA and DNA levels in different laboratories. Tumour heterogeneity explained by these findings and germline WES results were important considerations in the selection of negative and positive controls used in this study to evaluate the clinical relevance of *BRCA1/2* founder/recurrent mutation testing using POCT. The sequential informed consent process and ethics approval obtained for this process furthermore contributed to the development of a framework for tiered informed consent for genomic health research applicable to Africa (Nembaware et al. 2019).

Chapter 3

Experimental Procedures

3.1. Ethics approval

Ethics approval was obtained from the Health and Wellness Sciences Research Ethics Committee (CPUT/HW-REC 2018/H10) of the Cape Peninsula University of Technology. This project was also registered as a sub-study under reference number N09/06/166 by the Health and Research Ethics Review Committee (HREC) of Stellenbosch University.

3.2. Study design

A pathology-supported genetic testing (PSGT) platform established at the interface between the research laboratory and routine clinical practice was used to develop a genomics database (Kotze at el, 2015), which was mined in this study to extract information of eligible breast cancer patients (Figure 4). All study participants were previously referred for the 70-gene MammaPrint test (2008-2019), followed by *CYP2D6**4 genotyping performed between 2012 and 2019. *BRCA1/2* mutation status was determined using a point-of-care (POC) assay that can be performed within 1-2 hours.

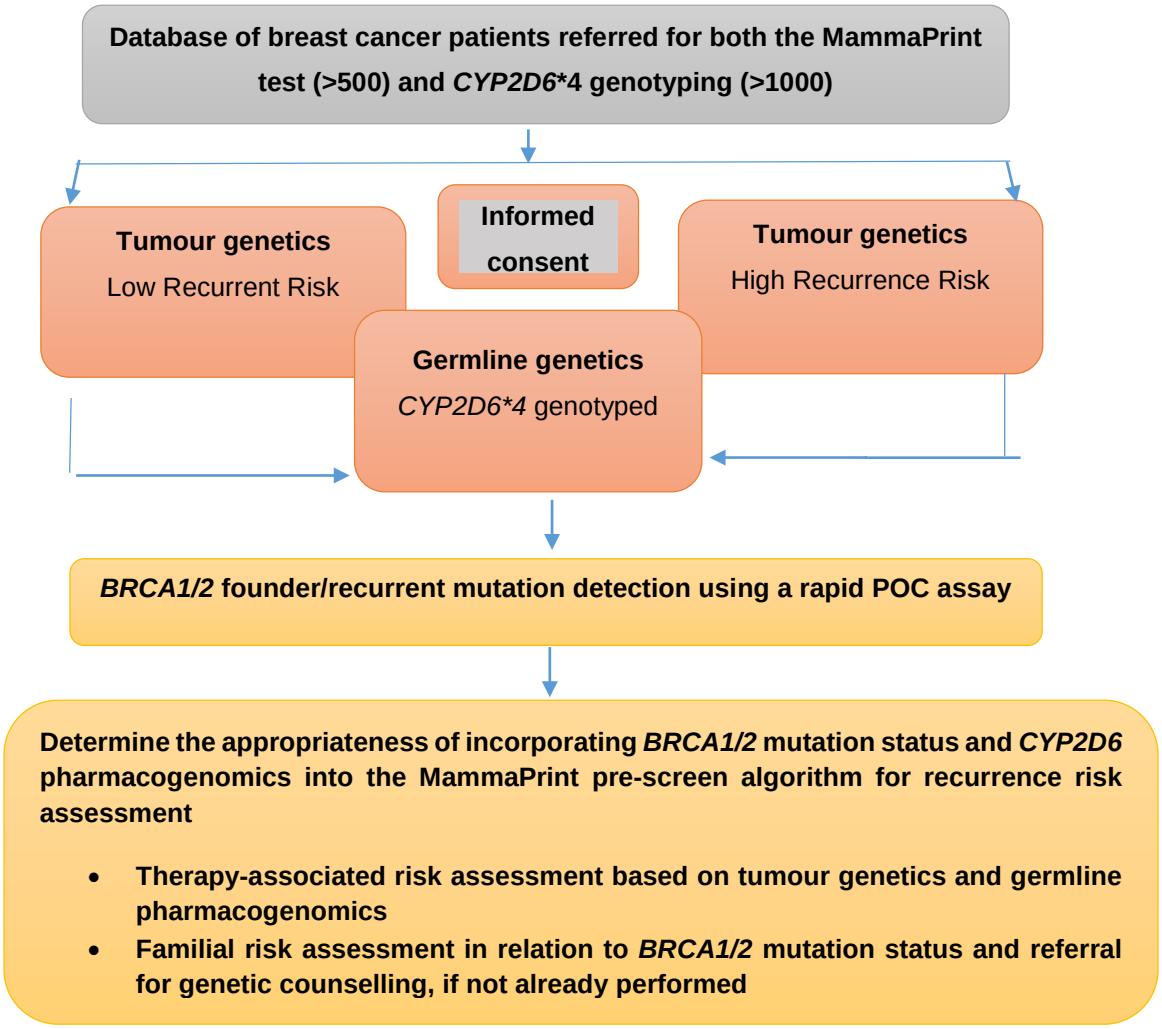

Figure 5: Flow chart illustrating the research plan using a pathology-supported genetic testing platform (PSGT) for data extraction and extended analysis of available DNA samples using a newly-developed point-of-care (POC) assay including eight pathogenic BRCA1/2 variants previously identified at an increased frequency in the South African population due to a founder/recurrent effect.

3.3. Data collection

This study was performed in South African patients with breast cancer previously subjected to tumour genomic risk profiling using the 70-gene microarray (MammaPrint®) test as previously described by Grant et al. (2013). Routine histopathology and immunohistochemistry (IHC) reports including assessment of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) status were reviewed and entered into the genomics database. From 2010, the 80-gene microarray (BluePrint®) was added to the MammaPrint service alongside ER, PR, and HER2 approximation of intrinsic molecular subtypes (Grant et al. 2015, 2019). From 2012, results from pharmacogenetics *CYP2D6* genotyping was added to the PSGT platform after obtaining informed consent for inclusion of information in the genomics database (van der Merwe et al. 2012a, 2017).Patients are recruited on an ongoing basis by treating clinicians and their information is available in the genomics database freely available to registered users at www.gknowmix.org (Kotze et al. 2013). New data are added to the PSGT platform as results of histopathology and laboratory tests become available during routine clinical practice (Kotze 2016). Written informed consent was obtained from all patients who provided blood or saliva for germline genetic testing.

Breast cancer patients with a low-risk MammaPrint profile were administered only endocrine therapy by their treating clinicians, without the addition of chemotherapy. Patient referrals from 2007 were closely monitored while awaiting the outcome of the prospective Microarray in Node Negative and 1 to 3 Positive Lymph Node Disease May Avoid Chemotherapy (MINDACT) trial, which demonstrated the highest level 1A evidence of clinical utility (Cardoso et al. 2016). Use of the PSGT platform for identification of patients with recurrent or a second primary cancer was an important consideration as mutation detection in tumour or germline DNA during follow-up may alter their treatment (Grant et al. 2013).

3.3.1. Inclusion criteria

- Samples from individuals diagnosed with breast cancer and subjected to tumour genomic testing, who subsequently relapsed or developed secondary tumours
- Samples from participants where other molecular cancer genetic testing results are available for comparison, including high-penetrance *BRCA1/2* mutations and/or polymorphisms in the *CYP2D6* gene, screened for in DNA extracted from the tumour, blood and/or saliva samples
- Samples with available genetic test results in the database approved for research under the parent gene profiling project (Number N09/06/166)

3.3.2. Exclusion criteria

- Use of samples from breast cancer patients who are non-consenting to germline genetic analysis

3.4. Pathology-supported genetic testing

ER, PR and HER2 status assessed by IHC were obtained from accredited pathology laboratories at referral of breast cancer patients for MammaPrint microarray testing performed at Agendia in the Netherlands. Hormonal status for ER and PR was based on the proportion of positive staining cells and staining intensity, while HER2 status was determined by IHC and/or fluorescence *in situ* hybridisation (FISH) in equivocal cases (2+). These results obtained at the protein level for approximation of the four major tumour subtypes, luminal A, luminal B, HER2-enriched and basal-type, were incorporated into the MammaPrint pre-screen algorithm (MPA) developed and implemented as a cost-saving strategy in South Africa (Grant et al. 2013; Myburgh et al. 2016). In this study, eight germline *BRCA1/2* founder/recurrent mutation status was assessed in early-stage breast cancer patients in relation to tumour subtype and *CYP2D6* pharmacogenetics in order to determine the appropriateness of including clinically relevant germline variants in these genes in the MPA for test selection and/or improved clinical interpretation/management. This was deemed necessary due to detection of both a pathogenic *BRCA2* c.7934delG founder/recurrent variant and the *CYP2D6**4 pharmacogenetics biomarker in tumour DNA of a South African female patient (index case) diagnosed with bladder cancer four years after referral for the MammaPrint test. Testing of two different tests in the same patient a few years apart was flagged on the PSGT platform, following next generation sequencing (NGS) of the patients' tumour DNA performed in conjunction with IHC (OncoDNA, Belgium). A database query resulted in 53 samples, of which 50 with both MammaPrint (tumour) and *CYP2D6* (germline) results were selected for extended *BRCA1/2* founder/recurrent mutation screening in this study. Two samples were excluded based on informed consent requirements. The third sample had a pathogenic *CHEK2* variant (rs555607708) previously detected by whole exome sequencing (WES) and was used as one of the negative controls for the eight *BRCA1/2* founder/recurrent mutations analysed in this study. Clinical outcome of patients with results obtained using the BRCA POC test were compared with data previously documented in the genomics database, including *BRCA1/2* results available for a small number of patients. Patients without follow-up data already captured in the genomics database were contacted, where possible, in collaboration with their treating oncologists to obtain current information for inclusion in the research database based on the informed consent provided.

3.5. DNA extraction

DNA was available for all 50 cancer patients and 10 control samples included in this study. For DNA extraction from whole blood the QIAamp DNA Mini Kit was used. Twenty microliters (20 µl) of protease was pipetted into a 1.5 ml microcentrifuge tube. Two hundred microliters of venous blood was

added to microcentrifuge tube, followed by 200 µl of AL Buffer and vortexed for 15sec. After incubation at 56°C for 10 min 200 µl of 100% ethanol was added to the sample and mixed by vortexing for 15 sec. The mixture was applied to a QIAamp Mini spin column and centrifuged at 8000 rpm for 1 min. The mini-spin column was placed in a clean 2 ml collection tube and the filtrate was discarded. Wash buffer (500 µl) was added into the mini-column and centrifuged at 8000 rpm for 1 min. The mini-spin column was placed in a clean 2 ml collection tube and the filtrate was discarded. Five hundred microliters (500 µl) of AW2 buffer was added to the mini-column, then centrifuged at 14000 rpm for 3 min. The mini-spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. After centrifuging the mini-column, 200 µl of Buffer AE was added and incubated at room temperature 15^{o}C - 25^{o}C for 1 min and centrifuged at 8000 rpm for 1 minute. The quality and concentration of the extracted DNA were assessed with a Nanodrop One spectrophotometer (Nanodrop Technologies, Wilmington, USA) before analysis. Samples analysed using Nanodrop had an optical density ratio $^{A260}/_{A280}$ >1.8.

3.6. *BRCA1/2* point-of-care genetic testing

PCR amplification and melting curve analysis of eight *BRCA1/2* founder/recurrent mutations previously identified in the South African population (Reeves et al. 2004; van der Merwe and van Rensburg 2009; van der Merwe et al. 2012b) were performed using the BRCA 1.0 POC Research Kit and instrumentation (Laboratory of Government Chemist Limited, LGC, Teddington, UK). Kit development by LGC was based on the ParaDNA POC genotyping principles as previously described (Pirmohamed et al. 2013). The reaction plate kits (BRCA 1.0) were stored at -20^{o}C and thawed at room temperature for 15-20 min before use. DNA samples were diluted to a concentration of 1 ng/ul, and 2 ul of each sample transferred into each well of the ParaDNA reaction plates. Prior to the analysis of 50 DNA samples available for this study, 1 blank, 2 negative controls, and eight positive control samples of known genotype for each founder/recurrent mutation were tested. The ParaDNA kits comprised all the reagents required for multiplex melt curve analysis of the eight *BRCA1/2* targets in a four-tube format (Table 6) using the fluorescent dyes FAM, CAL Fluor Orange 560 (CAL560) and CAL Fluor Red 610 (CAL610). Following an initial denaturation step (98°C for 1 min), the targets were amplified using 50 PCR cycles of 99°C for 7 sec, 62°C for 12 sec and 72°C for 12 sec, followed by denaturation at 95°C for 20 sec and probe annealing at 35°C for 30 sec. After PCR amplification, the reactions were denatured (95°C for 20 sec) and cooled (35°C for 30 sec). Melting curve analysis was performed by heating the samples from 35°C to 80°C using a 0.1°C/s ramp rate and fluorescence acquisition. The ParaDNA software (version 1.6.0.27) automatically analysed the sample melting curves and reported the *BRCA1/2* genotypes on the computer screen. Automated software calls were assessed using the ParaDNA Data Review software to examine sample melting curves.

Table 5: Multiplex analysis of eight BRCA1/2 founder/recurrent mutations in a four-tube ParaDNA format.

Tube	Gene	Founder/recurrent mutation	Variant	Probe label
A	*BRCA1*	c.1374delC	rs397508862	FAM
	BRCA2	c.7934delG	rs80359688	CAL560
B	*BRCA1*	c.2641G>T	rs39750888	FAM
	BRCA2	c.5771_5774delTTCA	rs80359535	CAL560
C	*BRCA1*	c.5266dupC	rs80357906	FAM
	BRCA1	c.68_69delAG	rs80357914	CAL610
D	*BRCA2*	c.6448_6449insTA	rs397507858	FAM
	BRCA2	c.5946delT	rs80359550	CAL560

3.7. Sanger sequencing

Sanger sequencing was used as the gold standard for confirmation of the genotypes of the control samples and to confirm the results obtained with the BRCA 1.0 POC Research assay. The standard operating procedure (SOP) developed at the Pathology Research Facility was followed before submission of samples for Sanger sequencing at the Central Analytical Facility, Stellenbosch University. Table 7 shows the oligonucleotide primers used for Sanger sequencing as obtained from the SOP. Electropherograms were analysed using the Ensembl human reference sequence for direct comparison.

Table 6: *Oligonucleotide primers used for conventional polymerase chain reaction application and Sanger sequencing of BRCA1 (NM_007300.4) and BRCA2 (NM_000059.3).*

Gene	Region	Variant	Primer	Oligonucleotide primers (5' to 3')	Size (bp)
BRCA 1	Exon2	c.68_69delAG	F	TGTGTTAAAGTTCATTGGAACA	149
			R	CATAGGAATCCCAAATTAATACA	
	Exon 11	c.1374delC	F	TCGCATGCTCAGAGAATCC	400
			R	TGTGGCTCAGTAACAAATGCTC	
	Exon 11	c.2641G>T	F	GCTCAGTATTTGCAGAATAC	253
			R	GCTTATCTTTCTGACCAACC	
	Exon 20	c.5266dupC	F	AGTCAGAGGAGATGTGGTCAATGG	236
			R	GTGGTTGGGATGGAAGAGTGAA	
BRCA 2	Exon 11	c.5946delT	F	CGAGGCATTGGATGATTCAGAG	394
			R	GAGCTGGTCTGAATGTTCGTTAC	
		c.6448_6449insTA	F	GAGAAACCCAGAGCACTGTG	404
			R	CTAAGATAAGGGGCTCTCCTC	
		c.5771_5774delTTCA	F	CGAGGCATTGGATGATTCAGAG	394
			R	GAGCTGGTCTGAATGTTCGTTAC	
	Exon 17	c.7934delG	F	GTAGTTGTTGAATTCAGTATC	354
			R	TGGCAACTGTCACTGACAAC	

The reagents and PCR conditions used for sanger sequencing is shown in table 8 and 9 respectively.

Table 7: Master mix preparation for c.7934delG

Reagents	
Nuclease Free Water	15.675
5x buffer	5
$MgCl_2$ (25mM)	1.5
dNTP mix (10mM)	0.5
Forward Primer (10 µM)	0.6
Reverse Primer (10 µM)	0.6
Go Taq Polymerase	0.125
DNA template (10ng/ µl)	1
Total	25

Table 8: Thermal cycling condition for c.7934delG

Steps	Cycles	Temperature (^{0}C)	Time(min)
Initial denaturation	1	95	02:00
Denaturation	30	95	00:30
Annealing	30	62	00:30
Final extension	30	72	05:00

3.8. Statistical analysis

Qualitative characteristics were described using cross tabulation and frequency tables analysed using the STATISTICA package. One-way ANOVA was used to compare average age between subgroups. The significance level was set at 0.05 for determination of statistical significance.

Chapter 4

Results

4.1. Characteristics of the study population

Table 10 shows the clinical characteristics of the study population, subdivided into 26 breast cancer patients with a low-risk MammaPrint profile (52%) and 24 with a high-risk profile (48%). Four (8%) of these patients also had bilateral or other forms of cancer, including the index case (MPR 055) diagnosed with bladder cancer four years after referral for the MammaPrint test. Two low-risk patients that are non-mutations carriers developed basal cell cancer and colon cancer respectively. One patient with a high-risk profile and a non-mutation carrier developed breast cancer on the opposite breast (left). The average age of the study cohort of predominantly Afrikaners of European ancestry, was 51 (34-74) years, with no substantial difference between the mean age of patients with low- and high-risk tumours. Notably, 6 (24%) premenopausal patients below the age of 40 years have a high risk for distant recurrence, while only one patient (4%) in this age group had a low-risk genomic risk profile. Most of the patients included in this study were diagnosed with ductal/carcinoma of no special type (82%), with a similar distribution of high- and low-risk MammaPrint profile. Of the six lobular cancers, four were reported as low risk and two as high risk. Only one patient had an ER-negative tumour, which was categorised as high-risk for recurrence. In the high and low-risk groups an equal number of patients had PR-negative tumours. Of the 50 patients included in the study, 16 of their tumours (38%) were scored by immunohistochemistry (IHC) as equivocal HER2 (2+) and referred for fluorescence *in situ* hybridization (FISH) analysis. Using FISH, 12 (75%) of the 16 tumours which were found to be 2+/3+ on IHC, were reported as HER2 negative, one remained equivocal and three (18.8%) demonstrated HER2 amplification (Table 11). Sixty percent (30/50) of the patients reported a family history of cancer (data not shown). After stratification based on genomic recurrence risk, a similar family history of cancer was seen in high and low-risk groups.

Table 9: Clinical characteristics of the study population of predominantly European ancestry stratified by MammaPrint recurrence risk profile.

Characteristics	All patients (n=50)	Low risk (n=26)	High risk (n=24)
Age mean (years)	50.68 years (36-74)	51.54 years (36-74)	49.75 years (34-73)
<40 41-50 >51	7 (14%) 19 (3%) 24 (48%)	1 (4%) 12 (46%) 13 (50%)	6 (25%) 7 (29%) 11 (45%)
IHC ER status Positive Negative	 49 (98%) 1 (2%)	 26 (100%) 0	 23 (96%) 1 (4%)
IHC PR status Positive Negative	 46 (92%) 4 (6%)	 25 (96%) 1 (4%)	 21 (88%) 3 (12%)
IHC HER2 status 1+ 2+ 3+ Not provided	 7 (14%) 11 (22%) 5 (10%) 27 (54%)	 3 (12%) 8 (30%) 1 (4%) 14 (53%)	 4 (17%) 3 (12%) 4 (17%) 13 (54%)
Tumour Type Lobular Ductal Not provided	 6 (12%) 41 (82%) 3 (6%)	 4 (15%) 21 (80%) 1 (4%)	 2 (8%) 20 (83%) 2 (8%)
Grade 1 2 3 Not provided	 16 (32%) 23 (46%) 2 (4%) 9 (18%)	 9 (35%) 11 (42%) 0 6 (23%)	 7 (29%) 12 (50%) 2 (8%) 3 (12%)
Family history of cancer Yes No Not provided	 30 (60%) 7 (14%) 13 (26%)	 15 (58%) 4 (15%) 7 (27%)	 15 (62%) 3 (12%) 6 (25 %)

IHC, immunohistochemistry; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2

Table 10: Fluorescence in situ hybridization (FISH) verification of 16 tumours assessed by immunohistochemistry (IHC) as equivocal (2+) or positive (3+).

			FISH		
		n=16	**Negative**	**Positive**	**Equivocal**
IHC	**2+**	11 (68.8%)	10 (62.5%)	-	1(6.2%)
	3+	5 (31.2%)	2 (12.5%)	3 (18.8%)	-
TOTAL			12 (75%)	3 (18.8%)	1 (6.2%)

4.2. *BRCA1/2* genotyping using the ParaDNA POC Assay

Before testing the DNA samples of patients included in this study, the performance of the BRCA 1.0 Research Kits was verified using 10 DNA samples of known genotype for each of the eight *BRCA1/2* founder/recurrent variants as controls, as well as a blank containing no DNA (Figure 5). All samples were genotyped using a 3-colour, 4-tube multiplex assays, after adding the extracted DNA to each of

the four plate wells. The test duration from sample-to-result was 60 minutes. The ParaDNA software automatically analysed the multiplex melting curve data and assigned sample genotype calls. The accuracy of software calls was assessed by adding different DNA samples, comprising known South African founder/recurrent mutations, to each well of ParaDNA plates. All of the samples and negative controls were assigned the correct software calls using 2 ng of input DNA.

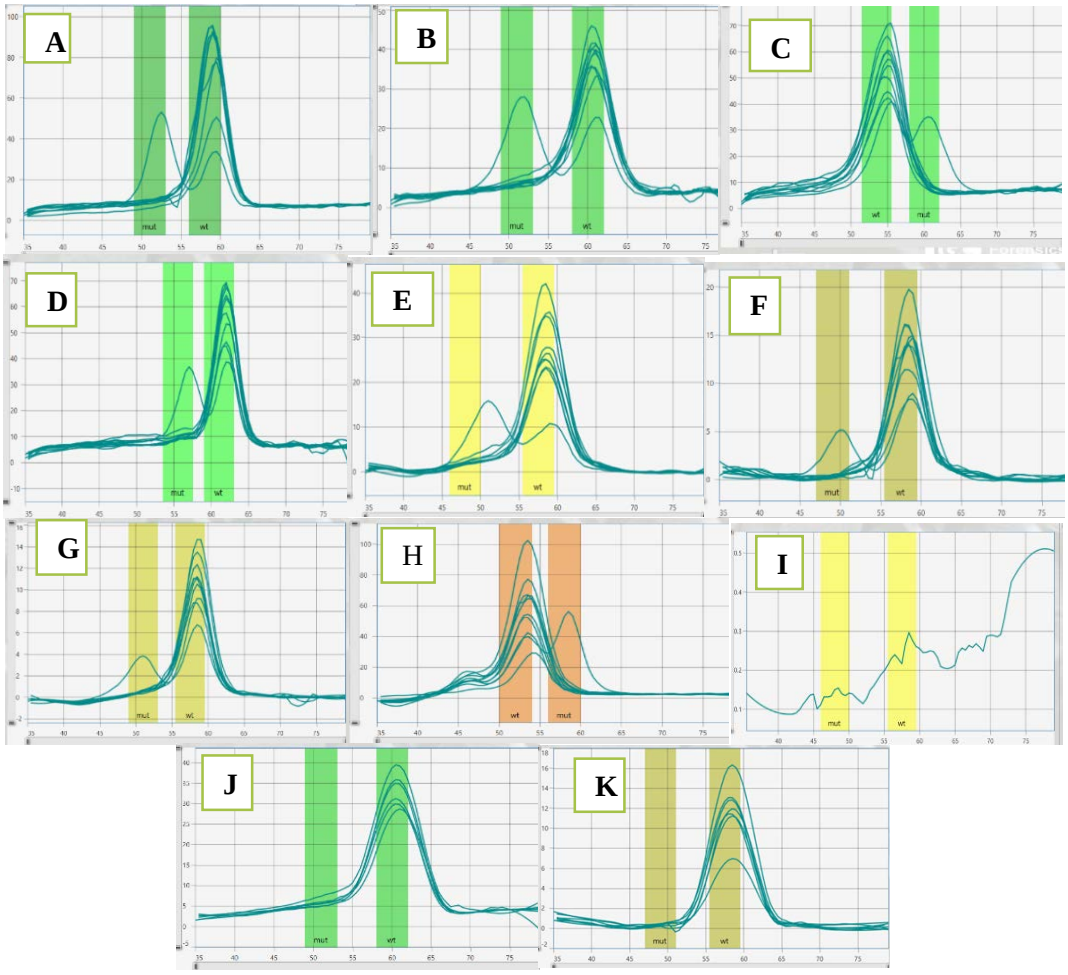

*Figure 6: BRCA1/2 genotyping of control samples using the BRCA 1.0 POC Kit. Melting curve analysis of founder/recurrent mutations BRCA1 c.68_69delAG, c.1374delC, c.2641G>T, c.5266dupC and BRCA2 c.5771_5774delTTCA, c.5946delT, c.6448_6449insTA, c.7934delG (A-H). By using FAM-labelled HyBeacon (green), melting peaks were correctly detected for pathogenic variants BRCA1 c.1374delC (rs397508862) (**A**), BRCA2 c.6448_6449insTA (rs397507858) (**B**), BRCA1 c.2641G>T (rs397508988) (**C**), BRCA1 c.5266dupC (rs80357906) (**D**), while CAL 560 probes (orange) detected BRCA2 c.5771_5774delTTCA (rs80359535) (**E**), BRCA2 c.5946delT (rs80359550) (**F**), BRCA2 c.7934delG (rs80359688) (**G**) and the CAL 610 probe identified the pathogenic variant BRCA1 c.68_69delAG (rs80357914) (**H**) using controls with known BRCA1/2 mutations. No peaks are shown in (**I**), corresponding to the blank sample with no DNA, while the two negative controls each generated one peak, corresponding to the samples without any of these pathogenic variants (**J** and **K**).*

After the accuracy of the method used for *BRCA1/2* founder/recurrent mutation detection was confirmed, germline DNA of 50 breast cancer patients previously analysed using the 70-gene MammaPrint were genotyped. Five of the patients tested positive for the pathogenic *BRCA2* c.7934delG variant. For one of the samples with a low risk MammPrint profile, four of the eight founder/recurrent mutations tested failed despite repeat analysis (two times), due to poor quality of the stored DNA used. Homozygous wild-type (wt) samples generated single melting peaks, whereas heterozygous samples

with pathogenic variants generated two peaks (wt & mut). All five samples (Peak 1) with the pathogenic *BRCA2* c.7934delG variant generated melting peaks at 50 ^{0}C (Figure 7), as confirmed by Sanger sequencing (Figure 8). The FAM rs397508862, FAM rs397508988, CAL560 rs80359535, FAM rs3 80357906, CAL610 rs80357914, FAM rs397507858 and CAL560 rs80359550 HyBeacon probes generated 44 single wt melting peaks indicating that none of the other *BRCA1/2* founder/recurrent mutations (Peak 2) tested for were present in the DNA samples successfully tested. The lower peaks were either due to poor sampling quality, low DNA concentration or mass loss from improper plate sealing.

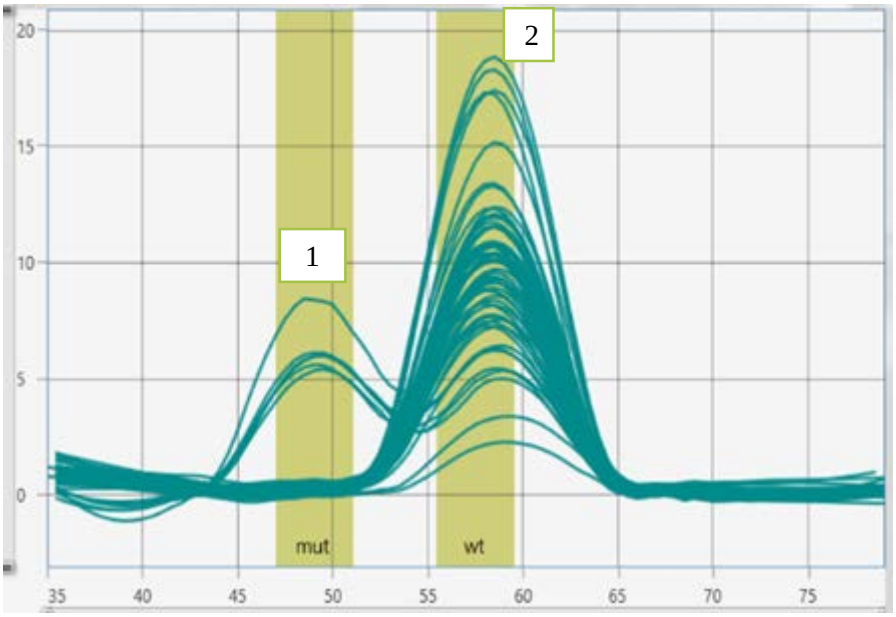

Figure 7: BRCA1/2 genotyping of breast cancer patient samples using the BRCA 1.0 POC Kit Melting curve analysis of eight BRCA1/2 founder/recurrent mutations BRCA1 c.68_69delAG, c.1374delC, c.2641G>T, c.5266dupC and BRCA2 c.5771_5774delTTCA, c.5946delT, c.6448_6449insTA, c.7934delG (A-H). CAL 560 probe detected five of the patients tested positive for the pathogenic BRCA2 c.7934delG variant (Peak 1). No pathogenic variants were detected in 44 DNA samples (Peak 2).

4.3. Confirmation by Sanger sequencing

Detection of *BRCA2* c.7934delG using the BRCA 1.0 POC Kit was confirmed by Sanger sequencing, as shown for the index case in Figure 8. This founder/recurrent mutation was initially detected in DNA extracted from the bladder tumour.

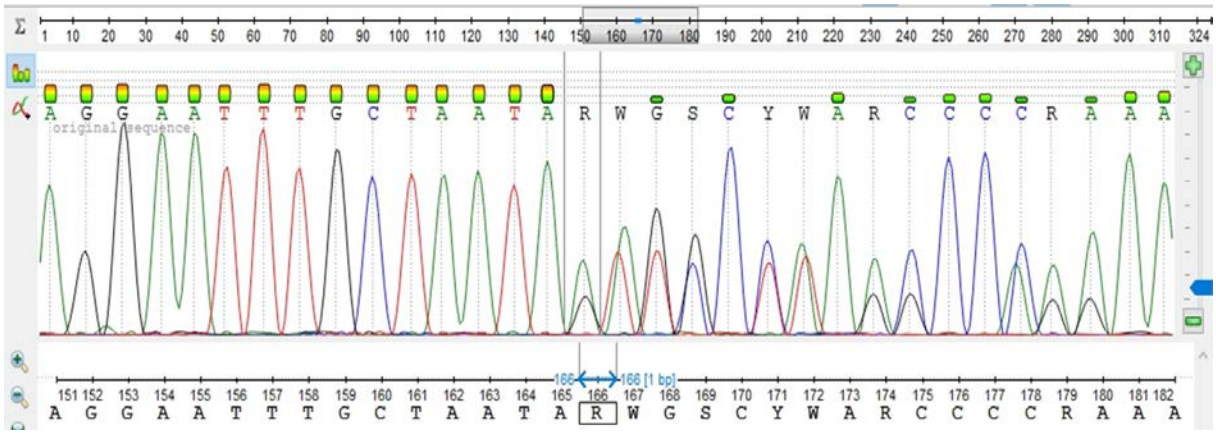

Figure 8: Confirmation of BRCA2 c.7934delG detected by the BRCA 1.0 POC Kit using Sanger Sequencing.

4.4. *BRCA2* c.7934delG, age of onset and MammaPrint risk profile

As shown in Figure 9, patients with the *BRCA2* c.7934delG founder/recurrent mutation were diagnosed with breast cancer at a significantly younger age than those without this pathogenic variant (p=0.02). Clinical features of 49 patients successfully genotyped for all eight *BRCA1/2* founder/recurrent mutations are compared in Table 12.

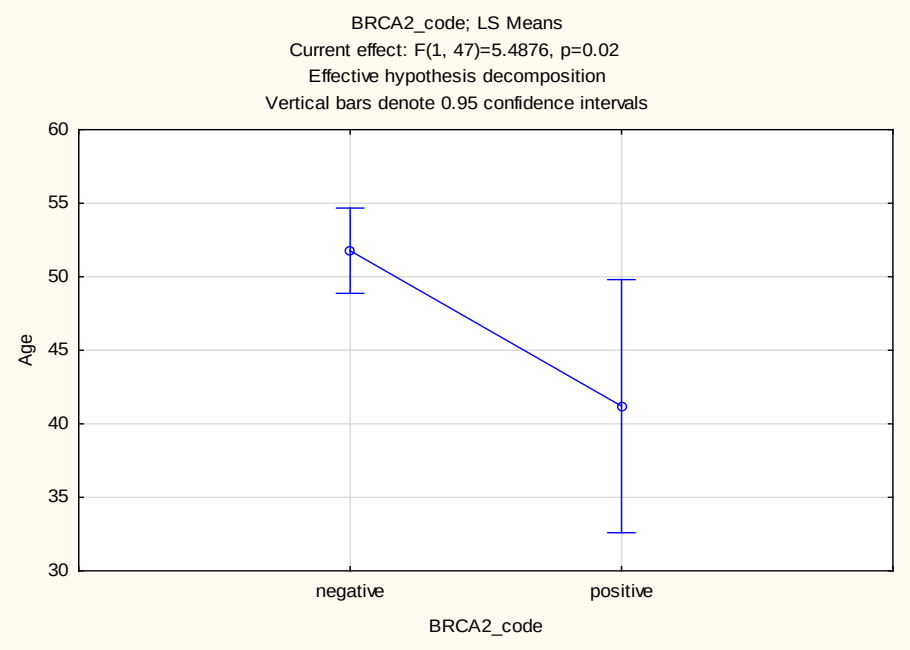

Figure 9: Comparison of the mean age between the five breast cancer patients identified with the BRCA2 c.7934delG founder/recurrent mutation versus the 44 non-mutation carriers.

Three of the 24 patients with a high-risk MammaPrint profile (12%) and two of the 26 low-risk cases (8%) tested positive for the *BRCA2* c.7934delG mutation. Use of the validated Manchester score including histopathology criteria to estimate the likelihood of harbouring a pathogenic *BRCA1/2*

variant, showed that only one patient with a high-risk MammaPrint profile would have qualified for *BRCA1/2* testing, with a score of 29.

Table 11: Comparison of clinical features between breast cancer patients with and without the BRCA2 c.7934delG founder/recurrent mutation, grouped according to their MammaPrint recurrence risk profile.

***BRCA1/2* Result**	**Negative (n= 44)**		**Positive (n=5)**	
70-gene Result	Low (n=23)	High (n=21)	Low (n=2)	High (n= 3)
Age, average (range)	52 years (36-74)	51years (34-73)	42.5years (36-47)	41 years (34-47)
Family history Yes No Not provided	 14 (61%) 2 (9%) 7 (30%)	 12 (57%) 3 (14%) 6 (29%)	 0 2	 3 0
Tumour Type Ductal Lobular Not provided	 19 (83%) 4 (17%) 0	 17 (81%) 2 (9.5%) 2 (9.5%)	 2 0 0	 3 0 0
ER status Positive Negative	 23 (100%)	 20 (95%) 1 (5%)	 2 0	 3 0
PR status Positive Negative	 22 (96%) 1 (4%)	 18 (86%) 3(14%)	 2 0	 3 0
HER2 status 1+ 2+ 3+ Not provided	 1 (4%) 7 (30%) 1 (4%) 14 (61%)	 3 (14%) 3 (14%) 2 (9.5%) 13 (62%)	 2 0 0 0	 1 0 2* 0
Ductal Ca Grade 1 2 3 Not provided	 9 (39%) 9 (39%) 0 5 (22%)	 7(33%) 10 (48%) 1 (5%) 3 (14%)	 0 2 0 0	 0 2 1 0

*Microarray analysis reported HER2-negative status, subsequently confirmed using reflex FISH (Grant et al. 2015). Case 291 with a low-risk MammPrint profile was excluded due to failure to detect four of the eight *BRCA1/2* variants tested for.

4.5. *BRCA2* c.7934delG and C*YP2D6* pharmacogenetics

*CYP2D6*4* genotyping previously performed in the study population detected one homozygote with the poor metabolizer status (2%) and 18 heterozygotes with the intermediate metabolizer status (36%). Of the five patients with the pathogenic *BRCA2* c.7934delG variant, three were heterozygous for *CYP2D6*4*, including the index case and two patients with a high-risk MammaPrint profile. Follow-up studies revealed that only the index case previously shown to have a low-risk MammaPrint profile is deceased. The two patients with a high-risk MammaPrint profile found to be heterozygous for

*CYP2D6*4* received this pharmacogenetic information relevant to the selection of Tamoxifen earlier in their cancer treatment process compared to the index case selected for this study after referral for NGS on tumour DNA.

The index case was diagnosed with ductal carcinoma *in situ* at the age of 47 years, and invasive ductal carcinoma of no special type (grade 1, ER/PR 3+, HER2 negative) at the age of 51 years, resulting in the initial referral for the MammaPrint test which showed a low-risk profile for breast cancer recurrence. Four years later, this patient, initially treated with Tamoxifen, was diagnosed with invasive urothelial (transitional) cell carcinoma grade III. *BRCA2* c.7934delG identified in the bladder tumour was confirmed in the patient's germline DNA (Figure 8), which also showed genetic variation in the *CYP2D6* drug metabolism pathway. The existence of two malignancies having different histopathology and at anatomically distinct sites, suggested the diagnosis of metachronous malignancy involving the breast and the bladder. IHC testing performed on the bladder biopsy tissue showed cytokeratin 20 and 7, p63, 34Be12 and p504s positive staining. Morphological characteristics of the bladder tumour was not consistent with that of an infiltrating ductal carcinoma of no special type, previously diagnosed in the breast biopsy. This confirmed a primary bladder cancer and ruled out breast cancer metastasis due to misclassification with use of the MammaPrint test.

Chapter 5

Discussion

The database query performed in this study was resticted to breast cancer patients referred for the 70-gene MammaPrint test using RNA extracted from tumour biopsies as part of routine clinical care, as well as germline *CYP2D6* genotyping following written informed consent for additional germline genetic testing. *CYP2D6*4* was initially detected in the presence of *BRCA2* c.7934delG in tumour DNA of a tamoxifen-treated patient with metachronous malignancies involving the breast and the bladder. These findings prompted confirmation of the *BRCA2* c.7934delG founder/recurrent mutation in germline DNA of this "index case" in 2018. This finding supported subsequent development of a rapid point-of-care (POC) BRCA assay used in this study to screen 50 breast cancer patients for eight relatively common *BRCA1/2* founder/recurrent mutations previously described in the South African population (Reeves et al. 2004; van der Merwe and van Rensburg 2009; van der Merwe et al. 2012b). Verification of the *BRCA1/2* results obtained for the eight founder/recurrent mutations tested was obtained blindly by DNA sequencing or comparison with previous patient reports provided by participating clinicians. Reports of previous *BRCA*/other gene panel testing was available for comparison in five patients with the *BRCA2* c.7934delG variant and eight patients without *BRCA2* variants based on founder/recurrent mutation testing or extended gene panels.

5.1. MammaPrint risk profile distribution in relation to *BRCA2* c.7934delG

BRCA2 c.7934delG was the only pathogenic variant detected in five of the study participants, with a slightly higher proportion of affected cases classified with a high recurrence risk based on the MammaPrint test. The age at diagnosis did not differ between South African breast cancer patients with a high- and low-risk MammaPrint profile, while patients with the *BRCA2* c.7934delG founder/recurrent mutation developed breast cancer at a significantly earlier age than non-carriers. It therefore seems unlikely that a large number of pathogenic variants other than *BRCA2* c.7934delG were missed in the study cohort due to use of the BRCA 1.0 Research POC assay limited to eight founder/recurrent mutations.

The MammaPrint risk score distribution in South African breast cancer patients with the *BRCA2* founder/recurrent mutation showed a similar pattern compared to that previously reported in oestrogen receptor (ER) positive *BRCA1/2* carriers screened with the 21-gene assay (OncotypeDX) assay (Lewin et al. 2016; Halpern et al. 2017). In the study performed by Lewin et al. (2016) assessing germline *BRCA1/2* mutation status in Israel, the 21-gene recurrence score distribution shifted towards high (*BRCA1* 50 % and *BRCA2* 29%) and intermediate (*BRCA1* 35% and *BRCA2* 52%) risk compared to a small proportion in low-risk cases (*BRCA1* 15 % and *BRCA2* 18.4%). It remains uncertain whether ER-positive early-stage breast cancer patients with pathogenic *BRCA1/2* germline variants could safely avoid chemotherapy based on tumour gene profiling, as gene targeted therapies such as PARP inhibitors may be more appropriate. Age is also an important consideration with use of the OncotypeDX

test as the TailorX trial showed that premenopausal women 50 years of age or younger at the higher extreme of the intermediate-risk range (16-25) may have a small benefit from chemotherapy (Sparano et al. 2018).

Detection of the same South African founder/recurrent mutation in breast cancer patients selected for microarray testing by clinico-pathological features incorporated in the MammaPrint pre-screen algorithm (MPA) using pathology-supported genetic testing (PSGT), has the advantage that a search for other risk modifiers in the causal pathway involving *BRCA2* c.7934delG can be further explored. Apart from Giusti et al. (2003) who found no differentiating clinical or pathological characteristics among prostate cancer patients with the same Ashkenazi Jewish founder mutation, when compared to non-carriers, this study is the first investigation of South African cancer patients selected by tumour subtype with the same founder/recurrent mutation. Detection of germline pathogenic *BRCA1* variants in patients with hormone-receptor positive cancer were initially disregarded as incidental or sporadic, but a more plausible explanation may be that age-related metabolic changes combined with environmental exposures predisposing to genomic instability may produce different tumour subtypes within the same genetic background (Tung et al. 2010). Indeed, Naushad et al. (2012) demonstrated that dysfunction of one-carbon metabolism as reflected by raised homocysteinaemia may predict breast cancer subtype and disease progression. Other subtype-specific factors include body mass index (BMI), age of cancer onset and vitamin D status being explored in South African breast cancer patients with hormone receptor-positive breast cancer (Baatjes et al. 2019; Okunola et al. 2019). These findings raised the question whether preventive strategies in cancer patients with the same pathogenic germline *BRCA1/2* variant will change tumour pathology and hence have a positive impact on clinical management, in a similar way that cholesterol levels and other biochemical blood tests are used in cardiovascular disease (CVD) risk management. RNA-based gene profiling assays such as MammaPrint have not previously been studied in relation to *BRCA1/2* mutation status using the PSGT platform to help overcome limitations of pathology and genetic tests when used in isolation.

5.2. Tumour pathology of patients with pathogenic germline *BRCA1/2* variants

The breast cancer patients included in this study were categorized by age, hormonal status, tumour type and grade and underwent RNA-based tumour gene expression profiling using the 70-gene microarray-based assay. On histopathology, *BRCA2* associated breast cancer is commonly categorised as luminal with few immunohistochemical (IHC) and morphological characteristics to distinguish it from sporadic disease. These cancers are generally hormone receptor-positive (ERα and PR), with low or negative HER2 expression (van der Groep et al. 2011). Numerous studies have reported a comparable incidence of ER/ PR-positive cancers in *BRCA2* carriers associated with sporadic cases (Armes et al. 1999; Lakhani et al. 2002; Palacios et al. 2005). In a large series of *BRCA2*-associated breast cancer, Bane et al. (2007) showed a luminal subtype despite a predominantly high-grade phenotype of invasive ductal

carcinomas of no special type. These tumours were also less likely than control tumours to overexpress HER2/neu or the basal cytokeratin CK5, with no difference in expression of cyclin D1, MIB1, p53 and bcl2.

In South Africa to improve cost-effectiveness, IHC ER/PR-positive and HER2-negative tumours are usually selected for genomic profiling using the MammaPrint and BluePrint assays, and therefore our cohort consisted predominantly of hormone receptor-positive tumours. As expected, most South African patients with a low recurrence risk had ER/PR-positive cancers, with one ER-negative case showing a high-risk MammaPrint profile. In some patients with HER2-positive tumours referred for the MammaPrint test, some were previously reclassified as negative using microarray analysis and vice versa (Grant et al. 2015). The differences in tumour characteristics were not associated with a distinctive pattern in all five South African breast cancer patients with the *BRCA2* c.7934delG variant, including one patient with IHC HER2 status reclassified by microarray analysis.

There is a notable difference in the distribution of tumour grade in ER-negative and ER-positive breast cancer in *BRCA1* carriers (Tung et al. 2010). One feature is that moderately or poorly differentiated tumours are more often reported in ER-positive *BRCA2* breast cancer carriers than in patients with sporadic disease. This was evident in our study where 80% of the patients with the founder/recurrent *BRCA2* c.7934delG variant versus 45% of the non-carriers, were diagnosed with grade 2/3 tumours. None of the breast cancer patients with the pathogenic *BRCA2* variant had well differentiated tumours (grade 1), however, despite 36% (16/44) of non-mutation carriers diagnosed with grade 1 cancers, seven of these patients were reported as having a genomic high risk for recurrence. Only one patient with sporadic breast cancer had a poorly differentiated tumour and was regarded as high-risk according to MammaPrint. Tumour type associated with *BRCA2* breast cancer is mostly infiltrating ductal carcinomas (van der Group et al. 2011), which was confirmed in our study.

In a study of 217 women recruited from various clinical sites, Beumer et al. (2016) validated the prognostic value of the MammaPrint test in patients with early-stage invasive lobular carcinoma. An association was found between the MammaPrint high risk profile and an unfavourable clinical outcome in distant metastasis-free and overall survival. True HER2-amplified breast cancer is rare in *BRCA1* and even less frequent in *BRCA2* mutation positive patients (Evans et al. 2016). However, this may be an underestimation due to enforcement of standard clinical guidelines for IHC staining used to determine the need for expensive anti-HER2 therapy. Current consensus is that only tumours reported as IHC 2+ are referred for FISH testing to determine the HER2 status when uncertain. In our previous study where IHC initially reported HER2 as negative while microarray analysis and reflex FISH testing revealed HER2 amplification (Grant et al. 2015), validity is supported by others who reported FISH amplification in 6% (Gown et al. 2008) and in up to 14% (Martin et al. 2012) of IHC 0/1+ samples. These discrepancies are not only due to pre-analytical factors or subjective interpretation of results, but

could be due to aberrant HER2 protein expression due to pathogenic germline variants not identified with routine immunostaining procedures. When comparing breast cancer patients in our study, *BRCA2* carriers with HER2-amplified cancers had predominantly high risk tumours based on MammaPrint scoring. Gene expression assessments propose that *BRCA1/2* related cancers and HER2-positive cancer are two different entities, therefore assessment of other tumour markers may be more significant than HER2 to help predict the risk of an actionable *BRCA1/2* variant in a patient to justify the cost of genetic testing (Maynes et al. 2010). The enrichment of the *BRCA2* c.7934delG variant among South African breast cancer patients referred for MammaPrint warrants further study in an extended sample to determine whether the MPA can be used to select breast cancer patients for *BRCA1/2* testing at the POC to facilitate clinical interpretation for improved clinical management.

5.3. *BRCA1/2* scoring algorithms and founder/recurrent mutation testing

The Manchester Scoring System was established on empirical information collected from the Manchester mutation screening programme. The purpose of the score is to restrict *BRCA1/2* testing to breast and ovarian cancer patients with the highest likelihood of carrying a pathogenic mutation, which in turn can be used for pre-symptomatic diagnosis in at-risk family members (Evans et al. 2004). Characteristics such as the number of affected family members and age of onset are given a score and these are added together to give a combined score for each of *BRCA1* and *BRCA2* regarding mutation detection probability. This total can be converted into a percentage chance of detecting a mutation in a single family member affected by breast or ovarian cancer (Evans et al. 2017). The updated pathology-adjusted Manchester score provides a valuable tool for estimation of the threshold for *BRCA1/2* probability. In unaffected family members, a 20-point score obtained in their affected first-line relatives indicate suitability at the 10% threshold for a positive *BRCA1/2* result. The individual Manchester scores calculated for the five South African patients with the pathogenic *BRCA2 c.7934delG* variant resulted in scores lower than 10 in four patients, including the index case who had no family history of cancer. Only one patient had a high score of 26 translating into a more than 10% chance that the patient has a *BRCA1/2* mutation, as previously shown at another laboratory prior to participation in this study.

In order for the Manchester scoring system to be more effective in the South African population, the inclusion of ancestry/founder mutation status might enhance the prediction of a *BRCA1/2* variant in an affected individual/family as evident from our results. In the South African population, at least eight *BRCA1/2* founder/recurrent mutations associated with development of the hereditary breast and ovarian cancer syndrome (HBOCS) were identified previously. In a study performed by Seymour et al. (2016) to determine the frequency of the three most common Afrikaner founder/recurrent mutations compared to non-founder pathogenic variants, testing for founder/recurrent mutations was recommended first, before further investigation or testing of other variants in cancer patients. These authors also reported

that the Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm (BOADICEA) was valuable for categorizing individuals at high risk for a *BRCA1/2* mutation and also helped with the decision for further testing.

The use of screening algorithms such as the Manchester score and BOADICEA, followed by genetic testing of a limited number of well-defined pathogenic variants present at an increased frequency in a given population due to a founder/recurrent effect, has traditionally been considered the most cost-effective way to perform genetic testing, before embarking on extended genetic testing in genetically uncharacterized patients. Recent use of a Markov model to compare the cost-effectiveness of population-based *BRCA1/2* testing with a clinical-criteria driven approach in Sephardi Jewish women based on breast/ovarian cancer outcome and excess deaths from heart disease supports the former approach, regardless of Ashkenazi/Sephardi ancestry (Patel et al. 2018). However, this dogma is increasingly challenged as the cost of NGS technologies decreases (Manchanda et al. 2018). According to Slade et al. (2016) when evaluating familial cancer screening in the UK, the most effective service approach was to screen at risk family members of cancer patients with pathogenic *BRCA1/2* variants detected by comprehensive screening of the index case unselected by age of onset or family history. The information gathered in the study was assessed over a 6-month period in comparison with patient-level data from the Royal Marsden Cancer Genetics audit, with testing offered to persons at ≥10% risk of having a pathogenic *BRCA1/2* variant. Approximately 40% of women who utilized the Cancer Genetics Service through the audit had breast and/ or ovarian cancer, although 62 % of unaffected women were worried about their family history. Application of a structured service strategy as an integral part of clinical oncology might be accomplished by including germline analysis with tumour pathology examination. Such integration with oncology practice was regarded most time-efficient with better delivery of equity of access to *BRCA1/2* screening than the standard highly selective service model based largely on family history and age of disease onset.

There is a lack of readily available reference costs for different methodologies used for *BRCA1/2* testing as a stand-alone test or as part of a clinical management algorithm in African countries. Analysis of breast cancer patients selected by the MPA in this study was the first step towards a risk-benefit analysis of combining the MammaPrint tumour gene profiling test with germline *BRCA1/2* and *CYP2D6* pharmacogenetics.

5.4. Incorporation of *BRCA1/2* POC testing into the MammaPrint care pathway

Numerous genetic risk-prediction models use a comprehensive approach that includes assessment of tumour pathology data (Evans et al. 2009, Tai et al. 2008). Some literature suggests using specified age pathological information in the risk prediction models might provide more precise mutation carrier prediction. Moreover, accurate classification of the distribution of tumour features in mutation carriers

might guide treatment strategies (Mavaddat et al. 2010). The National Comprehensive Cancer Network (NCCN) has established an all-inclusive set of clinical practice strategies to assist health providers in managing cancer patients. These endorsements require that any patient diagnosed with breast cancer or primary peritoneal/epithelial ovarian or fallopian tube cancer diagnosed, at or before the age of 50, must be referred for a genetic risk evaluation (Morgan et al. 2016). Genetic testing offers numerous benefits for women and their close family members, while genetic counselling helps patients make choices about their health and available therapies to improve understanding of cancer genetics (Schwartz et al. 2004). *BRCA1/2* mutation carriers might benefit from a prophylactic surgical procedure, use of chemoprevention as well as regular monitoring (Rebbeck et al. 2009). Adhering to recommendations improved after genetic testing and counselling (Burton-Chase et al. 2013), but despite patients' interest genetic counselling and testing endorsements have not been entirely incorporated into clinical practice. Since genetic testing could decrease deaths from breast and gynaecologic cancers, development of tools that can enable comprehensive cancer risk evaluations are important to make sure women with the disease are receiving proper treatment and care (Febbraro et al. 2015).

The rapid POC assay used in this study was developed based on previous identification of relatively common *BRCA1/2* founder/recurrent mutations in both tumour and germline DNA across ethnic groups in South Africa. This ParaDNA Screening System uses a disposable sample collector to convey the test material into the PCR consumable containing the pre-loaded reaction assay mix. DNA amplification with fluorescent HyBeacon probe detection of PCR amplicons was used to identify the target DNA sequences for eight *BRCA1/2* founder/recurrent mutations. Changes in fluorescence occurred as the HyBeacon probe melts away from an amplified allele at a specific temperature, ranging between 20-70^{o}C. This temperature variation correlates to a proportion of fluorescence as interpreted by the software and converted into colour-coded identification of the targeted DNA sequences. Standardisation of the POC assay employing multiplex PCR was first developed using the CFX96 Real-Time PCR detection system (Bio-Rad, Hemel Hempstead, UK), followed by transfer to the ParaDNA format. The ParaDNA BRCA 1.0 Research Assay comprises HyBeacon assays designed to simultaneously detect the founder/recurrent mutations in the *BRCA1* and *BRCA2* genes. DNA samples were genotyped using a 3-colour, 4-tube multiplex test for the *BRCA1/2* founder/recurrent mutations, which were accurately detected using melting curve analysis. Extracted DNAs can be manually added to each of the four plate wells or ParaDNA sample collectors (Blackman et al. 2015) used to transfer cells and DNA from buccal swabs to all plate wells simultaneously. The ParaDNA sample collectors or use of a drop of blood (diluted in water) allows immediate genetic testing of individual samples at or near the POC, without the not need to wait for DNA extraction or batching of samples for cost-effective genotyping on high-throughput laboratory-based apparatus. The test duration from sample-to-result is approximately 60 minutes and can provide same-day results in a clinic or a laboratory with relatively small numbers of samples received per day for a specific test.

POC testing using the ParaDNA platform has been exemplified by genotype guided dosing for warfarin, incorporating clinical and genetic factors to maintain the international normalised ratio within a therapeutic range (Jorgensen et al. 2019). Smooth implementation into routine clinical practice could be demonstrated, confirming the findings of a previous randomised controlled trial with a positive risk-benefit outcome in daily practice. Only minor adjustments were suggested by staff and patients who trusted the results obtained in clinical management as it was verified against standard real-time PCR including negative controls and duplicate samples with every run. We compared our results with alternative methods based on available genetic reports from other laboratories for a subset of patients, Sanger or NGS. Relevance of the Warfarin dosing POC kit to breast cancer was furthermore evident in at least one South African patient included in this study, who reported deep vein thrombosis (DVT) ascribed to hormone therapy. This could be explained by detection of the most common genetic risk factor for inherited thrombophilia (factor V Leiden mutation) routinely included in the CVD multigene assay illustrated in Figure 1. PSGT takes advantage of both founder/recurrent and pleiotropic gene effects to identify genetic variants in common pathways influencing multiple cancers and/or associated co-morbidities (Kotze 2016). Patients referred for the MammaPrint test who are at risk of therapy-induced comorbidities such as DVT, may therefore also benefit from Warfarin pharmacogenetics at or near the POC. Immobility peri-operatively and several anti-cancer therapies interact with genetic risk factors shared by cancer and associated co-morbidities. A multi-disciplinary approach may help to determine whether further testing using WES or other advanced technologies is necessary in patients with treatment failure, medication side-effects or co-morbidities that are not explained by the results of first-line POC assays.

Pharmacogenomics is undoubtedly one area of personalized medicine with proven clinical utility in the areas of cancer treatment and drug safety (Pirmohamed et al. 2014). Several translational research studies are focused on the introduction of pharmacogenetic analysis into clinical settings, targeting specific well-characterised genes such as *CYP2C19* for antiplatelet treatment (Empey et al. 2018, Cavallari et al. 2018) and *CYP2D6* genotyping for more than 20% of commonly prescribed drugs (Cavallari et al. 2019).

5.5. *BRCA2* c.7934delG and C*YP2D6* pharmacogenetics

The 70 genes included in the MammaPrint assay does not analyse genes such as *BRCA1/2* associated with development of familial breast cancer, or pharmacogenetic markers such as *CYP2D6* that may assist with the choice of endocrine treatment between Tamoxifen or aromatase inhibitors. Patients with two null alleles for *CYP2D6*4* or other null/reduced function variants in this gene are defined as poor metabolizers, while heterozygotes could be considered as intermediate metabolizers. In women

receiving adjuvant Tamoxifen treatment for familial breast cancer, Newman et al. (2008) showed that variation in the *CYP2D6* gene had a significant negative effect on overall survival in patients with pathogenic *BRCA2* variants which was not evident in *BRCA1* mutation carriers. This finding is of particular relevance in the South African population due to a founder/recurrent effect responsible for the increased *BRCA/2* mutation frequency as seen in this study, especially in hormone receptor-positive breast cancer patients using certain antidepressants that may inhibit CYP2D6 enzyme activity (van der Merwe et al. 2012a).

Confirmation of the pathogenic *BRCA2* c.7934delG variant in germline DNA of the index case after initial detection of this variant in DNA extracted from her bladder cancer biopsy, provided a likely explanation for development of a second cancer despite a MammaPrint low risk profile for breast cancer recurrence reported earlier. Detection of *CYP2D6*4* also confirmed in her germline DNA may furthermore explain the failure of Tamoxifen to prevent a second primary in this patient. The value of PSGT lies in the integration of results from multiple assays on the same platform, which started in the index case with referral for the MammaPrint test. Subsequently NGS of her tumour DNA identified eligibility for a PARP inhibitor and could explain failure of previous drugs used. If tumour sequencing was not performed first, this patient's familial risk would also not have been identified. Several studies have recently highlighted *BRCA2* as a potential predisposition gene for urothelial carcinoma (Nassar et al. 2019) that can predict the prognosis of bladder cancer (Kuang et al. 2019). These findings and the good response to Olaparib in *BRCA2*-altered urothelial carcinoma after chemotherapy and PD-L1 inhibitor failure highlight the potential benefit of *BRCA1/2* screening informed by intrinsic breast cancer subtype (Necchi et al. 2018).

The future of innovative technology development in the health care sector rests on consulting with health care professionals and understanding the challenges they are facing. By performing a clinical needs assessment, solutions can be developed to fill clinical gaps in disease management and treatment pathways. For this reason, prior to the introduction of POC DNA testing and genome sequencing training at a skills development workshop at the Tygerberg Academic Hospital (26-27 September 2019), a survey was performed at a pre-conference workshop of the Southern Africa Society of Human Genetics in Cape Town (August 2019). Questions were intended to assess the opinions of workshop participants consisting of medical scientists, genetic counsellors and treating clinicians. Results of the survey were published on the website of the Open Genome Project (https://www.gknowmix.org/opengenome/survey/), an initiative aimed at implementation of personalised medicine using an integrated service and research approach.

Of particular relevance to this study was that a large percentage of survey respondents (88%) who agreed with the potential benefits a pharmacogenetic POC test for *CYP2D6* genotyping may bring to identify increased risk for Tamoxifen resistance and potential interaction with anti-depressants, as well as Warfarin dosing (72%) already validated in the UK. Most respondents agreed (75%) that POC testing for *BRCA1/2* founder/recurrent mutations could augment the initiation of FDA approved PARP inhibitors for the treatment of hereditary breast and ovarian cancers.

Respondents to the survey were mostly supportive of a POC test which was affordable and could be used for intervention in the clinical care pathway as well as during genetic counselling (>90%), but that screening for founder/recurrent mutations should be led by taking ancestry and family history into account (78%). When patients cannot afford comprehensive panel gene testing, inexpensive POC founder/recurrent mutation testing would suffice (91%) if genetic counsellors, knowing the limitations of population based testing, would interpret the findings or refer patients for sequencing when the result is uninformative (94%). This approach where POCT for founder/recurrent variants followed by MinION or whole genome sequencing is implemented, would reduce the likelihood of missing those at risk of HBOC (78%), which may then be confirmed by Sanger sequencing (91%). Some respondents were of the opinion that receiving a mutation positive *BRCA1/2* POC testing results within an hour may be overwhelming for the unprepared patient (81%), as conventional testing provides time to mentally prepare for results (84%). Less respondents felt that providing the report after initiation of therapy defeats the purpose of genetic testing (34%), while most agreed (88%) that when patients need to pay for genetic testing themselves, they prefer more comprehensive gene panels rather than having another test later.

Although only 44% of workshop respondents were concerned that a population-specific POC test would not be clinically useful in a diverse population where reduced detection of *BRCA1/2* founder variants may occur over time, as well as infrequent requests for this testing in the private health care sector (66%), these analyses are still used as first line testing in the state sector where POCT could be widely utilised (75%). Where indication for *BRCA1/2* founder/recurrent mutation testing is uncertain due to lack of family history, some respondents (47%) felt that POCT could be used to detect cases of Lynch syndrome. Most respondents (84%) agreed that POCT is exciting and worth offering to other conditions where causative mutations are known such as in paediatric conditions, especially metabolic disorders where immediate treatment is lifesaving (88%).

In our study, a founder/recurrent *BRCA2* germline mutation was confirmed subsequent to tumour genomic identification, which supports incorporating appropriate POC BRCA DNA testing in oncology practice. This may benefit not only therapeutic decision making, but could present the opportunity for initiating cancer prevention strategies in affected family members (Veyseh et al. 2018). Careful

consideration is required about the level of expertise/qualification needed to perform and interpret the results obtained from germline DNA POC tests. Using moderately complex POCT, trained non-healthcare, non-medical staff are capable of generating similar results to those obtained by the laboratory (Laney et al. 2019). If maintaining strict quality assurance, nurses may be able to perform POC HIV testing as well as medical technicians (Gouws et al. 2016). These studies support the introduction and operation of routine pathology POC tests, but are not clear as to who should operate, interpret and report on results obtained from POC germline tests. As POC tests are designed to be operated easily with low technical requirements, a wide range of users have access to them with low error rates. However, some studies have highlighted user errors which when assessed, held no significant negative impact on patient health (O'Kane et al. 2011). However, this may not be the case in germline POC testing, where these errors may not be as negligible and could result in serious physiological trauma, unwarranted risk-reducing surgery or treatment with ineffective chemotherapeutic agents. It therefore becomes imperative that germline POC tests are performed and interpreted by medically trained operators on validated testing platforms with sufficient training and support, who will perform the assay according to the manufacturer's instructions under an established quality system. In South Africa, the SA Health Products Regulatory Authority (SAHPRA) is the statutory regulatory authority which replaced the Medicines Control Council for registration of *in vitro* diagnostics (IVDs), which covers POCT based on verification of registration in other countries or by the World Health Organisation.

5.6. Limitations and strengths

The small sample size and lack of direct comparison using Sanger sequencing or NGS in all 50 samples tested due to cost implications, were the major limitations of the study. With each new batch of BRCA POC kits to be manufactured in South Africa in future, at least ten control samples should be tested every time to first verify the accuracy of the assay, as described in the Results section. Similar to Sanger sequencing currently used in our laboratory to detect or confirm known pathogenic variants detected by NGS, it is not possible to include positive and negative controls when patient samples are screened using Hybeacon probes for POC testing on the ParaDNA apparatus. Failure of one of the 50 samples selected from the biobank for inclusion in this study alerted us to the need for improved quality assurance regarding the use of stored DNA samples. None of the results obtained in this study using the BRCA 1.0 Research Assay was reported back to clinicians or patients.

Detection of the same pathogenic *BRCA2* c.7934delG founder/recurrent variant among both MammaPrint high- and low-risk cases justifies further investigation in a larger study cohort. Stratification of patients with different tumour characteristics according to *BRCA1/2* status may identify modifier genes and environmental factors underlying differences in tumour pathology associated with

the same founder/recurrent mutation. Incorporation of *BRCA1/2* POC testing and genetic counselling into the MammaPrint care pathway may empower patients in high-risk populations with knowledge on the difference between tumour and germline genetics. Although ethnicity was not used to select the study cohort, the relatively high frequency of the *BRCA2* c.7934delG variant may relate to the fact that it is the most common founder mutation in the Afrikaner population of European descent.

Chapter 6

Conclusion

This study demonstrated that use of the MammaPrint prescreen algorithm (MPA) may identify patients who could benefit from pharmaco-diagnostic *BRCA1/2* screening at the point-of-care (POC), coupled with *CYP2D6* genotyping when tamoxifen is considered for endocrine treatment. Three of the patients with the same *BRCA2* founder/recurrent variant were also heterozygous for *CYP2D6**4 associated with an increased risk of tamoxifen resistance, an especially important consideration in patients with familial breast cancer (Newman et al. 2008). Two of these patients had a high risk MammaPrint profile and one a low-risk profile. Delayed detection of the cumulative risk scenario in the latter patient (index case) treated with tamoxifen for breast cancer, was a missed opportunity for preventing the onset of bladder cancer, which caused her death approximately four years after receiving a low-risk Mammaprint result. The histopatholgy results ruled out the possibility that development of bladder cancer in this patient was a result of risk misclassification using the Mammaprint test. Based on the literature, it is more likely that the defective *BRCA2* pathway represents a genetic link between breast and bladder cancer. Absence of a family history of cancer or other strong clinical indicators for *BRCA1/2* testing at the time of the MammaPrint test was performed, highlighted the importance of unrestricted screening of genes with pleiotropic function underpinning different forms of cancer. The unique genetic structure of the South African population makes cost-effective POC testing feasible as a first-line screening test towards comprehensive genome sequencing targeted at prevention of combined *BRCA/2* - *CYP2D6* effects.

The benefit of point-of-care technology (POCT) is that it provides quick access to potentially actionable information due to relative simplicity and wide accessibility to non-medical laboratory trained operators, especially valued in resource-limited settings. Generally, POC tests are of low complexity, for example urine dipsticks or pregnancy tests, while others such as disposable single-use tests for glucose are of medium complexity and can be performed by the patients themselves. The results of the BRCA POC assay used in this study should, however, be handled with extreme caution according to international genetic counselling guidelines. Despite the obvious advantages of *BRCA1/2* testing at the POC, a positive germline result has major consequences for both the patient and the extended family. In order to understand this issue, patients need time to discuss different clinical scenarios with educated healthcare workers. They need to disseminate and understand the information themselves before providing informed consent for *BRCA1/2* testing. Given the psychological burden of being a mutation carrier, the cost-effectiveness and accessibility of POC does not justify implementation outside a clinical setting. Up to four samples can be analysed in the 4-unit ParaDNA device, while multiple analytes as well as nucleic acid-based POC tests can be run simultaneously in new generation pocket-size sequencing devices such as the the MinION.

Selection of a test system should be based on intended clinical use and evaluated accordingly. Failure to place novel POCT in the clinical management care pathway is often due to the lack of clinical utility, possible suboptimal analytical or clinical validity, or due to a disconnect between the developer and the

needs of the test operator. The advantage of the ParaDNA POCT used in this study in relation to all three these aspects, became evident when the time needed to perform it and cost of DNA sequencing was considered for direct comparison of the BRCA 1.0 POC results obtained in all 50 samples analysed. This POC assay is considered suitable for evaluation in parallel with usual care to determine its true analytical and clinical validity.

Our finding that at least one of the South African *BRCA1/2* founder/recurrent mutations occurred in approximately 10% of hormone receptor-positive breast cancer patients, unselected for family history or age at diagnosis, warrants a pharmaco-diagnostic implementation study at the POC. Assessment of *BRCA1/2* founder/recurrent mutation status, alongside routine IHC assessment of ER, PR and HER2 status to approximate the luminal A, luminal B, HER2-enriched and basal molecular subtypes, could be performed during the time it takes to perform genetic counselling of patients considering Tamoxifen or gene-targeted therapies following MammaPrint gene profiling. Comprehensive assessment of recurrence risk in relation to both familial and therapy-related genetic risk factors on a single platform using PSGT, has the potential to translate into real-life clinical application of personalised genomic medicine.

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APPENDIX 1:

Letter OF ETHICAL APPROVAL 2018

Cape Peninsula University of Technology
HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)

Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South
Africa Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

20 June 2018
REC Approval Reference No: CPUT/HW-REC 2018/H10

Dear Mr Lwando Mampunye

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Mr Mampunye for ethical clearance on 29 March 2018. This approval is for research activities related to student research in the Department of Biomedical Technology at this Institution.

TITLE: MammaPrint risk score distribution in breast cancer patients with BRCA1/2 mutations.
Supervisor: Dr K. Grant and Prof M.J Kotze

Comment:

Approval will not extend beyond 21 June 2019. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Mr. Navindhra Naidoo

Chairperson – Research Ethics Committee

Faculty of Health and Wellness Sciences

APPENDIX 2:

Approval Letter Progress Report

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

25/10/2019

Project ID: 5190

Ethics Reference No: N09/06/166

Project Title: Analysis of the clinical utility of gene expression profiling in relation to conventional prognostic markers in South African patients with breast carcinoma

Dear Dr Kathleen Grant,

We refer to your request for an extension/annual renewal of ethics approval dated 19/09/2019 11:10.

The Health Research Ethics Committee reviewed and approved the annual progress report through an expedited review process. The approval of this project is extended for a further year.

Approval date: 25 October 2019

Expiry date: 24 October 2020

Kindly be reminded to submit progress reports two (2) months before expiry date.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: https://applyethics.sun.ac.za.

Please remember to use your Project ID **[5190]** and ethics reference number **[N09/06/166]** on any documents or correspondence with the HREC concerning your research protocol.

Mr. Francis Masiye, HREC Coordinator,

Health Research Ethics Committee 2 (HREC2).

National Health Research Ethics Council (NHREC) Registration Number: REC-130408-012 (HREC1)·REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372

Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number: IRB0005240 (HREC1)·IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the World Medical Association (2013). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects; the South African Department of Health (2006).Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa (2nd edition); as well as the Department of Health

(2015). Ethics in Health Research: Principles, Processes and Structures (2nd edition).

The Health Research Ethics Committee reviews research involving human subjects conducted or supported by the Department of Health and Human Services, or other federal departments or agencies that apply the Federal Policy for the Protection of Human Subjects to such research (United States Code of Federal Regulations Title 45 Part 46); and/or clinical investigations regulated by the Food and Drug Administration (FDA) of the Department of Health and Human Services.Yours sincerely,

APPENDIX 3

ETHICS RENEWAL

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)

Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South
Africa Symphony Road Bellville 7535
Tel: +27 21 959 6917
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16 August 2019

REC Approval Reference No: CPUT/HW-REC 2018/H10 (renewal)

Dear Mr Lwando Mampunye

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Mr Mampunye for ethical clearance on 29 March 2018. This approval is for research activities related to student research in the Department of Biomedical Technology at this Institution.

TITLE: MammaPrint risk score distribution in breast cancer patients with BRCA1/2 mutations. Supervisor: Dr K. Grant and Prof M.J Kotze

Comment:

Approval will not extend beyond 17 August 2020. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Dr. Navindhra Naidoo

Chairperson – Research Ethics Committee

Faculty of Health and Wellness Sciences