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

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# DECLARATION

**I the undersigned, hereby declare that the work contained in this thesis is my original work and that I have no previously submitted it, in its entirety or in part at any other University for a Degree.**

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# ABSTRACT

Breast cancer is a heterogeneous disease characterised by marked inter-individual variability in presentation, prognosis and clinical outcome. The recognition that morphological assessment has limited utility in stratifying patients into prognostic subgroups led to clinico-pathological classification of tumour biology, based on receptor expression using immunohistochemical (IHC) techniques. This standard is currently complemented by the development of gene expression profiling methodology that led to the identification of intrinsic molecular subtypes, reflecting tumour genetics as the true driver of biological activity in breast cancer.

The study was based on the hypothesis that molecular classification of breast carcinomas integrated with established clinico-pathological risk factors will improve current diagnostic and risk management algorithms used in clinical decision-making. A pathology-supported genetic testing strategy was used to evaluate microarray-based gene profiling against diagnostic pathology techniques as the current standard.

Clinico-pathological factors including age, number of positive axillary nodes, tumour size, grade, proliferation index and hormone receptor status was documented for 141 breast cancer patients (143 tumours) referred for microarray-based gene expression profiling between 2007 and 2014. Subsets of patients were selected from the database based on the inclusion criteria defined for three phases in which the study was performed, in order to determine 1) the percentage of patients stratified as having a low as opposed to high risk of distant recurrence using the 70-gene MammaPrint profile within the inclusion criteria, 2) correlation of HER2 status as determined by IHC and fluorescence in situ hybridisation (FISH) with microarray-based mRNA readout (TargetPrint), and 3) the relationship between hormone receptor determination as reported by standard IHC and molecular subtyping using the 80-gene BluePrint profile.

Similar distribution patterns for MammaPrint low- and high-risk profiles were obtained irrespective of whether fresh tumour biopsies or formalin-fixed paraffin embedded (FFPE) tissue was used. During the first phase of the study, 60% of the 106 tumour specimens analysed with MammaPrint were classified as low-risk and 40% as high-risk using a newly-developed MammaPrint pre-screen algorithm (MPA) aimed at cost-saving. In the second phase of the study, performed in 102 breast tumours, discordant or equivocal HER2 results were found in four cases. Reflex testing confirmed the TargetPrint results in discordant cases, achieving 100% concordance regardless of whether fresh tumour or FFPE tissue was used for microarray analysis. For the third phase of the study 74 HER2-negative tumour samples were selected for comparative analysis. Statistically significant positive correlations were found between protein expression (IHC score) and mRNA (TargetPrint) levels for estrogen receptor (ER) ($R=0.53$, $p<0.0001$) as well as progesterone receptor (PR) ($R=0.62$, $p<0.0001$), while combined ER/PR tumour status was reported concordantly in 82.4% of these tumours. BluePrint was essential for interpretation of these results used in treatment decision-making.

The MPA developed in South Africa in 2009 was validated in this study as an appropriate strategy to prevent chemotherapy overtreatment in patients with early-stage breast cancer. The use of microarray-based analysis proved to be a reliable ancillary method of assessing HER2 status in breast cancer patients. Risk reclassification based on the TargetPrint results helped to avoid unnecessary high treatment costs in false-positive cases, in addition to providing potentially life-saving treatment to those for whom it was indicated. While neither IHC nor TargetPrint estimation of intrinsic subtype correlated independently with the molecular subtype as indicated by BluePrint profiling, the ability to distinguish between basal-like and luminal tumours was enhanced when the combined protein and mRNA values was considered.

Genomic profiling provided information over and above that obtained from routine clinico-pathological assessments. This finding supports the relevance of a pathology-supported genetic testing approach to breast cancer management, whereby advanced genomic testing is combined with existing clinico-pathological risk stratification methods for improved patient management.

# OPSOMMING

Borskanker is ‘n heterogene siekte wat gekenmerk word deur merkbare inter-individuele variasie in kliniese beeld, prognose en uitkoms. Die beperkings van morfologiese klassifikasie vir identifikasie van prognostiese subgroepe het gelei tot klinies-patologiese tumor karakterisering op grond van reseptor uitdrukking deur gebruik van immunohistochemiese (IHC) toetse. Hierdie standaard word tans gekomplementeer deur ontwikkeling van geenuitdrukking tegnologie wat gelei het tot die identifikasie van intrinsieke molekulêre subtipes, wat die tumor genetika reflekteer as die ware drywer van biologiese aktiwiteit in borskanker.

Die huidige studie is gebaseer op die hipotese dat integrasie van die molekulêre klassifikasie van borskanker met konvensionele risiko klassifikasie skemas huidige diagnostiese en behandelings algoritmes kan verbeter vir kliniese besluitneming. ‘n Patologie-gesteunde strategie is gebruik om mikroplaat-gebaseerde geen profilering te evalueer teen standaard patologie diagnotiese tegnieke.

Kliniese-patologiese faktore insluitend ouderdom, aantal positiewe aksillêre limfnodes, tumor grootte, gradering, proliferasie indeks en hormoon reseptor status is gedokumenteer in 141 borskanker pasiente (143 tumore) wat verwys is vir mikroplaat-gebaseerde geenuitdrukking profilering tussen 2007 en 2014. Pasiënt subgroepe is geselekteer uit die databasis volgens die insluitingskriteria soos gedefiniëer in die drie fases waarvolgens hierdie studie uitgevoer is, om vas te stel 1) watter proporsie pasiënte geklassifiseer word as lae- of hoë-risiko vir latere herhaling van die borskanker deur gebruik van die 70-geen MammaPrint profile binne die insluitingskriteria, 2) hoe korreleer HER2 status soos vasgestel deur IHC en fluoreserende in situ hybridisasie (FISH) toetsing met mikroplaat-gebaseerde RNA lesings (TargetPrint), en 3)

wat die verwantskap is tussen hormoon reseptor status soos deur standaard IHC gerapporteer en molekulëre klassifikasie volgens die 80-geen BluePrint profiel.

Soortgelyke verdelingspatrone vir MammaPrint lae- teenoor hoe-risiko profiele is waargeneem ongeag of vars tumor biopsies of formalien-gefikseerde paraffin bevattende weefsel gebruik is. Tydens die eerste fase van die studie is 60% van die 106 tumore as lae-risiko en 40% as hoë-risiko geklassifiseer met toepassing van die nuwe MammaPrint Presifting Algoritme (MPA) wat ontwikkel is met die doel op kostebesparing. In die tweede fase van die studie waar 102 tumore ingesluit is, het die resultate van vier gevalle verskil van mekaar of was onbepaald ten opsigte van HER2 status. Refleks herevaluering het die TargetPrint resultate bevestig in alle nie-ooreenstemmende gevalle, en 100% ooreenstemming is bereik ongeag of vars tumor biopsies of formalien-gefikseerde paraffin bevattende weefsel gebruik is vir mikroplaat analise. In die derde fase van die studie is 74 HER2-negative tumore selekteer vir vergelykende analise. Statisties beduidende positiewe korrelasies is waargeneem tussen proteïen uitdrukking (IHC) en mRNA (TargetPrint) vlakke vir die estrogeen reseptor (ER) ($R=0.53$, $p<0.0001$) sowel as progesteroon reseptor (PR) ($R=0.62$, $p<0.0001$), terwyl gekombineerde ER/PR reseptor status ooreenstemming getoon het in 82.4% tumore. BluePrint was noodsaaklik vir die korrekte interpretasie van die resultate wat gebruik is in kliniese besluitneming vir behandeling van pasiënte.

The MPA wat in Suid Africa ontwikkel is in 2009, is gedurende hierdie studie bevestig as n toepaslike strategie om onnodige handeling met chemoterapie te voorkom in pasiënte met vroeë stadium borskanker. Die gebruik van mikroplaat-gebaseerde analise is aangetoon as ‘n betroubare aanvullende metode om HER2 status te evalueer. Risiko herklassifikasie gebaseer op TargetPrint resultate het onnodige hoë behandelingskoste in vals-positiewe gevalle vermy,

sowel as om die verskaffing van potensieël lewensreddende behandeling vir die toepaslike pasiënte te verseker.

Genomiese profilering het inligting addisioneel tot dit wat met roetine klinies-patologies metodes verkry kan word verskaf. Hierdie bevinding ondersteun die relevansie van ‘n patologie-gesteunde genetiese toets benadering tot hantering van borskanker, waardeur genomiese toetsing gekombineer word met bestaande klinies-patologiese risiko stratifisering metodes om pasiënt behandeling te verbeter.

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| | |
|---|---|
| **95% CI** | 95% Confidence Intervals |
| **ANOVA** | Analysis of Variance |
| **AO** | Adjuvant Online |
| **AOL** | Adjuvant Online! |
| **ASCO** | American Society of Clinical Oncology |
| **BIG** | The Breast International Group |
| **BRCA 1/2** | Breast Cancer gene 1/2 |
| **CAP** | College of American Pathologists |
| **CI** | Confidence Interval |
| **CISH** | Chromogenic in situ hybridization |
| **CVD** | Cardiovascular disease |
| **DFS** | Disease Free Survival |
| **DNA** | Deoxyribonucleic acid |
| **EORTC** | European Organisation for Research and Treatment of Cancer |
| **ER** | Estrogen receptor |
| **ESMO** | European Society for Medical Oncology |
| **FDA** | Food and Drug Administration |
| **FF** | Fresh Frozen Tissue |
| **FFPE** | Formalin fixed paraffin embedded |
| **FISH** | Fluorescence in-situ hybridisation |
| **HER2** | Human epidermal growth factor receptor-2 |
| **HTA** | Health Technology Assessment |
| **IHC** | Immunohistochemistry |

| | |
|---|---|
| **IMPAKT** | International Medical Products Anti-Counterfeiting Taskforce |
| **IVDMIA** | In Vitro Diagnostic Multivariate Index Assay |
| **MDD** | Major depressive disorder |
| **MINDACT** | **M**icroarray **I**n **N**ode-negative and 1 to 3 positive lymph node **D**isease may **A**void **C**hemo**T**herapy |
| **MP** | MammaPrint |
| **MPA** | MammaPrint Pre-screen Algorithm |
| **MRHM** | Metropolitan Health Risk Management (Pty) Ltd |
| **mRNA** | Messenger Ribonucleic acid |
| **NCCN** | National Comprehensive Cancer Network |
| **NCD** | Non-communicable disease |
| **NPP** | Negative predictive value |
| **PAM50** | Prediction Analysis of Microarray 50 |
| **PCR** | Polymerase chain reaction |
| **pCR** | pathologic complete response |
| **PPV** | Positive predictive value |
| **PR** | Progesterone receptor |
| **qRT-PCR** | Quantitative reverse transcriptase polymerase chain reaction |
| **RASTER** | Micro ar**RA**y progno**ST**ics in breast canc**ER** |
| **RNA** | Ribonucleic acid |
| **RT-PCR** | Reverse transcription polymerase chain reaction |
| **SERM** | Selective estrogen-receptor modulator |
| **SISH** | Silver enhanced in-situ hybridization |
| **SWOG** | Southwest Oncology Group |
| **TAILORx** | **T**rial **A**ssigning **I**ndividua**L**ized **O**ptions for **T**reatment (Rx) |

# CHAPTER 1
## INTRODUCTION

Breast cancer is the most common non-cutaneous neoplasm in women globally, with an estimated 1.4 million cases reported in 2008 based on data obtained in182 countries (Ferlay et al. 2013). Although breast cancer mortality rates have declined steadily over the past two decades, its incidence continue to increase worldwide, a trend which is particularly evident in developing countries, where the majority of cases are diagnosed at an advanced stage. Moreover, despite a good infrastructure typical of developed countries, low survival rates in the majority of South African breast cancer patients reflect late detection typical of resource-limited countries. The finding that stage at breast cancer diagnosis may depend on residential distance to a diagnostic hospital (Dickens et al. 2014) calls for more collaborative approaches in research and policy development to prevent the emerging cancer crisis in Africa (Busolo and Woodgate 2014).

Oncology is a prime example of a specialist discipline where the translation of molecular and genomic discoveries into real-world actionable benefits provides a framework for the routine implementation of a healthcare model which resonates with the principles and ideals of personalized medicine. The recognition that categorization according to histo-pathological criteria has limited utility in stratifying patients into meaningful prognostic subgroups laid the foundation for the establishment of classification schemes based on tumour characterization according to certain molecular phenotypes using laboratory techniques such as immunohistochemistry (IHC) (Viale 2012). Differentiation based on estrogen (ER) and progesterone receptor (PR) expression, as well as amplification of the HER2/neu oncogene and/or overexpression of its receptor (HER2), created an incentive to develop tailored treatment modalities including selective estrogen receptor modulators (SERMs) and HER2-targeted

therapies such as trastuzumab (Fisher et al. 1983; Slamon et al. 1987; Piccart-Gebhart et al. 2005).

These advances served to usher in the era of personalized breast cancer therapy, which until very recently, was largely restricted to classification based on ER and HER2 status. However, this prevailing standard has now been challenged by the emergence and increasing availability of novel technologies such as microarray analysis, which allowed for the identification of distinct disease subtypes based on intrinsic molecular activity, i.e. the luminal A, luminal B, HER2-enriched and basal-like phenotypes (Perou et al. 2000). It is therefore no longer appropriate to consider breast cancer as a single clinical entity, but rather a pathological spectrum characterized by marked variability in presentation, morphology, prognosis and therapeutic outcomes. The ability of gene expression profiling techniques to provide a comprehensive overview of multiple carcinogenic pathways may offer a more accurate estimation of recurrence risk compared to existing diagnostic standards, facilitating direction of treatment selection beyond their limited scope. There are now genome-based frameworks for the molecular categorisation of breast cancer including the development of prognostic and predictive signatures that potentially allow individualisation of treatment, which is of particular relevance in the context of directing the selection of patients eligible for chemotherapy.

Owing to the limited availability of gene expression profiling in the clinical setting, surrogate definitions for these subtypes based on IHC biomarkers have been proposed (Goldhirsch et al. 2011), as illustrated in Table 1. Although offering a convenient estimation, these surrogate panels lack standardization, and do not provide sufficient information to divide patients into meaningful prognostic and predictive subgroups (Guiu et al. 2012). A particularly noteworthy limitation is the limited ability of various proliferative biomarkers to distinguish between the luminal A and B subtypes. This is of significant clinical relevance: although luminal breast

cancer is per definition ER-positive, implying a favourable response to hormonal treatment, the more aggressive luminal B phenotype is not only relatively resistant to endocrine therapy, but less responsive to chemotherapy than other high-risk subtypes with which it shares striking pathogenic overlap (Tran and Bedard 2011). Moreover, although luminal B breast cancer is sometimes defined as IHC ER+/HER2- (Bhargava and Dabbs 2008), up to 20% of cases are IHC HER2-positive (Wiranpati et al. 2008). Furthermore, the IHC-based approximation of the basal-like subtype is equivalent to the definition of triple-negative breast cancer (TNBC); however, ~20% of basal-like breast cancers retain hormone receptor expression, while ~7% of triple-negative tumours are stratified as luminal (Prat et al. 2013).

**Table 1: Surrogate definitions for the intrinsic molecular subtypes of breast cancer based on immunohistochemistry biomarkers.**

| Molecular subtype | Prevalence (approximate) | Immunohistochemistry (IHC) biomarkers | Comments |
|---|---|---|---|
| **Luminal A** | 40% | ER+ and/or PR+, HER2-, low Ki67 | PR expression >20% may identify luminal A breast cancers with favourable prognosis |
| **Luminal B** | 20% | ER+ and/or PR+, HER2+ or HER2-, high Ki67 | Other proliferative markers which may help identify luminal B breast cancers include TP53, EGFR, CK5/6 and QSOX1 |
| **Basal-like** | 15-20% | ER-, PR-, HER2- | A minority of basal-like breast cancers retain hormone and HER2 receptor expression |
| **HER2-enriched** | 10-15% | ER-, PR-, HER2+ | HER2-postive tumours may also be of the Luminal B subtype |

Considering these findings in light of the limitations imposed by IHC testing, a pressing need exists to evaluate emerging genomic technologies against current diagnostic standards. In order to advance the routine clinical implementation of these novel genomic techniques, validation studies are essential in relation to a) analytical validity, i.e. its diagnostic accuracy and reproducibility, b) clinical validity, i.e. providing independent predictive and prognostic information, and c) clinical utility, i.e. predicting clinical outcomes in relation to changes in therapeutic decision making. Independent evaluation and quality assurance studies may promote the establishment of a standard practice platform; through credible testing and mitigation of incorrect or non-standardized testing results, potential detrimental consequences to the patient may thereby be prevented (Bartlett and Starczynski, 2011).

**Research focus**

Although the use of gene expression profiling has been shown to provide prognostic and predictive information above and beyond that for standard clinico-pathological risk stratification schemes relevant to breast cancer, most South Africa healthcare practitioners remain hesitant in embracing these emerging technologies as part of routine patient management. A growing recognition that standard methodologies used to assess hormone and HER2 receptor status may produce discordant results has sparked new interest in the use of RT-PCR and microarray-based signatures as potentially viable alternatives. Furthermore, it is increasingly appreciated that risk classification based on accurate molecular tumour subtyping may help assist clinical decision making by guiding the selection of appropriate emerging tailored treatments. While results from ongoing prospective clinical trials are eagerly awaited in order to conclusively affirm the value of gene expression profiles in breast cancer, a large body of retrospective data supports its use for disease prognostication and predicting therapeutic outcomes.

In the present study, gene expression profiling techniques are evaluated against conventional diagnostic standards with the aim of establishing their potential to add value by providing independent prognostic and predictive information. The evolution of laboratory techniques used in breast cancer risk stratification is reviewed in the next section (Chapter 2), followed by a description of the study population and methodology used (Chapter 3). The aims of the study are motivated by a description of the gap in current knowledge (Chapter 4) related to a versatile microarray platform that allowed three separate gene profile readouts evaluated:

1) MammaPrint: 70-gene assay used to predict response to chemotherapy according to recurrence risk
2) TargetPrint: single-gene mRNA readout which provides a quantifiable assessment of ER, PR and HER2 status
3) BluePrint: 80-gene profile used for tumour stratification according to molecular subtype

Findings obtained in relation to these analyses are presented separately in the results and discussion section (Chapter 5). This is followed by a general conclusion based on the new knowledge and database resource generated as a result of this investigation (Chapter 6) as well as references used (Chapter 7).

# CHAPTER 2
## LITERATURE REVIEW

There has been a steady evolution in the classification of breast cancer corresponding to increasing appreciation of its defining heterogeneity. Recognition that histo-pathological classification based solely on morphological criteria has limited utility in dividing patients into meaningful prognostic subgroups laid the foundation for the prevailing standard for stratification according to tumour characterization. The prognostic and predictive value of biomarkers assessed using IHC testing is well-evidenced. However, this prevailing standard has been challenged given more recent insights gathered from gene expression profiling studies, which allowed for a novel means of classification into distinct subtypes based on intrinsic molecular portraits.

While surrogate definitions for these intrinsic phenotypes have been proposed, they fail to consider the fact that the prognostic power of molecular subtyping is inherently based on a comprehensive and global evaluation of function. In this context, a pressing need exists to evaluate such emerging genomic applications against current diagnostic standards.

### 2.1 Diagnosis of breast cancer

The microscopic examination of biopsied breast tissue is the only diagnostic procedure that can determine with certainty if a suspicious lump is cancerous. The American College of Radiography developed standardised terminology to describe the findings of various breast imaging techniques used for diagnosis, namely mammography, ultrasound, and magnetic resonance imaging (MRI) (Thomassin-Naggara et al. 2014). Based on the Breast-Imaging Reporting and Data System (BI-RADS) lexicon, a seven-level positive predictive value of

malignancy classification system has been developed, giving imaging a central role in the diagnostic strategy.

Laboratory tests using either fresh biopsies or formalin fixed paraffin embedded (FFPE) samples could identify breast cancer subtypes with different survival rates and response to therapy. Although genetic testing is used increasingly in clinical practice, the precise diagnosis and prognosis of breast cancer still relies heavily on descriptive histo-pathological data (Luo et al. 2003). The observation that morphologically similar cancers may have diverse clinical outcomes highlights the important role robust molecular markers can play in diagnosis and risk assessment.

### 2.2 Risk of breast cancer

The combined risk of developing breast cancer by the age of 75 years is approximately one in 35 for South African women (NCR Report, 2007). More than 4 000 women are diagnosed with breast cancer every year in our country. Inherited gene defects explain about 5% of the total breast cancer incidence and approximately 20% of the familial risk, while acquired mutations account for the majority of disease (>80%). The best-known inherited breast cancers are caused by mutations in the BRCA1 and BRCA2 genes. Acquired genetic abnormalities result from error during gene reproduction or from interaction with environmental factors such as diet, hormones and other toxic exposures.

In settings where pre-symptomatic screening mammography is available, an increasing number of women are diagnosed with early disease and no nodal involvement. Most of these women enjoy long-term survival; however, 20-30% of cases relapse and die of their disease. In these patients, distant metastases are accountable for the majority of deaths and are the reason for

administering adjuvant systemic chemotherapy to all women considered at risk of relapse. The established clinico-pathological risk factors considered include age, number of positive axillary nodes, tumour size, grade, proliferating index, ER, PR and HER2/neu status. These factors are important in assessing risk of relapse but do not fully explain the biological complexity of breast carcinoma.

### 2.3 Introduction of microarray technology for breast cancer prognosis

The advent of microarray assays has enhanced our understanding of malignancies by allowing the simultaneous analysis of the activity of thousands of genes involved in different cancer-related pathways. Genes control the cellular function and activity of some important genes that may determine whether a tumour will metastasize. A microarray is numerous spots of highly concentrated fragments of DNA arranged in an ordered pattern of grids on a solid surface or "chip". These sequences map to specific genes and are the target for hybridisation of test RNA/cDNA from tissue and cellular samples. Application of microarray technology confirmed the concept that breast cancer is a heterogeneous disease comprising several histological types that have distinct biological features and clinical behaviour (Cleator and Ashworth 2004).

The most comprehensive test used for chemotherapy selection, called MammaPrint, allows highly 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). In the reference group, patients classified as "high-risk" using the MammaPrint test had 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" had a 97% chance of survival after 10 years and 87% chance to be metastasis free after 10 years without adjuvant treatment (van de Vijver et al. 2002). In an independent external patient group (Buyse et al. 2006) the 10 year survival prediction for low-

risk patients was 88% (81%-95%) using the MammaPrint test compared with 71% (63%-78%) for high-risk patients.

Oncologists are aware of an almost assured death from metastatic breast cancer and generally use the Adjuvant! Online software and the St. Gallen guidance criteria in an attempt to avoid under treating affected women. This results in many patients with early breast cancer suffering the side effects of over treatment, as well as increasing the economic burden on health care and the state. A significant number of patients can now be saved the toxic effects of chemotherapy without apparent benefit by applying gene-expression profiling.

### 2.4 Quality assurance

In the rapidly developing area of breast cancer treatment, continuous monitoring of new technologies against current standards is advisable. Assessment of the clinical utility and analytical validation (quality assurance) of transcriptional profiling in South African breast cancer patients formed an important aspect of this study. Clinical utility depends on the following parameters: 1) Prevalence if the disease in the tested population, 2) availability of effective interventions in genetic subgroups and 3) cost-effectiveness that is largely determined by the ability to isolate a target population that will benefit most.

### 2.5 From Immunohistochemistry to microarrays

Recognition of marked inter-patient diversity led to the development of clinico-pathological classification schemes complemented by evaluation of ER, PR and HER2 status as prognostic and predictive biomarkers using IHC techniques. In HER2 equivocal cases, DNA-based FISH is generally used to identify the 15-20% of breast carcinomas with a poor prognosis due to oncogene amplification. Confirmation of HER2-positive status plays a central part in determining

eligibility for HER2-targeted treatments such as trastuzumab (Herceptin), shown to reduce recurrence risk by up to 50% when combined with chemotherapy. Efforts to increase the prognostic and predictive value of laboratory tests led to the development of RNA-based gene expression profiling techniques using reverse transcription polymerase chain reaction (RT-PCR) and microarray analysis. The evolution from IHC and FISH to RT-PCR and microarray technology is highly relevant to introduction of MammaPrint as the most comprehensive multi-gene test used to predict risk of distant metastases and chemotherapy responsiveness. The addition of separate genetic profiles to the same microarray platform, which allows for hormone and HER2 receptor assessment as well as molecular subtyping at no additional cost, greatly enhanced the clinical utility of the MammaPrint service. The performance and added value of gene profiling in relation to IHC and FISH as the current diagnostic standards supported the implementation of a pathology-supported genetic testing strategy in the resource-limited South African setting (Kotze et al. 2013).

## 2.6 Immunohistochemistry

Assessment of tumour receptor status is routinely performed in all newly diagnosed breast cancer patients. Immunohistochemistry (IHC) is an inexpensive antibody-based assay where hormone receptor-specific antibodies and improved antigen retrieval methods allow ease of application to FFPE specimens. Despite the robustness and reproducibility of the biomarkers this technique assesses, IHC testing is limited by multiple pre-analytical, analytical and post-analytical factors which may contribute to variability in testing results reported.

### *2.6.1 Steroid hormone receptors*

The mandatory assessment of hormone receptor status is considered the standard of care in all newly diagnosed breast cancer patients. Therapeutic agents which target the ER receptor were

the first tailored treatment options for breast cancer, and it is now well-established that protein expression correlates with the likelihood of a favourable response to endocrine therapy and prolonged disease-free survival (Harvey et al. 1999). Confirmation of PR co-expression in ER-positive breast cancer implies a particularly favourable expected response to hormonal therapy while PR-negativity may correspond to a more aggressive phenotype less sensitive to such treatment (Cui et al. 2005; Nguyen et al. 2008).

Despite evidence supporting the robustness and reproducibility of ER and PR as prognostic and predictive biomarkers, limitations inherent to IHC testing, including sampling error, methodological variability, lack of assay standardization and observer variability in interpretation, contribute to increased risk of inaccurate reporting of hormone receptor status in breast cancer. Given the dependency of therapeutic decision making on these results, the American Society of Clinical Oncology / College of American Pathologists (ASCO/CAP) published guidelines aimed at addressing these shortcomings with the goal of reducing variability in the reporting of ER and PR status (Hammond et al. 2010). Suggestions outlined include standardization of pre-test tissue handling procedures, use of external controls, assay validation and the preferential use of a semi-quantitative rather than dichotomous method of interpretation. The threshold for determining ER positivity was previously recommended by ASCO/CAP as ≥10%; currently, it is defined as tumours with ≥1% positively staining cells.

### *2.6.2 Human epidermal growth factor receptor 2 (HER2)*

Evaluation of HER2/neu oncogene amplification and/or protein overexpression has important clinical implications, as it provides prognostic information and guides the selection of HER2-targeted therapy. Confirmation of HER2-positive status infers a significantly higher recurrence risk, with a shorter disease free and overall survival rate (Montemurro et al. 2013, Dowsett et al.

2008, Burstein, 2005). A lack of consensus regarding certain aspects of HER2 evaluation (Lee et al.2011, Bartlett and Starczynski, 2011, Vogel et al.2011, Viale, 2011) are a source of ongoing debate (Perez et al. 2012; Wolff et al. 2014), as testing variability may result in inaccurate reporting of HER2 status.

Perez et al. (2006) noted a discordance of 18% for IHC and 12% for FISH despite the analyses being performed on the same tumour specimen. Using conventional IHC testing, results for approximately 20% of samples are reported as equivocal (Sapino et al. 2013). Concerns that application of ASCO/CAP cut-off criteria could exclude certain patient groups who may have benefitted from HER2-targeted treatment support ongoing research into alternate techniques which may accurately and reproducibly measure HER2 status (Perez et al. 2014). The most recent ASCO/CAP update places emphasis on reducing false-negative HER2 results (Wolff et al. 2014) which could result in unnecessary and costly repetition of tests with a nominal increase in HER2 positive breast cancer cases (Rakha et al. 2014). Conversely, retesting of tumours reported as IHC 0/1+ or FISH-negative for HER-2 especially in younger patients with early-stage breast cancer is considered be a cost-effective approach (Garrison et al. 2013).

The complex interactions between ER and HER2 signalling pathways could account for marked inter-patient variability in responsiveness to hormonal and HER2-targeted treatments. ER- and HER2-positivity are inversely correlated (Quenel et al. 1995; Andrulis et al. 1998), while ER protein expression in hormone sensitive breast cancer is higher in patients who are HER2-negative (Konecny et al. 2003). Pinhel et al. (2012) investigated the relationship between ER and HER2 mRNA levels using RT-PCR in different tumour subtypes based on IHC surrogate definitions, and noted that, while mRNA levels were positively correlated in HER2-negative patients, the inverse was true of HER2-positive ones. HER2 mRNA levels were also higher in HER2-positive tumours that were ER-positive compared to ER-negative based in IHC results.

### 2.7 In-situ hybridisation

Fluorescence (FISH), chromogenic (CISH) and silver in-situ hybridisation (SISH) have been approved by the FDA for the determination of HER2 status in all 2+ IHC-equivocal cases, and to confirm gene amplification in 3+ IHC-positive cases.

These techniques are regarded as more discriminatory with less observer variation (Gutierrez and Schiff. 2011, Ellis et al. 2004) and considered the gold standard for determining eligibility for HER2-targeted treatment in HER2-positive patients (Meijer et al. 2011). However, up to 30% of tumours may retain equivocal reporting of HER2 status despite in situ testing (Clay et al. 2013). Although FISH is the method of choice for determining HER2 status on surgical specimens, there are several limitations, including requiring a high level of expertise in malignant cell recognition and differentiation between areas of invasive carcinoma versus that of normal tissue or in situ carcinoma (Moelans et al. 2011). Both IHC and FISH are slide-based; however, as the latter requires a fluorescence microscope, it is a more expensive and time-consuming test to perform. CISH/SISH have the advantage over FISH of using a standard microscope with a more robust DNA target and well-visualised malignant cells; however, all ISH techniques rely on subjective scoring. About 1 in 10 breast cancer tumours referred for ISH testing due to HER2 equivocal results (IHC 2+), pose diagnostic difficulties due to heterogeneous HER2 gene amplification, co-amplification of both HER2 and CEP17 regions (Starczynski et al. 2012) and overestimation of polysomy 17 (Marchiò et al. 2009).

### 2.8 Gene expression profiling techniques

Global gene expression studies performed over the last two decades have led to the development of genomic signatures which may be used to guide therapeutic management in early-stage breast cancer (Parker et al. 2009, Cornejo et al. 2014). A comparison of the two

genomic platforms currently available in South Africa, i.e. Oncotype DX using RT-PCR methodology (Paik et al. 2004, 2006) and MammaPrint using a microarray-based platform (van 't Veer et al. 2002, van de Vijver et al. 2002), is presented in Table 2.

**Table 2: Comparison between the Oncotype DX RT-PRC assay and the MammaPrint microarray platform.**

| Characteristic | Oncotype DX test (ODX) | MammaPrint platform (MP) |
|---|---|---|
| **Application** | Prognostic and predictive | Prognostic and predictive |
| **Test selection criteria (not influenced by BRCA mutation status)** | Stage I or II ER-positive lymph-node negative or positive (up to 3 nodes positive) breast cancer treated with tamoxifen for 5 years | Stage I or II lymph-node negative or positive (up to 3 nodes positive and 4- 9 nodes for prognostic purposes) breast cancer with a tumour size of 5 cm or less, regardless of ER status or tamoxifen treatment |
| **Tissue required** | FFPE | FFPE (minimum 30% tumour required) |
| **Assay platform** | RNA-based RT-PCR | RNA-based microarray |
| **Development strategy** | Developed from 250 breast cancer-related genes with known function at the time of test development | Developed from all ~25 000 genes in the human genome chosen blindly according to their biological effect |
| **Risk categories** | High (RS<18), intermediate (RS 18-30) and low risk (RS>30) | High and low risk (provided with recurrence arrow relative to cut-off point) |
| **Validation population** | Tamoxifen-treated patients (risk implications valid after five years of tamoxifen treatment) | Untreated patients after surgery |
| **Number of genes** | 16 genes (covers 3 cancer pathways)<br>5 reference genes | 70 genes for MammaPrint (covers all known cancer pathways)<br>3 genes for TargetPrint (ER, PR, HER2)<br>80 genes for BluePrint (tumour |

| | | |
|---|---|---|
| | | subtyping) |
| **ER, PR and HER2 status** | Forms part of the gene profile to calculate the recurrence score and also reported separately in the report | Reported through TargetPrint as a separate read-out as part of the MammaPrint microarray |
| **Molecular subtyping** | No | Yes, in addition Blue Print identifies patients who will not respond to hormonal therapy due to ER-positive tumours (~2%) lacking ERα function (yet expressing ERα at the protein and mRNA level) and distinguishes the basal-like subtype found to be more sensitive to specific systemic therapy regimes. |
| **Prospective trials** | No follow-up data available | Excellent 5-year survival in low-risk cases from RASTER trial |
| **FDA Approval** | No | Yes |

*2.8.1 Reverse transcriptase polymerase chain reaction technology (Oncotype DX test)*

Oncotype DX is a 21-gene assay based on RT-PCR methodology and includes ER, PR and HER2 assessment as an integral part of the recurrence score (RS). Patients eligibility for Oncotype DX testing are those with stage I or II ER-positive lymph-node negative or positive (up to 3 nodes positive) breast cancer treated with tamoxifen for 5 years. The assay was developed from 250 candidate genes, which identified 16 cancer-related genes of known function for inclusion together with five reference genes. Risk-associated genes reflect a limited number of pathological processes including proliferation, apoptosis survival and inhibited detoxification of carcinogens. Low ER-expression and high proliferation/invasion are associated with a greater risk of recurrence, while high ER, GSTM1 and BAG1 expression are associated with a better

prognosis. Oncotype DX assigns a continuous RS with three risk categories: low (RS<18), intermediate (18≤RS≤30) and high (RS≥31), based on the need for chemotherapy. The test uses FFPE specimens. The analytical reliability of the RT-PCR method was questioned based on the high false-negative rate of HER2 status in comparison with fluorescence in situ hybridization (FISH) considered the gold standard (Dabbs et al. 2011). Oncotype DX has not filed for clearance by the Food and Drug Administration (FDA) that serves as an independent opinion of analytical and clinical validation.

### *2.8.2 Microarray-based platform (MammaPrint service)*

Microarray-based testing platforms such as the MammaPrint service have moved to the forefront as the most comprehensive next-generation genomic application used to direct clinical management of patients with early stage breast cancer. This is based on their ability to provide 1) independent prognostic information and predict the responsive to chemotherapy based on risk classification, 2) a high-quality, quantifiable and objective evaluation of hormone and HER2 receptor status, and 3) a means of tumour stratification according to intrinsic molecular subtypes. The 70-gene microarray-based MammaPrint test, which has been available in South Africa since 2007, provides independent predictive and prognostic information above and beyond that for standard clinico-pathological risk stratification schemes, which may be used to determine eligibility for neoadjuvant chemotherapy in early-stage breast cancer (Straver et al. 2010, Iwamoto et al. 2011; Grant et al. 2013, Drukker et al. 2013).

#### *2.8.2.1 70-gene MammaPrint assay*

MammaPrint test is a 70-gene microarray-based assay used to guide therapeutic decision making concerning adjuvant chemotherapy based on recurrence and metastasis risk (van de

Vijver et al. 2002). A highly versatile microarray platform is used that reflects the critical hallmarks of cancer-related biology for classification of early-stage breast cancer into low- or high-risk groups for chemotherapy selection (Tian et al. 2010). It has been validated in at least 3 independent studies, with clinical utility confirmed by a recent prospective 5-year follow-up trial (Drukker et al. 2013). MammaPrint is offered to patients with stage I or II lymph-node negative or positive breast cancer with a tumour size of 5 cm or less, regardless of ER status or Tamoxifen treatment. The MammaPrint signature provides a binary stratification based on recurrence risk, i.e. low-risk or high-risk of distant recurrence (Figure 1).

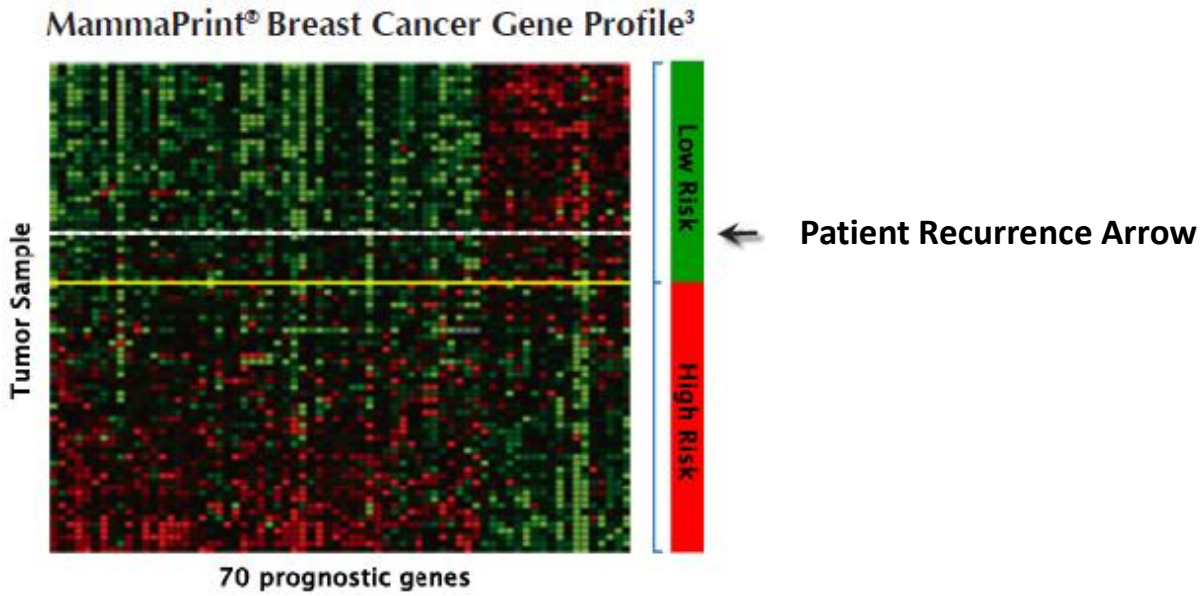


**Figure 1: Microarray analysis using the 70-gene MammaPrint profile**

The previous requirement for fresh tumour biopsies has been replaced by use of FFPE tumour specimens successfully used for MammaPrint since 2012 (Sapino et al. 2014). Use of microarray testing for risk stratification in early-stage breast cancer patients has been cleared by the FDA as an In Vitro Diagnostic Multivariate Index Assay that provided an independent opinion on the analytical and clinical validation of the test.

*2.8.2.2 Single-gene TargetPrint assay*

Although ER, PR and HER2 are not included as part of the 70-gene MammaPrint profile, their RNA expression profiles are routinely provided as a separate microarray readout, the TargetPrint test (Figure 2) when the MammaPrint service is requested (no additional cost). Exclusion of HER2 from the 70-gene profile has the benefit that 10% of HER2 positive patients may be identified as low-risk based on the MammaPrint 70-gene (Knauer et al. 2010).

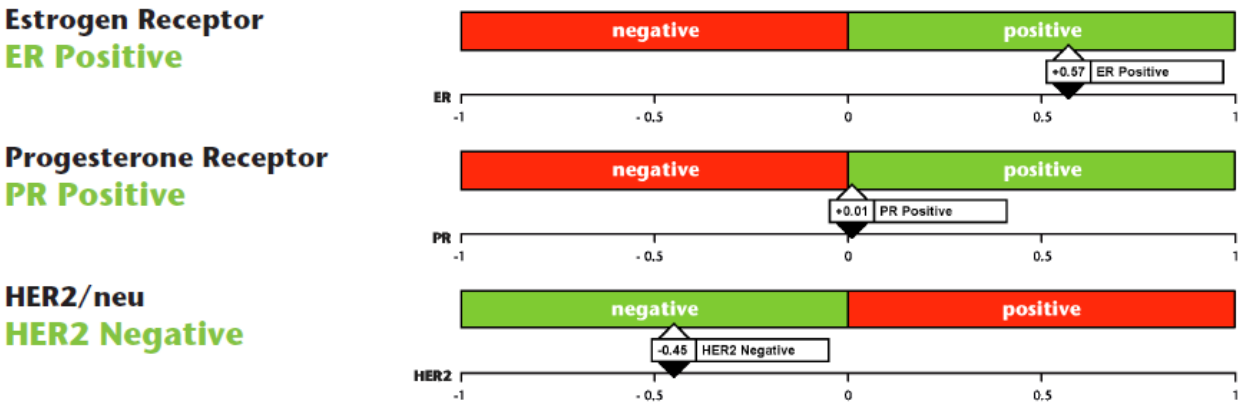


**Figure 2: Microarray analysis using the TargetPrint test**

TargetPrint proved highly accurate as a second opinion for receptor status and to resolve borderline or equivocal cases, given the growing recognition that standard IHC methodologies may provide discordant results. Comparative analyses indicated very high concordance between hormone receptor and HER2 status results provided by TargetPrint and standard IHC/FISH methodologies respectively. Comparably, assessment of HER2 status using conventional techniques has yielded results discordant with those provided as part of the Oncotype test in more than one study (Dabbs et al. 2011, Dvorak et al. 2013, Park et al. 2014).

This may be indicative of low quality precision, necessitating further extensive analytical validation as RT-PCR-based assays are not recommended for the assessment of HER2 status (Yamamoto-Ibusuki et al. 2013). As such, clinicians have to be aware that inaccurate determination of HER2 status by m-RNA-based assays such as Oncotype DX may lead to inappropriate treatment decisions (Dabbs et al. 2011, Christgen et al. 2012). These findings highlight the important role of the TargetPrint microarray read-out to support standard IHC/FISH tests used to guide expensive HER-targeted treatment. To improve cost-effectiveness in the resource-poor African context, HER2–positive breast cancer patients are not eligible for MammaPrint according to the reimbursement policy applied in South Africa (Grant et al. 2013), which should be revisited regularly as more data on the correlation of IHC and TargetPrint become available.

#### *2.8.2.3 80-gene BluePrint assay*

Different molecular subtypes of breast cancer vary in their response to chemotherapy. Addition of the 80-gene BluePrint profile as an extension of the MammaPrint service allows for the differentiation of luminal breast cancer into the A and B subtypes (Glück et al. 2013). This distinction cannot be achieved by standard pathology and demonstrates the power of the microarray platform as a discovery tool for ongoing research, based on the ~25 000 genes in the human genome evaluated during development of the MammaPrint test.

BluePrint recently identified the majority of discordant MammaPrint high-risk and Oncotype DX low-risk cases as being of the more aggressive luminal B subtype (Shivers et al. 2014). Luminal B is characterized by increased proliferation, higher recurrence rates and worsened overall prognosis, compatible with a high-risk MammaPrint profile (Creighton 2012). Use of the MammaPrint signature has also been shown to provide independent prognostic information in patients with the luminal A subtype and involvement of four to nine lymph nodes (Saghatchian

et al. 2013). More recently, BluePrint has also been shown to correctly reclassify patients with a higher pCR as having the HER2-enriched and basal subtypes (Bender and de Snoo et al. 2014). Table 3 shows the relationship between the four major breast cancer subtypes determined by IHC and the Blueprint molecular profiler.

**Table 3: Major breast cancer subtypes determined by IHC and microarray analysis**

| **SUBTYPE** | **PREVALENCE** (approximate) | **MOST COMMON IHC PROFILES FOR EACH SUBTYPE** (not all tumours will have these features within the subtypes) | **MICROARRAY PROFILING** (using MammaPrint, TargetPrint and BluePrint microarray platform) |
|---|---|---|---|
| Luminal A | 40% | ER+ and/or PR+, HER2-, low Ki67 | Important to distinguish patients with Luminal A and Luminal B subtypes as they are treated differently in relation to hormone therapy |
| Luminal B | 20% | ER+ and/or PR+, HER2+ (or HER2-), high Ki67 | |
| Basal-like | 15-20% | ER-, PR-, HER2- | Identification of basal-like subgroup important for selection of specific systemic therapy regimen |
| HER2-enriched | 10-15% | ER-, PR-, HER2+ | Patients with the HER2-enriched subtype respond better to trastuzumab than HER2-positive cases identified with standard IHC/FISH |

*2.8.3 Limitations of gene expression profiling*

A significant advantage of gene expression profiling techniques over standard IHC testing is the ability to assess a wider array of genes and therefore greater pathogenic diversity. The advantage of more comprehensive assessment of overall functionality provided by gene expression profiling is however offset by the requirement for more complex statistical evaluation required for the interpretation and therefore reporting of testing results, which currently lack standardization. In addition, assay and methodological variability between different platforms are important limitations that need to be considered in the context of RT-PCR (Nolan et al. 2006). Differences in sub-cellular receptor distribution poses a concern in IHC diagnostics; however, intra-tumour diversity in mRNA expression is a shortcoming inherent to more complex genomic technologies as well, particularly with regards to hormone and HER2 receptor mRNA variation in larger, morphologically heterogeneous neoplasms, on which the Oncotype DX RS (RT-PCR) is heavily based. The predictive results yielded by microarray-based tests are derived as a composite of all mRNA contained in a particular tissue sample; their estimation is therefore partly a reflection of the method by which the specimen was obtained (fine needle aspiration/core biopsy/tumour resection) as well as the proportion of malignant as opposed to benign cells (Ross et al. 2008).

## 2.9 Potential advantages of next-generation microarrays over RT-PCR technology

Many of the potential advantages of the microarray-based MammaPrint assay over the RT-PCR-based Oncotype DX test may be attributable to the selection of the former's constituent genes from the entire human genome (~25 000 genes) without a priori conception concerning their significance. The biological processes represented in the MammaPrint profile represent all known carcinogenic pathways implicated in neoplastic transformation, including invasion,

proliferation, extravasation, survival of apoptosis and angiogenesis (Tian et al. 2010, Kittaneh et al. 2013).

A growing body of evidence suggests that utilization of MammaPrint profiling provides valuable prognostic and predictive information above and beyond that for standard clinico-pathological risk stratification schemes. Direct comparative studies to date have however failed to demonstrate that Oncotype DX test offers such additional benefits independent of that for hormone and HER2 receptor status assessed using standard IHC methodologies (Cuzick et al. 2011, Iwamoto et al. 2011; Mattes et al. 2013). Studies have shown that determination of HER2 status using RT-PCR technology produces results discordant with those of IHC testing. Oncotype DX is currently not recommended for the independent determination of HER2 status; this has significant implications for the accuracy of the overall RS, since it is heavily reliant thereupon. This however does not pose a concern in the context of recurrence risk estimation by the microarray-based MammaPrint test, since HER2/neu is not included in the 70-gene assay, but rather provided as a separate readout.

BluePrint profiling may further identify high-risk patients with the basal-like subtype, who may require addition of an alkylating agent and/or platinum agent to taxane and anthracycline-based chemotherapy regimens. Low ER/PR expression, positive HER2 status, triple-negative disease (~70-80% being of the basal-like subtype) and luminal B subtype were found to be more responsive to chemotherapy. For luminal B and HER2-enriched subtypes, both anthracyclines and taxanes should ideally be included in the chemotherapy regimen, which can result in a pathological complete response (pCR) of 30-40% (Sikov 2014). The basal-like subtype is more sensitive to anthracycline-based neoadjuvant chemotherapy than luminal B breast cancer, with a poorer prognosis in the former ascribed to a greater likelihood of relapse in patients where complete pCR was not achieved (Rouzier et al. 2005). The addition of cyclophosphamide or

platinum agents to anthracycline-based neoadjuvant chemotherapy regimens has been associated with more favourable long-term health outcomes in patients with triple-negative breast cancer (Goldhirsch et al. 2011, Gelmon et al. 2012, Giacchetti et al. 2014). In patients treated with chemotherapy in the neoadjuvant setting where recurrence or treatment insensitivity is noted, HER2 status retesting may be necessary, since elimination of HER2-positive tissue may account for changes in IHC findings and the development of treatment resistance (Quddus et al. 2005).

There is significant overlap between BRCA1-mutated breast cancer and the basal-like phenotype (Turner and Reis-Filho 2006). BRCA1 dysfunction could relate to a possible favourable response to platinum agents evident for this subtype. This phenotypic overlap may further account for basal-like breast cancer being more sensitive to poly-(ADP) ribose polymerase (PARP) inhibitors than other molecular subtypes (Toft and Cryns 2011). A more recent clinical trial however failed to show that the addition of platinum-based treatments to chemotherapy regimens improves their efficacy in these patients (Alba et al. 2012). A number of novel subtype-specific treatments have been developed; for example, agents targeting insulin-like and fibroblast growth factor as well as phosphoinositide 3-kinase signalling pathways may prove useful in patients with luminal B breast cancer (Tran and Bedard 2011). Anti-angiogenic agents and drugs that target epidermal growth factor signalling may be particularly useful in patients with basal-like breast cancer (Toft and Cryns 2011)

### 2.10 Clinical utility and randomized control trials (RCTs)

While data from retrospective studies indicates that molecular profiling signatures provide useful predictive and prognostic information in early-stage breast cancer, validation thereof is often subject to bias and interpretation and may be clouded by failed methodology. Mixing of training

and test datasets in validation studies is problematic as it may result in overestimation of the discriminatory value of a gene profile. In order to overcome such limitations previously reported for Oncotype DX (Ionnidis 2007), large phase III prospective clinical trials are required to definitively establish clinical value. In this context the TAILORx trial may not be informative as it offers no comparison with currently used risk stratification parameters to conclusively determine predictive benefits. Concerning the design of this trial, patients with a RS ranging between 11 and 25 are randomly assigned to either hormonal therapy alone or in combination with chemotherapy, while those with a RS < 11 are assigned hormonal therapy for up to 5 years. This conflicts with the RS classification provided in the patient report, with a RS < 18, between 18-30 and ≥ 31 corresponding to low-, intermediate- and high-risk categories respectively. The number of genes and RS has been adopted for analysis of patients with ductal carcinoma in situ (DCIS). Furthermore, results from the SWOG 8814 trial, which aimed to determine whether this assay could predict the value of chemotherapy in lymph node positive breast cancer, showed no significant difference between low- and intermediate-risk patients. The 50-point increment suggested as indicative of its predictive value (Albain et al. 2010) is however not indicated in the report. The predictive utility of the RS may differ at present from the results obtained with anthracycline-based chemotherapy and those of the NSABP trial with CMF, which were based on older standards of chemotherapy than currently used in oncology practice regarding types of chemotherapy and dosing.

For the MINDACT trial which aims to further evaluate the predictive value of the MammaPrint test in relation to clinical risk indicators, patient enrolment was completed in 2011 (Rutgers et al. 2011). Prospective results from the RASTER trial were found to be in line with previous retrospective validation and proved that patients with a low-risk MammaPrint profile can safely be spared chemotherapy (Drukker et al. 2013). These findings are in accordance with local data confirming that MammaPrint reclassification of clinically high-risk patients to low-risk spares

unnecessary, costly and potentially lethal chemotherapy while maintaining excellent clinical outcome (Grant et al. 2013).

### 2.11 Prospective-retrospective studies as alternative to RCTs

The prospective randomized controlled study is considered the gold standard to conclusively determine the clinical value of a novel medical intervention prior to its adoption as part of existing practice. However, their necessity remains the subject of heated debate. In addition to raising complex ethical issues relating to the process of withholding treatment considered the standard of care from patients, these trials require a very large sample size and are extremely expensive and time-consuming to conduct, which are particularly noteworthy limitations in resource-limited environments. At the current rate of technological development in this emerging field, conclusive evidence for the clinical utility of a particular test under investigation may only become available once it is already obsolete. It therefore seems apparent that prospective clinical trials have their shortcomings and are not always practical or feasible. As recommended in the consensus statement of the IMPAKT 2012 Working Group, patient registries may represent a viable alternative to prospective clinical trials (Azim et al. 2013). Although no single approach is currently considered either standard or superior (Faulkner et al. 2012), the value of patient registries was demonstrated by Drukker et al. (2014), demonstrating excellent 5- and 25-year survival rates in MammaPrint low-risk patients spared unnecessary chemotherapy exposure. No prospective follow-up data has yet been published for the Oncotype DX test currently further evaluated in the TAILORx (Trial Assigning IndividuaLized Options for Treatment) trial to determine which intermediate-risk patients will respond to chemotherapy.

### 2.12 Concluding remarks

Although the use of gene expression profiles has been shown to provide prognostic and predictive information above and beyond standard clinico-pathological risk stratification schemes for breast cancers, most local healthcare practitioners remain hesitant in embracing these emerging technologies as part of routine patient management. A growing recognition that standard methodologies used to assess hormone and HER2 receptor status may produce discordant results has sparked new interest in the use of RT-PCR and microarray-based signatures as viable alternatives. Furthermore, it is increasingly appreciated that risk classification based on accurate molecular tumour subtyping may help assist clinical decision making by identifying patients eligible for neoadjuvant chemotherapy, in addition to guiding the selection of appropriate emerging tailored treatments. While results from ongoing prospective clinical trials are eagerly awaited in order to conclusively affirm the value of gene expression profiles in breast cancer, a large body of retrospective data supports its use for disease prognostication and predicting therapeutic outcomes.

# CHAPTER 3
## RATIONALE AND AIMS OF STUDY

The present study was based on the hypothesis that molecular classification of breast carcinomas integrated with established clinico-pathological risk factors will improve current diagnostic and risk management algorithms used in clinical decision-making.

### 3.1 Aims

The specific aims of the study were to address the following aspects investigated in three phases of the study:

Phase 1: Lack of proof that **MammaPrint** can reduce use of unnecessary chemotherapy in the South African population; this test was introduced in clinical practice based on international data published in the scientific literature (clinical validation).

Phase 2: Uncertainty about the performance of **TargetPrint** in comparison with standard IHC and FISH for determination of HER2 status using FFPE specimens (analytical validation).

Phase 3: Paucity of the added value provided in the local setting with use of **BluePrint** for identifying intrinsic subtypes for targeted treatment (clinical utility).

### 3.2 Rationale

Breast cancer is the most common cause of cancer-related mortality in women worldwide and places an increasing burden on health services in Western as well as non-occidental regions (Benson and Jatoi, 2012). Although mortality rates have decreased over the past two decades, the incidence of breast cancer continues to increase, particularly in developing countries, where the majority of cases are diagnosed at an advanced stage. Chemotherapy is generally the only treatment option available for patients with the most aggressive subtype, known as basal-like

breast cancer, which appears to predominate in Africa. In approximately 20% of breast cancer patients, HER2/neu amplification and/or overexpression is associated with a poor prognosis and resistance to tamoxifen as well as methotrexate-based chemotherapy regimens, while targeted immunotherapy with Herceptin (trastuzumab) reduces the recurrence rate by approximately 50%. Patients with hormone-dependent breast cancer usually respond to a 5-year course of selective ER modulators (SERMs), ovarian suppression or aromatase inhibitors. While the benefit of targeted therapy in triple-negative and HER2-positive breast cancer is well documented, only a small minority of patients with double hormone receptor positive and HER2-negative tumours will benefit from chemotherapy. However, all patients with such tumours offered chemotherapy are exposed to its side-effects.

Conventional treatment guidelines used to define patient eligibility for adjuvant chemotherapy consider tumour extent, lympho-vascular invasion and cellular morphology as surrogate markers for tumour biology. This approach may however overestimate the requirement for systemic treatment. Over the past 20 years, extensive research into the genetic mechanisms underlying the development and progression of breast cancer has allowed for the accurate identification of high-risk subtypes associated with poorer overall outcomes. Several genomic tests have been, of which MammaPrint and Oncotype Dx, are commercially available in South Africa.

### 3.2.1 MammaPrint

A critical step during the implementation phase of new technologies is to understand where any additional information provided by a genomic test could fit into the context of the current clinicopathological prognostication of early-stage breast carcinoma. Traditionally, tumours with ER and/or PR overexpression that are HER2-negative, have shown the lowest benefit to the

addition of adjuvant chemotherapy. The addition of anthracycline chemotherapy to Herceptin is considered standard of care, and HER2-positive tumours were expected to benefit little by further genetic profiling. Due to the lack of alternative treatment options in ER-, PR- and HER2-negative tumours, chemotherapy remains the only adjuvant option and the use of MammaPrint would not alter the treatment plan. Therefore, the ability to base clinical decision-making on microarray analysis after exclusion of triple-negative and HER2-positive patients following standard IHC and FISH assessments was an important consideration.

The likelihood that it would be most cost-effective to use MammaPrint in such a clinical intermediate group (ER-positive and HER2-negative) in the resource-poor South African context, prompted a local medical insurer to subject the 70-gene MammaPrint test to a health technology assessment (HTA) in 2009. The results of the HTA indicated a break-even-point for cost-effectiveness of the test (at R22 000 per test) at approximately R88 000 for the cost of chemotherapy (Bateman. 2009). In this model, conventional criteria for chemotherapy treatment, namely the St Gallen Index and Adjuvant! Online, were replaced with a newly defined MammaPrint Pre-screen Algorithm (MPA) which was further evaluated in Phase 1 of the study (Grant et al. 2013).

### *3.2.2 TargetPrint*

The HER2 subtype accounts for approximately 15% of all invasive breast cancers (Hanna et al. 2014). Quantification of HER2 status plays an integral role in breast cancer prognostication and prediction of the response to HER2-targeted therapies, shown to result in a 30-50% improvement in disease-free and overall survival when combined with chemotherapy. Assessment of HER2 status is routinely performed using IHC, while FISH is generally reserved for IHC (2+) equivocal cases. However, up to 20% of test results may be inaccurate and

standard IHC/FISH techniques cannot predict resistance to HER2-targeted therapy (Wolff et al. 2007, 2013). These findings highlight the need to analytically validate new methods that can be used in conjunction with standard pathology for establishing HER2 status.

RT-PCR methods using RNA extracted from FFPE samples is considered unsuitable for determination of HER2 status (Yamamoto-Ibusuki et al. 2013). This limitation was highlighted by concerns over the value of assessing HER2 status as part of the RT-PCR-based 21-gene Oncotype DX assay, which led to inappropriate HER-2 targeted treatment in some patients (Dabbs et al. 2011, Park et al. 2014) Determination of HER2 status using multi-gene profiling tests is currently not recommended due to potential clinical implications and the impact on cost-effectiveness (Park et al. 2014, Milburn et al. 2013). However, whether this also applies to microarray-based multi-gene assays remains unclear. HER2 status is provided as a separate read-out (TargetPrint) from the microarray-based 70-gene MammaPrint test, which was shown to consistently outperform existing clinico-pathological risk stratification schemes to accurately identify a subgroup of low-risk patients (including HER2-positive breast cancer) in whom chemotherapy can be safely avoided without compromising long-term clinical outcomes (Knauer et al. 2010, Drukker et al. 2014).

In Phase 2 of study, we investigated whether TargetPrint could improve quality assurance by serving as a second opinion for selection of patients for HER2-targeted treatment, supported by objective microarray-based analysis. To our knowledge, the clinical utility of TargetPrint using FFPE tumour specimens for the majority of samples tested has not previously been investigated in a clinical setting. Since none of the methods used for predicting the response to HER2-targeted therapy is currently considered optimal, we implemented the use of a combination of independent predictive indicators of tumour biology to complement standard HER2 assessment methodologies.

*3.2.3 BluePrint*

The growing recognition that breast cancer encompasses a heterogeneous disease spectrum characterized by marked variability in clinical presentation, morphology, prognosis and therapeutic outcomes necessitates the development and validation of scientifically sound classification systems in order to optimize patient management (Polyak, 2011). An appreciation for the limited utility of clinico-pathological stratification schemes laid the foundation for the prevailing use of immunohistochemical techniques to characterize tumours based on the routine assessment of hormone receptor status and HER2 overexpression/gene amplification in all newly diagnosed cases (Viale, 2012). This current standard has however been challenged by the emergence of novel genomic applications which allowed for the molecular classification of breast cancer into distinct biological subtypes.

In a seminal study by Perou et al. (2000) gene expression profiling led to the description of four intrinsic disease phenotypes, namely luminal A, luminal B, HER2-enriched and basal-like breast cancer. Luminal breast cancer is defined by ER positivity, and patients with the low-risk luminal A subtype are expected to respond favourably towards endocrine therapy. In contrast, the more aggressive luminal B phenotype, which is associated with a poorer overall prognosis and unfavourable clinical outcomes, not only displays relative resistance to hormonal treatment compared to luminal A breast cancer, but is consistently less responsive to chemotherapy than the high-risk HER2-enriched and basal-like subtypes (Tran and Bedard, 2011). The accurate delineation of luminal tumours into useful prognostic subgroups therefore has important implications for the selection of appropriate treatment.

Given the limited availability of genetic testing in the clinical domain, surrogate definitions based on IHC biomarker panels have been suggested as an alternative means of subtyping. In

particular, the use of proliferative markers as well as confirmation of PR negativity are proposed as a means of differentiating between the luminal A and B phenotypes (Cui et al. 2005, Cancello et al. 2013, Feeley et al. 2014). There is however still a lack of consensus regarding the utility of extended immunohistochemical panels, which although offering a convenient approximation thereof, is not necessarily a reflection of the true underlying molecular subtype (Goldhirsch et al. 2011, Guiu et al. 2012). Viewed in relation to the restrictions imposed by pre-analytical errors, lack of assay standardization and observer variability in interpretation, this creates an incentive to validate emerging genomic technologies against current diagnostic standards in early-stage breast cancer.

Despite promising findings, recent studies discourage the preferential use of RT-PCR technology above IHC testing for the assessment of hormone and HER2 receptor status (Kraus et al. 2012, Park et al. 2014). Multiple studies have however shown that microarray-based mRNA readout (TargetPrint), provided in a separate readout as extension of the MammaPrint service, is highly comparable to IHC testing for determination of ER and PR status (Roepman et al. 2009, Gevensleben et al. 2010, Viale et al. 2014). Most recently, the 80-gene microarray-based BluePrint profile, which utilizes molecular profiling to accurately stratify patients according to breast cancer phenotype, (Glück et al. 2013), was also been made available in the local setting.

In Phase 3 of the study, the potential added value of microarray-based molecular profiling using the 80-gene BluePrint assay was investigated in relation to ER/PR status which served as an approximation of the predicted breast cancer subtype. Insights gathered as a result of the present investigation may ultimately facilitate the development of decision making algorithms incorporating extended microarray-based assessment as ancillary to existing clinically

orientated risk stratification schemes. Ultimately, the implementation of these guidelines may serve to improve the standard of care for breast cancer in routine clinical practice.

# CHAPTER 4
## SUBJECTS AND METHODS

Tumour biopsies included for analysis in this translational research study became available within the framework of routine patient care.

### 4.1 Ethics approval

This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Ethical approval was granted by the Health and Research Ethics Committee (HREC) of the University of Stellenbosch (reference number N09/06/166).

#### *4.1.1 Informed consent*

The informed consent form approved by HREC is provided in the Appendix. In order to identify discordance between test results based on different methodologies including DNA- and RNA-based tests relevant to breast cancer, a waiver of consent was requested. This was approved without limitation to the method used or laboratories where the breast cancer related tests were performed due to low perceived risk associated with routine laboratory tests. Since return of research results may impact patient management, any information deemed important by the scientists were provided to the treating oncologist to be used according to their discretion.

### 4.2 Study population

Prospective study participants were selected from an initial cohort of 141 South African patients (140 females, 1 male; aged 27-78) with a histologically confirmed diagnosis of breast carcinoma.

Patient inclusion criteria: Patients older than 18 years with early-stage breast cancer (I to II A)

Patient exclusion criteria: Receiving neo-adjuvant chemotherapy.

For Phase 1 of the study, 104 patients referred for MammaPrint profiling by participating clinicians, as previously described by Grant et al. (2013). Two of these patients presented with >1 tumour. For Phase 2, a total of 127 specimens collected from 125 patients (40 fresh tumour biopsies and 62 FFPE specimens) were selected, as outlined in Figure 3. For Phase 3, of the study only HER-2 negative samples (74 FFPE tumour samples from 73 patients) previously subjected to the full genomic profiling array (MammaPrint, TargetPrint and BluePrint) were selected.

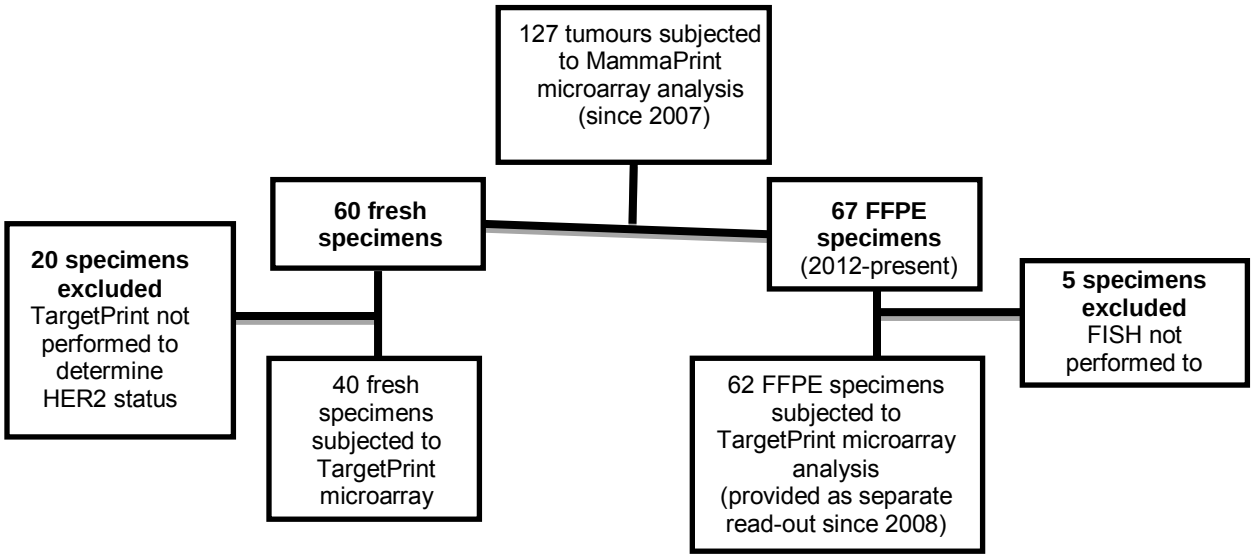


**Figure 3: Selection of 40 fresh and 62 FFPE tumour specimens for comparative analysis of HER2 status between microarray analysis (TargetPrint) and standard IHC/FISH.**

*4.2.1 Flow chart illustrating work plan*

Patient referred (Mammographically/clinically malignant and/or FNAB diagnosis)

↓ OR

Patient referred with no diagnosis

↓

FNAB/CNB done → diagnosis made including ER/PR/HER2 performed on slides

↓

Surgical procedure planned (no neoadjuvant therapy)

↓

Consent from patient obtained*

↓

Pre surgery, patient under general anaesthetic

↓

Tumour excision/ CNB sent for transcriptional profiling

↓

Surgical specimen for histopathology and correlation with transcriptional profiling

↓

* Since transcriptional profiling is already used routinely in clinical practice patient consent for participation in the study may be requested before or after surgery. For auditing of results between different laboratories/methods a waiver of consent was obtained.

## 4.3 Methods

### *4.3.1. Pre-test documentation of clinical and pathological data*

Clinical information and pathology test results including tumour classification, TNM stage, histological grade, lympho-vascular invasion status, and the presence of multi-centric pathology were obtained from patient records for inclusion in a central database. Relevant clinical and pathological data was then used for risk stratification to determine recurrence and mortality risk according to St Gallen or Adjuvant! Online guidelines. Details concerning the types of treatment provided to the patient were also documented by participating clinicians, including chemotherapy (whether in the neo-adjuvant or adjuvant setting), hormonal therapy, radiation therapy and surgery (including type of treatment and whether sentinel node biopsy or axillary dissection was performed).

The combined information gathered from this multidisciplinary managerial approach was firstly entered into the front-end of the research database (accessed at www.gknowmix.com). This data is utilized to determine whether the patient is eligible for microarray analysis, as may be reflected in the test quote automatically generated at referral. The provision of the test report following microarray analysis is in keeping with the principles of informed consent, analytical validity of the assay in question, as well as the notion that the information provided by such investigation are clinically actionable. Data obtained from patients who provided written informed consent for research participation were filtered through to the back-end of the patient database (accessed at www.gknowmix.org).

### *4.3.2 Immunohistochemistry (IHC) testing*

Standard pathology reporting of hormone and HER2 receptor status using IHC to measure protein expression levels varied amongst different laboratories. To standardize the data for

statistical analysis, estimation of semi-quantitative ER and PR expression was performed using the intensity score (0-3). This was multiplied by the reported proportion of positively stained nuclei, thereby calculating a final ER and PR score (0-300).

*4.3.3 Assessment of HER2 status using IHC and FISH*

Assessment of HER2 status by protein expression using IHC was performed during routine analysis according to local laboratory procedures. In cases where equivocal IHC scoring (2+) was reported and in 3+ cases samples were investigated for HER2 gene amplification using Locus Specific HER2 and chromosome 17 control FISH probes (Vysis). Silver enhanced in-situ hybridization (SISH) was performed in one sample with equivocal HER2 status following IHC and FISH, to allow for quantitative scoring of the gene copy number.

HER2 status was compared between locally performed IHC/FISH and microarray-based mRNA readout (TargetPrint). Concordance was regarded as the agreement between different testing methods concerning both positive and negative results. If different tests provided results which were negative versus equivocal or positive versus equivocal, these results were not regarded as discordant. Equivocal is not the same as discordant, but poses a clinical dilemma regarding treatment decisions for breast cancer patients with HER2 equivocal status.

*4.3.4 Sample collection for RNA extraction*

For analysis of fresh tumours, samples for RNA-based microarray testing were taken within an hour postoperatively. A single unfixed representative tumour sample was collected using a punch with a diameter of 6 mm (except for tumours less than 1 cm in diameter where a punch of 3 mm was used). Tumour samples were placed directly in preservative solution in a provided sample tube marked with an ID-sticker. Specimens were stored at 4ºC overnight (or -20ºC for

longer periods) before transportation at room temperature to Agendia in the Netherlands where the microarray analysis was performed. Since 2012, microarray-based analysis became available using FFPE tissue. With compliance to the MPA criteria, tumours were assessed by a local pathologist to determine eligibility for microarray-based analysis. Suitable tissue specimens were transported to the Netherlands under an export permit obtained from the South African Department of Health.

*4.3.5 Evaluation of tumour quality for microarray analysis*

Microarray-based gene expression profiling was performed at the centralized Agendia Laboratory in accordance with standard testing protocols (Glas et al. 2006). An experienced pathologist evaluated tumour suitability for genomic analysis based on confirmation of a minimum tumour cell content of 30% in accordance with compliancy criteria laid out by the US Food and Drug Administration (FDA). Samples that contain less than 30% tumour cells were excluded due to an inadequate yield of representative RNA from such specimens.

*4.3.6 Microarray-based gene expression profiling*

After tissue was micro-dissected from sections, RNA was extracted from fresh or FFPE tissue samples for microarray analysis using the 70-gene MammaPrint test, in combination with assessment of ER, PR and HER2 status using the TargetPrint test, and the BluePrint, 80-gene molecular sub-typing assay. Briefly, the mRNA was labelled with fluorescent dyes and hybridised against reference RNA. Two independent assays were performed for each patient, with triplicate measurements each against control genes. The quality of the RNA was determined using the Agilent bioanalyzer. Six replicate measurements of HER2 mRNA expression were consolidated in a single score considered positive for expression when a value of ≥0 was achieved on TargetPrint. Hormone receptor mRNA expression was reported on a

continuous exponential scale ranging from -1 to 1, with a value equal to less than 0 being considered the equivalent of ER- or PR-negative status.

*4.3.7 Defining the MammaPrint criteria*

The international MammaPrint selection criteria, that allow inclusion of ER-negative and HER2-positive breast cancer, were modified for use in the HTA being evaluated in Phase 1 of the study (Table 4).

**Table 4: Modification of the international criteria for MammaPrint for reimbursement purposes in South Africa.**

| | International | SA |
|---|---|---|
| Tumour size (cm) | <5.0 | ≤4 |
| Lymph nodes, *n* | ≤3 | ≤3 |
| Stage | I – II | I - II |
| Hormone receptor | ER-positive/-negative<br>PR-positive/-negative | ER-positive |
| HER2 | Positive/negative | Negative |
| Therapy | Tamoxifen independent | No neo-adjuvant therapy |

*SA = South Africa; ER = oestrogen receptor; PR = progesterone receptor; HER 2 = human epidermal growth factor receptor 2*

*4.3.8 Statistical analysis*

All statistical analyses were performed using the Statistica software and R Studio package (freely available at www.r-studio.org) were used in the study. Cross-tabulation frequencies were calculated for MammaPrint low-risk vs. high-risk classification between fresh tumour biopsy and FFPE tissue samples and compared using the Chi-square test. A one-way analysis of variance (ANOVA) was conducted to test for a relationship between risk classification and age of breast cancer diagnosis at the time that the MammaPrint test was performed. Observer agreement

measures for IHC and microarray-based mRNA readout (TargetPrint) assessment were calculated from two-way contingency table analysis, and included 1) sensitivity and specificity, 2) proportion positive and negative agreement and 3) Cohen's kappa (κ) scores (Landis and Koch 1977). The relationship between protein expression (IHC) and mRNA (TargetPrint) levels was assessed using Spearman rank correlation analysis. Results corresponding to a p-value of $< 0.05$ were deemed statistically significant.

# CHAPTER 5
## RESULTS AND DISCUSSION

The project was performed in three phases aimed to fill important knowledge gaps related to the MammaPrint test approved for clinical use by the FDA in 2007, as well as TargetPrint added from 2009 and BluePrint from 2011 as separate readouts from the same microarray.

### 5.1 Phase 1 Results: MammaPrint focus

The HTA performed by a medical insurer in 2009 introduced a set of test eligibility criteria – the MammaPrint Pre-screen Algorithm (MPA) – applied in the first phase of the study to determine the clinical usefulness of a pathology-supported genetic testing strategy, aimed at the reduction of healthcare costs. This implementation study took advantage of the fact that the 70-gene profile excludes analysis of hormone receptor and HER2 status, which form part of the MPA based partly on immunohistochemistry routinely performed in all breast cancer patients.

#### *5.1.1 Baseline characteristics*

Table 5 summarises the clinico-pathological characteristics of the study population including 104 female patients with a confirmed diagnosis of early-stage breast cancer. Of the tumour specimens analysed, MammaPrint classified ~ 60% of patients as low-risk and ~ 40% as high-risk for distant recurrence. Similar distribution patterns for low-risk compared to high-risk profiles were obtained irrespective of whether fresh tumour biopsies or FFPE tissue was used (Figure 4).

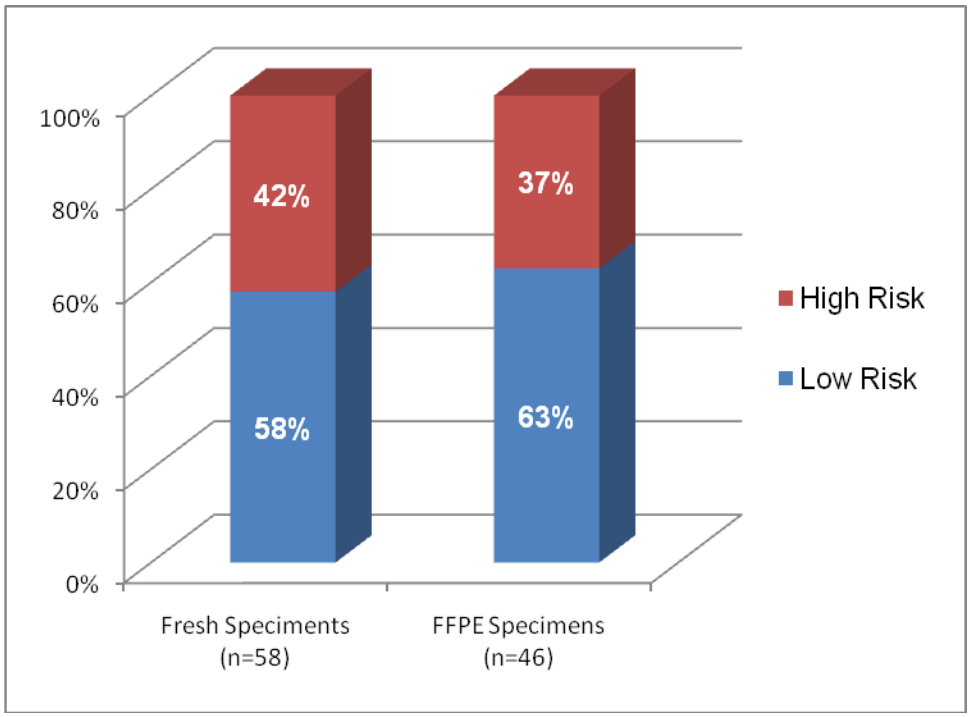


**Figure 4: MammaPrint high versus low risk profile distribution between FFPE and fresh tumour specimen types (*n*=104)**

No statistically significant relationship was found between risk classification and age at diagnosis (p=0.19), although younger patients tended to have a high-risk MammaPrint profile. The youngest patient (24 years old) had a high-risk profile and the oldest (78 years old) had a low-risk profile. Two patients (age >55 years) had multi-centric lobular carcinomas. One patient was identified as low-risk for both tumours, while the second patient had both a high- and low-risk tumour according based on MammaPrint profiling. The tumours of these two patients were not graded. IHC results for hormone and HER2 receptor status are not shown in Table 5, as these assessments formed part of the selection criteria for MammaPrint profiling using the MPA.

**Table 5: Clinical characteristics of tumours from female breast cancer patients in relation to the 70-gene MammaPrint profile.**

| | | 70-gene profile | |
|---|---|---|---|
| | **Total** | **Low-risk** | **High-risk** |
| | ***N*** | ***n* (%)** | ***n* (%)** |
| **Total** | 104 | 62 (60) | 42 (40) |
| **Specimen** | | | |
| Fresh | 58 | 33 (58) | 25 (42) |
| FFPE | 46 | 29 (63) | 17 (37) |
| **Age (years)** | | | |
| mean | 54 | 55 | 52 |
| <36 | 4 | 1 | 3 |
| 36 - 45 | 16 | 9 | 7 |
| 46 - 55 | 40 | 24 | 16 |
| >55 | 44 | 28 | 16 |
| **Pathology** | | | |
| Ductal | 85 | 51 | 34 |
| Lobular | 12 | 7 | 5 |
| Mucinous | 2 | 2 | 0 |
| N/A | 5 | 2 | 3 |
| **Tumour grade** | | | |
| 1 | 36 | 24 | 12 |
| 2 | 36 | 21 | 15 |
| 3 | 9 | 3 | 6 |
| N/A | 23 | 14 | 9 |

*FFPE = formalin fixed, paraffin-embedded, N/A = not available.*

*5.1.2 Effect of MammaPrint Pre-screen Algorithm*

When applying the criteria for selection of South African patients eligible for MammaPrint testing (Table 4), 95/104 patients qualified. In this subgroup, 59 (62%) were classified as low-risk and 36 (38%) as high-risk based on the 70-gene profile. The remaining 9 patients were referred for

MammaPrint before implementation of the MPA in 2009, or opted for genetic profiling outside the approved algorithm for medical aid reimbursement, as they refused chemotherapy based on clinico-pathological features alone.

*5.1.3 Discussion*

The South African MPA, developed with the aim of reducing healthcare costs, was validated in Phase 1 of this translational research study, as an appropriate strategy for selection of patients with early-stage breast cancer eligible for chemotherapy. The process used to introduce the 70-gene MammaPrint test into the local healthcare system included modification of the test selection criteria for local use, based on a) clinical experience, and b) establishment of an online database tool to provide support for reimbursement by funders and to facilitate long-term health outcome studies.

A total of 104 early-stage breast cancer patients were included in the present study. When we applied the MPA for determination of MammaPrint testing eligibility, 95 patients qualified for gene profiling. Using the MammaPrint test, 62% of patients in this clinical intermediate subgroup were re-classified as low-risk for recurrence. The HTA, using a model that replaced the conventional criteria for chemotherapy treatment (i.e. the St Gallen Index and Adjuvant! Online) with the MammaPrint profile, initially indicated a theoretical increase from 15% to >40% of patients who could be spared adjuvant chemotherapy. The fact that HER2-positive breast cancer accounts for approximately 20% of all breast cancers and that these cases, as well as ER-negative tumours on IHC, are excluded from genomic testing when applying the SA criteria, could explain the relatively high percentage of low-risk tumours in our study cohort.

Early-stage breast cancer patients with low-risk tumours can safely be spared chemotherapy, as demonstrated by Drukker et al. (2013) in the first prospective 5-year follow-up study performed for gene expression profiling in breast cancer. One subject in the present study cohort with bilateral, MammaPrint low-risk tumours has remained disease free since 2008, despite a family history of early-onset breast cancer. The patient tested negative for mutations in the BRCA1 and BRCA2 genes, analysed separately using DNA extracted from blood. She was included in our clinical outcome study performed in the first 50 SA breast cancer patients (Apffelstaedt and Kotze, 2011),which showed that two high-risk tumours and none of the low-risk tumours have recurred. This finding supported the treatment changes reported in 46% of cases discordant with conventional criteria, leading to a reduction in chemotherapy recommendations.

The proportion of low-risk patients identified using the MPA (62%) is in accordance with the results of Hartmann et al. (2012), who reported 38/60 (63%) female patients as low-risk using similar eligibility criteria for the 70-gene MammaPrint test, i.e. pT1c-3, pN0-1a, grade 2/3, hormone receptor-positive, HER2-negative tumours. While previous studies suggested that the amount of prognostic information provided using a basic IHC panel is similar to that of the Oncotype DX RS (Cuzick et al. 2011; Hartmann et al. 2012), the authors confirmed that MammaPrint provides further risk stratification over and above that obtained by standard pathology tests. These findings supported the development of the MPA as a pre-screen prior to MammaPrint testing in the resource-poor South African context. In a direct comparison of the cost effectiveness of the two different genomic profiling tests using a Markov model, Yang et al.(2012) demonstrated MammaPrint to be the most cost-effective. Several studies have demonstrated greater cost-effectiveness in patients with ER-positive tumours, which is in accordance with the HTA performed in the local population, based partly on the exclusion of patients with ER-negative and HER2-positive tumours for MammaPrint testing.

For breast cancer patients to benefit from new technologies, an increased focus on translational research is required to move basic science into clinical and public medical practice (Schully et al. 2011).The first step to incorporate gene expression profiling into clinical management of early-stage breast cancer patients in South Africa involved a feasibility study of the surgical procedure for specimen collection for the MammaPrint test (Kotze et al. 2005).The 70-gene MammaPrint test initially required a fresh tumour biopsy taken during surgery for microarray analysis, which was placed into a preservative solution and shipped to the reference laboratory at room temperature. Since the beginning of 2012, the option of using FFPE tumour tissue has also become available for the MammaPrint test. Initially, using a fresh biopsy to perform the test, a relatively high failure rate of 23% (18/78) was experienced, mainly due to the inability to meet the FDA requirement of at least 30% tumour cell tissue. It also required the pre-operative planning for tissue collection and storage of the tissue in an appropriate medium until the final tumour histopathology became available. In comparison, all 46 specimens tested to date using FFPE have been successfully analysed using fixed tissue collected during surgery or core needle biopsies.

The use of FFPE tissue instead of fresh surgical biopsies greatly enhanced the accessibility and convenience of the MammaPrint test both locally and abroad. Our finding of a similar distribution for MammaPrint low-risk and high-risk profiles using fresh tumour biopsies or FFPE tissue confirmed the feasibility of RNA extraction from FFPE specimens for use in microarray analysis. Analytical validation using FFPE specimens was confirmed by excellent agreement with IHC/FISH results for the determination of HER2 status using microarray analysis and was found to be particularly useful to help resolve borderline cases (data not shown).

Discrepancies may arise due to subjective interpretation of IHC and FISH results, arbitrary cut-off levels for positive results that may differ between commercial kits, or due to a low percentage

of invasive tumour in the tissue block when performing RT-PCR (Dabbs et al. 2011, Dvorak et al. 2013).Our pathology-supported genetic testing strategy enables the evaluation of a combination of tests most likely to overcome the limitations of a single test procedure. For both specimen types the low-risk MammaPrint profile was shown to be approximately 60%, which implies safe avoidance of chemotherapy in this subgroup.

The HTA-based recommendations for use of the MammaPrint 70-gene profile in SA breast cancer patients initially included a requirement that the breast tumour should range between 1 and 4 cm in diameter. Recently, this requirement was modified to include tumours <1 cm and core biopsies, due to the change of sampling from fresh biopsies to FFPE specimens for microarray analysis. The international criteria for MammaPrint include tumours up to 5 cm and are independent of ER/HER2 status and treatment regimes. Furthermore, stage I or II disease, with a maximum of 3 nodes positive for disease, are eligible for MammaPrint referral. Although axillary lymph node status is an important prognostic factor, 25-30% of node-positive patients remain free of distant metastasis. Mook et al. (2009) have demonstrated that the 70-gene signature can predict disease outcome in breast cancer patients with 1-3 positive lymph nodes. The conclusion was that MammaPrint accurately identifies patients with an excellent disease outcome in node-positive breast cancer, who may be safely spared adjuvant chemotherapy. Some patients in our cohort refused chemotherapy, yet opted to have gene profiling done outside the approved protocol for medical aid reimbursement. These included patients with 4 nodes positive for disease, triple-negative or HER2-positive tumours. Those found to be low risk despite a clinical high-risk profile could be reassured that chemotherapy might be safely avoided, as supported by the findings of Glück et al. (2013). The results of this retrospective analysis on prospectively collected tumour specimens accurately predicted response to chemotherapy and showed a 5-year survival benefit in luminal B, HER2-positive and basal-type tumours (Glück et al. 2013).The identification of a biological HER2-negative subgroup despite

IHC/FISH positivity may potentially lead to an extension of the MPA and further saving of healthcare costs in South Africa.

Use of gene profiling raises the confidence levels of clinicians regarding their treatment decisions (Bateman, 2009).Traditionally, results generated in the laboratory rely on the clinician to make a diagnosis and provide the appropriate treatment. Evaluation of the MPA in routine clinical practice performed in this study led to the development and implementation of an integrative pathology-supported genetic testing service for MammaPrint, whereby gene profiling is combined with pathological measurements to identify subgroups of patients requiring different treatment strategies. An online database tool is used to provide support for reimbursement by funders and to facilitate long-term health outcome studies. This integrative software tool, freely available to clinicians, should be seen as a platform where biology and the clinical components of medical judgement converge to assist clinicians in planning treatment of their patients.

The continuing rise in healthcare costs is unsustainable without changes in how cancer care is provided and reimbursed by funders. A process has therefore been initiated to develop a coverage policy for genomic tests by insurers in South Africa, based on the MammaPrint experience. Implementation of the MPA as a screening step for selection of patients for MammaPrint had a significant impact on reducing chemotherapy in early-stage breast cancer patients. The cost-saving implications of this approach support incorporation of the comprehensive microarray platform into treatment planning to (i) select chemotherapy in relevant early-stage breast cancer patients, (ii) confirm receptor status by providing quantitative gene expression assessment, as well as (iii) provide molecular subtyping of luminal A and B verifying receptor pathway activity.

## 5.2 Phase 2 Results: TargetPrint focus

Phase 2 of the study was conducted at the interface between the laboratory and oncology practice in an attempt to address the growing concern over the accuracy of standard techniques used for determination of HER2 status in breast cancer patients.

### *5.2.1 Baseline characteristics*

The baseline characteristics of 102 tumour specimens are presented in relation to MammaPrint test results in Table 6.

**Table 6: Clinical characteristics and HER2 status presented in relation to the 70-gene MammaPrint microarray profile performed in 102 tumours of breast cancer patients.**

| | | 70-gene profile | |
|---|---|---|---|
| | Total | Low Risk (%) | High Risk (%) |
| Total | 102 | 60 (58.8) | 42 (41.2) |
| Specimen | | | |
| Fresh | 40 | 24 (60) | 16 (40) |
| FFPE | 62 | 36 (58.1) | 26 (41.9) |
| Age (years), mean | 53.1 | 54.2 | 51.5 |
| <36 | 5 | 2 | 3 |
| 36-45 | 21 | 10 | 11 |
| 46-55 | 37 | 25 | 12 |
| >55 | 39 | 23 | 16 |
| Pathology type | | | |
| Ductal | 82 | 47 | 35 |
| Lobular | 16 | 10 | 6 |
| Mucinous | 3 | 3 | 0 |
| Medullary | 1 | 0 | 1 |
| Pathology grade | | | |
| 1 | 28 | 19 | 9 |
| 2 | 45 | 23 | 22 |
| 3 | 11 | 4 | 7 |
| N/A | 16 | 14 | 4 |
| HER 2 | | | |
| Positive | 5 | 0 | 5 (100) |
| Negative | 97 | 60 (61.9) | 37 (38.1) |

N/A= not available

### *5.2.2 Comparison of HER2 status determined by IHC and FISH/SISH*

FISH was performed on 19 (18.6%) of the 102 samples, based on IHC positive (3+) or equivocal (2+) results (Table 7). A 50% concordance rate between IHC 3+ and FISH amplification of HER2/neu was demonstrated. In the IHC 2+ group, 86% (12/14) of tumours were negative for HER2/neu amplification as assessed by FISH. Only one (5%) tumour reported as IHC 2+ remained FISH/SISH equivocal for HER2 status.

**Table 7: Comparison of HER2 gene amplification determined by in situ hybridisation and HER2 protein expression determined by immunohistochemistry in 19 tumour specimens of breast cancer patients.**

| FISH | IHC Results | | | |
|---|---|---|---|---|
| | **Negative (%)** | **Equivocal (%)** | **Positive (%)** | **Total (%)** |
| | **0/1+** | **2+** | **3+** | |
| **Positive** | 1(100%)[a] | 1 (7%) | 2 (50%) | 4 (21%) |
| **Equivocal** | 0 (0%) | 1 (7%) | 0 (0%) | 1 (5%)[b] |
| **Negative** | 0 (0%) | 12 (86%) | 2 (50%) | 14 (74%) |
| **Total** | **1 (5%)** | **14 (74%)** | **4 (21%)** | **19 (100%)** |

[a]FISH performed in IHC 0 case due to microarray result which confirmed accuracy of TargetPrint; [b]IHC 2+ remained equivocal on FISH and repeated SISH

*5.2.3 Comparison of HER2 status between IHC/FISH and TargetPrint*

In three tumours, TargetPrint HER2 status and the original IHC/FISH reports were discordant, with one case reported as HER2 negative by IHC. Resolution of an equivocal IHC/FISH testing report was provided by TargetPrint in a fourth sample (Figure 5).

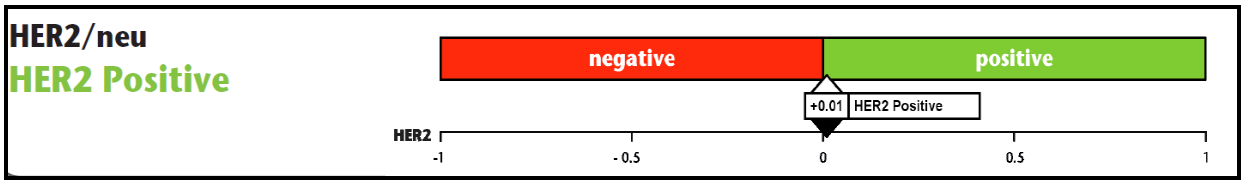


**Figure 5: TargetPrint result showing HER2 status of a patient with early-stage breast cancer. No definitive result for HER2 status could be provided for 1 sample using IHC/FISH, while the quantitative RNA expression using microarray analysis (TargetPrint) showed a slightly positive value of +0.01.**

Reflex FISH/SISH testing at two different reference laboratories after sample exchange confirmed the accuracy of the negative TargetPrint results in two borderline FISH-positive cases. A ratio of 2.98 was demonstrated with FISH in the IHC negative (0) case with a HER2 positive TargetPrint result. A comparison of IHC/ISH and TargetPrint HER2 assessment in the total 102 tumour specimens analysed is shown in Table 8. As an equivocal result is not discordant, the equivocal case was not included when the correlation between IHC/ISH and microarray HER2 receptor status was determined, demonstrating 100% agreement.

**Table 8: Comparison of HER 2 status between IHC/FISH and TargetPrint results after reflex testing and exclusion of one equivocal case resolved by microarray analysis.**

| **Pathology** | **TargetPrint (mRNA)** | | |
|---|---|---|---|
| **HER2 IHC/FISH** | **HER2 positive (*n*=5)** | **HER2 negative** | **Total** |
| **Positive** | 4 (100%) | 0 (0%) | **4 (4%)** |
| **Negative** | 0 (0%) | 97 (100%) | **97 (96%)** |
| **Total** | **4 (4%)** | **97 (96%)** | **101 (100%)** |

### 5.3 Discussion

In phase 2 of the study we demonstrated the added value TargetPrint provided with the 70-gene MammaPrint microarray analysis in 102 tumour specimens, to definitively establish HER2 status in equivocal or discordant cases using conventional IHC/FISH methodologies. Risk reclassification in 4 patients (3.9%) based on HER2 status resolved by TargetPrint microarray analysis led to a change in treatment decisions supporting the clinical utility of the test in the following cases: two HER2-negative patients with previous borderline-positive FISH results, one false-negative case on IHC subsequently confirmed by FISH as HER2-positive in line with the TargetPrint result, and one equivocal case resolved as HER2-positive by TargetPrint. We therefore demonstrated a 100% concordance rate between IHC/FISH and TargetPrint results for HER2 tumour status irrespective of whether fresh surgical biopsies (40 samples) or FFPE

specimens (62 samples) were used. This finding proved that use of FFPE tumour specimens provides a reliable source of RNA for microarray analysis of HER2 status, analytically validated for the first time in South African breast cancer patients using TargetPrint.

Our results are in general agreement with those reported in a comparative analysis of HER2 status using TargetPrint considering the first 800 patients enrolled in the MINDACT (Microarray In Node-negative and 1 to 3 positive lymph node Disease may Avoid ChemoTherapy) trial (Viale et al. 2014), with cases considered HER2 positive when scored 3+ by IHC and/or amplified by FISH (ratio ≥2). A lack of consensus regarding the optimal cut-off value for HER2 testing in breast cancer is a source of on-going debate (Wolff et al. 2013, Perez et al. 2014), which highlights the clinical relevance of our findings. Although current testing techniques for HER2 receptor status are limited in their ability to identify patients likely to benefit from HER2-targeted therapy (Hurvitz et al. 2013), retesting of IHC 0/1+ or FISH-negative cases is projected to be a cost-effective clinical strategy (Garrison et al. 2013). For patients enrolled in our study, microarray HER2 tumour status was determined in addition to MammaPrint at no extra costs.

Concerns that the evidence is insufficient to base clinical decision making concerning eligibility for trastuzumab therapy on multi-gene testing (Dabbs et al. 2011, Park et al. 2014, Milburn et al. 2013)do not apply to the advanced microarray technology evaluated in this study in comparison with standard IHC/FISH. Despite limited sample size, our study demonstrated that microarray-based assessment of HER2 status using either fresh biopsies or FFPE specimens is highly reproducible and accurate, effectively reducing the number of false-negative and false-positive IHC/FISH results. To our knowledge, this is the first study which initiated independent FISH/SISH retesting on the same tumour specimen used for TargetPrint when a discordant HER2 microarray result was obtained.

Analytical validation of MammaPrint (Sapino et al. 2014) and TargetPrint (present study) microarray analysis on FFPE tissue is an important milestone in the era of personalised genomic medicine. Our pathology-supported genetic testing approach led to safe avoidance of chemotherapy in more than 60% of patients previously identified as low-risk for distant metastasis based on the MammaPrint profile (Grant et al. 2013),with no distant recurrence of disease or breast cancer-related deaths reported to date in the 7-year follow up data from the low-risk group. The use of microarray analysis in the present study furthermore proved to be a reliable ancillary method of assessing HER2 status in breast cancer, demonstrating the clinical utility of this assay as evidenced by changes in therapeutic decision making. By considering the TargetPrint results in the context of those provided by conventional testing and MammaPrint, an improved level of confidence in HER2 tumour status could be achieved on which to base treatment decisions.

### 5.4 Phase 3 Results: BluePrint focus

#### *5.4.1Description of tumour morphology in relation to molecular subtype*

The baseline pathological characteristics of FFPE tissue specimens (n=74) subjected to microarray-based assessment are presented in relation to tumour subtype in Table 9. Molecular profiling stratified 49 tumours (66.2%) as luminal A subtype, 21 tumours (28.4%) as luminal B and the remaining four tumours (5.4%) as basal-like. For one patient with a diagnosis of multi-focal pathology, one tumour was designated luminal A and the other luminal B.

**Table 9: Comparison of tumour morphology and grade between FFPE samples (n=74) obtained from 73 South African breast cancer patients in relation to molecular subtype**

| | Total<br>n (%) | Luminal A<br>n (%) | Luminal B<br>n (%) | Basal-like<br>n (%) |
|---|---|---|---|---|
| **Total (n)** | 74(100) | 49(66.2) | 21(28.4) | 4(5.4) |
| **Tumour pathology** | | | | |
| Ductal | 62(83.8) | 41(67.2) | 17(27.9) | 4 (6.5) |
| Lobular, Pleomorphic | 3 (4.1) | 1(33.3) | 2(66.7) | 0 (0.0) |
| Lobular, Classic | 9(12.2) | 7(77.8) | 2(22.2) | 0 (0.0) |
| **Tumour grade** | | | | |
| 1 | 17(23) | 14(82.4) | 3(17.6) | 0 (0.0) |
| 2 | 34(45.9) | 23(67.6) | 10(29.4) | 1(2.9) |
| 3 | 15(20.3) | 6(40.0) | 6(40.0) | 3(20) |
| N/A | 8(10.8) | 6(75.0) | 2(25.0) | 0 (0.0) |

*5.4.2 Comparative analysis of ER and PR status*

Microarray-based mRNA readout (TargetPrint) had a sensitivity of 0.92 (95% CI: 0.83-0.96) and specificity of 0.67 (95% CI: 0.21-0.94) for ER; for PR, the sensitivity was 0.94 (95% CI: 0.85-0.98) and specificity 0.63 (95% CI: 0.31-0.86) for PR. The proportion of positive and negative agreement for ER was 0.95 and 0.36; for PR, the proportion of positive and negative agreement was 0.90 and 0.59 respectively. Microarray-based mRNA readout showed fair to moderate agreement with IHC testing respectively for determination of ER ($\kappa$=0.32) and PR status ($\kappa$=0.53).

Significant positive correlations were further noted between protein expression (IHC score) and mRNA (TargetPrint) levels for ER (R=0.53, p<0.0001) as well as PR (R=0.62, p<0.0001), as illustrated in Figures 6 and 7. These associations retained significance in a separate sub-analysis according to recurrence risk in both high-risk (ER: R=0.63, p=0.0008; PR: R=0.79, p<0.0001) and low-risk (ER: R=0.41, p=0.0036; PR: R=0.45, p=0.001) cases.

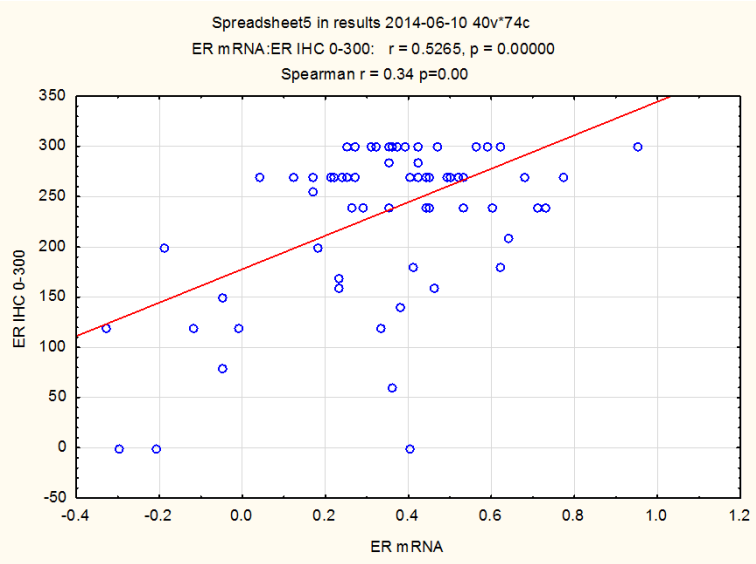


**Figure 6: Scatter plot diagram illustrating a significant positive correlation between protein expression (IHC score) and mRNA (TargetPrint) levels for ER.**

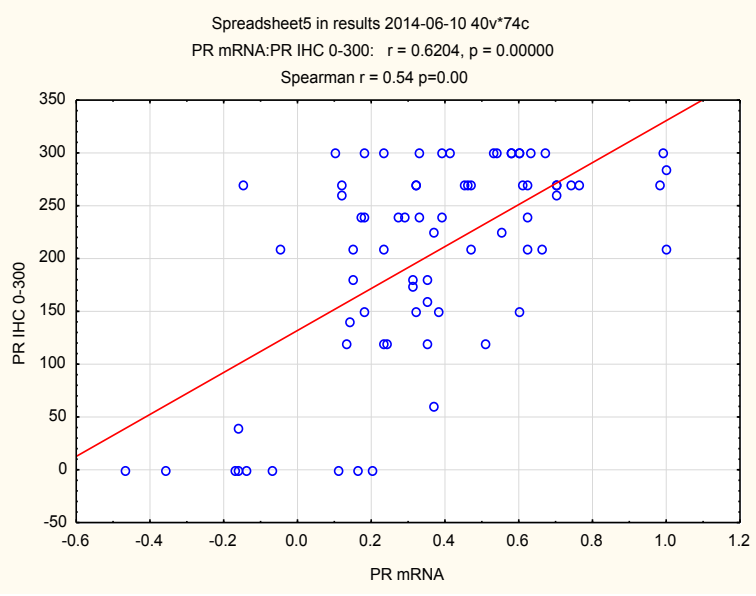


**Figure 7: Scatter plot diagram illustrating a significant positive correlation between protein expression (IHC score) and mRNA (TargetPrint) levels for PR.**

*5.4.3 Relation of molecular subtype to combined tumour ER/PR status*

The clinical relevance and potential added value of microarray-based molecular profiling using the 80-gene assay (BluePrint) was further assessed in relation to the combined hormone receptor status (ER/PR) as determined by IHC testing in addition to microarray-based mRNA readout (TargetPrint) analysis, which provided an approximation of the expected subtype (Table 10).

**Table 10: Tumour classification according to molecular subtype stratified in relation to combined ER/PR status.**

| **IHC testing** ***n*(%)** | **mRNA readout (TargetPrint)** ***n*(%)** | | **Molecular profiling (BluePrint)** ***n* (%)** | | |
|---|---|---|---|---|---|
| | | | **Luminal A** | **Luminal B** | **Basal-like** |
| **Concordance** | **61 (82.4)** | | | | |
| ER+/PR+ | 56 (91.8) | | 39 (69.6) | 17 (30.4) | |
| ER+/PR- | 3 (4.9 ) | | 0 | 2 (67.7) | 1 (33.3) |
| ER-/PR+ | 2 (3.2 ) | | 0 | 0 | 2 (100) |
| **Discordance** | **13 (17.6)** | | | | |
| ER+/PR+ | 7 (9.5) | ER-/PR+ | 3 (42.8) | | |
| | | ER+/PR- | 2 (28.6) | 1(14.3) | |
| | | ER-/PR- | 1 (14.3) | | |
| ER+/PR- | 5 (6.8) | ER+/PR+ | 3 (60) | | |
| | | ER-/PR- | | 1 (20) | 1 (20) |
| ER-/PR+ | 1 (1.4) | ER+/PR+ | 1(100) | | |

Agreement between microarray-based mRNA readout (TargetPrint) and IHC testing for ER/PR status was noted for 61 tumours (82.4%), of which 56 (91.8%) were classified as double hormone receptor positive (ER+/PR+). When referring only to the IHC determination of tumours as ER+/PR+, a total of 63 (85.1 %) were reported with this hormone receptor status, as illustrated in Figure 8. These were all luminal tumours, of which 45 were stratified by BluePrint as luminal A and 18 as luminal B molecular subtypes. In the 11 cases where IHC determination of ER or PR was negative, the presence of ER+/PR+ status determined by TargetPrint in 4 of those cases, correlated with a Luminal A subtype. In the remaining 7 of these 11 cases determined by IHC as either ER or PR negative, subsequently determined as ER and/or PR negative on TargetPrint, indicated that 3 tumours were Luminal B and 4 were basal-subtypes when analysed using the 80-gene BluePrint assay.

When tumour ER/PR status based on TargetPrint analysis alone was assessed in relation to the molecular subtype (BluePrint), the 60 ER+/PR+ tumours were still stratified as luminal. However, in tumours where ER and/or PR expression was lost, agreement with the molecular phenotype was poor, further evident from the diverse classification of three ER-/PR- tumours (TargetPrint) as luminal A, luminal B and basal-like respectively. A flow diagram outlining the possible clinical positioning of gene expression profiling as ancillary to standard IHC testing to direct patient management is presented in Figure 8.

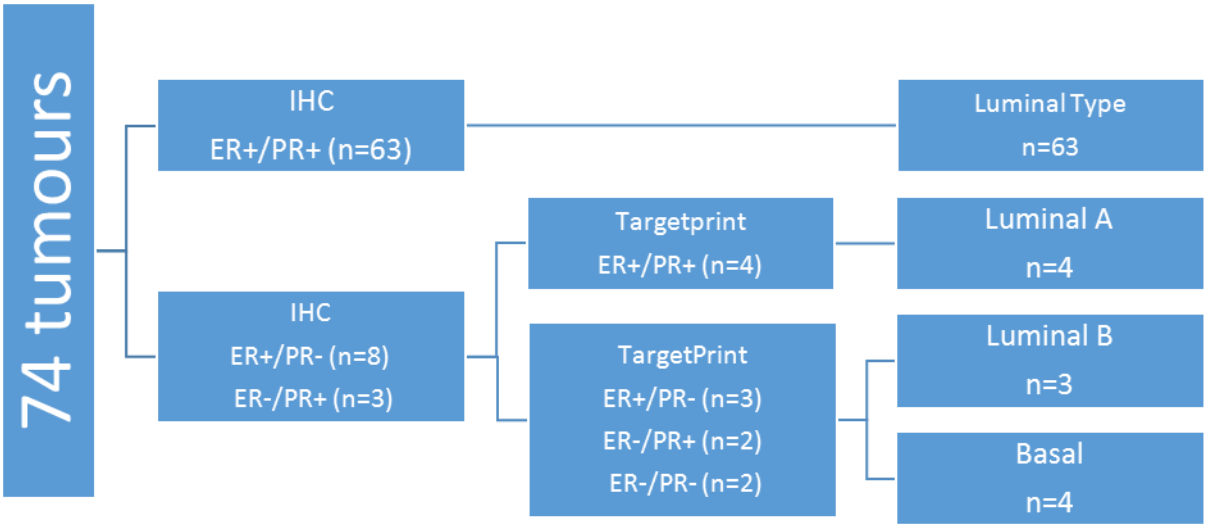


**Figure 8: Flow diagram illustrating the proposed clinical positioning of gene expression profiling in relation to standard immunohistochemistry (IHC) testing as ancillary to existing classification schemes in early-stage breast cancer.**

### 5.5 Discussion

In Phase 2 of this study, microarray-based mRNA readout (TargetPrint) was evaluated against IHC testing as the current standard used to assess ER and PR receptor status in patients with early-stage breast cancer. The potential added value of microarray-based molecular profiling using the 80-gene assay (BluePrint) was further investigated in this 3rd Phase of the study, by correlating the molecular tumour subtype with that predicted based on ER/PR status, as determined by IHC testing as well as TargetPrint analysis. In accordance with previous studies (Roepman et al. 2009, Gevensleben et al. 2010, Viale et al. 2014), contingency-based analysis of observer agreement measures indicated that microarray-based mRNA readout (TargetPrint) analysis was comparable to IHC testing for assessment of hormone receptor status. However, in contrast to our previously reported 100% concordance between TargetPrint and FISH for HER2 status (Grant et al. 2014), a 17.6% discordance rate for combined ER/PR status was

evident between TargetPrint and IHC from the current data. Despite the significant positive correlations noted between mRNA and protein expression levels for ER as well as PR, it is emphasized that an elevation in mRNA does not necessarily translate to protein expression or reflect underlying protein functionality (Itoh et al. 2014).

Several plausible explanations which could underlie the abovementioned discordance include intra-tumour heterogeneity, sampling error, as well as variable false positive and negatives rates reported for IHC testing based on the type of tissue analysed (Seferina et al. 2013, Welsh et al.2011, Gown 2008). In contrast to the established predictive and prognostic value of ER and PR based on IHC assessment, there is still a paucity of data available concerning the utility of microarray-based analysis of these biomarkers. In accordance with the need to evaluate ER status as a biological threshold assessed in relation to clinical outcomes, future prospective studies aimed at defining standard cut-off values for protein expression levels should ideally apply the same reasoning to mRNA levels as well (Harbech and Rody 2012, Brouckaert et al. 2013).

The potential added value of microarray-based gene expression profiling (BluePrint) was further assessed by correlating the molecular subtype which was predicted based on ER/PR status evaluated using a combination of IHC testing and microarray-based mRNA readout (TargetPrint) analysis. Agreement between the molecular and predicted subtype was noted for all but three ER/PR concordant cases; however, in 46.2% of discordant cases, such disparity resulted in disagreement between the predicted and molecular subtype. While all ER+/PR+ tumours were accordingly classified as luminal breast cancers, BluePrint profiling added value by stratifying 17 tumours as the high-risk luminal B phenotype, in keeping with findings reported by Glück and colleagues (Glück et al. 2013). The ability of microarray-based molecular profiling to delineate between the luminal A and B subtypes lies in its capacity to measure the functional

integrity of multiple oncogenic pathways, which is not possible using standard laboratory techniques. The observation that the majority of luminal B tumours were ER+/PR+ concordant argues against the notion that loss of PR expression in ER-positive breast cancer is indicative of a more aggressive phenotype. However, this is opposed by the finding that all ER+/PR- concordant tumours were stratified as high-risk based on molecular subtyping.

The term triple-negative breast cancer (TNBC) is sometimes used interchangeably with the basal-like subtype when referring to a more aggressive phenotype which per definition lacks hormone receptor expression. However, a minority of TNBC is stratified as luminal, while some basal-like breast cancers show positive hormone receptor expression. In keeping with this notion, two triple-negative tumours from the present study were classified as luminal, while two hormone receptor positive concordant tumours were stratified as basal-like. The former example is in keeping with the observation that most ER-/PR+ tumours are non-luminal, while confirmation of this molecular phenotype in the latter case (ER+/PR-) may be ascribed to a splice variant in the ER gene, as previously described by Groenendijk et al (2013). Several ER splicing variants have been reported in the literature, resulting in one or more exons being omitted from the ER mRNA. Antibodies used to detect ER protein during routine IHC assessment rely on epitope recognition encoded by the first exon of the ER gene; therefore, antibody binding at these sites will produce a positive ER result, despite the lack of normal ER functionality in the splice variant.

The primary limitations of this study include a relatively small sample size as well as pre-selection of hormone receptor positive and HER2-negative patients, in compliance with the MPA developed for reimbursement purposes in South Africa (Grant et al. 2013). This restricted the potential to assess the ability of microarray-based mRNA readout (TargetPrint) to reclassify discordant or borderline cases for HER-targeted treatment, as demonstrated by Grant et al.

(2014). However, the main strength of this study lies in the evidence provided in support of the analytical validity of FFPE tissue samples used since 2012 for microarray-based assessment of hormone receptor status, shown to be comparable to standard IHC testing for this purpose. Despite these reassuring findings, the limited added predictive value of TargetPrint analysis fails to substantiate its utility above and beyond existing diagnostic standards. By comparison, BluePrint profiling proved invaluable for the accurate identification of the high-risk luminal B and basal-like phenotypes, which would not otherwise have been suspected if molecular subtyping was not performed. This has significant implications for the selection of appropriate treatment in early-stage breast cancer and illustrates the importance of the clinical positioning of gene expression profiling in relation to standard immunohistochemistry (IHC) testing as ancillary to existing classification schemes in early-stage breast cancer.

Overall, the relevance of a pathology-supported genetic testing approach to breast cancer management, combining microarray-based analysis as ancillary to existing clinico-pathological risk stratification and prognostication tools, is supported. The routine implementation of genomic profiling alongside standard pathology tests may increase clinician confidence in treatment decision making and ultimately optimize individualized management of early-stage breast cancer patients by identifying molecular subgroups more accurately. When viewed in the context of IHC/FISH and other available genomic assays, the data presented in this study provide further support for microarray analysis to aid patient management in early-stage breast cancer.

Collectively, the findings discussed above resulted in a critical understanding of the limitations and benefits of different laboratory tests used in the prognostication and prediction of recurrence risk in early-stage breast cancer.

# CHAPTER 6
## CONCLUSIONS AND FUTURE PROSPECTS

Whilst several genetic signatures have been developed for chemotherapy selection (Ross et al. 2008) only two are currently available in South Africa, i.e. MammaPrint and Oncotype Dx. The MammaPrint service has the added benefit of providing ER/PR/HER2 tumour status in a separate microarray readout (TargetPrint) not included in the 70-gene risk score, and also defines the molecular subtypes (Luminal A, Luminal B, Basal like and HER2 type) based on the 80-gene BluePrint profile (Viale et al. 2014). Implementation of the MammaPrint service evaluated in this study is supported by several studies confirming the prognostic (Drukker et al. 2013, 2014) and predictive value of MammaPrint in both the adjuvant and neo-adjuvant settings (Glück et al. 2013, Knauer et al. 2010, Straver et al. 2010). These findings were in accordance with the initial clinical validation studies which demonstrated that MammaPrint outperforms standard clinical-pathological risk assessment for all endpoints: time to distant metastasis and overall survival (van't Veer et al. 2002, van de Vijver et al. 2002, Buyse et al. 2006).

The laboratory tests used in this study to distinguish between breast cancer subtypes ranged from protein-based IHC, DNA-based FISH and RNA-based microarray-based analyses. The finding that tests performed at different laboratories could result in discordance due to analytical variables including methodology and arbitrary cut-off values, may explain part of the heterogeneity in treatment response. Analytical validation of microarray analysis in this study using HER2 status based on TargetPrint as a marker in comparison with IHC/FISH was very important due to replacement of fresh tumor biopsies used from 2007 for microarray analysis by FFPE tissue from 2012, as previously described by Sapino et al. (2014).

The prevailing standard in laboratory diagnostics are challenged by an increasing number of complex genomic tests that need to be interpreted within the context of the existing clinic-pathological parameters for clinical application on which optimal treatment decisions are based. Implementation of a pathology-supported genetic testing service for breast cancer patients provided solid proof of the clinical utility of microarray analysis in early-stage breast cancer, enabled by the Gknowmix database that served as an interface between the laboratory and clinic. The significant impact of MammaPrint on treatment decisions has recently been confirmed by Pohl et al. (2014) in an audit of the Gknowmix database. Although these authors selected only those patients considered least likely to gain any benefit from additional chemotherapy, whilst still having the option of endocrine therapy, gene profiling could potentially change the treatment of one in two (52%) patients irrespective of 10-year mortality based on clinical parameters as determined by Adjuvant! Online (www.adjuvantonline.com).

### 6.1 Clinical utility demonstrated

The ability to read out multiple genetic profiles from the versatile MammaPrint microarray platform used in this study expanded the clinical utility of this assay. As experience grows, an improved level of confidence has been achieved among South African clinicians for implementation of the 70-gene MammaPrint assay used for risk stratification, TargetPrint as a second opinion of ER, PR and HER2 status, and BluePrint to identify the functional pathways in each individual tumour on which to base choice of treatment. Confirmation of the clinical utility of these microarray tests for improved management of breast cancer as a major cause of cancer-related mortality and morbidity (deSantis et al. 2014) was an important outcome of this study.

The term clinical utility reflects to what extent a particular test may improve clinical outcome as compared to the gold standard (Bossuyt et al. 2012). Although no clinical outcome data was

available in February 2007 when the 70-gene MammaPrint profile was approved for clinical use by the FDA, recent studies confirmed the benefit of this test in relation to breast cancer survival. MammaPrint remains the only genomic profiling test for early-stage breast cancer with 5 and 25-year prospective clinical data showing that chemotherapy may be safely avoided in low-risk patients without compromising health outcomes. In a recent study by Drukker et al. (2014), following a median follow-up of 18.5 years, a significant difference was observed in long-term distant metastasis-free survival for patients with a high- and low-risk MammaPrint profile ($p<0.0001$) (Drukker et al. 2014). The strongest prognostic power was found in the first 5 years after diagnosis. Results from the RASTER trial showed that patients who were discordantly labelled as MammaPrint low / Adjuvant! Online high-risk and did not receive adjuvant systemic therapy had a 100% 5 year disease free interval (Drukker et al. 2013). It has therefore been suggested that use of the MammaPrint signature in a clinical setting may assist the timely implementation of tailored yet standardized treatment in specific subgroups of breast cancer patients (Drukker et al. 2014). These findings are in accordance with local data confirming that MammaPrint reclassification of clinically high-risk patients to low-risk spares unnecessary, costly and potentially lethal chemotherapy while maintaining excellent clinical outcome (Grant et al. 2013, Phase 1 of study).

The MammaPrint profile provides hormone receptor and HER2 status as a separate readout, termed TargetPrint. The clinical utility of TargetPrint was demonstrated in Phase 2 of the study in South African early-stage breast cancer patients. Comparative analyses indicated very high concordance between hormone receptor and HER2 status results provided by TargetPrint and standard IHC/FISH methodologies respectively. Use of TargetPrint proved to be particularly useful as a second opinion and to resolve borderline cases. Based on these results, certain patients were spared expensive and potentially hazardous treatment, while others were provided with lifesaving therapy which would not have been considered based on standard

assessment of HER2 status. To improve cost-effectiveness in the resource-poor African context, HER2–positive breast cancer patients are also not eligible for MammaPrint according to the reimbursement policy applied in South Africa (Grant et al. 2013), which should be revisited in future based on the aforementioned findings. Comparably, assessment of HER2 status using conventional techniques has yielded results discordant with those provided as part of the Oncotype test in more than one study (Dabbs et al. 2011; Dvorak et al. 2013; Park et al. 2014). This may be indicative of low quality precision, necessitating further extensive analytical validation as RT-PCR-based assays are not recommended for the assessment of HER2 status (Yamamoto-Ibusuki et al. 2013).

The addition of BluePrint profiling allows for the classification of breast cancer into different molecular subtypes, which are not possible using standard pathological tests. This plays a key role in predicting the efficacy of systemic therapy. BluePrint accurately discriminates between the luminal A and B subtypes (Glück et al. 2013), the latter being characterized by increased proliferation, higher recurrence rates, worsened overall prognosis, compatible with a high-risk MammaPrint profile (Leo et al. 2012). This test was also recently shown to identify the majority of discordant MammaPrint high-risk and Oncotype DX low-risk cases as being of the luminal B subtype (Shivers et al. 2014). Furthermore, BluePrint correctly reclassifies patients with a higher pCR as having the HER2-enriched and basal-like subtypes (de Snoo et al. 2014). Sub-stratification of triple negative breast cancer into the basal-like subtype identifies a specific subgroup set to derive greatest benefit from targeted systemic therapies, including PARP inhibitors, FGFR2, TRAIL and anti-angiogenic agents. Also, the addition of cyclophosphamide or platinum agents to anthracycline-based neoadjuvant chemotherapy regimens has been associated with more favourable long-term health outcomes in patients with triple-negative breast cancer (Goldhirsch et al. 2011; Gelmon et al. 2012; Giacchetti et al. 2014). Inclusion of BluePrint for microarray analysis in a subgroup of ER-positive breast cancer tumors lacking ERα

(~2%) function, yet expressing ERα at the protein (determined by IHC) and mRNA levels (determined by TargetPrint), can furthermore identify a subgroup who will not respond to hormonal therapy.

### 6.2 Research translation

Oncology is a prime example of a medical discipline where genomic discoveries are translated into tangible healthcare benefits, and oncogenomics has the potential to greatly advance the development and implementation of a personalized multidisciplinary approach to breast cancer management. However, the inherent complexity of genomic tests may be considered an impediment to their more widespread application, complicated by a lack of confidence among clinicians regarding the interpretation and reporting of these results (Chin et al. 2011). These recognitions have led to a growing incentive to establish to what extent and under what conditions incorporating personalized genomics as part of standard clinico-pathological risk stratification schemes may benefit patient care (Amir et al. 2010; Wacholder et al. 2010).

The prospective randomized controlled study is considered the gold standard to conclusively determine the clinical value of a novel medical intervention prior to its adoption as part of existing practice. The results of two large trials designed to evaluate the clinical utility of two gene expression profiling signatures indicated for patients with early-stage breast cancer, namely MammaPrint and Oncotype DX, are currently underway. However, as pointed out by Azim et al. (2013), the design of these trials may prohibit assessment of the relationship between the gene expression profile in question and the “magnitude” of the therapeutic benefits related to chemotherapy. This prospective interventional approach has a number of other important shortcomings. Firstly, they raise fundamental ethical concerns, since they involve withholding treatment considered standard care from patients. Secondly, they require a very

large sample size and are extremely expensive and time-consuming to conduct, which are particularly noteworthy limitations in resource-limited environments. At the current rate of technological development in genomics, conclusive evidence for the clinical utility of a particular test under investigation may only become available once it is already obsolete. It therefore seems apparent that prospective clinical trials have their shortcomings and are not always practical or feasible, and the question of their necessity remains subject to debate.

Multiple retrospective studies have demonstrated that gene expression profiling tests provide independent prognostic and predictive value above and beyond that for standard diagnostic tools (Parker et al. 2009; Cuzick et al. 2011; Iwamoto et al. 2011; Cornejo et al. 2014). Although growing evidence supports their analytical as well as clinical validity (Bueno-de-Mesquita et al. 2007; Drukker et al. 2013, 2014; Sapino et al. 2014), the perception exists that there is currently still insufficient data substantiating their clinical utility, which is conventionally obtained from performing prospective trials. This has hampered both their widespread implementation as part of routine practice, as well as reimbursement as part of oncology benefits. Many frameworks have been developed in an attempt to provide viable and robust alternatives for evaluating the clinical utility and cost-effectiveness of novel genetic tests. However, no single approach is currently considered either standard or superior (Faulkner et al. 2012). Chang et al. (2013) proposed that pooled meta-analyses considering existing large-scale data may negate the need for prospective randomized control trials. Similarly, Parker et al. (2009) proposed that retrospective analysis of prospectively collected and archived tissue samples may offer a more suitable alternative to fully prospective evaluation. In 2012, the IMPAKT Working Group delivered a consensus statement in which the establishment of patient registries consisting of large-scale longitudinally collected data was proposed as a cost-effective complementary alternative to randomized prospective studies (Azim et al. 2013).

In view of the above the breast cancer patient database developed during the course of this study is considered a valuable resource for future research and to assess patient outcome over time in collaboration with the treating clinicians. By providing local evidence substantiating the clinical utility of microarray-based gene expression profiling tests, such research may add confidence in conservative therapeutic decision making and allow for careful monitoring of health outcomes and patient follow-up over time. Its continuous updating is anticipated to advance the implementation of a truly individualized approach to breast cancer prevention and therapeutic management. Following the introduction of MammaPrint profiling to the South African healthcare fraternity in 2007 as complementary to existing clinico-pathological risk stratification and management schemes the use of this database as resource for translational health outcome studies have yielded evidence supporting the analytical validity, clinical utility and cost-effectiveness of microarray-based gene expression profiling signatures, as well as enabling the development of eligibility criteria and reimbursement policies for such testing (Figure 9).

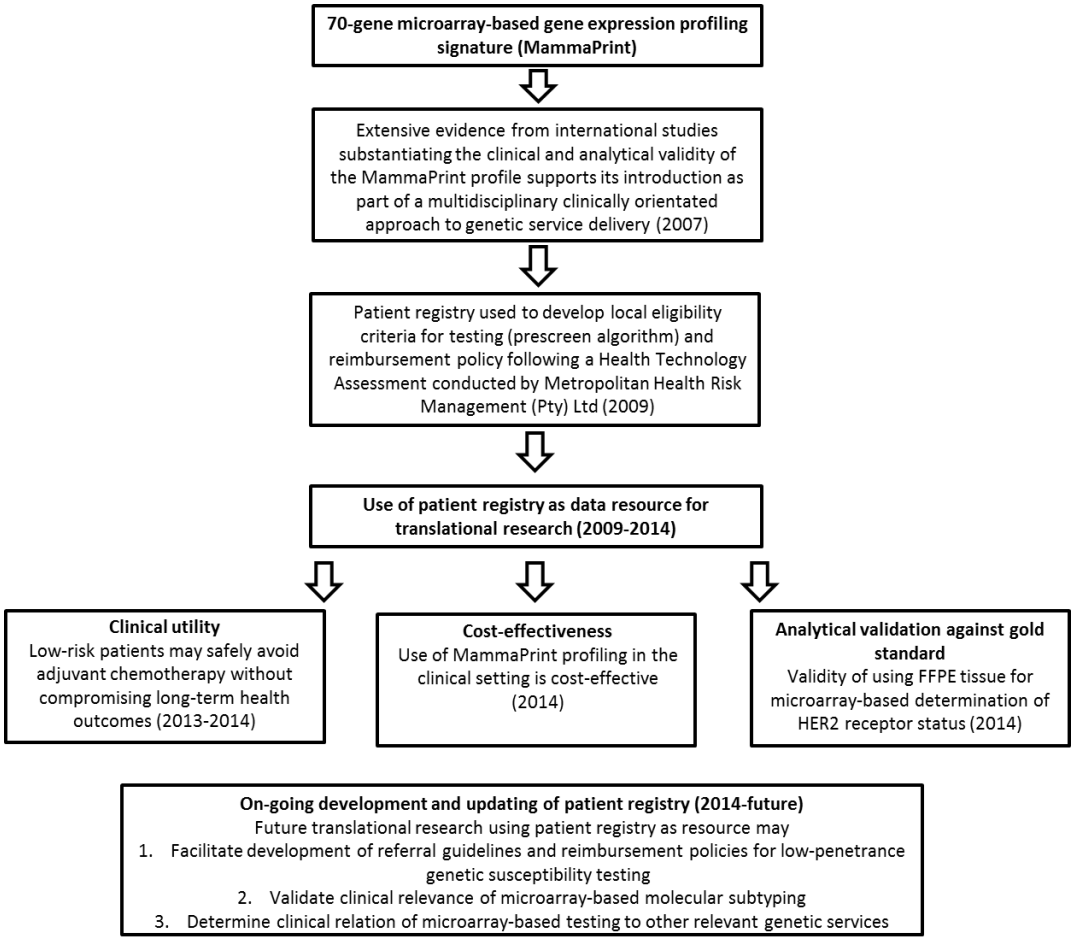


**Figure 9: Introducing the MammaPrint test into the South African healthcare system by establishing a patient database at the interface between the laboratory and the clinic. A systematic approach was undertaken by using a pathology-supported genetic testing (PSGT) strategy to complement current testing procedures and establish clinical utility of gene profiling in early-stage breast cancer.**

While the clinical validity of the MammaPrint signature is well-established, future studies using the database resource may provide additional evidence substantiating the analytical and clinical utility of microarray-based gene expression profiling performed in the context of breast cancer

management in the local setting. Additionally, such investigation may allow for the development of eligibility criteria and reimbursement policies for wider implementation of microarray-based testing. Prospective evaluation of the collected patient information may serve to validate the clinical utility of gene expression profiling in the local population, by determining whether patients designated as having a low risk for recurrence in whom adjuvant chemotherapy was foregone did so without compromising long-term health outcomes. The clinical value of extended microarray-based gene expression profiling to facilitate molecular tumor subtyping in guiding the selection of appropriate chemotherapy in high-risk patients is an important consideration. The patient database developed during the course of this study may also be used in future to establish how gene expression profiling relates to other relevant genetic services offered in the context of breast cancer management, as well as facilitate the development of standardized referral guidelines and reimbursement policies for low-penetrance genetic susceptibility screening in the local setting.

Based on extensive local research using the Gknowmix database as resource, future studies may ultimately advance the implementation of a truly individualized clinical approach to breast cancer risk management in South Africa.

### 6.3 Ethical considerations

Tumour biopsies used in this study became available within the framework of routine patient care as the project included both a service and research component. Patients were informed that their tumour specimens would be shipped abroad for genetic analysis and approval was requested to include relevant data in a central database for comparative analysis.

The request to perform an audit as part of this study was considered a low risk in relation to potential patient benefit as it formed part of a laboratory study and reflex testing as a result of the findings could improve patient management. Clinically relevant information obtained in this study was therefore provided to the treating oncologists to be used according to their discretion. To our knowledge this approach, which required careful data management at the interface between clinical practice and the participating laboratories, has not previously been reported with proven patient benefit as evidenced by endorsement of MammaPrint as part of oncology benefits by several medical schemes in South Africa. Breast cancer patients designated as having a low risk of recurrence based on MammaPrint profiling could safely avoid adjuvant chemotherapy without compromising long-term health outcomes. The analytical validity of using FFPE tissue for microarray-based determination of HER2 receptor status, provided as a separate readout to the MammaPrint service, was also demonstrated in the local population. Moreover, risk reclassification based on these results in addition to BluePrint profiling significantly altered the clinical management in nearly 60% of patients subjected to microarray analysis since 2007 in Southern Africa.

### *6.3.1. Relevance of genetic counselling*

Gene expression profiling has no direct implications for familial breast cancer risk as the analysis is performed on RNA extracted from the tumour specimen, and not whole blood or saliva routinely used for detection of high-penetrance germline mutations in genes such as BRCA1 or BRCA2. However, genetic counselling may need to be provided to patients who request additional information or are deemed eligible for further genetic testing if indicated. Similar to the evolving nature of genomic technologies, the scope of genetic counselling is changing from a family-centred to treatment-based approach. In Table 11, a list of questions raised by one of the breast cancer patients with discrepant HER2 status reported in the 2nd

phase of the current research project is presented, along with responses provided by the primary author of this study. Once these were answered, more questions were raised by the patient on additional tests that may be appropriate to the treatment algorithm developed by the treating oncologist based on available information, as well as the relevance of diet and lifestyle factors which may influence recurrence risk.

**Table 11: Questions raised by a breast cancer patient and the response provided**

| Questions | Answers |
|---|---|
| **According to my MammaPrint I have too many HER receptors or more than normal?** | HER2 positive means the tumour tests positive for a protein called human epidermal growth factor receptor 2 (HER2). This protein promotes the growth of cancer cells. We should only have 2 copies of the HER2 gene, but cancer cells make an excess of HER2 due to a gene mutation. These cancer cells encode more mRNA to make more protein. So TargetPrint provided at no additional cost to MammaPrint measures the amount of mRNA made by the gene, not the protein where additional factors contribute to its expression levels measured by the pathology lab. Using sophisticated software and repeated readings the amount of mRNA is quantified and measured against that produced by normal cells. When this is more than what is expected, the tumour is regarded as HER2 overexpressed. Alternately, the tumour cell may express more epitopes (binding sites), enabling more binding than normal of the protein and thereby promoting abnormal cell growth. This is not measured by gene expression profiling with the microarray used for 70-gene MammaPrint (high/low risk) and TargetPrint (ER, PR, HEH2). |
| **How many receptors are normal?** | This is measured differently with each of the three tests used: 2) protein-based IHC , DNA-based FISH and RNA-based microarray/TargetPrint. Using IHC, when the amount of cells staining positive is >10%, the tumour is regarded as over expressing |

| | |
|---|---|
| | HER2 protein, therefore HER2 positive. These are always tested using FISH to check if there really is more gene copies in the cell nucleus. For FISH, the amount of nuclei per cell are counted and the amount of signals (fluorescent "dots" which represent the HER2 gene) in each nucleus are counted. For greater accuracy, a centromeric probe (to indicate the number of chromosomes) is also used, reported as the HER2/CEP 17 ratio. One centromeric probe to two HER2 gene copies is what we expect (representing one copy each from Mom and Dad). Sometimes we have more copies of the gene or of chromosome 17, but there is not an overproduction of protein. Here gene profiling with TargetPrint is helpful as it measures the amount of mRNA expressed by the HER2 gene not possible with standard testing. BluePrint is useful too, as it will be able to distinguish if a tumour, found HER positive using routine testing (IHC/FISH), is of a molecular HER2 enriched subtype or more of the luminal B high-risk type. These tumours may show a different response to trastuzumab therapy and is helpful in assisting the oncologist in treatment planning. |
| **What is my average number HER2 ?** | Because 3 different tests was done for the same purpose the results cannot be combined to work out the average – each need to be evaluated on its own and since two of the three were HER2 positive this is the correct result. |
| **How do I know for certain that polyploidy chromosome does not mimic as HER receptors?** | Use of the centromeric probe as explained above with the HER2 probes is used to determine the HER2/CEP 17 ratio. There are a few combinations used but basically amplification (when there is genuinely too many copies) is defined as (i) HER2/CEP17 >2 or HER2 copy number>4 (ii) HER2/CEP17 >2.2 or HER2 copy number>6. The FISH HER2 positive result is confirmed by TargetPrint. |
| **Do I have a CEP17 average of > 3.0 per** | The FISH report say CEP17 ratio of 2.98 with a mean value of 3.2 in the 35% of cells analysed that showed gene amplification. |

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| **nucleus?** | |
| **Does the amplified HER receptors make my profile High Risk or is this just a factor?** | HER2 amplification/overexpression occurs in about 20% breast tumours and is associated with a poor prognosis but treatment with Herceptin at least half the increased risk for recurrence. The HER2 gene is not evaluated as part of this MammaPrint risk score, however the profile is thought to capture gene expression of relevant HER2 pathways to formulate a risk score. The 70 genes tested for MammaPrint is separate from HER2 as this profile is only used for chemotherapy selection and not to decide on the need for Herceptin. |
| **How does the Blueprint indicate that the "receptor pathways" are active?** | BluePrint uses 80-gene, 4 of which are the same as those used in the MammaPrint profile. This profile is enriched with estrogen receptor pathway genes, which are known to respond to estrogen stimulation. If the tumour tested does not show expression in these genes, the BluePrint profile will recognise this tumour as basal-type. Normal laboratory testing, including TargetPrint, only tests the presence of the protein or mRNA of one gene (HER2), but cannot measure all the genes in the pathway to check if the protein produced is actually functioning. There can be many mutations in one gene and many genes in the same pathway so the more genes we test the better the test. |
| **Why is it important to know whether the receptor pathways are active?** | If the pathway is functioning, the tumour can respond to estrogen stimulation. So when treating the tumour, the drug can block that pathway and cut off the route of stimulation causing the tumour to shrink. If the tumour does not have a functioning ER pathway, it will continue growing even if agents blocking the ER pathways are given and the patient then needs to be treated with different chemotherapeutic agents to ensure tumour shrinkage. |
| **What is a molecular pathway?** | This is a step by step process of conveying messages from the nucleus to the cell surface and cytoplasm. It's an ordered series of events among the molecules in the cells which leads to a certain |

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| | end point or to a cell function or a product. Other alternate pathways may exist, so when we block one, another may be available to produce the product we need. This is the case in cancer sometimes, where agents are found not to be effective and alternate drugs have to be used to block the alternate pathway that may be of relevance in a subgroup of patients depending on their unique genetic make-up. |
| **Why is it shown which one is dominant?** | So that we know which one to target and design a treatment strategy most suited to gain therapeutic advantage. |
| **How is this indicated?** | BluePrint reports indicates if the tumour is luminal A, luminal B, HER2 or basal. Luminal A cancers have the most functioning pathway of all and therefore respond well to hormonal therapy but not to chemotherapy, whereas the basal tumours do not respond to hormonal therapy but very well to chemo. Luminal B tumours, less well to hormonal and better to chemo that luminal A, and the HER2 respond to trastuzumab-chemo regimes. |
| **How does the MammaPrint test for HER+ differ from my IHC found to be zero and the FISH ratio which was 2.98?** | Each test is good at testing (measuring) what it is designed to test, but each has its limitations and this is why obtaining information from the protein (IHC), DNA (FISH) and RNA (TargetPrint) is ideal. These tests all measure the same HER2 gene expression but use different methods developed by scientists who are trained to try and develop better and better methods all the time. The IHC measures the protein made by the mRNA which was measured by TargetPrint. FISH on the other hand, allows one to count the amount of genes in the nucleus of the cell by using fluorescent markers which bind to the HER2 gene. Because the cell has mutated, the processes are abnormal, so if we measure all aspects of the same gene form DNA-to-RNA-to-protein (first transcription then translation), we get a more comprehensive picture of what is actually happening. The oncologist then evaluates all the information available to decide on the best treatment option. |

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| **Should I have a Spot-Light HER CISH test done?** | This is another method of counting how many copies of the HER2 gene are present. It is the same as FISH but use other markers called chromogenic markers (staining), not fluorescent markers to see the HER2 number. This should not provide a different result in your case, as we have had 2 tests done which show that the HER2 gene is producing too much mRNA (TargetPrint) and that there are too many copies of the HER2 gene (FISH) as well. |
| **Do I have according to my MammaPrint a "tumour with a poor prognosis signature"?** | If the report say High Risk the tumour has a poor prognosis ONLY if left untreated. These signatures are worked out as if they would not be treated, then if they are treated with hormonal therapy alone, and then when they are treated with hormonal and chemotherapy. Knowing the tumour type is of a poor prognosis, gives more information about the biology of the tumour and gives the opportunity to plan highly effective treatment which has been shown to improve prognosis significantly. |
| **Determine the tumour signature my survival instead of how early the cancer was detected?** | Yes, early detection means the tumour is small in size and has not spread to other parts of the body. Most of these tumours can be removed and the patient will not have recurrent breast cancer, however, some patients will relapse and die of their disease. To prevent this most breast cancer patients are over treated with chemotherapy because we don't know who will survive and who will not. With genetic testing using MammaPrint, we can determine which of these small, early stage breast cancer tumours will need to be treated, and predict how they will respond (using TargetPrint and BluePrint) to this treatment, to ensure long term survival. |

*6.3.2. Data mining*

In an attempt to maintain anonymity, each patient was assigned an automatically generated reference number with patient data therefore being de-identified after uploading to the research database. The type of biological specimen provided (surgical or core biopsy), as well as the

preferred handling thereof subsequent to testing (store sample, decodified/anonymized or destroy), is also documented based on patient preference. Researchers may request access to the database for translational research that has been ethically approved for each individual application. After the necessary data fields are selected for a specific study, patient information is extracted and exported to an Excel document.

### 6.4 Future developments based on past experience

The central database developed in parallel to this study facilitated the development of a reimbursement policy for MammaPrint based on the test eligibility criteria evaluated in a real-world situation during Phase 1 of this study, as recently reported by Grant et al. (2013). Metropolitan Health Risk Management (Pty) Ltd (MHRM) became the first health risk management organization in South Africa to endorse the MammaPrint profile as part of oncology benefits, following a Health Technology Assessment (HTA) performed in South Africa in 2009, shortly before commencement of this study. The reimbursement model for this test was developed using a combined research and service delivery approach, as illustrated in Figure 10. In accordance with recommendations set out as part of the HTA, the MammaPrint service was linked to the on-going development of the patient database, which now includes data from all patients referred for MammaPrint in Southern Africa since the introduction of the test in 2007.

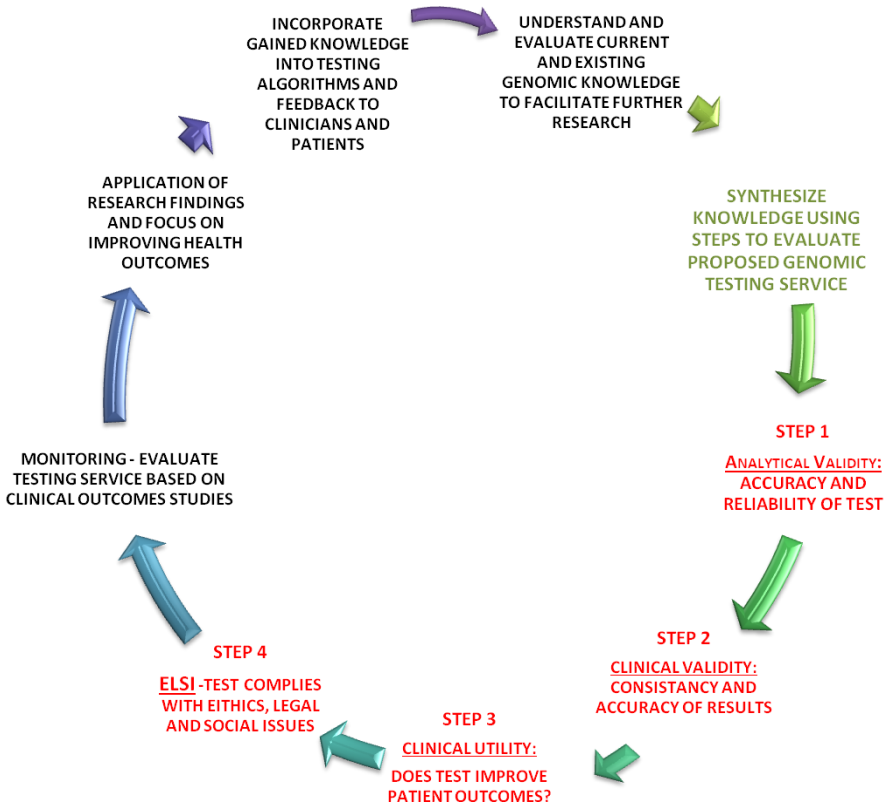


**Figure 10: Combined service and research approach towards development of a genomics reimbursement model.**

Accurate determination of HER2 receptor status plays a key role in disease prognostication and selection of appropriate therapy in patients with breast cancer. Although historically performed using standard IHC methodologies, the growing recognition that such standard diagnostic techniques may produce inaccurate results as also evidenced in this study, led to the development and implementation of advanced genomic risk stratification tests. The MammaPrint microarray platform has successfully been transferred from initial use of fresh tissue to FFPE samples (Sapino et al. 2014). FFPE tissue was found to be a reliable source of RNA for microarray-based assessment of tumour receptor status. Following reflex testing via FISH testing for discordant or equivocal cases, 100% concordance was achieved in this study

regardless of whether fresh tumor or FFPE tissue was used for microarray analysis. Risk reclassification based on the results obtained in South African breast cancer patients helped to avoid unnecessary high treatment costs in false-positive cases and withholding of potential life-saving treatment in false-negative cases.

The identification of intrinsic breast cancer subtypes which are highly heterogeneous in terms of their etiology, presentation and prognosis (Parker et al. 2009) provided an example of how novel genomic technologies advance our understanding of the underlying pathological basis of this disease spectrum. The recognition that different breast cancer subtypes vary in their response towards and tolerance of different chemotherapeutic agents supports the use of BluePrint provided as an extension of the MammaPrint platform applied in this study to optimize treatment and limit side-effects and therapeutic failure in the adjuvant setting.

### *6.4.1 Value of patient database as resource for future health outcome studies*

Continual updating and improvement of the breast cancer patient database is anticipated to advance a number of initiatives in the near future. While indications for inclusion in familial breast cancer screening programs (Kotze et al. 2013), as well as microarray-based gene expression profiling (Grant et al. 2013), have been developed in the local context, standardized referral guidelines for polygenic susceptibility screening performed as a component of breast cancer risk management are currently lacking. Furthermore, it remains unclear where such testing, and indeed high-penetrance diagnostics, lie in relation to gene expression profiling in the clinical domain. The observation that the spectrum of penetrance for most risk-associated polymorphic variants remains unknown casts doubt over the clinical utility of these tests. Similar to gene expression profiling, patient registries may serve as a valuable resource for translational research aimed at clarifying this issue. If correctly applied, personalized genotyping may

facilitate the development of individualized strategies aimed at decreasing cumulative risk for disease progression or recurrence as well as associated comorbidities, and optimizing patient therapy in accordance with this knowledge.

*6.4.2 Genetic susceptibility testing in the pharmacogenomics context*

The vast majority of the global breast cancer burden (~90%) is attributed to sporadic disease. As such, most breast cancers are considered polygenic in nature and classified as complex phenotypic traits, with clinical emergence dependent on the interaction between multiple risk-associated polymorphic variants in low-penetrance genes and environmental factors which trigger their expression. Considering genetic testing results in isolation therefore profoundly limits the capacity for clinical research translation, with a multidisciplinary approach to testing posited as a means of overcoming these limitations. It is increasingly being recognized that the majority of chronic non-communicable diseases (NCDs), including lifestyle-related neoplasms, neuropsychiatric illnesses and cardiovascular disease (CVD), share common pathogenic mechanisms and overlapping genetic risk factors, which relate to the dysfunctional regulation of core multifunctional metabolic pathways. Comprehensive genetic assessment considering multiple risk-associated polymorphic variants implicated in these processes, when performed as part of a clinically orientated, multidisciplinary approach to NCD risk management, may therefore facilitate the identification of a high-risk genetic subgroup of patients set to derive the greatest therapeutic benefit from the timely implementation of individually tailored lifestyle-cantered harm reduction strategies aimed at decreasing cumulative disease risk across the diagnostic spectrum.

There exists significant inter-ethnic and inter-individual variation in drug pharmacokinetics, including their absorption, metabolism and excretion, conferring risk for treatment failure or

therapeutic intolerance. Although this may be influenced by clinical and physiological factors as well as drug-drug interactions hereditary polymorphisms in drug-metabolizing enzyme genes also play a key role. Variation in the gene encoding the cytochrome P450 enzyme CYP2D6 is of particular importance as this ubiquitous enzyme catalyzes the hydroxylation and demethylation of many drugs routinely used in clinical practice. These include certain antidepressants and hormonal therapies such as the selective estrogen-receptor modulator (SERM) pro-drug tamoxifen, the active metabolites of which are hydroxytamoxifen and endoxifen. Major depressive disorder (MDD) is a frequently encountered comorbidity in patients with breast cancer. As such, the treatment of MDD is an important clinical consideration, with ~20-30% of breast cancer patients receiving tamoxifen treatment also being prescribed antidepressants (Kim et al. 2010). Certain antidepressants, such as selective serotonin reuptake inhibitors (SSRIs), may decrease the therapeutic efficacy of tamoxifen by interfering with CYP2D6 enzymatic activity, thereby increasing risk for disease recurrence or treatment failure. This topic was reviewed by van der Merwe et al. (2012). The authors conclude that personalized genotyping for the common CYP2D6*4 inactivating allele may be indicated in breast cancer patients receiving tamoxifen therapy, who are at an increased risk for tumor recurrence, or are on concurrent treatment with antidepressants which compete for the CYP2D6 allele.

In addition to polygenic susceptibility testing, screening for mutations in the high-penetrance BRCA1 and BRCA2 tumor suppressor genes may also play a role in predicting resistance to hormonal therapy in early-stage breast cancer. Therapeutic failure in response to estrogen-targeted therapies may be attributed to estrogen receptor (ER) negative status as an intrinsic property of BRCA1-mutated breast cancers (Foulkes et al. 2004). This mirrors the high prevalence of BRCA mutations observed in patients with triple-negative disease (Peshkin et al. 2010), as well as the pathogenic overlap between BRCA1 mutations and basal-like breast cancer (Santarosa and Maestro 2012). In light of the above, there currently exists not only a

demand for standardized referral guidelines for low-penetrance genetic testing aimed at predicting therapeutic failure or intolerance in the context of breast cancer management, but also a need to possibly extend the existing role of BRCA mutation screening beyond its current diagnostic application.

### 6.5 Conclusion

The effective implementation of screening programs has greatly benefited the early detection of as well as timely initiation of treatment for breast cancer. Despite these advances, therapeutic decision making still largely follows a “trial-and-error” approach, with many patients either unnecessarily exposed to hazardous and potentially lethal treatments, or failing to receive lifesaving treatment where indicated. Use of clinically validated biomarkers to facilitate a more effective approach to treatment prioritization is now widely integrated as part of routine cancer management and care. Since its introduction in the South African setting in 2007, the clinical utility of the 70-gene MammaPrint gene expression profiling signature has been questioned despite approval by the FDA. However, it is now showing greater acceptance in the medical fraternity, owing to a growing body of local evidence supporting its clinical utility, while evidence substantiating the analytical validity of using FFPE samples for microarray-based determination of hormone and HER2 receptor status has enabled greater accessibility and ease of use in this regard. The initial establishment and on-going development of a local patient database, which served as a basis for such translational research, has further lent itself to a number of valuable applications over recent years. The development of locally applicable eligibility criteria (MPA) for MammaPrint profiling has allowed approximately ~60% of patients with early-stage breast cancer tested to safely forego chemotherapy, sparing these patients unnecessary and expensive treatment associated with harmful and potentially lethal side-effects. Additionally, the prospective evaluation of patient data contained in this database has provided evidence

substantiating the clinical utility of microarray-based gene expression profiling performed in the context of comprehensive breast cancer management. Translational research utilizing this database as resource further supports the clinical relevance of microarray-based determination of hormone and HER2 receptor status (TargetPrint) as well as molecular subtyping (BluePrint) to optimize treatment in South African patients with early-stage breast cancer.

The initial establishment and on-going development of the breast cancer database has and will continue to provide a valuable resource for health outcomes studies providing benefit to patients and their family members. Future translational research may add confidence in conservative therapeutic decision making and allow for careful monitoring of health outcomes and patient follow-up over time. This novel approach to integrating research and service delivery may itself provide a screening step to determine eligibility for next-generation whole exome or genome sequencing for genetically uncharacterized patients or those not responding to treatment. Ultimately, it is envisioned that the patient database which enabled extensive local research to date will continue to inform clinical decision making and optimize delivery of a comprehensive and truly individualized genomics-based healthcare service for breast cancer patients based on disease patterns relevant to the South African population.

# CHAPTER 7
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# APPENDIX I
## LETTER OF ETHICAL APPROVAL

UNIVERSITEIT•STELLENBOSCH•UNIVERSITY
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**Ethics Letter**

27-Feb-2014

**Ethics Reference #:** N09/06/166
**Title:** Analysis of the clinical utility of gene expression profiling in relation to conventional prognostic markers in South African patients with breast carcinoma

Dear Ms K Grant Smit,

At a review panel meeting of the Health Research Ethics Committee that was held on 19 February 2014, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 19 February 2014 Expiry Date: 19 February 2015

If you have any queries or need further help, please contact the REC Office 0219389207.

Sincerely,

REC Coordinator
Mertrude Davids
Health Research Ethics Committee 2

# APPENDIX II

## PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

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**TITLE OF RESEARCH PROJECT:** Analysis of the clinical utility of gene expression profiling in relation to conventional prognostic markers in South African patients with breast carcinoma.
REFERENCE NUMBER: N09/06/166

PRINCIPAL INVESTIGATOR: Kathleen Grant

**ADDRESS:** Department Biomedical Sciences, Faculty of Health and Wellness, Cape Peninsula University of Technology, Bellville & the Department of Pathology, Faculty of Health Sciences, University of Stellenbosch, Tygerberg.

**CONTACT NUMBER:** Tel: (021) 959 6411 / 0834583880

We would like to invite you to participate in a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part initially.

This research study has been approved by the ethics **Committee for Human Research at Stellenbosch University** and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes.*

**What is genetic research?**
Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Occasionally genetic material is obtained from other sources such as saliva or biopsy specimens. (A biopsy is a tiny piece of tissue that is cut out e.g. from the skin or from a lump, to help your doctor make a diagnosis.) Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

**What does this particular research study involve?**
This study will analyze genetic factors that may influence the development, treatment response or recurrence rate of breast or related cancer types and may also look for environmental factors (e.g. smoking, alcohol intake, medication) that may interact with genetic risk factors. The information will be used to develop genetic counselling and clinical guidelines for doctors to use in the prevention or treatment of cancer. Individuals without breast cancer may also be included

as controls to identify factors that could influence development of this cancer in generations to come.

**Why have you been invited to participate?**
You are either a sufferer from breast cancer or someone without any form of cancer that could form part of the control group (to allow us to make comparisons). We would like to test for various potential risk factors to find out if patients have these risk factors more often than the control group without breast cancer, or how knowledge of specific genetic risk profiles affect clinical management of breast cancer patients.

**What procedures will be involved in this research?**
You will be asked to fill in a questionnaire with questions pertaining to your health status and relevant environmental factors. Your length and weight may be measured and three small blood bottles of 5 ml each may be taken for laboratory determinations. Alternatively, a saliva sample or cheek swab will be taken for genetic analysis. In patients with breast cancer, biopsies may be collected for research when you have surgery for a specific medical purpose. A fine needle attached to a syringe will be used to remove cells from the tumour. If your specimen(s) has already been used for genetic testing (e.g. MammaPrint test) as part of a routine service, you may be approached to participate in the research in which case the information obtained as part of your clinical work-up will be applied in the research project. The operation is not changed at all and follows normal practice.

**Are there any risks involved in genetic research?**
If a specimen is taken for research during surgery no additional risks are involved. If blood is collected you may experience minor pain and discomfort during the procedure or bruising at the site. Some insurance companies may mistakenly assume that taking part in genetic research indicates a higher risk for disease. Thus, no information about you or your family will be shared with such companies.

**Are there any benefits to your taking part in this study and will you get told your results?**
Your genetic material will be stored and may be tested immediately or at a later time when batches of samples are available; this will be done to limit testing time and costs involved. This research may benefit people with breast cancer in the future as the results will be used to improve clinical decision making.

Research results will be made known to you only **if they indicate** that you may:

- Have a definite risk for developing (a second) breast cancer.
- Need specific medical treatment to reduce recurrence risk (e.g. chemotherapy vs hormonal therapy)
- Have a predisposition or a risk factor for developing breast cancer that is treatable or avoidable e.g. by a lifestyle or dietary modification.
- Need genetic counselling.

If your need genetic counselling you may be referred to a registered genetic counsellor or consulted by another qualified health care practitioner who may charge you for this service, which is not included in the research.

**How long will your specimen be stored and where will it be stored?**
Your specimen may be stored at the University of Stellenbosch Medical School and/or transferred to the Netherlands for genetic analysis. After completion of the study your specimen

could be returned to South Africa and stored in a dedicated fridge or freezer indefinitely or destroyed on your request.

**If your specimen is to be stored is there a chance that it will be used for other research?**
Your specimen will only be used for genetic research that is directly related to breast cancer or cancer risk. Also if the researchers wish to use your stored blood or tissue for **additional research in this field** they will be required to apply for permission to do so from the Human Research Ethics Committee at Stellenbosch University that can be contacted at telephone number 021 938 9657. If you do not wish your blood specimen or tissue sample to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

**How will your confidentiality be protected?**
Each specimen will be given a number and only the clinicians, researchers and support staff involved in the study will have access to the original questionnaires with identifying information. If ever information comes to light that could be important for the individual or her descendants, all possible attempts will be made to contact these participants and counsel them. The results of the study will be included in scientific manuscripts without revealing the identity of the study participants.

**Will you or the researchers benefit financially from this research?**
You will not be paid to take part in this study.
Important information: In the unlikely event that this research leads to the development of a commercial application or patent, you or your family will not receive any profits or royalties but the researchers (inventors) may benefit.

**Declaration by participant**

By signing below, I ……..……. agree to take part in this **research study on breast cancer genetics.**

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I have received a signed duplicate copy of this consent form for my records.

Tick the option you choose:
☐ I agree that my blood or tissue sample can be stored **indefinitely** but I can choose to request at any time that my stored sample be destroyed. My sample will be identified with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out.
OR
☐ I agree that my blood or tissue sample can be stored **indefinitely** after the project is completed but that it is anonymised with all possible links to my identity removed, and that the researchers may then use it for additional research in this or a related field. Once my sample is

anonymised by deleting my name from the data base and destroying this consent form, my rights to the sample labelled with only a unique ID number, are waivered. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field

OR

☐ Please destroy my blood or tissue sample as soon as the current research project has been completed.

I may be contacted again in future by my doctor, the study nurse/assistant and/or the researcher (please underline relevant option) to document response to treatment: □ YES □ NO

Signed at (*place*) on (*date*)

Signature of participant Signature of witness

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Declaration by investigator

I *(name)* …… declare that:

- I explained the information in this document to the study participant
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- (If an interpreter is used then the interpreter must sign the declaration below.

Signed at (*place*) ..... on (*date*) …….. .

Signature of investigator /
Trained study nurse/research assistant Signature of witness

Declaration by Interpreter

I *(name)* …… declare that:

I assisted the investigator (*name*) …… to explain the information in this document to (*name of participant*) …….. using the language medium of Afrikaans/Xhosa.
We encouraged him/her to ask questions and took adequate time to answer them.
I conveyed a factually correct version of what was related to me.
I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ..... on (*date*) …….. .

Signature of interpreter Signature of witness

# APPENDIX III
## PUBLICATIONS AND CONGRESS PRESENTATIONS

### Article Published

Grant KA, Apffelstaedt JP, Wright CA, Myburgh E, Pienaar R, De Klerk M, Kotze MJ. MammaPrint Pre-screen Algorithm (MPA) reduces chemotherapy in patients with early-stage breast cancer. S Afr Med J. 2013 Jul 3;103(8):522-6. doi: 10.7196/samj.7223.

Article submitted for publication

### Manuscripts Submitted for Publication

Grant KA, Pienaar R, Brundyn K, et al. Incorporating microarray assessment of HER2 status (TargetPrint) in clinical practice supports individualized therapy in early-stage breast cancer. Multidisciplinary Oncology Congress, 14-15 February 2014, Sandton, Johannesburg, South Africa (manuscript submitted for publication, *Breast*, May 2014).

EH Pohl, Myburgh E, Grant KA, et al. Cost-effective introduction of the 70-gene MammaPrint assay in South Africa and the impact on treatment decision making. (manuscript submitted, *JNCI*, August 2014).

### Abstracts Published

Grant KA, Apffelstaedt JP, Wright CA, Myburgh E, Pienaar R, de Klerk M, Kotze MJ (2012) Development and implementation of the MammaPrint Algorithm (MPA) to reduce chemotherapy overtreatment in South African patients with early stage breast cancer. Histopathology 61(1): 20-21

Grant KA, Apffelstaedt JP, Wright CA, Myburgh E, Pienaar R, de Klerk M, Kotze MJ (2013) MammaPrint® Prescreen Algorithm (MPA) reduces chemotherapy in South African breast cancer patients. The Breast 22S1: S92

Grant KA, Cronje FJ, Botha K, Apffelstaedt JP, Kotze MJ (2013) Development of a genetic database resource for monitoring of breast cancer patients at risk of physical and psychological complications. South African Journal of Psychiatry, Vol 19, Issue 3: 91-126

## Congress Presentations

Grant KA, Apffelstaedt JP, Wright CA, Myburgh E, Pienaar R, de Klerk M, Kotze MJ. Transcriptional Profiling in South African Breast Cancer Patients. International Human Genetics Conference, March 2011, CTICC, Cape Town, South Africa.

Grant KA, Apffelstaedt JP, Wright CA, Myburgh E, Pienaar R, de Klerk M, Kotze MJ. Clinical Utility of Transcriptional Profiling in South African Breast Cancer Patients. Laboratory Medicines Congress, 3 – 7 September 2011, Sandton Convention Centre, Johannesburg, South Africa.

Grant KA, Botha K, Apffelstaedt JP, Kotze MJ. Clinical Utility of Transcriptional Profiling: Potential to reduce anxiety caused by breast cancer diagnosis and treatment. Biological Psychiatry Congress 22 – 25 September 2011, Protea Hotel Stellenbosch, South Africa.

Grant KA, Apffelstaedt JP, Wright CA, Myburgh E, Pienaar R, de Klerk M, Kotze MJ: Development and implementation of the MammaPrint Algorithm (MPA) to reduce chemotherapy overtreatment in South African patients with early stage breast cancer.29$^{th}$International IAP Conference, Sept /Oct 2012,Cape Town International Conference Centre, Cape Town, South Africa.

Grant KA, Apffelstaedt JP, Wright CA, Myburgh E, Pienaar R, de Klerk M, Kotze MJ. MammaPrint® Pre-screen Algorithm (MPA) reduces chemotherapy in South African breast cancer patients.13th St Gallen International Breast Cancer Conference, 13-16 March,2013: St Gallen, Switzerland.

Grant KA, Cronje FJ, Botha K, Apffelstaedt JP, Kotze MJ: Development of a genetic database resource for monitoring of breast cancer patients at risk of physical and psychological complications. SASOP Biological Psychiatry Congress, 29 August - 1 September 2013, Wild Coast Sun, Port Edward, South Africa.

Grant KA, Apffelstaedt JP, Wright CA, Myburgh E, Pienaar R, de Klerk M, Kotze MJ. Analytical validation of HER2/neu overexpression using formalin-fixed paraffin embedded tissue (FFPE)

for microarray analysis in early stage breast cancer patients.15th Biennial Congress of Southern African Society for Human Genetics: 6-9th October 2013, Johannesburg, South Africa.