Comparison of Xpert® Breast Cancer STRAT4 Assay and Immunohistochemistry for the Evaluation of Breast Cancer Biomarkers in South African Patients.

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Thesis presented in fulfilment of the requirements for the Master's degree in Anatomical Pathology in the Faculty of Medicine and Health Sciences at Stellenbosch University

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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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Summary

Background: Breast cancer is one of the most common cancers diagnosed in women and approximately 60% of breast cancer related deaths are reported in low- and middle-income countries. Breast cancer is a highly heterogeneous disease, and molecular subtyping is paramount for effective treatment of patients. Therefore, it is important to validate new molecular methods for assessing cancer biomarkers for cost-effective use in resource-poor settings.

Aim: A retrospective study was performed to determine the concordance between a Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) CE-IVD assay (Xpert® Breast Cancer STRAT4*) and the current gold standard methods of immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) for determining estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and proliferation index (KI-67) expression in breast carcinomas.

Method: One hundred and one cases of breast carcinoma were retrieved from the archives of the Division of Anatomical Pathology, Tygerberg Academic Hospital. The original stained slides were reviewed and IHC expression of ER, PR, HER2 and KI-67 scored. Three-micron sections were cut from formalin-fixed paraffin embedded (FFPE) tissue blocks and processed according to the instructions of the manufacturer. The assay was run on the resultant lysates.

Results: The overall percentage agreement between the Xpert® STRAT4 assay and IHC / FISH results were 85.15% for *ESR*, 89.90% for *PGR*, 91.09% for *ERBB2*, 90.72% for *MKI67* (when using a cut off of 10%) and 84.54% for *MKI67* (when using a cut-off of 20%). The positive percentage agreement for *ESR, PGR, ERBB2, MKI67* with 10% cut-off and *MKI67* with 20% cut-off were 82.76%, 94.64%, 68.97%, 91.30% and 96.05%, respectively, and the negative percentage agreement were 100%, 84.09%, 91.67%, 80.00% and 42.86%, respectively.

Conclusion: The study has shown that the Xpert® Breast Cancer STRAT4 assay shows good concordance with IHC and FISH in detecting breast cancer biomarkers, and may become a supplementary or alternative standard of care after validation studies are performed.

*CE-IVD. In vitro diagnostic medical device. Not available in all countries. Not available in the US.

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Opsomming

Agtergrond: Borskanker is een van die algemeenste kankers wat by vroue gediagnoseer word en ongeveer 60% van sterftes wat met borskanker verband hou, word in lande met lae en middle-inkomste aangemeld. Borskanker is 'n hoogs heterogene siekte, en molekulêre subtipes is van uiterse belang vir die effektiewe behandeling van pasiënte. Daarom is dit belangrik om nuwe molekulêre metodes te evalueer vir die bepaling van kankerbio-merkers vir koste-effektiewe gebruik in hulpbron-arm instellings.

Doel: 'n Terugwerkende studie is uitgevoer om die ooreenkoms tussen 'n RT-qPCR CE-IVD-toets (Xpert® Borskanker STRAT4*) en die huidige goudstandaardmetodes van immunohistochemie (IHC) en fluoresensie in situ-hibridisasie (FISH) om die uitdrukKIng van estrogeen reseptor (ER), progesteroon reseptor (PR), menslike epidermale groeifaktor reseptor 2 (HER2) en proliferasie indeks (KI-67) te bepaal in borskarsinoom.

Metode: Honderd-en-een gevalle van borskarsinoom is uit die argiewe van die Afdeling Anatomiese Patologie, Tygerberg Akademiese Hospitaal, geselekteer. Die oorspronklike gekleurde skyfies is hersien om die IHC-uitdrukKIng van ER, PR, HER2 en KI-67 te bepaal. Drie-mikron snitte is van die formalien vaste paraffien-ingebedde weefselblokke gesny en volgens die instruksies van die vervaardiger verwerk. Die toets is uitgevoer op die resulterende lisate.

Resultate: Die algehele persentasie-ooreenkoms tussen die Xpert® STRAT4-toets en IHC / FISH-resultate was 85,15% vir *ESR*, 89,90% vir *PGR*, 91,09% vir *ERBB2*, 90,72% vir *MKI67* (wanneer 'n afsnypunt van 10% gebruik is) en 84,54% vir *MKI67* (met 'n afsnypunt van 20%). Die positiewe persentasieooreenkoms vir *ESR*, *PGR*, *ERBB2*, *MKI67* met 10% afsnypunt en *MKI67* met 20% afsnyspunt was onderskeidelik 82,76%, 94,64%, 68,97%, 91,30% en 96,05%, en die negatiewe persentasieooreenkoms was 100%, 84,09%, 91,67%, 80,00% en 42,86%, onderskeidelik.

GevolgtrekKIng: Die studie het getoon dat Xpert® borskanker STRAT4-toetsing goeie ooreenstemming met IHC en FISH toon vir die opsporing van biomerkers in borskanker, en dit kan 'n aanvullende of alternatiewe standaard vir sorg word nadat meer valideringstudies gedoen is.

* CE-IVD. In vitro diagnostiese mediese toerusting. Nie in alle lande besKIkbaar nie. Nie besKIkbaar in die VSA nie.

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Abbreviations

ADH	Alcohol dehydrogenase
ASCO/CAP	American Society of Clinical Oncology/College of American Pathologists
BI-RADS	Breast Imaging Reporting and Data System
BRCA1/2	Breast Cancer susceptibility gene
BSE	Breast self-examination
CBE	Clinical breast examination
cDNA	Complimentary DNA
DCIS	Ductal carcinoma *in situ*
ER	Estrogen receptor
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin embedded
FISH	Fluorescence *in situ* hybridization
GDPR	General data protection regulation
GEP	Gene expression profiling
GLOBOCAN	Global Cancer Observatory
HER2	Human epidermal growth factor receptor 2
HIC	High income countries
HPF	High power field
HRT	Hormone replacement therapy
IARC	International Agency for Research on Cancer
IBC-NST	Invasive breast Carcinoma of no special type
IHC	Immunohistochemistry
LCIS	Lobular carcinoma *in situ*
LMIC	Low-medium income countries
MTA	Material transfer agreement
POPI	Protection of Personal Information Act
PR	Progesterone receptor
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
TBH	Tygerberg Hospital
TNBC	Triple negative breast cancer
WHO	World Health Organization

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Chapter 1

Introduction

Cancer has become an epidemic with an increasing global burden (WHO, 2018). Research leading to the control of cancer prevalence and its resultant mortality and morbidity is critical to relieving the burden of this disease on the population of any nation (Rafiemanesh *et al*., 2018). Higher incidence rates of cancer in Asia and Africa, as reported by the World Health Organization (WHO), is a result of failure to diagnose cancer early and lack of resources for holistic treatment in these regions (Bray *et al*., 2018; WHO, 2018). However, it is noteworthy that breast cancer incidence is highest in high income countries (HIC) in comparison with low- to medium income countries (LMIC), predominantly in Africa (Boyle, 2012; Akarolo-Anthony *et al*., 2012). In contrast, the death rate from breast cancer in LMIC is higher as compared to HIC (Jedy-Agba *et al*., 2016).

Reports from GLOBOCAN (2018) indicate that the top five most frequent cancers (excluding non-melanoma skin cancer) are lung, breast, colorectal, prostate and stomach cancer. Cancer of these five organs accounted for 46% of cancer prevalence and 43% of cancer deaths in 2018. Breast cancer results in a high mortality rate amongst women globally (Jedy-Agba *et al*., 2016; Stefan, 2015). Approximately 60% of breast cancer related deaths are reported in developing or LMIC, while 40% occur in developed countries (Rivera-Franco and Leon-Rodriguez, 2018; da Costa Vieira *et al*., 2017; Narod *et al*., 2015). This reduced mortality rate in developed countries is largely due to early diagnosis and treatment at state-of-the-art facilities specializing in the management of breast cancer (Jemal *et al*., 2011).

Socio-economic factors and limited access to health care facilities may be contributing factors to the increased incidence of breast cancer in developing countries (Rivera-Franco and Leon-Rodriguez, 2018; Akarolo-Anthony *et al*., 2010). In addition, urbanization, reproductive cycle alteration (from use of contraceptives, exposure to carcinogens etc.), environmental risk factors (pollution), lifestyle (alcohol use, smoking) and increasing life expectancy are associated with the increasing incidence of breast cancer occurring in LMIC (Hadgu *et al*., 2018; Akarolo-Anthony *et*

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al., 2010;). Tefferi *et al*. (2015) in their study projected that two-thirds of new cancer diagnoses will occur in developing countries by 2035.

Breast cancer management is a major challenge due to the heterogeneity of the disease. There are many different subtypes of carcinoma with diverse attributes, both biologically and clinically (Rivenbank *et al*., 2013). Classification of breast carcinoma according to the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2) and proliferation index Ki-67 (proteins encoded by *ESR1*, *PGR*, *ERRB2* and *MKI-67* genes, respectively*)* is an essential step in the treatment of the disease, prognostication and predicting the response to treatment (Dai *et al*., 2016). Breast carcinoma biomarker status assessment can be performed at various molecular levels using different laboratory techniques (Eswarachary *et al*., 2017). Currently, the clinical gold standard for measuring breast cancer biomarkers ER, PR and HER2 status is immunohistochemistry (IHC) on formalin-fixed paraffin embedded (FFPE) tissue (Müller *et al*., 2011). Breast carcinomas reported to have an equivocal HER2 IHC score undergo fluorescence *in situ* hybridization (FISH) testing to determine the HER2 status as recommended by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) (Eswarachary *et al*., 2017; Wolff *et al*., 2013).

The evaluation of IHC and FISH is performed using light and fluorescent microscopy, respectively (Bogdanovska-Todorovska *et al*., 2018). It needs to be recognized, however, that there may be discrepancies due to many pre-analytical factors such as the technique of the tissue sample acquisition, fixation and preparation. This may lead to challenges in interpretation of results using these techniques as previous described by Grant *et al* (2015;2019) in the South African context. These authors used a microarray platform to explore different characteristics of tumors and to predict prognosis in specific groups of breast carcinoma patients, based on level 1A evidence for determination of chemotherapy benefit (Cardoso *et al*., 2016). Microarray testing lead to the discovery of four major intrinsic subtypes of breast cancer (Perou, *et al*., 2000). This resulted in the development of commercially available RNA-based gene profiling tests such as Mammaprint (70 genes) and BluePrint (80 genes). These tests which are currently only available in the private health care sector in South Africa, are used in combination with IHC/FISH to help inform effective

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chemotherapy and anti-HER2 therapy using microarrays (Grant *et al*., 2019; Myburgh *et al*., 2016).

Recently, the above mentioned microarrays has been transferred onto a next generation sequencing platform (Mittempergher *et al.,* 2019). High-throughput technologies assessing transcriptional profiling have ignited the ability of researchers to study breast cancer at the molecular level, with the ultimate objective of early detection and targeted treatment (Ho *et al*., 2020). However, the availability and affordability of high-quality diagnostic technologies is relatively limited in LMIC (Nelson *et al*., 2016). Exploring these technologies in LMIC depends on the incidence of the disease in the targeted population and availability of resources (Clifford, 2016) for analytical validation prior to implementation.

There are three categories of test performance to be evaluated for new tests: analytic validity, clinical validity, and clinical utility (Holtzman and Watson, 1999). These categories are linked together and may overlap (**Figure 1-1).** A major goal of analytical validity is the ability of a test to accurately and reproducibly measure an analyte (Burke, 2014). Holtzman and Waston (1999) suggested that prior to use of a newly developed test, clinical validity and utility must be taken into consideration. Clinical validity refers to the accuracy with which the assay identifies a particular clinical outcome. There are important variables to consider for clinical validity, such as the type of assay used and its analytic validity. On the other hand, clinical utility would refer to the risks and benefits resulting from using a test. This aspect involves the medical and social outcomes associated with the test. The determination of clinical validity and utility was not part of the scope of this present study, which only focused on the analytical validity.

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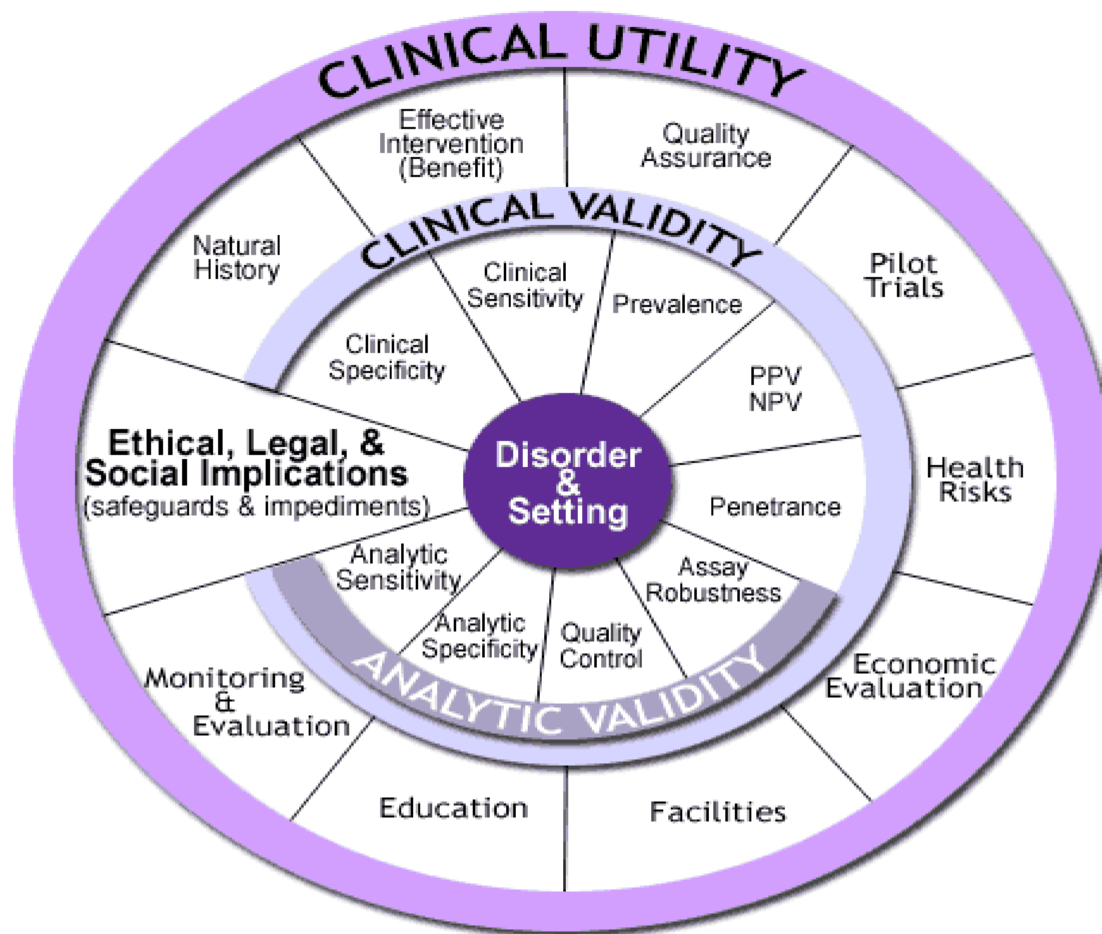

Figure 1-1: Evaluation process for genetic testing. *Source*: Centers for Disease Control and Prevention (CDC).

1.1 Rationale

Introduction of advanced molecular technologies alongside standard pathology services faces a number of challenges in LMICs which may include the lack adequate infrastructure and skilled personnel at all levels (Patel *et al*., 2016). Although immunohistochemistry testing is readily available in academic and private histology laboratories in South Africa, at Tygerberg Hospital (TBH), FISH testing (required for equivocal HER2 results), is referred to another laboratory due to these complexities. This may generate inconsistencies between results produced with the use of the same samples at different laboratories. The optimization and standardization of tests along with

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a high degree of human intervention (performing the test and interpretation of results) may all play a role in producing discrepant results (Wu *et al*., 2018; Wolff *et al*., 2013). Despite attempting to overcome these challenges associated with referral of samples, the risk of specimen loss during transit and long turn-around times for results can hamper appropriate patient care. Therefore, development and validation of rapid laboratory techniques that minimise human intervention and is easy to use for optimising patient management is a priority. In this study will evaluate a real time polymerase chain reaction (RT-qPCR) assay, the Xpert® Breast Cancer STRAT4 assay (STRAT4), in order to quantitatively assess mRNA of *ESR1, PGR, ERBB2* and *MKI67* in invasive breast carcinoma and compare these results to IHC and FISH biomarker assessment.

1.2 Aim of study

In this study ER, PR, HER2 and KI-67 status was evaluated in breast carcinomas of patients at TBH using the Xpert® Breast Cancer STRAT4 assay. The specific objectives were as follows:

- To measure mRNA transcripts of *ESR1, PGR, ERBB2 and MKI67* using the Gene Xpert® instrument in FPPE samples.

- To correlate the results of different techniques to (IHC, FISH & RT-qPCR) used to assess breast carcinoma subtypes at the protein, DNA and mRNA levels.

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Chapter 2

Literature Review

The International Agency for Research on Cancer (IARC) reported over 47 000 deaths from cancer in South Africa in 2012 (Ferlay *et al*., 2013). Several factors such as an ageing, demography, infectious diseases, abnormal weight gain and abuse of narcotics amongst the female population have been attributed to an increased incidence of breast cancer and resultant death from this disease (Torre *et al*., 2015). Similarly, Norman *et al*. (2007) had also listed the aforementioned factors to the cancer morbidity and mortality indices in the South African population. Previous studies have reported some decline in mortality rates for other forms of cancer, especially internal malignancies such as tracheal, bronchial and lung cancers between 2001 to 2006 (Dela Cruz *et al*., 2011; Mayosi *et al*., 2009). However, an increased mortality was recorded for breast cancer, cervical cancer and prostate cancer around the same period (Mayosi *et al*., 2009). According to the South African National Cancer Registry (NCR), increased incidence rates were observed for breast and cervical cancer in women, and lung and colorectal cancer in men (South African National Cancer Registry, 2012). Cancers of these organs accounted for 46% of cancer prevalence and 43% of cancer deaths in 2018 (Bray *et al*., 2018).

Sixty percent of breast cancer deaths and a half of new breast cancer diagnoses are observed in developing countries, while a noticeably lesser mortality rate is observed in developed countries (Bray *et al*., 2018; Jemal *et al*., 2011). The mortality rates for breast cancer ranged from 40% to 60% in low- and middle-income countries (LMIC) compared to 40% in the United States of America (USA) (Rivera-Franco and Leon-Rodriguez, 2018; Narod *et al*., 2015).

2.1 Breast Cancer Risk Factors

Epidemiological findings revealed many risk factors for the increased incidences and death related to breast cancer in the South African population (Torre *et al*., 2015). Risk factors include genetic susceptibility, early menarche, lower parity, older age at first birth, reduced breastfeeding periods, use of contraceptives and hormone replacement therapy (HRT), obesity after menopause, lack of physical activity and narcotics/alcohol consumption (Shoemaker *et al*., 2018; Travis and Key, 2003). Identification of factors associated with an increased incidence of breast carcinoma

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development is important in general health screening for women. These risk factors may be divided into: (a) non-modifiable factors such as sex, a personal or family history of breast carcinoma, menopausal status, genetic risk and reproductive risk factors; and (b) modifiable factors which include lifestyle and exogenous hormone use (Nindrea *et al*., 2017; Majeed *et al*., 2014).

2.1.1 Modifiable

Lifestyle risk factors which include an increased dietary fat intake, smoKIng and excessive alcohol consumption are the most important modifiable risks for breast cancer (McDonald *et al*., 2013; Dumitrescu and Cotrla, 2005). Normally, pre-menopausal women produce most of their circulating estrogen in the ovaries and only a minute amount from fatty stores (Key *et al*., 2013). However, in overweight and post-menopausal women, higher circulating estrogen is produced from adipose tissue that can lead to an increased risk of breast cancer (Dumitrescu and Cotarla, 2005). Breast cancer risk is increased in obese women who do not use HRT, and for every 5kg of weight gained, the risk of breast cancer increases by 8% (Dumitrescu and Cotarla, 2005). Kori *et al*. (2018) also found that an important source of estrogen is synthesized from cholesterol in adipose tissue.

Diet influences cancer in about 35% of cancer cases (Kotepui *et al*., 2016). Reducing red meat, high fat and elevating fiber and vitamin D intake are preventive dietary measures associated with breast cancer. Studies have reported that meat cooked at high temperatures is a risk factor for breast cancer (Zheng *et al*. 1998, Sinha *et al*., 2000; Fu *et al*., 2011). The cooking duration and temperature of red meat have been reported to be associated with the amount of meat derived mutagens which have been shown to induce mammary gland tumors (Fu *et al*., 2011). One study reported that women who had a consistent intake of well-done meat had a 4.6-fold increased risk of breast cancer (Sinha *et al*., 2000). Nonetheless, postmenopausal women who consistently eat red meat have higher risk of breast cancer as compared to premenopausal women (Fu *et al*., 2011). Diets high in polyunsaturated fat have been reported to increase the occurrence of mammary tumors in animal models (Kotepui, 2016).

A meta-analysis of large prospective cohort studies showed that high dietary fiber intake is a protective factor for breast cancer (Dong *et al*., 2011). A study reported 7% reduction in risk of

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breast cancer associated with every 10-g/d incremental increase in dietary fiber intake (Dong *et al* 2011). Faecal fiber can inhibit absorption of estrogen in the gut which leads to reduction of increased estrogen circulation. Another proposed mechanism is the binding of unconjugated estrogen to fiber in the gut, thereby decreasing estrogen reabsorption (Moore *et al*., 1998)

Isoflavones found in soy products have been reported to have a similar molecular structure to mammalian estrogen (Peeters *et al*., 2003). Therefore, isoflavones can competitively bind to ER resulting in blocking estrogen from binding to its receptor (Peeters *et al*., 2003). Moreover, isoflavones are the most potent inhibitors of aromatase, the enzyme that converts androgen to estrogen (Rice and Whitebread, 2008). It has been hypothesized that vitamin D can reduce the risk of breast cancer. Vitamin D can inhibit the estrogen pathway leading to the expression of the aromatase gene (Krishnan *et al*., 2012).

An increased risk of breast cancer is also reported in women with a longstanding history of smoking (Hashemi *et al*., 2014). In a study by Bishop *et al*. (2014), it was found that women who smoke have a 6.7 times higher chance of developing recurrent breast carcinoma after partial mastectomy, than women who had never smoked. Active smoking and passive smoking are some of the most important risk factors for breast cancer and its recurrence (Bishop *et al*., 2014; Hashemi *et al*., 2014).

Excessive alcohol consumption is linked to an approximately 30 to 50% increased risk for breast cancer (McDonald *et al*., 2013). Alcohol elevates the level of estrogen-related hormones in the blood which often leads to signaling of estrogen receptor pathways and increase breast density (Seitz *et al*., 2012; Boyd *et al*., 2011, Fernandez, 2011). Alcohol consumption of two to three units per day poses a 20% relative risk for breast cancer incidence, as compared to women who do not consume alcohol (Feng *et al*., 2018). Ethanol is metabolized by alcohol dehydrogenase (ADH) into acetaldehyde (Seitz *et al*., 2012). Acetaldehyde binds to proteins and DNA, thus interfering with DNA synthesis and repair (Seitz *et al*., 2012). In addition, alcohol increases circulating estrogen levels which are thought to induce hormone-receptor mediated cell proliferation and cause genetic alterations (Dumitrescu and Cotarla, 2005). Ethanol increase transcriptional activity of ERα, a key estrogen receptor, by down-regulating the tumor suppressor gene BRCA1, which in turn leads to increased cell proliferation (Fan *et al*., 2006).

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Hormones circulating in the blood bind to receptors found on the surface of cells to facilitate cell proliferation. This is an important factor in ageing and development of cancer (Dehkhoda *et al*., 2018). Kamińska *et al*. (2015) reported on risk factors of breast cancer in relation to different ages, and found that both endogenous and exogenous hormones are important factors associated with breast cancer. Normal breast epithelial cells express nuclear receptors for estrogen and progesterone. Progesterone receptors function as critical regulators of transcription, as well as activating signal transduction pathways which are necessary precursors for pro-proliferative signalling in the breast (Daniel *et al*., 2011).

The cycles of endogenous estrogen levels play a role in either the development of breast cancer or protection from it (Rosato *et al*., 2014; Shah *et al*., 2014). An early menarche and late menopause expose a woman to a longer period of circulating estrogen, which in turn increase the risk for developing breast carcinoma (Shah *et al*., 2014). Dall and Britt (2017) found that every year menarche is delayed in a woman; the risk of developing breast carcinoma is reduced by 5%. In a similar vein, an early first, full-term birth was reported as an effective measure in breast cancer prevention, with a potential of halving a woman's lifetime risk (Katz, 2016). Therefore, a first birth at a younger age or multiple pregnancies has an overall protective effect against breast cancer (Katz, 2016; Shah *et al*., 2014). Similarly, another factor worthy of consideration is breastfeeding. According to the Collaborative Group on Hormonal Factors in Breast Cancer (2002), breastfeeding has a protective effect against the development of breast carcinoma. Breastfeeding may delay the return of regular ovulatory cycles and decrease endogenous sex hormone levels. It has been estimated that there is a 4.3% reduction in breast cancer incidence for every one-year of breastfeeding (Collaborative Group on Hormonal Factors in Breast Cancer, 2002).

Hormone replacement therapy (HRT) is known to relieve menopausal symptoms and may reduce osteoporosis, however, it is the main source of exogenous estrogen for post-menopausal women (Sun *et al*., 2017; Liu *et al*., 2016). Liu *et al*. (2016) in a cohort study of 22,929 women in Asia, demonstrated hazard ratios of 1.48 and 1.95 after HRT use for 4 and 8 years, respectively. However, this risk is decreased after cessation of HRT. Unfortunately, the risk is reported to increase and is irreversible for long term use (more than 15 years) (Feng *et al*., 2018). There was a decreased risk of developing breast carcinoma when HRT is stopped, i.e. breast carcinoma

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development is positively correlated with HRT use (Katz, 2016). Furthermore, a study in USA reported that the incidence of breast cancer decreased by approximately 7% in 2003 as compared to 2002 due to the reduction in the use of HRT (Ravdin *et al*., 2007). The use of oral contraceptives has also been found to increase breast cancer risk by up to 24% when compared to women who have never used oral contraceptives (Ban and Godellas, 2014).

2.1.2 Non-Modifiable

Several studies have reported the incidence of breast cancer to gradually increase with age (Feng *et al*., 2018; Sun *et al*., 2017). Sun *et al*. (2017) reported that in all the cases of cancer incidences in their study, 99.3% were over the age of 40 years. Also, in the same report, 71.2% of mortality due to breast cancer occurred among women over the age of 60 years (Siegel *et al*., 2017). Furthermore, according to a study breast cancer and age-related mortality rate, Abdulkareem (2013) asserted that by the age of 90 years, one-fifth of woman have been affected by some form of breast cancer related disease. Even though breast cancer incidence is relatively low in Sub-Saharan Africa, disease survival is generally poor on the African continent, which may largely be due to late diagnosis. There is generally a low cure rate due to late detection (Jedy-Agba *et al*., 2016). A relatively low breast cancer incidence in parts of Africa, compared to other developed countries, is likely due to a lower life expectancy (Kantelhardt and Grosse 2016; Brinton *et al*., 2014).

Approximately 15% of women in the USA diagnosed with breast cancer have a family history of the disease (American Cancer Society, 2019). Women with close relatives who have been diagnosed with breast cancer have a high risk depending on the degree of relation (Colditz *et al*., 2012). A study performed in the United KIngdom (UK) reported a 1.75-fold higher risk of developing breast cancer with one first degree relative with the disease (Brewer *et al*., 2017). Among women with a family history of breast cancer, the prevalence of benign breast disease was substantially higher (47.6%) than among women without a family history (37.9%) (Colditz *et al*., 2012). Women with a family member who has been diagnosed with breast cancer before the age of 50 years, has an increased risk of developing breast cancer compared to women with family members diagnosed at older ages (Anders *et al*., 2009). Overall, 15.38% of women reported a family history of breast cancer diagnosed in either a mother or sister; 3.4% had a family member

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with first diagnosis before age 50 and 11.94% had a family member with first diagnosis at age 50 or older (Colditz *et al*., 2012).

The most common cause of hereditary breast cancer is an autosomal dominant inherited mutation in the *BRCA1/2* high penetrance genes (Halperin & Edward, 2008) which accounts for 20–25 % of all hereditary breast cancer tumors (Paulch-Shimon *et al*., 2016; Balmana *et al*., 2011). Approximately 5–10% of breast carcinomas are linked to patient germline mutation in tumor suppressor genes, *BRCA1/2* (Paulch-Shimon *et al*., 2016; Majeed *et al*., 2014). The *BRCA1* gene is located on chromosome 17q21 and the deficiency of its protein leads to the dysregulation of cell cycle checkpoints, abnormal centrosome duplication and genetic instability (Dine and Deng, 2013; Deng, 2006). The *BRCA2* gene is located on chromosome 13q12 and its protein regulates recombination repair in DNA double-strand breaks (Wooster *et al*., 1994). Multiple high to moderate-penetrance mutations have also been identified in the *TP53, CHEK2, ATM, BRIP1, PALB2, RAD51C RAD50* cancer susceptibility genes (Han *et al*., 2017).

2.2. Pathophysiology of Breast Cancer

Breast carcinoma consists of a group of biologically and molecularly heterogeneous diseases originating from breast epithelium (Feng *et al*., 2018; Rakha *et al*., 2010). Normal breast development and mammary stem cells are regulated by several signalling pathways which control stem cell proliferation, cell death, cell differentiation and cell motility (Feng *et al*., 2018). However, mutations which lead to activation of oncogenes and inactivation of tumor suppressor genes may lead to deregulation of these signalling pathways (Lee and Muller, 2010).

Women with either of the *BRCA1/2* mutations have about 70% chance of developing breast carcinoma by the age of 80 years (Feng *et al*., 2018; Majumder *et al*., 2017). *BRCA1/2* mutation prevalence varies across populations and geographic distribution (Schlebusch *et al*., 2010; Ford *et al*., 1998). A study performed by the Breast Cancer Linkage Consortium (BCLC) in the United KIngdom on a total of 237 families reported that overall *BRCA1* mutations account for 52% of all familial breast cancer cases and *BRCA2* mutations for 32% (Ford *et al*., 1998). An increased frequency of at least eight *BRCA1/2* mutations have been identified in South Africa due to a founder effect (van der Merwe *et al*., 2012). This resulted in the development of a cost effective

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founder mutation test used as first line screening step to determine the need for extended germline genetic testing. Advanced next generations sequencing (NGS) technologies such as whole exome sequencing (WES) are increasingly used to distinguish between familial and lifestyle related causal pathways targeted for optimal treatment (van der Merwe *et al*., 2012).

Emerging evidence has indicated that epigenetic alterations and non-coding RNAs may play important roles in breast carcinoma development and may contribute to the heterogeneity and metastatic potential of breast carcinoma (Feng *et al*., 2018).

2.3 Diagnosis of Breast Cancer

A total of 5% of all worldwide expenditure for breast cancer screening takes place in developing countries (da Costa Vieira *et al*., 2017). Therefore, imaging techniques used for screening, such as mammography, may be limited, yet its deployment has been reported to help in significantly reducing mortality from breast cancer (Sun *et al*., 2017). However, breast self-examination (BSE) and clinical breast examination (CBE) is key to the diagnosis in LMIC when mammographic screening is not feasible (da Costa Vieira *et al*., 2017). The purpose of mammography is to identify breast cancer at an early stage, prior to symptoms and while the cancer is still curable. In symptomatic patients, the sensitivity of mammography is 90% and the specificity is 94% (Joy *et al*., 200). The positive predictive value (PPV) is 84% for all screened patients (Harris *et al*., 2004). The categories of the Breast Imaging Reporting and Data System (BI-RADS) is shown below (**Table 2-1**).

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Table 2-1: The BI-RADS scoring system (Magny *et al.,* 2020)

BI-RADS Score		
Category	**Assessment**	**Recommendation**
0	Incomplete study	Need additional imaging or prior studies
1	Negative: no masses, suspicious calcifications or areas of architectural distortion.	Routine screening
2	Benign: include secretory calcifications, simple cysts, fat-containing lesions, calcified fibroadenomas, implants and intramammary lymph nodes	Routine screening
3	Probably benign: a non-palpable, circumscribed mass on a baseline mammogram; a focal asymmetry, which becomes less dense on spot compression images, or a solitary group of punctate calcifications	Short-term follow-up establish stability
4	Suspicious abnormality: subdivided into a, b, and c. The subcategory of (a) has a low probability of malignancy with a 2% to 10% chance of malignancy. The subcategory of (b) has an intermediate change of malignancy ranging from 10% to 50%. The subcategory of (c) has a high probability of malignancy ranging from 50% to 95%.	Biopsy should be considered
5	Highly suggestive of malignancy	Surgical consultation
6	Known malignancy	Appropriate action should be taken

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2.4 Classification of Breast Cancer

Breast cancer, by definition, refers to any malignant neoplasm of the breast (American Cancer Society, 2019). Vinay *et al*. (2010) reported that most malignancies are adenocarcinomas which account for over 95% of breast malignancies. Other malignancies, although rare, that may primarily involve the breast are lymphomas or sarcomas (Acevedo *et al*., 2019) Carcinoma originates from epithelial cells lining the lobules and ducts. Furthermore, breast carcinoma may be classified as *in situ* or invasive carcinoma. Non-invasive breast carcinoma, also referred to as *in situ* carcinoma, is a malignant neoplasm which is confined to the ductal-lobular system and which does not invade beyond the surrounding myoepithelial cells and basement membrane into the connective tissue of the breast (American Cancer Society, 2019). Non-invasive breast carcinoma accounts for 15–20 % of all breast cancers. Ductal carcinoma *in situ* (DCIS) accounts for 90% of the non-invasive breast carcinoma cases (Sharma *et al*., 2010). In lobular carcinoma *in situ* (LCIS), the malignant cells are usually contained within the acini of lobules, but pagetoid spread into distal ducts may occur. LCIS is a relatively uncommon carcinoma type which accounts for 1–4.3% cases of breast carcinoma (Mo *et al*., 2018; Karakas, 2011).

Invasive carcinomas are morphologically subdivided into histological subtypes according to their growth patterns and cytological features (Rakha *et al*., 2010). Although, histological subtype provides useful prognostic information, the majority (60%–75%) of breast carcinomas have no specific morphological characteristics and are called invasive breast carcinoma of no special type (IBC-NST), also referred to previously as infiltrating ductal carcinoma (Makki, 2015). Carcinomas with “special” morphological characteristics, and which have differing prognostic significance, are relatively uncommon (Rakha *et al*., 2010). As a consequence, the role of histological typing in clinical management decision making is limited (Pereira *et al*., 1995). The second most frequent invasive carcinoma is invasive lobular carcinoma (ILC), which accounts for approximately 10-15% of carcinoma cases, and are prevalent in postmenopausal women, likely due to HRT (Makki, 2015; Sharma *et al*., 2010). Other histological subtypes of carcinomas which have been reported to have a better prognosis, and which account for a small proportion of all breast carcinomas, include tubular carcinoma (2%) and mucinous carcinoma. A study in Kenya reported 2.6% of carcinomas to be of the mucinous type (Sayed *et al*., 2014).

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2.4.1 Grading

Histological grading is based on the pattern of growth and degree of differentiation, relative to normal breast epithelium (Rakha *et al*., 2010). In breast carcinoma, histological grading refers to the semi-quantitative assessment of morphological features. Currently, histologic grading is done according to the Nottingham combined histologic grading system. Grading is one of the best-established prognostic factors in breast cancer (Rakha *et al*., 2010). In the Nottingham combined histologic grading system, three morphological characteristics are appraised: (a) degree of tubule or gland formation, (b) nuclear pleomorphism and (c) mitotic count. Grade 1: A well-differentiated tumor that demonstrates homology to the normal breast terminal duct lobular unit, tubule formation (>75%), a mild degree of nuclear pleomorphism, and low mitotic count. Grade 2: A moderately differentiated tumor and Grade 3: A poorly differentiated tumor with a marked degree of cellular pleomorphism, frequent mitoses and no tubule formation (<10%) (Rakha *et al.,* 2010) (**Figure 2-1**).

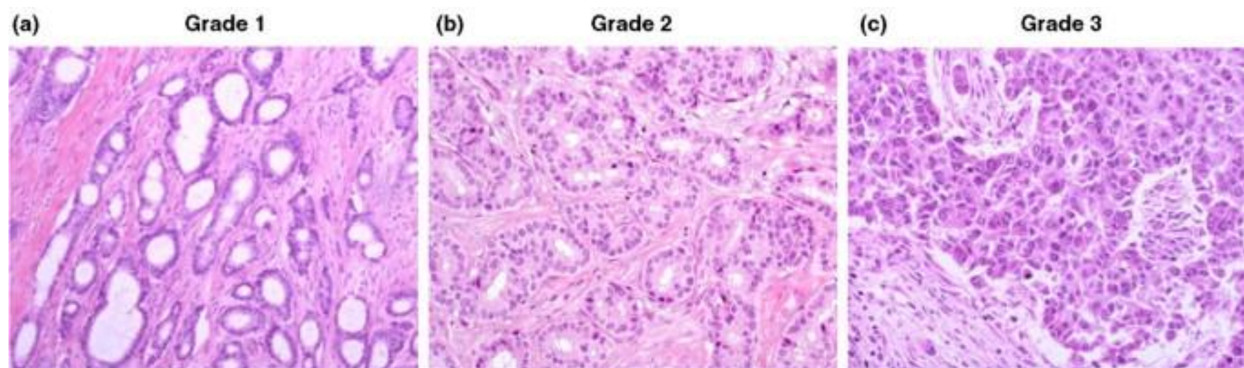

Figure 2-1: Schematic representation of the Nottingham combined histologic grading system. (Rakha *et al.,* 2010).

2.5 Breast Cancer Biomarkers

From the 1980s onwards, several strides have been made in researching breast carcinoma biomarkers and the correlation of biomarker expression and therapeutic response (Nomura *et al*., 1984). The histopathological assessment of biomarkers ER, PR, HER2 and KI-67 have been widely adopted in the past few decades for subtyping breast carcinoma, prognostication and

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prediction of therapeutic responses (Ulaner *et al*., 2016; Spitale *et al*., 2009). Light microscopy is still the foundation of pathological diagnosis, but in the era of modern personalized medicine, a number of molecular classification systems have been developed and introduced. American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommends routinely testing hormone receptors (ER and PR) and HER2 status on all primary invasive breast carcinomas and on recurrent or metastatic tumors (Hammond *et al*., 2010; Harris *et al*., 2007).

2.5.1 Estrogen Receptor (ER)

Estrogen is implicated in the development or progression of numerous diseases including breast carcinoma (Deroo and Korach, 2006). Binding of estrogen to the ER stimulates cell division and DNA synthesis. An increased rate of DNA synthesis leads to a higher risk of replication errors (mutations) that may disrupt normal cellular processes. Furthermore, estrogen metabolism leads to the production of genotoxic by-products that can directly damage DNA (Yue *et al*., 2005). Estrogen receptor expression in breast carcinoma is a favourable prognostic factor and strongly predictive of a response to hormonal therapy (Colomer *et al*., 2017; Nicolini *et al*., 2017). For ER expression to be regarded as positive, at least 1% of tumor cells must show nuclear staining of any intensity (Hammond *et al*., 2010). Up to 75% of breast carcinomas are ER-positive, and the majority occurs in postmenopausal women (Anderson *et al*., 2014). This is similar to high proportions of ER-positive breast carcinoma as reported in Nigeria, where 50% of tumors from a study were ER-positive (McCormac *et al*., 2013; Adebamowo *et al*., 2008). However, another study from Nigeria reported only 25% ER-positive cases (Hou *et al*., 2009).

The two independent studies showed significantly different results, and raise the possibility of variation in the disease due to social attributes and geographical location. ER-positive tumors are generally well-differentiated, less aggressive, and associated with a better outcome after surgery (Dunnwald *et al*., 2007). However, ER simultaneously down regulates epidermal growth factor receptor (EGFR) and HER2 while inducing IGF1R (Paplomata and O'Regan, 2013). In swift response, activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-KInase (PI3K) pathways by growth factor receptors down regulates estrogen receptor signalling (Osborne and Schiff, 2011). Recent gene expression profiling (GEP) studies have shown that ER expression

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status is a major clue to the molecular "portrait" of breast carcinoma (Dai *et al*., 2014). Carcinomas with differing ER expression are fundamentally different at the transcriptional level (Dai *et al*., 2014).

2.5.2 Progesterone Receptor (PR)

Understanding progesterone receptor (PR) action is of critical relevance in breast cancer study, as demonstrated by large-scale clinical trials conducted for over 10 years which findings reported that PR actions fuel breast cancer development (Hagan and Lange, 2014). Studies have shown that both ER and PR are important as predictors of response to adjuvant hormonal therapy (Sawe *et al*., 2016; Mirza *et al*., 2002). PRs are activated once the naturally occurring ovarian steroid hormone, progesterone or synthetic ligands (progestins) bind to it (Lange and Yee, 2008).

PR-positive tumors are almost never ER-negative (0.2% to 10%), and tumors with this immunophenotype may indicate a technical laboratory error (Allred, 2008; Olivotto *et al*., 2002). PR-positive tumors comprise 60% to 65% of breast carcinomas as reported in literature for Asian breast cancer incidences (Shah *et al*., 2014). These findings are in line with several African studies. Basro and Apffelstaedt, (2010) reported 60% PR-positive carcinomas in South Africa and Sayed *et al*. (2014) reported 64.8% positivity in Kenya. Approximately 40% of ER-positive carcinomas are PR-negative (Dai et a., 2016). Fohlin *et al*. (2020) identified novel prognostic factors for patients with ER-positive breast cancers and investigated if these factors have prognostic value in subgroups categorized by PR status. The results of their study therefore contributed to the understanding of biological heterogeneity within ER+/PR− tumors. Adjuvant tamoxifen or aromatase inhibitors (AIs) are the widely used anti-hormonal therapy with a strongly associated survival benefit for ER-positive tumors (Tremont and Cole, 2017).

2.5.3 Human Epidermal Growth Factor Receptor 2 (HER2)

Human epidermal growth factor receptor 2 (HER2) is an oncogene located on chromosome 17q12 (Iqbal and Iqbal, 2014). The HER2 protein is a member of the epidermal growth factor receptor family with tyrosine KInase activity. Dimerization of the receptor with other members of the family results in the autophosphorylation of tyrosine residues which stimulates a variety of

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signaling pathways leading to cell proliferation and tumorigenesis (Harbeck and Gnant, 2017). The clinical implications of HER2 amplification have been recognized since 1987 (Slamon *et al*., 1987). HER2 gene amplification or protein over-expression is associated with a poor prognosis, but predicts a good clinical outcome with systemic chemotherapy treatment (Dauda *et al*., 2011; Ikpat *et al*., 2002).

The protein over-expression and gene amplification of HER2 occur in 15% to 30% of all primary breast carcinomas. Eng *et al*. (2014), reported that the proportion of HER2-positive tumors varied markedly between studies, ranging between 40% and 80% in North Africa and between 20% and 70% in sub-Saharan Africa. This was attributed to the variation in number of women with breast cancer (over 12,000) in North Africa (Egypt and Tunisia) which was more than those with breast cancer in mainly sub-Saharan Africa (Nigeria and South Africa) (4,737) (Eng *et al*., 2014). With the above inference, it therefore appears that there are lower frequencies of HER2-positive carcinomas in West African countries (Sayed *et al*., 2014; Basro and Apffelstaedt, 2010; Bird *et al*., 2008).

Assigning HER2 status in breast cancer patients is imperative, and has been established as routine clinical practice before treating advanced tumors with trastuzumab or using adjuvant treatment for HER2-positive early stage patients (Mirza *et al*., 2002). In addition, HER2 is an important target of a variety of novel cancer therapies including vaccines and a drug, lapatinib, which is directed at the internal tyrosine KInase portion of the HER2 protein (Jiang *et al*., 2018). The prognostic value of HER2-positivity is higher in node-positive than node-negative patients. For example, in the retrospective study of Dovnik *et al*. (2016) in Slovenia, the results showed that anti-HER2 treatment changed the natural course of breast cancer in the targeted node positive patients as well as in the adjuvant setting in node-negative patients. HER2-positive patients who did not receive adjuvant trastuzumab had significantly worse disease-free survival (DFS) than HER2-negative patients (Dovnik *et al*., 2016).

2.5.4 Proliferation Index (KI-67)

Cell cycle analysis in cell nuclei has revealed the presence of KI-67 protein during the G1, S and G2 phases of the cell cycle and not in the quiescent G0 phase, indicative of its role as cell

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proliferation marker in many cancers (Urruticoechea *et al*., 2005). KI-67 protein has been widely used as a proliferation marker for human tumor cells for several decades (Sun and Kaufman, 2018). During mitosis, KI-67 is essential for formation of the perichromosomal layer (PCL), a ribonucleoprotein sheath coating the condensed chromosomes (Urruticoechea *et al*., 2005; Scholzen and Gerdes, 2000). KI-67 is reportedly active against aggregation of mitotic chromosomes (Sun and Kaufman, 2018). A high KI-67 index generally portends a poor prognosis (Ács *et al*., 2017). KI-67 expression has been found to be a prognostic and predictive marker and its assessment is used to determine the proliferation index of tumor cells (Li *et al*., 2014, Scholzen and Gerdes, 2000). When the KI-67 level is above 14% breast cancer patients are defined as being high-risk for aggressive and quick spread (Soliman and Yussif, 2016). KI-67 expression is an additional independent prognostic parameter for disease free survival (DFS) and overall survival (OS) in breast cancer patients in clinical trials of breast cancer treatments (Inwald *et al*., 2013).

2.6 Breast Cancer Subtype Classification

A combination of various IHC markers including ER, PR and HER2 with or without additional markers such as basal and proliferation markers, have been used to define breast carcinoma subtypes. Several gene expression profiling studies have classified breast cancer into molecular subtypes (Jiang *et al*., 2018; Kondov *et al*., 2018; Vasconcelos *et al*., 2016). According to the St. Gallen Classification System, the four breast cancer subtypes approximated by IHC/FISH are: luminal A = (ER+ and/or PR+, HER2-, KI-67 < 14%); luminal B = with HER2-negativity (ER+ and/or PR+, HER2-, KI-67 ≥ 14%), Luminal B with HER2-positivity (ER+ and/or PR+, HER2+, any KI-67), HER2-enriched (ER-, PR-, HER2+), and basal-like (ER-, PR-, HER2-) (Kondov *et al*., 2018; Vasconcelos *et al*., 2016). This classification system uses IHC expression as a surrogate for molecular subtyping. Several studies have shown trends in the risk of recurrence, prognosis and response to therapy between the different molecular subtypes (Guler, 2017; Ribelles *et al*., 2013; Blows *et al*., 2010). The luminal A subtype accounts for approximately 40% of all breast carcinomas (Guler, 2017). They are low-grade, slow growing and tend to have the best prognosis (Feng *et al*., 2018). Treatment typically involves hormonal therapy (Feng *et al*., 2018). Luminal A carcinomas have been reported to have a better prognosis and are more sensitive to hormonal

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therapy when compared to luminal B carcinomas, which require addition of chemotherapy (Guler, 2017; Blows *et al*., 2010).

A study done by Ribelles *et al*. (2013) showed a low risk of recurrence in the first three years of therapy of luminal A carcinomas, while luminal B carcinomas, demonstrated a high frequency of relapse in the first five years of therapy. Luminal B carcinomas grow slightly faster than luminal A carcinomas, and their prognosis is worse (Feng *et al*., 2018). According Somalin and Yussif, (2016) the luminal B subtype accounts for approximately 10% of all breast cancers. This subtype has also shown a higher index of proliferation compared to luminal A carcinomas (Bustreo *et al*., 2016).

HER2-enriched carcinomas have a poor prognosis (Al-Mahmood *et al*., 2018; Guler, 2017), although, it is highly responsive to anti-HER2 therapies (Huszno and Nowara *et al*., 2016). This subtype accounts for 10% to 15% of breast carcinomas (Feng *et al*., 2018). HER2-enriched carcinomas grow faster than both types of luminal carcinomas and have a generally worse prognosis (Fragomeni *et al*., 2018). However, they can be successfully treated with targeted therapies aimed at the HER2 protein such as trastuzumab. While about 50% of clinical HER2-positive breast carcinomas are HER2-enriched and hormone receptor negative, the remaining 50% may include luminal carcinomas with HER2 overexpression (Feng *et al*., 2018).

Triple negative breast cancer (TNBC) is a highly heterogeneous group (which includes the basal-like breast cancer subtype) and is the most aggressive, with limited treatment options (Hubalek *et al*., 2017; Prodehl and Benn, 2017; Lehmann and Pietenpol, 2014). In addition, it is associated with a poor prognosis, high risk of recurrence and a high proliferation index (Guler, 2017; Shim *et al*., 2014; Ribelles *et al*., 2013). TNBCs account for 12–20% of all breast carcinomas (Hubalek *et al*., 2017). By definition, TNBCs lack expression of hormone receptors and do not demonstrate HER2 overexpression (Lehmann and Pietenpol, 2014). A higher prevalence of TNBCs is found in Africa (Lebert *et al*., 2018; Anders and Carey, 2009; Anyanwu, 2008). TNBC is associated with advanced stage at presentation, aggressive tumor biology and poor outcomes in a study by Prodehl and Benn (2017). Fourteen percent of the patients in their study had TNBC (Prodehl and Benn, 2017).

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2.7 Treatment

Breast cancer treatment should follow a multidisciplinary approach (Eustachi *et al*., 2009). In South Africa, widely used, conventional methods for breast cancer treatment involve surgical excision, chemotherapy, radiotherapy and/or hormonal therapy (Govender, 2014). Hormonal therapy is an attractive modality which halts or slows tumor growth, reduces the risk of recurrence and decreases mortality in breast cancer patients (Govender, 2014; Rampurwala *et al*., 2014). Tamoxifen and aromatase inhibitors (AIs) are the most commonly used drugs for ER-positive and early stage breast carcinoma (Martei *et al*., 2017). The type of hormonal therapy depends on the patient's ovarian function. Tamoxifen may be administered as primary treatment in premenopausal women. Tamoxifen, a selective estrogen receptor modulator (SERM), when administered for 10 years, shows a greater reduction in recurrence of ER-positive breast carcinoma than when it is given for five years (Davis *et al*., 2011). Treatment for postmenopausal patients with ER-positive carcinomas consists of aromatase inhibitors (AIs), except where contraindications or intractable side effects are found (Younus and Kligman, 2010). Burstein *et al*. (2010) in their work suggested the use of tamoxifen alongside AIs in ER-positive postmenopausal patients. AIs decrease the levels of estrogen by blocKIng the enzyme aromatase (Scharl and Salterber, 2016; Hadji *et al*., 2011). Furthermore, a study by Huiart *et al*. (2011) reported that among older women, the use of AIs showed high rates of compliance.

However, these two hormonal therapies (tamoxifen and AIs) have different side-effect profiles (Fleming *et al*., 2018; Colleoni and Giobbie-Hurder.2010). A meta-analysis of tamoxifen therapy trials has shown an increase in the risk of developing endometrial cancers (Fleming *et al*., 2018). On the other hand, AIs have been reported to increase bone loss (Perez and Weilbaecher, 2006) in contrast to tamoxifen which protects against bone loss (Ding and Field, 2007). Genetic variation has been considered as one of the most important non-modifiable risk factors of bone loss with use of AIs in South African breast cancer patients (Baatjes *et al*., 2018; 2019).

2.8 Diagnostic Techniques in Breast Cancer

The most widely used Food and Drug Administration (FDA) approved diagnostic techniques for analysis of breast carcinoma biomarkers in the South African public sector are IHC and FISH

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(Nasrazadani *et al*., 2018; Moelans *et al*., 2011). IHC assesses ER, PR, HER2 and KI-67 protein expression using different antibodies, while FISH identifies *ERBB2* on chromosome 17q21 and polysomes using a DNA dual probe (Moelans *et al*., 2011). A novel platform for the assessment of ER, PR, HER2 and KI-67 has recently been developed which involves quantification of mRNA transcripts using real-time RT-qPCR techniques (Xpert® Breast Cancer STRAT4 assay). RT-qPCR is a laboratory technique based on amplifying and simultaneously quantifying a targeted DNA molecule. Xpert® Breast Cancer STRAT4 assay (STRAT4) is an assay for detection and quantification of *ESR1*, *PGR*, *ERBB2*, *MKI67* mRNA transcripts isolated from formalin-fixed embedded (FFPE) invasive breast carcinoma tissue. The Xpert® Breast Cancer STRAT4 assay is a one-step assay in a self-contained, single-use, disposable cartridge which combine the RT-qPCR reagents and host the RT-qPCR process. There are two phases involved in RT-qPCR. Firstly, in the reverse transcription phase, mRNA is used as a template to synthesize complimentary DNA (Mo, *et al*., 2012). The second phase involves amplification of the cDNA and analysis of the products generated during the reaction (Mo *et al*., 2018). During the extension step, the enzyme Taq polymerase synthesizes two new strands of DNA, using the cDNA as template (Garibyan and Avashia, 2013). The STRAT4 assay is 80% automated and has a run-time of less than two hours.

Studies have demonstrated correlation between the mRNA and protein expression (Wu *et al*., 2018; Wasserman *et al*., 2017). There is a high correlation between results obtained with IHC/FISH and RT-qPCR. A study done in USA reported a concordance between RT-qPCR and IHC/FISH to be 91.25% with a sensitivity of 0.87, specificity of 0.94, a positive predictive value (PPV) of 0.89 and a negative predictive value (NPV) = 0.92 (Wasserman *et al*., 2017). Similarly, Wu *et al*. (2018) reported an overall concordance between STRAT4 and IHC/FISH of ER=97.8%, PR=90 – 91%, HER2=95% and 93.3% (IHC and FISH, respectively) and KI-67=73%.

2.8.1 Immunohistochemistry

Semi-quantitative IHC is a technique based on the principle that antibodies bind to antigens. Proteins of interest are identified via labelled conjugates. When a primary antibody has a label attached, it is directed to an antigen epitope of the protein of interest and allowed to bind (Quintero-Ronderos *et al*., 2013). This is referred to as direct IHC. Indirect IHC is when the primary antibody

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is not labelled, but a labelled secondary antibody is added which triggers the signal (Greenwood *et al*., 2015; KIm *et al*., 2016) (**Figure 2-2)**.

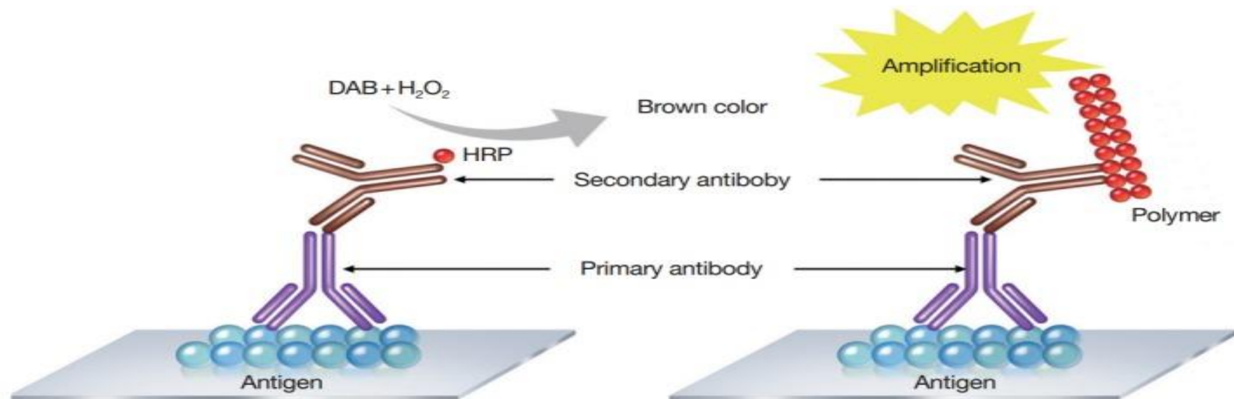

Figure 2-2: Schematic representation of the indirect IHC method using secondary antibodies tagged with various labels of immunostaining in the process of detecting specific antigen-antibody interactions (Kim *et al*., 2016).

The immunohistochemical evaluation of ER, PR and HER2 is reported as recommended by ASCO/CAP guidelines (Wolff *et al*., 2013). For interpretation of ER and PR, the Allred scoring system is employed, which combines the percentage of positive cells and intensity as shown in **Table 2-2** (Hammond *et al*., 2010), for a final score with 8 possible values Scores 0 and 2 are considered negative and 3 – 8 are considered positive. HER2 results are scored from 0 to 3+, visually assessing the amount of HER2 protein. This system evaluates the intensity of staining on the tumour cell membrane (**Table 2-3**).

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Table 2-2: Reporting of ER and PR testing by IHC assessment (Fitzgibbons *et al.*, 2018).

Proportion Score	Positive Cells, %	Intensity	Intensity Score
0	0	None	0
1	<1	Weak	1
2	1 to 10	Intermediate	2
3	11 to 33	Strong	3
4	34 to 66		
5	≥67		

Table 2-3: Reporting results of HER2 testing by Immunohistochemistry (IHC) (Fitzgibbons *et al.,* 2018).

Result	Criteria
Negative (Score 0)	No staining observed
	or
	Membrane stating that is incomplete and is faint/barely perceptible and within ≤10% of tumor cells
Negative (Score 1+)	Incomplete membrane staining that is faint/barely perceptible and within >10% of tumor cells
Equivocal (Score 2+)	Weak to moderate complete membrane staining in >10% of tumor cells
	or
	Complete membrane staining that is intense but within ≤10% of tumor cells
Positive (Score 3+)	Complete membrane staining that is intense and >10% of tumor cells

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KI-67 scoring is performed using the hot spot method as described by Penault-Llorca and Radosevic-Robin, 2017. The hot spot method is defined as the percentage of invasive tumor cells positively stained in the field with the highest number of positive nuclei (Leung *et al.,* 2016; Penault-Llorca and Radosevic-Robin, 2017). Only nuclear staining is considered positive, and was defined as any brown stain in the nucleus. Staining intensity is irrelevant during KI-67 scoring according to Leung *et al.,* 2016. According to Penault-Llorca and Radosevic-Robin (2017) aat least three high power fields (HPFs) including a hot spot are selected to represent the spectrum of staining as observed on the initial overview of the entire section (**Figure 2-3 B**). The St Gallen consensus proposed three categories: low (15%), intermediate (16–30%) and high (>30) (Nguyen *et al.,* 2019)

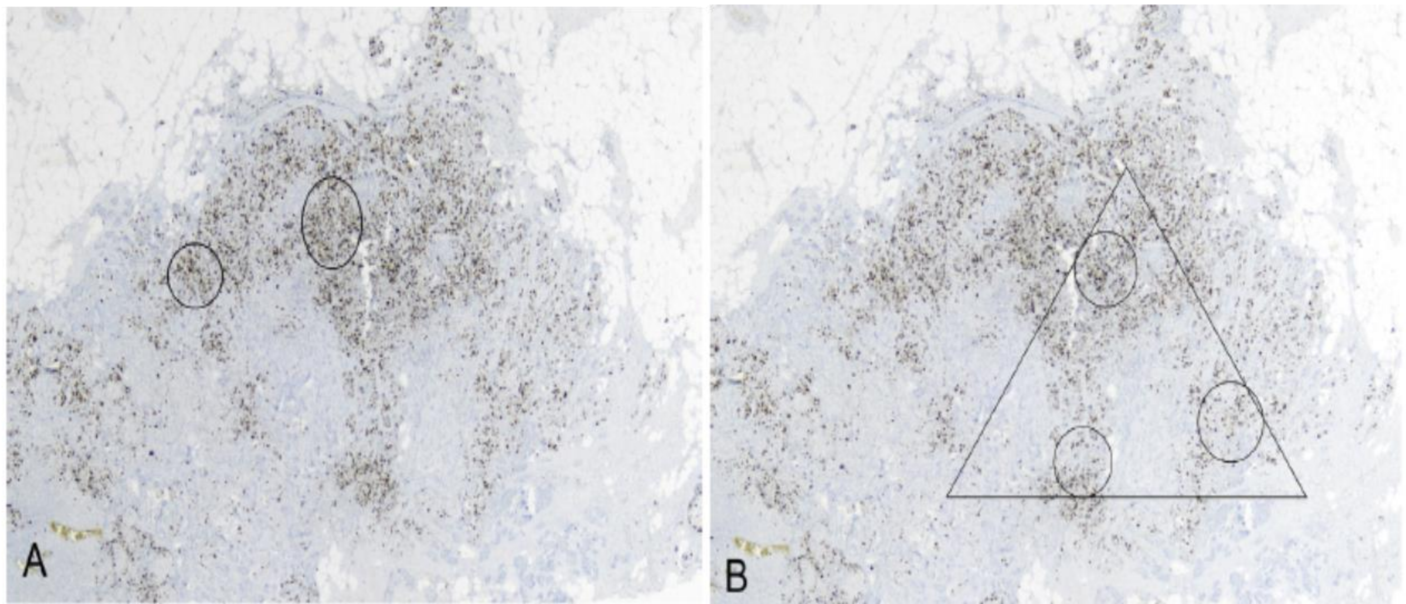

Figure 2-3: KI-67 scoring. (A) Hot spot fields with the highest number of positive nuclei. (B) Three high power fields (HPFs) including a hot spot. At least three HPFs should be selected to represent the scale of staining seen across the whole field of the invasive carcinoma (Penault-Llorca and Radosevic-Robin, 2017).

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2.8.2 Fluorescence In Situ Hybridization

Fluorescence in situ Hybridization (FISH) is a procedure that uses a probe to identify a target gene or DNA sequence. The probe is an oligonucleotide incorporated with fluorophore-coupled nucleotides that is complementary to a target gene or DNA sequence (Cui *et al*., 2016). The labelling of the probe can be direct (i.e. it produces a signal immediately after binding to the targeted DNA sequence) or indirect (i.e. a trigger is required to produce a signal). The target sequence is denatured using high temperatures which break the hydrogen bonds between the nucleotides (Jensen, 2014). Combining the labelled probe and the denatured targeted DNA sequence or gene allows the annealing of the complimentary strands, therefore producing a signal that is brighter compared with background levels (Ratan *et al*., 2017).

There are two types of HER2 assays that can be used: a single probe assay and a dual probe assay which have different interpreting guidelines (**Figure 2-4** and **2-5)**. A single probe only identifies the HER2 gene, whereas the dual probe identifies the HER2 gene and the centromere of chromosome 17 (Furrer *et al*., 2015). The main advantage with the dual probe is that it singles out the polysomes and these aids in the identification of heterogeneity of the tumour (Hu *et al*., 2017).

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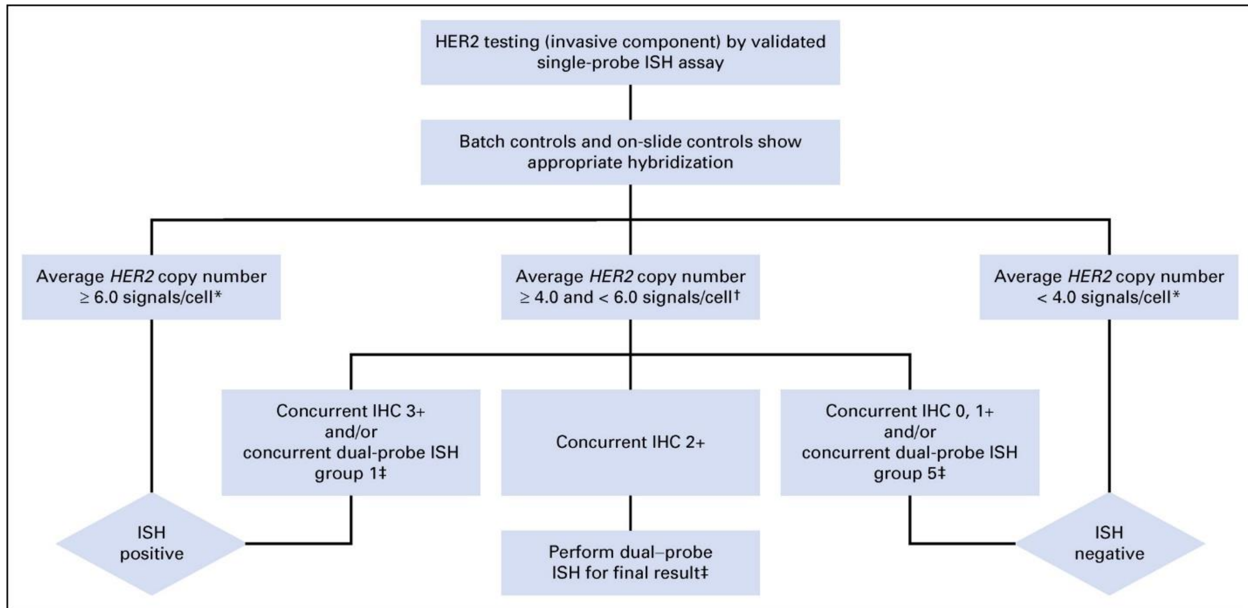

Figure 2-4: Schematic diagram for evaluation of human epidermal growth factor receptor 2 (HER2) by in situ hybridization (ISH) assay using a single-signal (HER2 gene) assay (single-probe ISH) (Wolff *et al*., 2018).

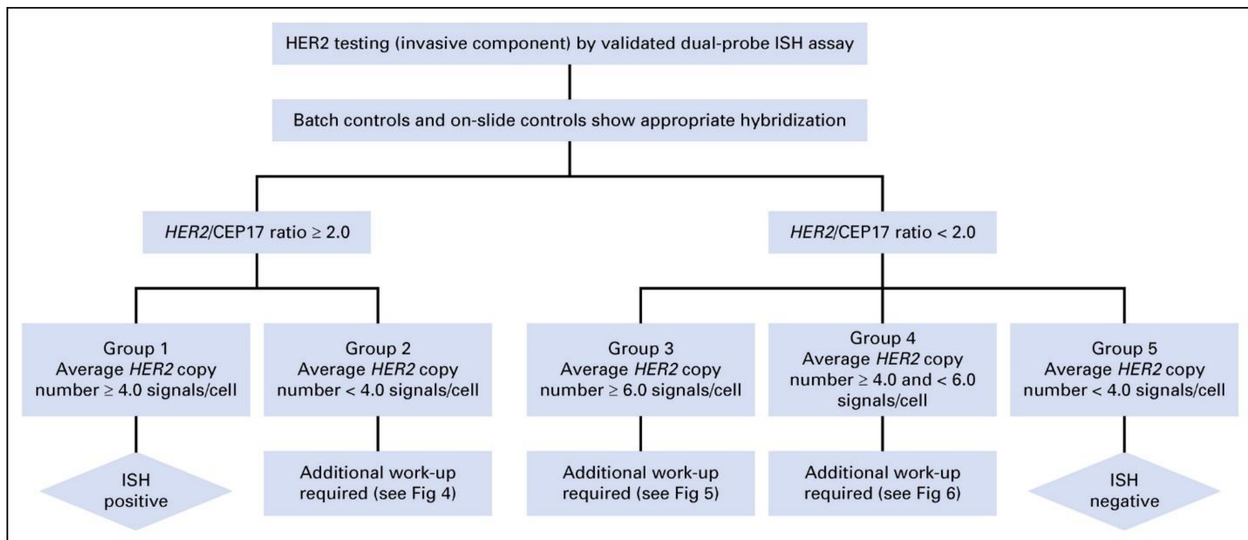

Figure 2-5: Schematic diagram for evaluation of human epidermal growth factor receptor 2 (HER2) gene amplification by in situ hybridization (ISH) assay using a dual-signal (HER2 gene) assay (dual-probe ISH) (Wolff *et al.,* 2018).

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Chapter 3

Methodology

3.1 Ethical consideration

Ethical approval was obtained from the Human Research Ethics Committee (HREC) at the Faculty of Health Sciences, University of Stellenbosch (Ethics Reference Number: *S19/05/095*) before the study commenced.

The study followed the Declaration of Helsinki code of conduct developed in 1964 by the World Medical Association. This code serves to protect the rights of participants and ensure they are not exposed to unnecessary harm, and ensure that methodological approaches are appropriate to the study aims. The Helsinki code of conduct provides principles for which medical research involving humans is to be managed.

3.2 Study design

This study included 101 retrospective cases of adult (>18 years) breast carcinoma that had been diagnosed at the Division of Anatomical Pathology, TBH. TBH is affiliated with the Faculty of Medicine and Health Sciences of Stellenbosch University.

A SNOMED search of the laboratory information system, TRAKCare was conducted from 01/01/2018 to 01/06/2018 (**Table 3-1**). Excel spreadsheets were generated, and sequential breast carcinoma cases which met the inclusion criteria were selected (**Table 3-2**).

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Table 3-1: SNOMED codes and corresponding descriptions.

SNOMED CODE	DESCRIPTION
• T-04000 • T-04030 • T-04020 • T-04007 • M-85003 • M-85213 • M-85202 • D0-F0357 • D0-F0367	• Breast structure • Left breast • Right breast • Breast NEC • Infiltrating duct carcinoma • Infiltrating ductular carcinoma • Lobular carcinoma • Carcinoma of breast • Carcinoma of breast, NOS

Table 3-2: Inclusion and exclusion criteria for study cases.

Inclusion	Exclusion
1. Core needle biopsy or excision specimens of breast carcinoma 2. Cases have available FFPE tissue blocks and more than 1 x $5mm^2$ area of residual carcinoma 3. The following data is available for each case: • Sex and age • Tumor histology (invasive breast carcinoma, NST, invasive lobular carcinoma, etc.) • Tumor stage at diagnosis (TNM) • Tumor grade	• Tumor section for study contains less than ~1 x 5 mm^2 invasive breast carcinoma area • Post-neoadjuvant therapy residual tumor (surgical) specimens • The specimen was fixed in fixative other than 10% neutral buffered formalin (NBF) or was known to be fixed in NBF for < 6 or >72 hours.

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3.3 Study population:

There were 473 cases found using the SNOMED search and 101 were reviewed and matched the inclusion and exclusion criteria.

3.4 Analysis

Each of the 101 cases had haematoxylin and eosin (H&E) and immunohistochemistry-stained slides (ER, PR, HER2 and KI-67) retrieved from the archives of the Division of Anatomical Pathology, and reviewed with the help of the study supervisor. The morphology of these tumors was reviewed, and a histological subtype of each carcinoma was determined. Histological grading was done and immunohistochemical stains were re-scored according to ASCO/CAP guidelines. Cases with a HER2 immunohistochemistry score of 2+ (equivocal), were previously sent for HER2 fluorescence *in situ* hybridization (FISH), and these results were documented. The FFPE tissue blocks were retrieved from archives and sections were cut at 3µm and placed on glass slides. H&E staining was performed on one section, and two other sections were left unstained on slides.

3.4.1 Immunohistochemistry (IHC)

Immunohistochemical staining was performed previously at the time of reporting the core needle biopsy or surgical excision specimens. The Leica BOND III machine was used for immunohistochemical staining.

3.4.1.1 Slide Preparation: Three-micron FFPE sections were fixed to Super Frost slides (Thermo Scientific, USA), and baked for 30 min at 70 °C.

3.4.1.2 Slide Staining: The slides were stained using the Leica BOND III staining program (**Table 3-3**). The primary antibodies used were diluted as follows: ER: Novocastra Estrogen Receptor diluted 1:250, PR: Novocastra Progesterone Receptor diluted 1:500; HER2 Novocastra HER2 oncoprotein diluted 1:250, KI-67: Dako KI-67 diluted 1:100. Unbound primary antibodies were removed with wash buffer which is prepared by adding 100 ml of BOND Wash concentration to 900ml of deionised water.

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3.4.1.3 Antigen retrieval: For ER, PR & KI-67 EDTA buffer base was used at pH 8.9-9.1 and for HER2 citrate base was used at pH 5.9-6.1.

Table 3-3: Leica BOND III staining program.

	Cycles (Ce)	**Time (min)**	**Temperature (°C)**
Dewax	3		72
100% Alcohol	3		
Bond wash	3		
Retrieval ER, PR & KI-67	2	20	100
Retrieval HER2	2	20	100
Bond wash	4		
Bond wash	1	3	
Antibodies	1	15	
Bond wash	1	2	
Bond wash	2	1	
Post primary	1	8	
Wash	3		
Polymer	1	8	

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Wash	2	2	
Deionized water	1		
Perioxide block	1	5	
Wash	1	1	
Wash	2		
Distilled water	1		
Mixed DAB refined	1		
Mixed DAB refined	1	10	
Deionized water	3		
Haematoxylin	1	5	
Deionized water	1		
Wash	1		
Deionized water	1		

Interpretation: For ER and PR, the Allred scoring system was employed, which combines the percentage of positive cells and intensity as shown in **Table 2-2** of Chapter 2 (Hammond *et al*., 2010)**.**

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3.4.2 Fluorescence in situ hybridization HER2 Analysis

All cases which had an immunohistochemical score of 2+ for HER2 were sent to the cytogenetics laboratory at Charlotte Maxeke Academic Hospital for FISH analysis. FISH was performed with a Path-Vysion HER2 DNA KIt (Abbott Pharmaceutical Co., Ltd., Lake Bluff, IL, USA).

3.4.2.1 Deparaffinising Slide:

Slides were immersed twice in new Hemo-De for 10 minutes at room temperature. Slides were dehydrated in 100% ethanol for five minutes at room temperature. Repeated and air-dried slides or placed slides on a 45 to 50°C slide warmer.

3.4.2.2 Pre-treating Slides:

Table 3-4: Slide pre-treatment procedure (Abbott Path-Vysion HER2 DNA Kit 30-608377/R7).

Immersed in	Reagents	Cycles	Time (min)	Temperature (°C)
	0.2N HCl	1	20	RT
	Purified water	1	3	RT
	Wash Buffer	1	3	RT
	Pre-treatment solution	1	30	80
	Purified water	1	1	RT
	Wash Buffer	2	5	RT

*RT: Room Temperature

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3.4.2.3 Protease treatment

Firstly, excess buffer was removed by blotting edges of the slides on a paper towel. Protease treatment was done by immersing slides in protease solution at 37± 1°C for 10 to 60 minutes, then in wash buffer for 5 minutes. The slides were dried on a 45 to 50°C slide warmer for 2 to 5 minutes.

3.4.2.4 Fixing the specimen

The slides were immersed in neutral buffered formalin at room temperature for 10 minutes. Thereafter, the slides were placed in wash buffer for five minutes. This procedure was repeated. Thereafter slides were dried on a 45 to 50°C slide warmer for two to five minutes.

Probe Preparation: The probe was allowed to warm at room temperature so that the viscosity could decrease sufficiently to allow accurate pipetting. Afterwards, it was vortexed to create a homogenous mix. Each tube was centrifuged for two to three seconds in a bench-top micro-centrifuge to bring the contents to the bottom of the tube. Lastly the probes were gently vortexed.

*Note***:** Denaturation of specimen DNA. The timing for preparing the probe solutions was carefully coordinated with denaturing the specimen DNA so that both would be ready for the hybridization step at the same time.

3.4.2.5 Hybridization

Following drying, 10μL of probe mixture was applied to the target area of the slide. A coverslip was immediately placed over the probe to allow for even spread under the coverslip. The coverslip was sealed with rubber cement using a 5ml syringe. Slides were placed in the pre-warmed humidified hybridization chamber. The chamber was covered with a tight lid and incubated at 37 ±1°C overnight (14 to 18 hours).

3.4.2.6 Post-hybridization washes and counterstaining

The rubber cement seal was removed from the first slide by gently pulling up on the sealant with forceps. The coverslip was removed by immersing the slide in a Coplin jar with post-hybridization (2X SSC/0.3% NP-40) wash buffer at room temperature allowing the clover slip to float off. After

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the coverslip had been carefully removed, excess liquid was removed by wicking off the edge of the slide and immersing the slide in post-hybridization wash buffer at 72°C for two minutes (six slides/jar).

The slide was counterstained by applying 10µL of DAPI to the target area of the slide and applying a glass coverslip. The slides were stored in the dark prior to signal enumeration.

Interpretation of FISH status was done using the ASCO/CAP guidelines **(Table 3-5).**

Table 3-5: Reporting of HER2 testing by FISH assessment using dual probe (Wolff *et al.*, 2018).

Group	Ratio	HER2 copy number	Status
1	> 2.0	> 4.0	Positive
2	> 2.0	< 4.0	Positive
3	< 2.0	> 6.0	Positive
4	< 2.0	> 4.0 < 6.0	Equivocal
5	< 2.0	< 4.0	Negative

3.4.3 Xpert® Breast Cancer STRAT4 Assay

3.4.3.1 Materials:

The Xpert® STRAT 4 assay kit consists of:

- Cartridges (10 units)

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- Lysis
- Protein kinase
- 15 ml tubes (10 units)
- 1.5 ml eppendorf (10 units)

All reagents required for sample preparation and RT-qPCR analysis are preloaded in the cartridge. However, other additional equipment was required **(Table 3-6**). Nucleic acids in the lysate are captured on a filter, washed, and eluted by sonication. The purified nucleic acid is mixed with dry RT-qPCR reagents, and the solution is transferred to the reaction tube for RT-qPCR and detection.

Table 3-6: List of extra equipment required for the STRAT4 assay.

Equipment	**Units per batch (4 samples)**
Gloves	1 pair
Razor blade	4
Ethanol (>95%)	2 ml
Pre-heated block (80°C)	-
Pipettes (1000 ul and 20ul)	-
Pipette tips	20

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Quantification and Detection of mRNA transcripts

Quantification: Changes in sample gene expression are measured based on a reference gene, also known as control 2 (sample adequacy control): Reference gene (*CYFIP1*) PCR cycles are characterized by the point in time (or PCR cycle) where the amplification curve crosses a signal threshold (an arbitrary level of fluorescence chosen on the basis of the baseline variability) during the reaction. This point is usually referred to as cycle threshold (Ct), the time at which fluorescence intensity is greater than baseline. Baseline is the background fluorescence which accumulates, but is beneath the limit of detection of the instrument. Cycle threshold (Ct) values are available to calculate the mean normalized gene expression from relative reference gene that determine "positive" (POS) vs "negative" (NEG) results from a given target (**Equation 1**), based on pre-defined dCt cut-offs (**Table 3-7**). A lower cycle threshold (Ct) value means a large amount of template (in sample) at the beginning of the reaction, therefore less cycles will be required to amplify the target genes. Conversely, a smaller amount of template generates larger Ct values. The Gene Xpert® software automatically reviews the signal from both the *CYFIP1* endogenous control and the transcripts for acceptability, and calculates the difference in cycle threshold (Ct) between the 2 signals, yielding a delta Ct (dCt) result.

Equation 1: $\boldsymbol{\Delta Ct = Ct\ of\ reference\ gene\ (CYFIP1) - Ct\ of\ the\ target\ gene}$

Table 3-7: STRAT4 assay targets dCt cut-off values.

Gene	Negatives	Positive
ESR1	< -1	≥ -1
PGR	< -3.5	≥ -3.5
ERBB2	< -1	≥ -1

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MKI67	< -4	≥ -4

Delta Ct values for each assay target was displayed by using a software program (ONCore Software) provided by Cepheid. The software yielded a report with a semi-quantitative result (sliding scale) for each analyte (**Figure 3-1**).

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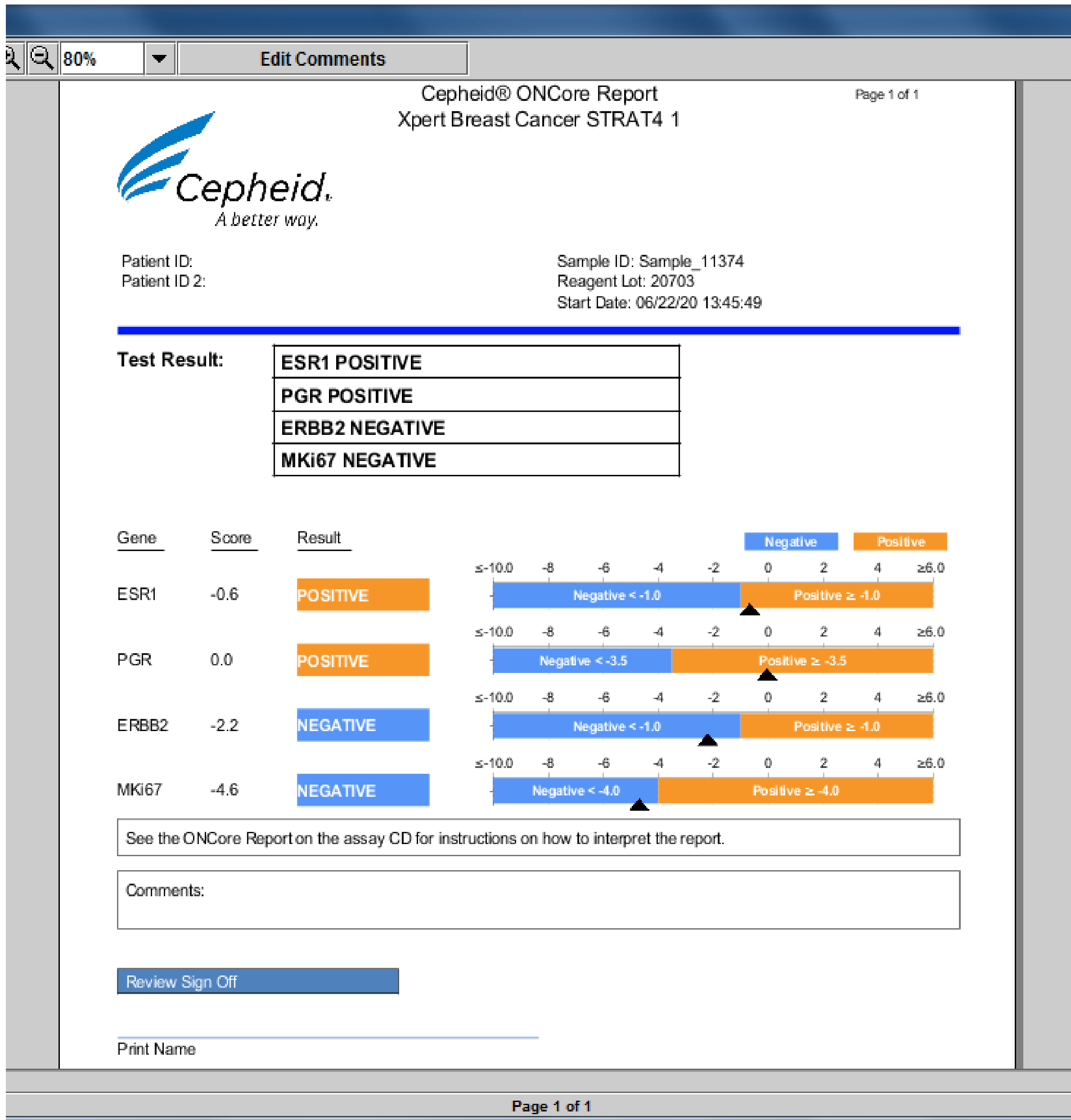

Figure 3-1: An illustration of results displayed on ONCore software.

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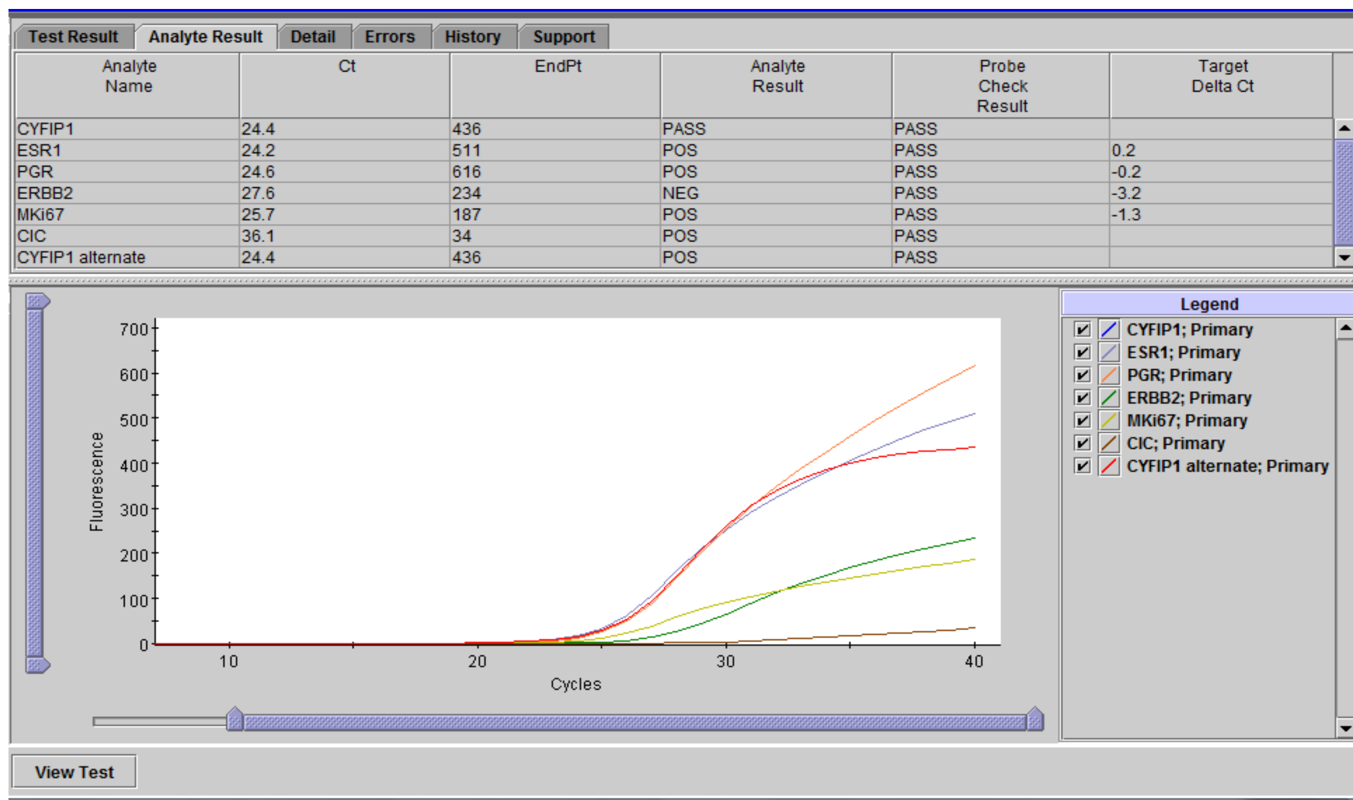

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result	Target Delta Ct
CYFIP1	24.4	436	PASS	PASS	
ESR1	24.2	511	POS	PASS	0.2
PGR	24.6	616	POS	PASS	-0.2
ERBB2	27.6	234	NEG	PASS	-3.2
MKi67	25.7	187	POS	PASS	-1.3
CIC	36.1	34	POS	PASS	
CYFIP1 alternate	24.4	436	POS	PASS	

Figure 3-2: The PCR curve for one patient showing the Ct values of each target mRNA transcript.

Detection: This was achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity.

3.4.3.1 Gene Xpert® Work flow

Regarding and microdissection: Haematoxylin and eosin (H&E) stained slides were examined under supervision of the study supervisor and carcinomas were regraded.

Slide Preparation (2X unstained slides): A 4-5µm thick section was cut using a microtome. Afterward sections were floated in a water bath at 40 °C. Sections were mounted on positively charged glass microscopic slides. The slides were air dried overnight.

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Tissue removal from the slides: The invasive tumor tissue was scraped from the slide and transferred of to a labelled 1.5ML lysis tube using a fresh razor blade for every scraping.

Tissue Processing: Five hundred microliters of FFPE lysis reagent was added to a 1.5mL lysis tube containing the FFPE section. Afterwards 20µL of Proteinase K (Cepheid) was added to the same 1.5mL lysis tube to de-crosslink and release nucleic acids from the tumor cells. The lid was closed and the mixture vortexed continuously at a maximum setting of five seconds. Lastly the sample was micro-centrifuged to remove any liquid from the lid. Using a pre-heated block (80°C) the 1.5ml lysis tube containing a sample lysis reagent was incubated for 30 minutes. The sample was vortexed at a maximum setting of five seconds and lastly micro-centrifuged to remove liquid from the lid. Five hundred microliters of ≥ 95% ethanol was added to the same 1.5 mL lysis tube. The lid was closed and the sample was vortexed at a maximum setting for 15 seconds. Thereafter the sample was briefly centrifuged to remove any liquid from the lid.

Preparing cartridge: The cartridge was removed from the cardboard packaging. The lysate was vortexed again for 15 seconds prior to use. The lid of the cartridge was opened and 520 µL of lysate was transferred to the sample chamber of the cartridge. The cartridge lid was closed by firmly snapping the lid into place (**Figure 3-4**)

The cartridge was inserted into a module of the Gene Xpert® instrument (**Figure 3-4**) where nucleic acid purification, amplification and real-time detection would take place.

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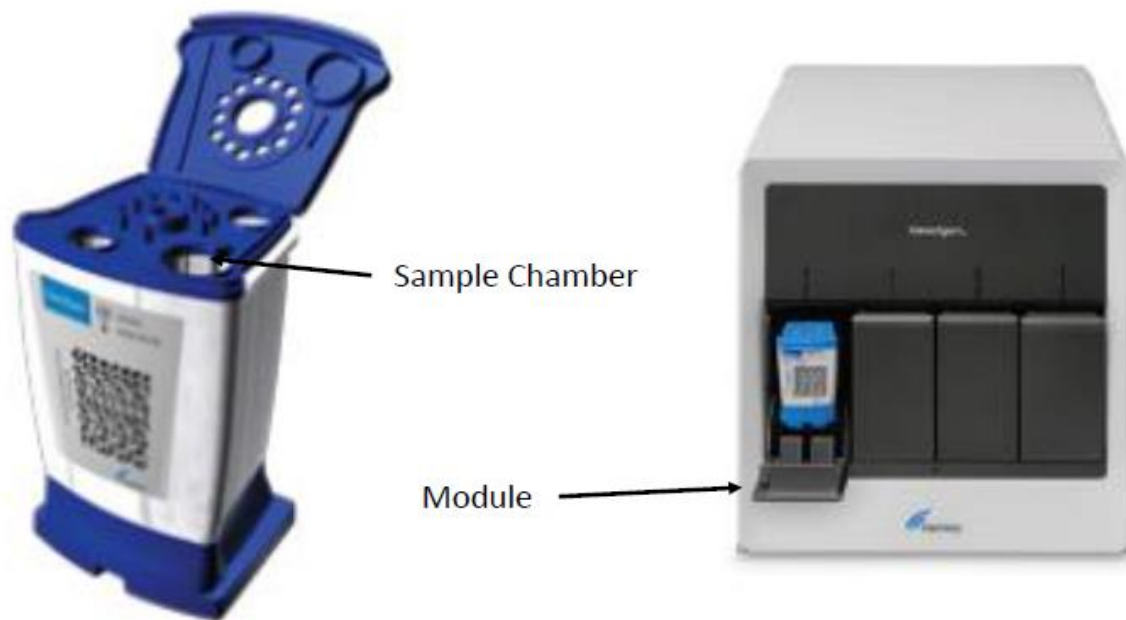

Figure 3-3: The cartridge and the module of the Gene Xpert® instrument.

3.5 Reliability and Validity

Reliability of the study was considered by using inclusion and exclusion criteria in the selection of cases, as well as using a standard operating procedure for specimen preparation and analysis. A consistent naming convention was used for sample testing in the assay, so that re-testing on the same patient ID could be easily differentiated from the original test results. Therefore, in cases where the same patient sample was re-tested (either another aliquot from the original lysate, or as a concentrated lysate from a new tissue section or additional tissue sections), the repeat sample was designated as such by using the following naming convention:

- “Sample No” for all initial tests run using lysate
- “Sample No_R” for a re-test run using another aliquot from the original lysate
- “Sample No_4xR” for the re-tests run using the “4x” concentrated lysate

The assay was repeated by either preparing a fresh lysate using an additional tissue section(s) or following the concentrated lysate procedure per the Xpert® FFPE Lysis kit Package (**Figure 3-4**

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in the following circumstances: (1) an "INVALID" result which indicated that the *CYFIP1* gene had failed due to the sample not being properly processed, PCR was inhibited or RNA quality of the tumor was inadequate (2) an "INDETERMINATE" result which indicated that the *CYFIP1* reference gene had a Ct that was not within the valid range (above Ct of 35.1) or the endpoint was below the threshold setting required for *PGR* or *MKI67* status determination.

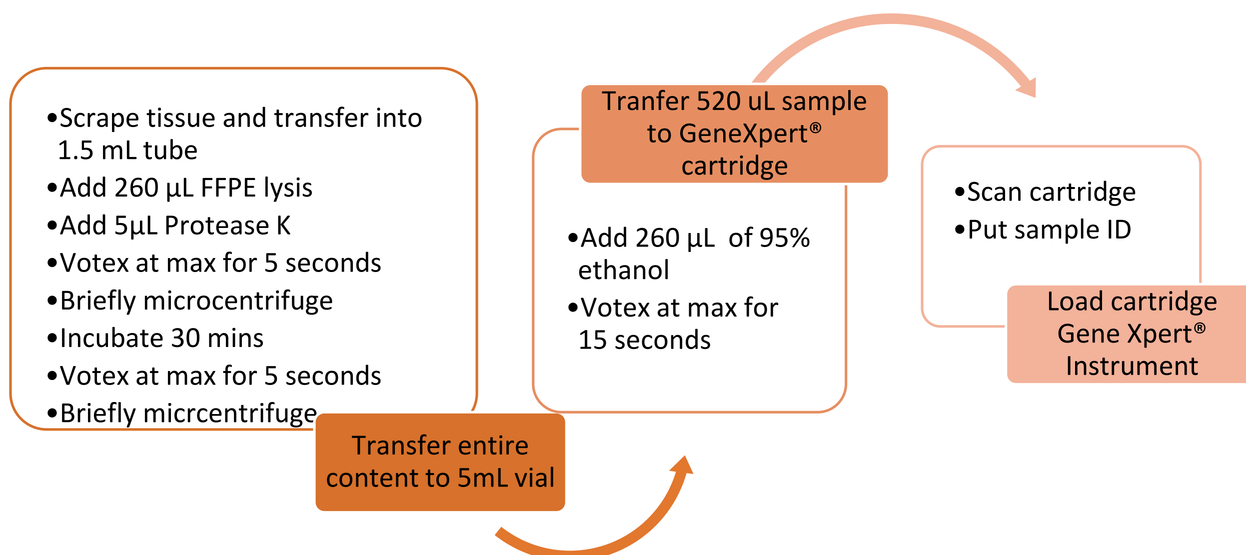

Figure 3-4: The concentrated lysate procedure as per the Xpert® FFPE Lysis KIt Package for INVALID and INDERTEMINATE results.

3.6 External Control Testing

FFPE controls were tested along with specimens once every batch and when a new lot of cartridges or FFPE lysis reagents were used. External controls were treated as specimens and processed according to the STRAT4 protocol.

If any of the control results were invalid (INVALID, ERROR, NO RESULT), or incorrect (e.g., a positive control produced a negative result), a repeat was conducted using a new aliquot of the same external control, or a fresh lysate prepared from the external control and new Xpert® cartridge used.

1. Control 1 (Reagent control): Probe Check Control (PCC)
   - Missing Target Specific Reagent (TSR) and/or Enzyme Reagents beads, which contain all primers, probes and internal control templates

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- Incomplete reagent reconstitution
- Incomplete reaction tube filler
- Probe degradation

2. Control 2 (Sample Adequacy control): Reference Gene (*CYFIP1*)

   - Verifies human cells and mRNA have been added into the sample chamber
   - To normalize the expression levels
   - To ensure that the sample contains sufficient mRNA

3. Control 3 (Cepheid Internal Control): Armored RNA (CIC)

   - Ensures that the sample was processed correctly
   - Verifies that the RT-PCR reaction proceeded with minimal inhibition

3.7 Data collection:

Laboratory data were collected in excel spreadsheets, updated by the researcher and controlled by the principal investigator (PI) from the records of the hospital information system. Cepheid was the sole provider of the platform.

3.8 Data analysis

The sample size calculations for the study primary objective (concordance between STRAT4 and IHC and/or FISH methods) are based on the performance goals for the study, which are the lower 95% 2-sided confidence interval (LCI) for positive percentage agreement (PPA) and negative percentage agreement (NPA) for each of the four assay targets. The performance goals are listed in **Table 3-8**.

Table 3-8: Performance goals for Gene Xpert® Breast Cancer biomarker assay.

IHC	**H_0**	**H_A**
ER – PPA	< 80%	≥ 80%

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ER – NPA	< 80%	≥ 80%
PR – PPA	< 70%	≥ 70%
PR – NPA	< 65%	≥ 65%
HER2 – PPA	< 75%	≥ 75%
HER2 – NPA	< 80%	≥ 80%
KI-67 – PPA	< 65%	≥ 65%
KI-67 – NPA	< 65%	≥ 65%
FISH	$\mathbf{H_0}$	$\mathbf{H_A}$
HER2 – PPA	< 75%	≥ 75%
HER2 – NPA	< 80%	≥ 80%

The hypothesis testing framework above was only used to estimate the sample size and conditional probabilities of passing acceptance criteria given designed performance as truth. The estimated total number of cases needed to be at least 100 cases with true positive (TP), true negative (TN), false negative (FN), and false positive (FP) targets as estimated in **Table 3-9**, based on product performance characteristics as specified in the Xpert® Breast Cancer STRAT4 CE-IVD package insert. With an estimated total sample size of 100, there is some risk for under-representation of some biomarkers. In the event of under-representation, performance assessment would focus on the point estimate of performance. Data from this evaluation would be compared to the pre-clinical data used to establish the assay cut-offs.

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Table 3-9: Sample size testing framework for Xpert® Breast Cancer STRAT4 assay.

	ER		**PR**		**HER2**		**KI-67**	
	(IHC)		(IHC)		(IHC & FISH)		(IHC)	
	PPA	NPA	PPA	NPA	PPA	NPA	PPA	NPA
Total SS	100	100	100	100	100	100	100	100
Probability of Passing	56.80%	43.10%	95.30%	40.00%	41.10%	56.40%	30%	54.20%
Point Estimate Required	90.00%	90.00%	80.00%	75.00%	85.00%	90.00%	75.00%	75.00%
LCI Requirement	80.00%	80.00%	70.00%	65.00%	75.00%	80.00%	65.00%	65.00%
Type I error (Alpha)	0.10%	1.80%	0.70%	2.80%	1.10%	0.03%	3.00%	0.40%
Required Pos / Neg	75	25	65	35	15	85	20	80
Required Min TP/TN	68	23	52	22	14	77	16	60
Required Max FN/FP	8	2	13	8	1	9	4	20
Point Estimate	90.00%	92.00%	80.00%	77.10%	93.30%	90.00%	80.00%	75.00%
Lower 95%CI	84.20%	81.20%	70.30%	66.00%	78.70%	84.60%	65.20%	67.80%
Upper 95%CI	93.90%	96.90%	86.00%	85.40%	98.20%	93.70%	89.50%	81.10%

3.9 Statistical Method

For assessment of agreement with the reference method(s) (IHC or FISH, as applicable), standard 2x2 tables were utilized along with calculation of the PPA, NPA, positive predictive value (PPV), negative predictive value (NPV) (**Table 3-10).** Receiver operator characteristic curves (ROC) was also used to plot the data for each analyte (*ESR1, PGR, ERBB2, MKI-67*) and includes the area under the curve (AUC), the standard error, 95% confidence interval.

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Table 3-10: Standard two by two table.

		IHC	
		POSITIVE	**NEGATIVE**
STRAT4 assay	**POSITIVE**	a = True Positive	b = False Positive
	NEGATIVE	c = False Negative	d = True Negative

Equation 2: Formulas of calculating sensitivity, specificity and the predictive values (Trevethan, 2017).

$$Sensitivity = \frac{a}{a+c} \times 100$$

$$Specificity = \frac{d}{b+d} \times 100$$

$$Positive\ Predictive\ Value\ (PPV) = \frac{a}{(a+b)} \times 100$$

$$Negative\ Predictive\ Value\ = \frac{d}{c+d} \times 100$$

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Chapter 4

Results

A total of 101 specimens from January to June 2018 fulfilling the study's inclusion criteria were included in the study. **Table 4-1** depicts the age distribution and grades of carcinomas. The majority of the samples belonged to the age group of 35-65 years, followed by the group above 65 years. The mean age was 53.69 years (range 22–90 years). More than half (70.3%) of the carcinomas were grade 2; 13.9% and 15.8% were grade 1 and 3, respectively.

Table 4-1: Age group distribution and grade of carcinomas.

Characteristics		**N**	**Percentage (%)**
Age (years)	< 35	3	3.0
	35 – 65	75	74.3
	> 65	23	22.8
Carcinoma Grade	I	14	13.9
	II	71	70.3
	III	16	15.8

All 101 samples yielded valid test results ("POSITIVE" or "NEGATIVE") for at least three assay targets. Five specimens had no or insufficient PCR amplification signal for the reference RNA *CYFIP1* (*CYFIP1 C*t > 35) "INDETERMINATE'' in one of the target assays. Out of the total 101 samples, 87 (86.10%) and 72 (71.28%) were positive for estrogen receptor status on IHC and STRAT4, respectively. Fourteen samples were negative for ER on IHC and 29 (28.71%) samples

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negative for ER on STRAT4. Fifty-six (55.44%) samples were positive for PR and 45 (44.55%) samples negative for PR on IHC. Sixty (59.40%) samples were positive for PR on STRAT4, while 40 (39.60%) were negative and 1 (0.99%) indeterminate on STRAT4. Twenty-nine (28.71%) specimens were HER2-positive while 72 (71.28%) were negative for HER2 on IHC. Twenty-six (25.74%) of samples were positive for HER2 while 75 (74.25%) were negative for HER2 on STRAT4. Twenty-three samples (22.77%) were positive for HER2 on FISH while 78 (77.22%) were negative for HER2 on FISH. KI-67 expression > 10% was present in 95 cases (94.05%) on IHC of which 85 (84.15%) were positive on STRAT4. Eighty-five cases (84.15%) were positive for *MKI-67* status on STRAT4 of which 79 (78.21%) were positive using 20% cut off on IHC. These findings are summarized in **Table 4-2**.

Table 4-2: ER, PR, HER2 and KI-67 status determined by IHC, FISH and STRAT4 assays.

	Assay	**IHC**		**STRAT4**		**FISH**	
Biomarkers		N	%	N	%	N	%
ER	Positive	87	86.13	72	71.28		
	Negative	14	13.86	29	28.71		
PR	Positive	56	55.44	60	59.40		
	Negative	45	44.55	40	39.60		
	Indeterminate		0	1	0.99		
HER2	Positive	29	28.71	26	25.74	23	22.77
	Negative	72	71.28	75	74.25	78	77.22

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KI-67 _1	Positive	95	94.05	85	84.15		
	Negative	6	5.94	12	11.88		
	Indeterminate		0	4	3.96		
KI-67 _2	Positive	79	78.21	85	84.15		
	Negative	22	21.78	12	11.88		
	Indeterminate		0	4	3.96		

4.1 Estrogen Receptor (ER)

More than two thirds of the samples were true positives, 72 (71.28%) and 14 (13.86%) were true negatives. There were no false positives recorded, whereas 15 were false negatives (**Table 4-3**).

Table 4-3: Two by two table for ER status.

	IHC		
STRAT4	Positive	Negative	Total
Positive	72	0	72
Negative	15	14	29
Total	87	14	101

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The specificity and PPV were both 100.00% and the sensitivity 82.76%. However, the NPV was 48.28%. The overall percentage agreement (OPA) between IHC and STRAT4 in determining ER status was 85.15% (**Table 4-4**).

Table 4-4: Concordance between IHC and STRAT4 for ER status.

Statistic	Value	95% CI
Sensitivity	82.76%	73.16% to 90.02%
Specificity	100.00%	76.84% to 100.00%
Positive Likelihood Ratio	48.3%	
Negative Likelihood Ratio	0.17	0.11 to 0.27
Positive Predictive Value	100.00%	
Negative Predictive Value	48.28%	37.07% to 59.66%
Overall Percent Agreement	85.15%	76.69% to 91.44%

Receiver operating characteristic (ROC) curve for STRAT4 *ESR1* AUC = 0.91 (**Figure 4-1**). Comparison of STRAT4 *ESR1* dCt values with -1 cut off as positive ≥ 1 or as negative < -1 vs ER status determined by immunohistochemistry (IHC) categorized according to Allred Score defined as the sum of the percentage of positive cells (X/5) and the intensity of staining (X/3) with possible values from 0 – 8, as negative ≤ 2 or positive >2 (**Figure 4-2**).

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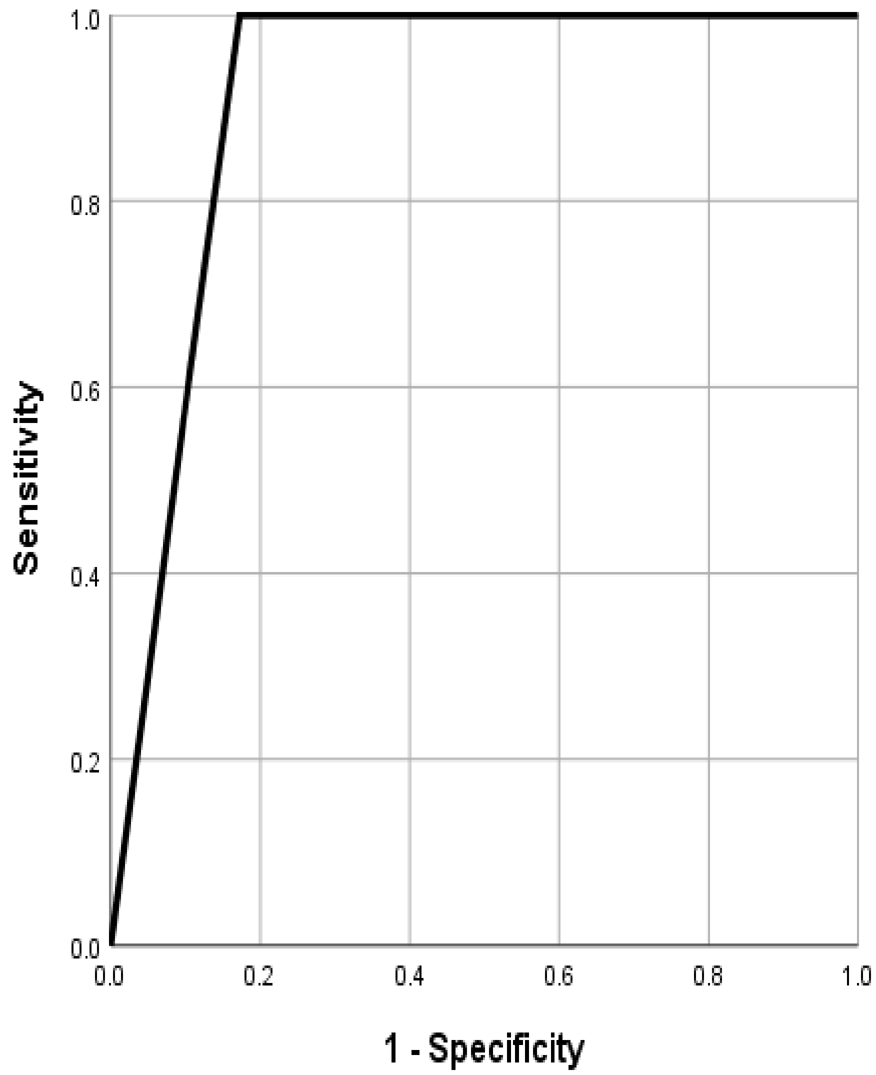

Figure 4-1: ROC curve for STRAT4 *ESR1* AUC = 0.91.

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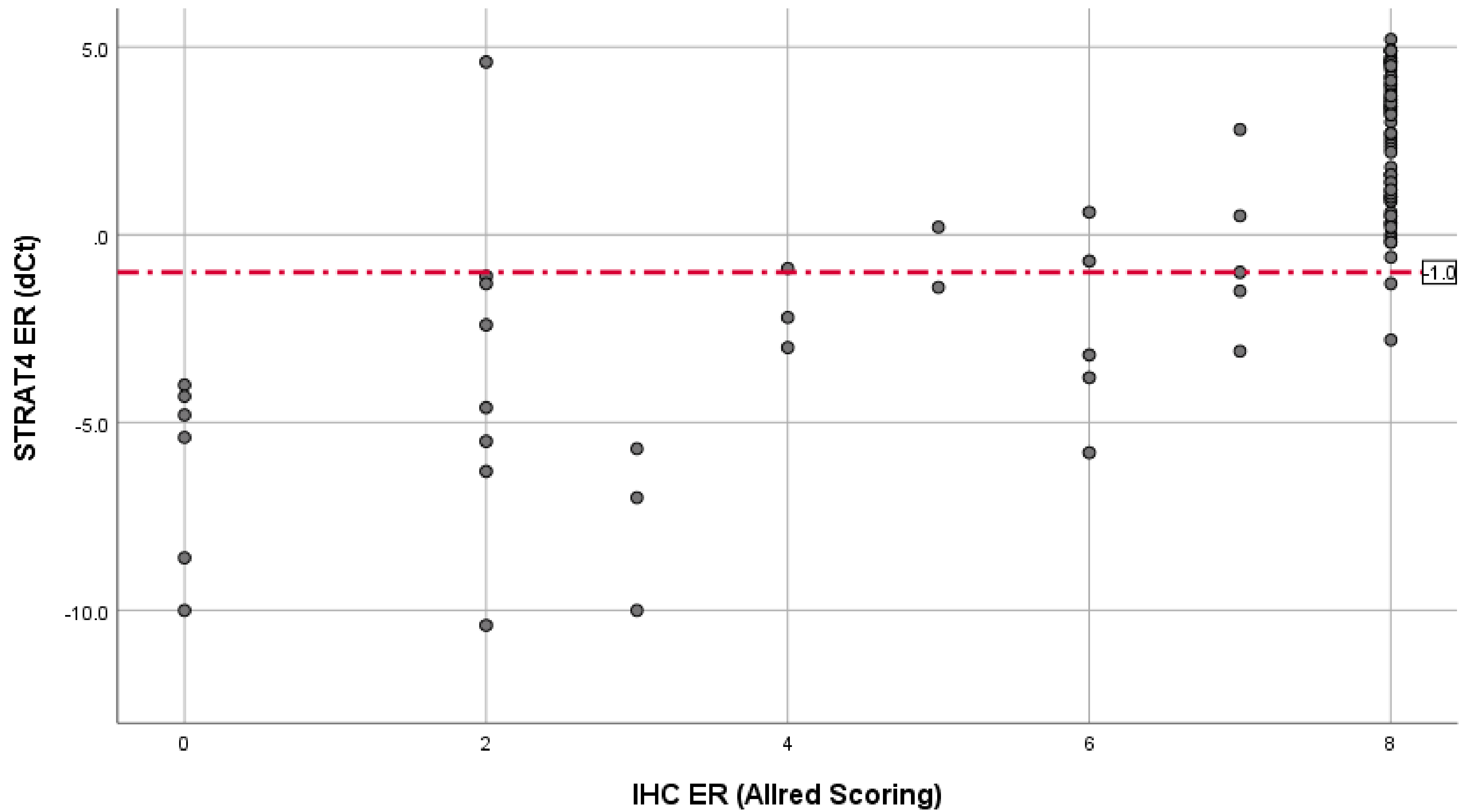

Figure 4-2: Graph of STRAT4 *ESR1* d*C*t values by ER IHC Allred score.

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4.2 Progesterone Receptor (PR)

Fifty-three cases (52.47%) were true positives for PR, while 37 (36.63%) were true negatives. There were three false negatives and seven false positives (**Table 4-5**).

Table 4-5: Two by two table for PR status.

	IHC		
STRAT4	Positive	Negative	Total
Positive	53	7	60
Negative	3	37	40
Indeterminate	0	1	1
Total	56	45	101

The OPA between STRAT4 *PGR* and PR IHC was 90.00%. STRAT4 showed a 94.64% sensitivity and 84.09% specificity for PR. The PPV recorded was 88.33% and the NPV was 92.50% (**Table 4-6**).

Table 4-6: Concordance between IHC and STRAT4 for PR status.

Statistic	**Value**	**95% CI**
Sensitivity	94.64%	85.13% to 98.88%
Specificity	84.09%	69.93% to 93.36%
Positive Likelihood Ratio	5.95	3.01 to 11.77
Negative Likelihood Ratio	0.06	0.02 to 0.19

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Positive Predictive Value	88.33%	79.29% to 93.74%
Negative Predictive Value	92.50%	80.28% to 97.39%
Overall Percent Agreement	90.00%	82.38% to 95.10%

Receiver operating characteristic (ROC) curve for STRAT4 *PGR* AUC = 0.89 (**Figure 4-3**). Comparison of STRAT4 *PGR* dCt values with -3.5 cut off as positive ≥ -3.5 or < -3.5 as negative vs PR status determined by immunohistochemistry (IHC) categorized according to Allred Score defined as the sum of the percentage of positive cells (X/5) and the intensity of staining (X/3) with possible values from 0 – 8, as negative ≤ 2 or > 2 as positive (**Figure 4-4**).

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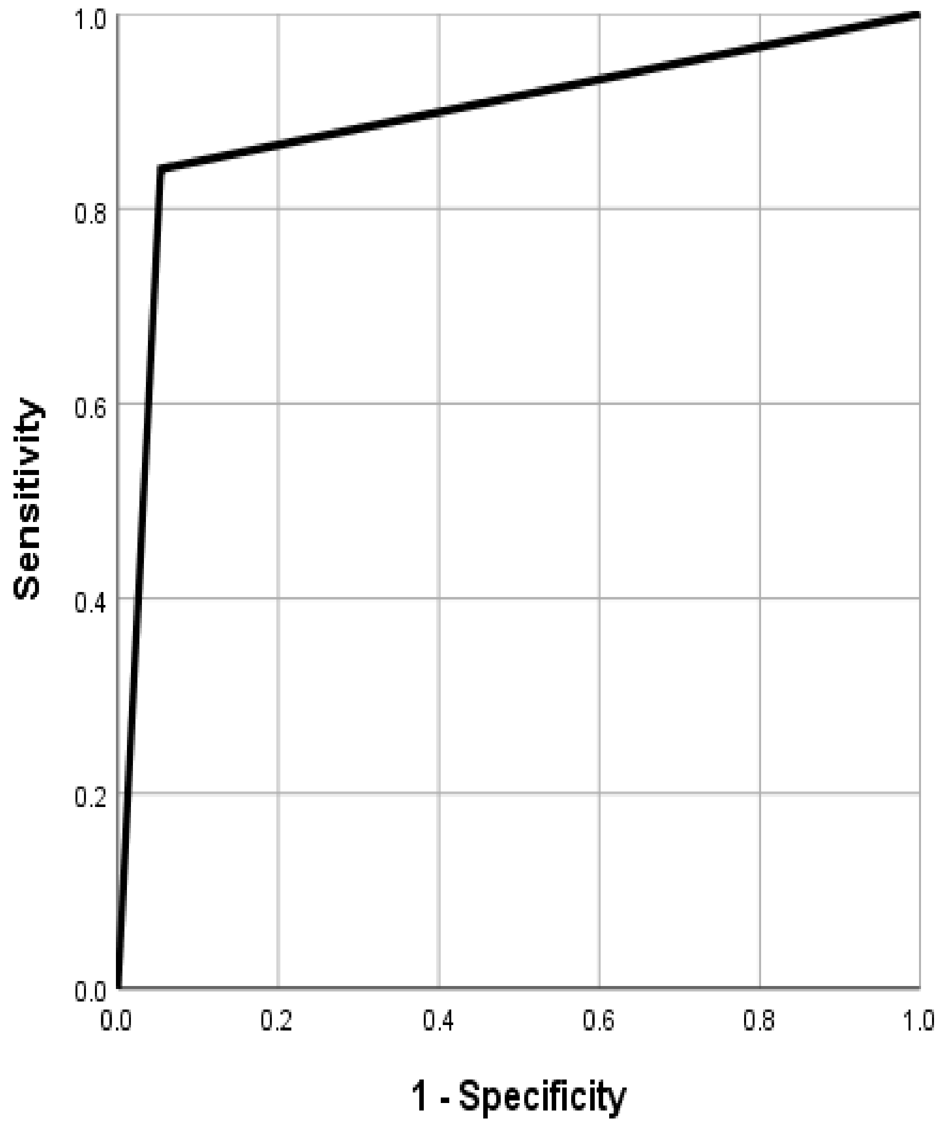

Figure 4-3: ROC curve for STRAT4 *PGR* AUC = 0.89.

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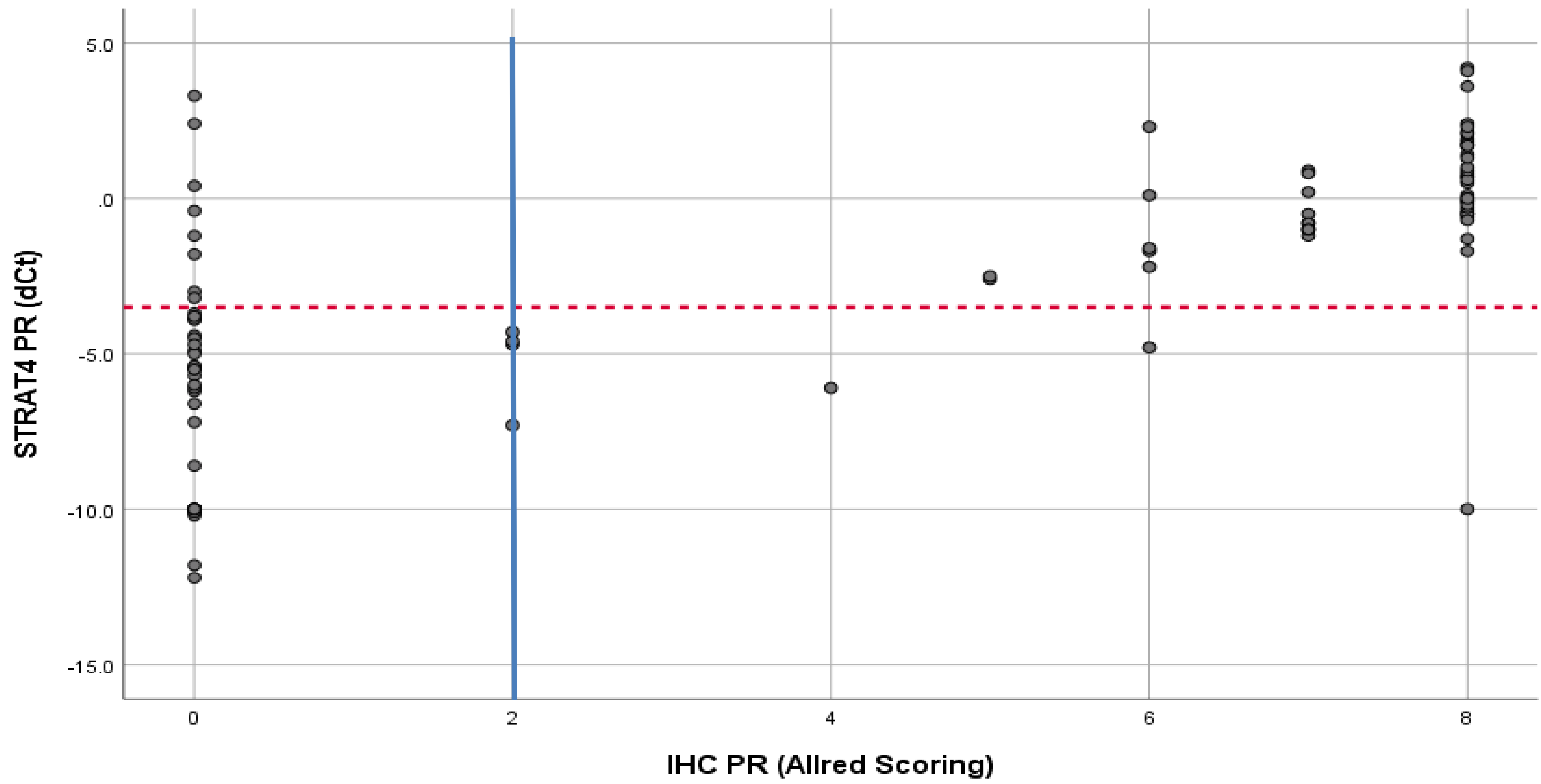

Figure 4-4: Graph of STRAT4 *PGR* d*C*t values by PR IHC Allred score**.**

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4.3 Human Epidermal Growth Factor Receptor 2 (HER2)

4.3.1 HER2 IHC

Twenty samples (19.80%) were true positives and 66 (65.35%) were true negatives. There were nine false negatives (8.91%) and six (5.94%) false positives (**Table 4-7**).

Table 4-7: Two by two table for HER2 status.

	IHC		
STRAT4	Positive	Negative	Total
Positive	20	6	26
Negative	9	66	75
Total	29	72	101

The sensitivity (68.97%) was lower than the specificity (91.67%). The NPV was 88.00% and the PPV 76.92%. The OPA between STRAT4 and IHC was 85.15% (**Table 4-8**).

Table 4-8: Concordance between IHC and STRAT4 assay for HER2 status.

Statistic	Value	95% CI
Sensitivity	68.97%	49.17% to 84.72%
Specificity	91.67%	82.74% to 96.88%
Positive Likelihood Ratio	8.28	3.70 to 18.49
Negative Likelihood Ratio	0.34	0.20 to 0.59

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Positive Predictive Value	76.92%	59.87% to 88.16%
Negative Predictive Value	88.00%	80.93% to 92.69%
Overall Percent Agreement	85.15%	76.69% to 91.44%-

4.3.2 HER2 IHC/FISH

The study showed 72 (71.28%) true negative and 20 (19.80%) true positive cases. Six samples (5.94%) were false positive and three (2.97%) samples were false negative (**Table 4-9**).

Table 4-9: Two by two table for human epidermal receptor 2 (HER2).

.	IHC/FISH		
STRAT4	Positive	Negative	Total
Positive	20	6	26
Negative	3	72	75
Total	23	78	101

The specificity, NPV and the OPA were all above 90% (96.00, 92.31% and 91.09, respectively). However, the sensitivity was lower at 76.92%, while the PPV was 86.96% (**Table 4-10**).

Table 4-10: Concordance between IHC/FISH and STRAT4 for HER2 status.

Statistic	**Value**	**95% CI**

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Sensitivity	86.96%	66.41% to 97.22%
Specificity	92.31%	84.01% to 97.12%
Positive Likelihood Ratio	11.30	5.16 to 24.78
Negative Likelihood Ratio	0.14	0.05 to 0.41
Positive Predictive Value	76.92%	60.33% to 87.96%
Negative Predictive Value	96.00%	89.29% to 98.57%
Overall Percent Agreement	91.09%	83.76% to 95.84%

Receiver operating characteristic (ROC) curve ROC curve for STRAT4 *ERBB2* AUC = 0.80 (**Figure 4-5**). Comparison of STRAT4 *ERBB2* d*C*t values with -1 cut off as postive ≥ -1 or ≤ -1 as negative vs progesterone receptor status (ER) determined by immunohistochemistry (IHC) categorized according to +1 as negative, +2 as equivocal (positive) and +3 as positive (**Figure 4-6**).

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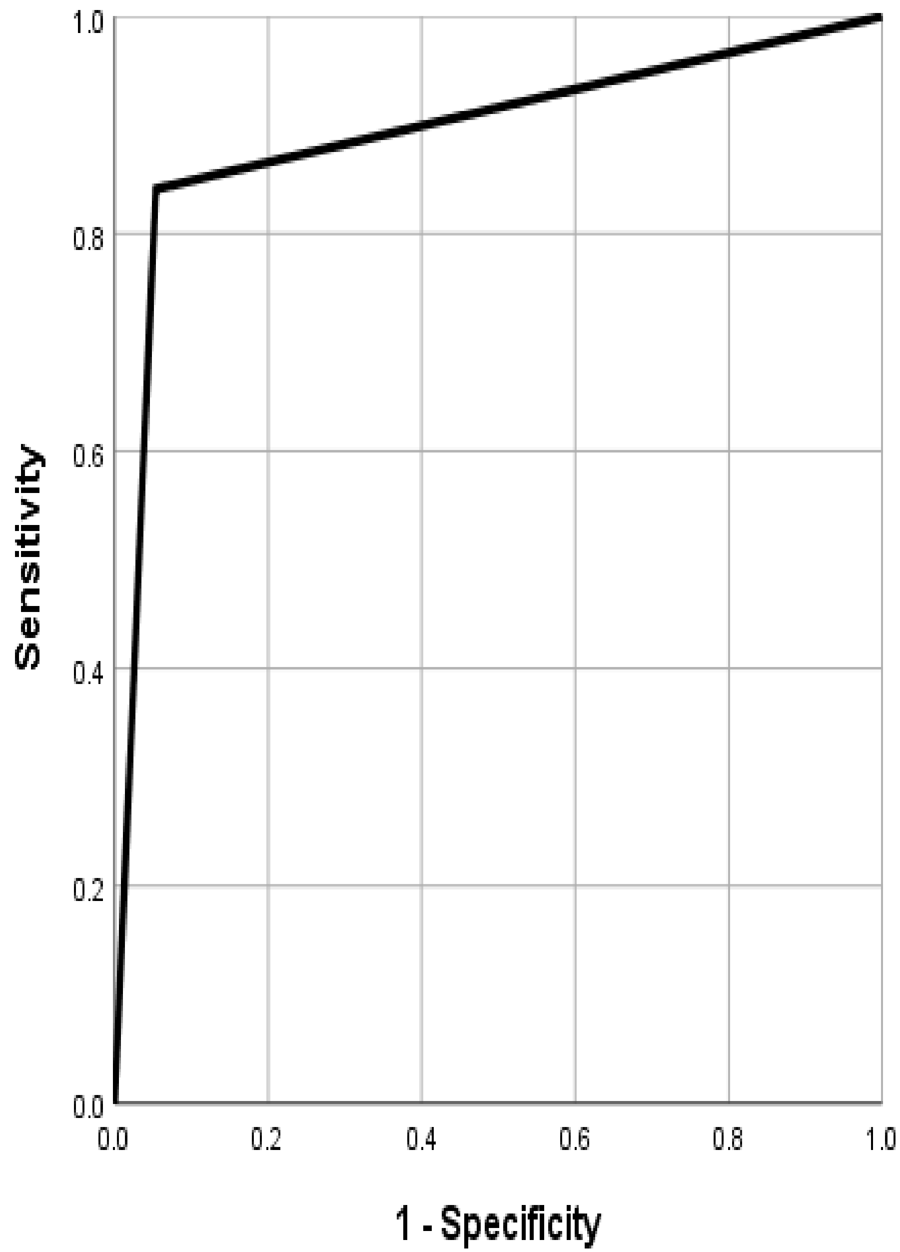

Figure 4-5: ROC curve for STRAT4 *ERBB2* AUC = 0.80.

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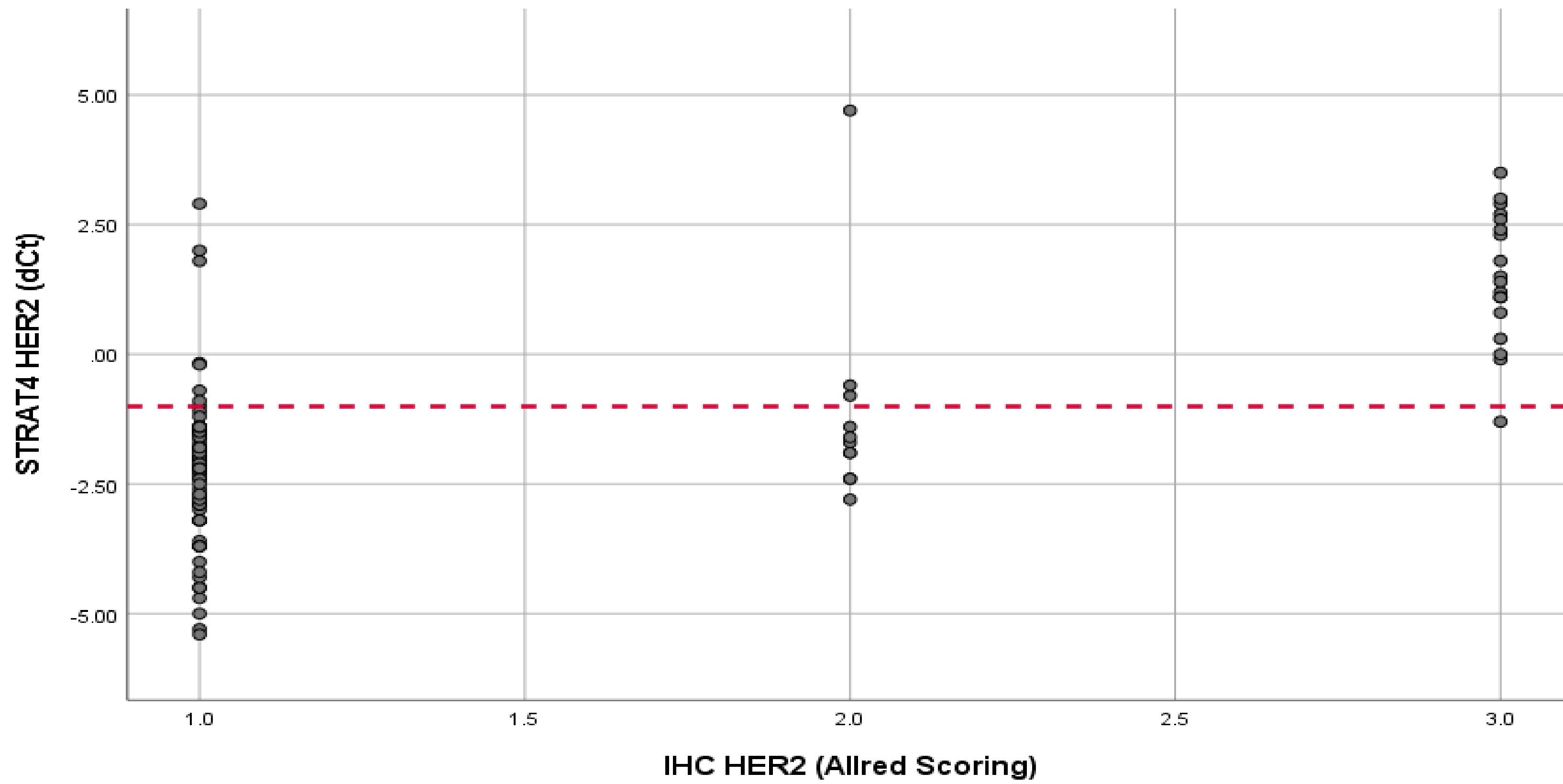

Figure 4-6: Graph of STRAT4 *ERBB2* d*C*t values by HER2 IHC Allred score

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4.4 Proliferation Index (KI-67)

4.4.1 KI-67 at 10% cut-off

True negatives and the indeterminate (excluded) results were four (3.96%) and the false negatives were eight (7.92%). The true positives were 84 (83.16%) with only one false positive (**Table 4-11**).

Table 4-11: Two by two table for proliferation index (KI-67) at 10% cut-off.

	IHC		
STRAT4	**Positive**	**Negative**	**Total**
Positive	84	1	85
Negative	8	4	12
Indeterminate	3	1	4
Total	85	12	101

The OPA between STRAT4 and IHC was 90.72%, sensitivity 91.30%, specificity 80.00%, PPV 98.82% and NPV 33.33% using a pre-defined IHC cut-off of 10% (**Table 4-12**).

Table 4-12: Concordance between IHC and STRAT4 for proliferation index status (KI-67_1) at 10% cut-off.

Statistic	**Value**	**95% CI**
Sensitivity	91.30%	83.58% to 96.17%
Specificity	80.00%	28.36% to 99.49%

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Positive Likelihood Ratio	4.57	0.79 to 26.38
Negative Likelihood Ratio	0.11	0.05 to 0.24
Positive Predictive Value	98.82%	93.56% to 99.79%
Negative Predictive Value	33.33%	18.43% to 52.52%
Overall Percent Agreement	90.72%	83.12% to 95.67%

4.4.2 KI-67 at 20% cut-off

When using a pre-defined cut-off of 20% for KI-67 positivity on IHC, 73 of the samples were true positive and only nine were true negative (**Table 4.13**). Twelve samples were false positive and only three were false negative. Four samples were excluded as they were indeterminate by STRAT4 (**Table 4-13**).

Table 4-13: Two by two table for proliferation index (KI-67) at a 20% cut-off.

	IHC		
STRAT4	**Positive**	**Negative**	**Total**
Positive	73	12	85
Negative	3	9	12
Indeterminate	3	1	4
Total	79	22	101

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The sensitivity recorded for KI-67 at 20% cut-off was high at 96.05%. However, the specificity was very low at 42.86%. The PPV and the NPV were 85.88% and 75.00%, respectively. The OPA between IHC and STRAT4 was 84.54% (**Table 4-14**).

Table 4-14: Concordance between IHC and STRAT4 for proliferation index status (KI-67_2) at 20% cut-off**.**

Statistic	Value	95% CI
Sensitivity	96.05%	88.89% to 99.18%
Specificity	42.86%	21.82% to 65.98%
Positive Likelihood Ratio	1.68	1.16 to 2.44
Negative Likelihood Ratio	0.09	0.03 to 0.31
Positive Predictive Value	85.88%	80.73% to 89.83%
Negative Predictive Value	75.00%	47.12% to 90.99%
Overall Percent Agreement	84.54%	75.78% to 91.08%

Comparison of STRAT4 *MKI67* d*C*t values with - 4 cut off, ≥ - 4 as postive or <- 4 as negative vs poliferation index (KI-67) determined by immunohistochemistry (IHC) at cut offs 10% and 20% of positive cells (**Figure** 4-7).

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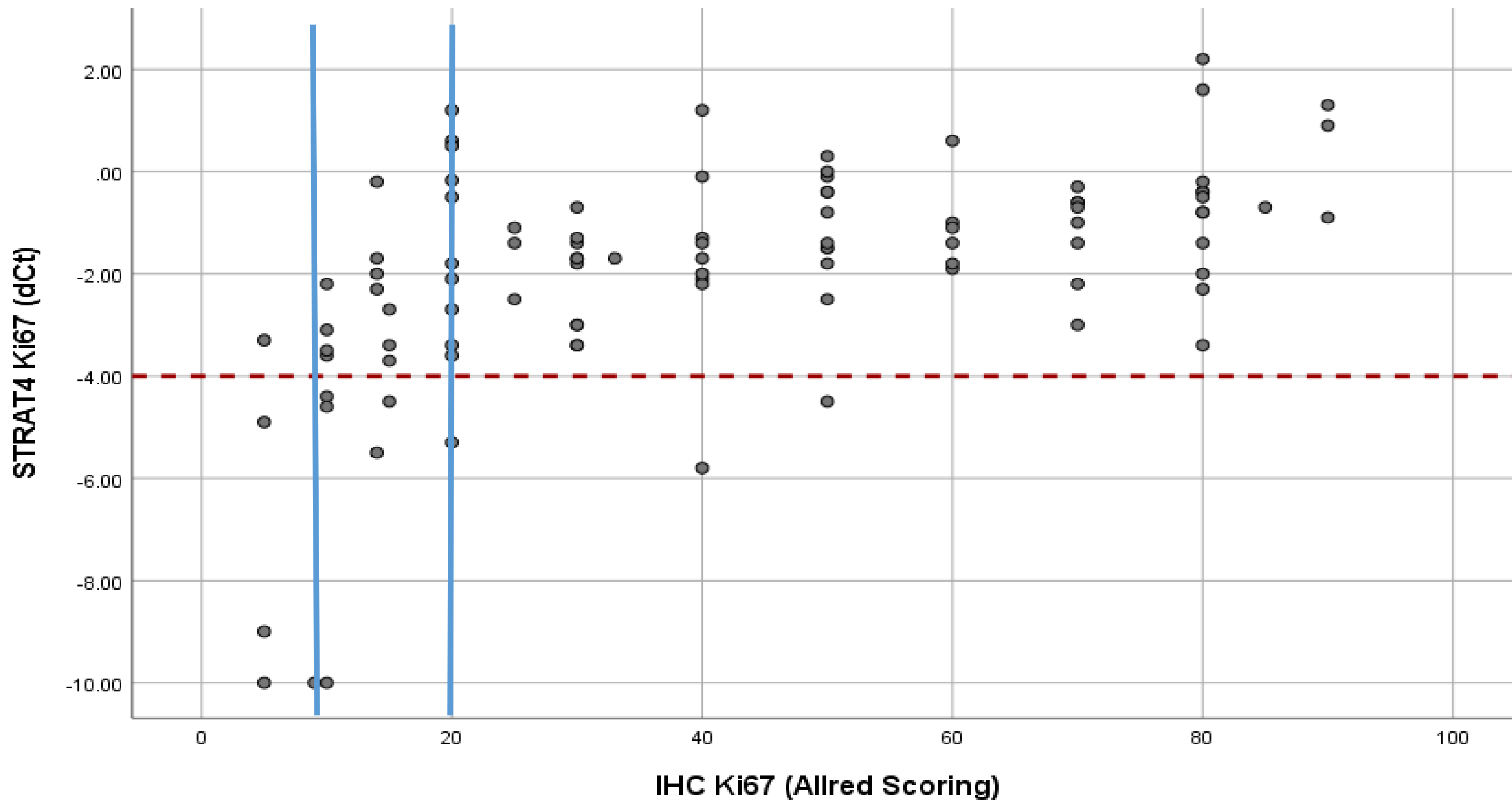

Figure 4-7: Graph of STRAT4 *MK167* d*C*t values by KI67 IHC at 10% and 20% cut off.

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4.5 Breast cancer subtyping

From the luminal A carcinomas, four were classified as different subtypes after analysis on STRAT4. Three were classified as luminal B and one as HER2-enriched. Nine of the luminal B carcinomas were classified as triple negative, two as HER2-enriched and six as luminal A (**Table 4-15**).

Table 4-15: Different breast cancer molecular subtypes according to IHC vs STRAT4 assay.

	STRAT4			
IHC	LUMINAL A	LUMINAL B	HER2-ENRICHED	TRIPLE NEG
LUMINAL A		3	1	
LUMINAL B	6		2	9
HER2 RICH				
TRIPLE NEG				

4.6 Turnaround Time

The TAT of STRAT4, from the time the sections are cut and ready for processing, to the availability of results, was approximately two hours for one batch (i.e. four samples). The average time taken on sample preparation per batch ranges from 45 to 50 minutes including 30 minutes of incubation. There is no loading of reagents as it is a preloaded cartridge. The Gene Xpert® instrument runs for 1 hour 5 minutes per run, and the results are available immediately. The Leica BOND III machine for IHC takes approximately two hours 10 minutes per run. This excluded time taken to load slides, load different reagents for different tests, and applying coverslips. In addition, afterwards, slides are taken to the pathologist for slide analysis under the microscope.

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Chapter 5

5.1 Discussion

Health service providers, researchers and especially clinicians are faced with the important task of breast cancer diagnosis and subtyping, to allow for directed treatment (Adebamowo *et al.*, 2014; Schwartz and William, 1998). This challenge has therefore necessitated the embrace of all available scientific techniques that could ease the process by evaluating biomarkers in an efficient way with a shorter turnaround time. In this study we evaluated the analytical validity of the new RT-qPCR CE-IVD assay (Xpert® Breast Cancer STRAT4*) against the standard-of-care, IHC and FISH techniques.

The most important reason for analytical validity is to build confidence in using a test. The important parameters in evaluating evidence about analytical validity include specificity and sensitivity (Lavanya *et al* 2013). However, several technical challenges may arise in evaluation of analytical validity, from sample requisition to the complexity of result interpretation (Burke *et al* 2002). A suitable test for clinical use is one that meets acceptable standards for analytical validity (Burke, 2014). The delta *C*t cut-offs used for all four biomarkers (*ESR1, PGR, ERBB2, MKI67)* for the STRAT4 assay were pre-specified based on prior testing in small datasets. This study provides the parameters of analytic validity for the STRAT4 assay, specificity and sensitivity in measuring the four breast cancer biomarkers (ER, PR, HER2 and KI-67).

Sensitivity was an important consideration in the study as it indicates the ability of a test to detect a true positive (Lavanya *et al* 2013; Trevethan, 2017). This informs us on whether the foundations of the test are satisfactory (Trevethan, 2017). Positive predictive value (PPV) measures the true positive rate among the positive results on the STRAT4 assay. This parameter assesses the practical usefulness of the test in clinical practice. According to Trevethan (2017) specificity measures the ability of a test to detect a true negative. Negative predictive value (NPV) is defined as the rate of true negatives among negative results of the STRAT4 assay.

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5.1.1 Turnaround time

During the study, the turn-around time recorded for the STRAT4 assay was on average two hours (i.e. from the time the sectioning of tissue is available on the bench to the availability of the results) for the analysis of four samples per run. One main advantage of the STRAT4 assay is that it is only dedicated to breast carcinoma and there is no loading of reagents, as the reagents are prepacked in the cartridge. On the other hand, IHC takes approximately two hours on the Leica BOND III machine (in addition to 30 minutes of baking slides prior to running the test). This reported turnaround time excludes the time taken to load slides into the Leica BOND III machine, load different reagents for different tests, setting up the system to recognise the position of slides as per test and lastly covering of slides with a coverslip before taking it to the pathologist for analysis under the microscope. Furthermore, availability of results is highly dependent on the laboratory workload. as other more urgent tests may take precedence. Interpretation of IHC staining by the pathologist may also take longer in certain cases. In context these factors can increase the turnaround time significantly.

5.1.2 Estrogen Receptor (ER/*ESR1*)

Sensitivity in our study for ER status is 82.76% which was similar to that of Denkert *et al*. (2019 (83.7%) while the study of Wu *et al.,* (2018) recorded a higher sensitivity of 98.3%. The lower sensitivity recorded in this present study is attributed to the number of cases which tested negative on the STRAT4 assay, but were reported as positive on the IHC (i.e. false negatives). The Allred scoring used to interpret ER and PR for IHC was done manually by a pathologist, and therefore contains a degree of subjectivity. Occasionally, a very low percentage of tumor cells (<1%) showed moderate or strong staining, and the case was scored as three (or more) out of eight, classifying it as ER-positive. There are no clear guidelines by ASCO/CAP on the response of these tumors from hormonal therapies. We recorded a PPV of 100%, meaning that there were no false positives in our study.

The results of this study showed that STRAT4 recorded the highest specificity (100%) for ER, as there were no false positive results recorded. This is comparable to the findings of Erber *et al*., (2020) where their specificity was 100%. Wu *et al*. and Denkert *et al*. reported lower specificities

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of 98% and 95.9%, respectively. However, it is worth noting that the sample sizes differ between studies. The sample size of our study is 101 cases, while that of Wu *et al.* was 523, and that of Denkert *et al.* and Erber *et al*. was 80 and 10, respectively. In context, this shows that the STRAT4 test is satisfactory in determining a true negative rate. However, we recorded a low NPV (48.2%), which means that false negatives were present. The OPA recorded in this study for ER status was 85.15%. Meanwhile, Denkert *et al*. recorded a slightly higher OPA of 89.7%, while, Wu *et al*. recorded 97.8% and Erber *et al.* recorded 100%. The concordance rate recorded in our study was supported by the ROC AUC value of 0.91.

5.1.3 Progesterone receptor (PR/*PGR)*

We recorded a sensitivity of 94.64% for PR, due to the few false negative cases. The sensitivity recorded in our study is in line with that of Wu *et al*. (93.5%), while Denkert *et al.* reported a lower sensitivity of 82.7%. Our study showed that STRAT4 was satisfactory to determine the true positive rate. In our study, specificity for PR was 84.09%, which is similar to that reported by Denkert *et al*. (83.7%) while Wu *et al*. reported a lower specificity of 81.1%. The low specificity recorded for PR in our study is due to seven cases with false positive results. In the case of low specificities or sensitivities, the predictive values (i.e. NPV or PPV) may be used in determining the treatment for breast cancer patients (Trevethan, 2017). In context, the low specificity recorded for PR status (despite the high sensitivity), may be ignored/overlooked in decision maKIng, due to a high NPV (92.50%) which equates to very few false negative cases. NPV is defined as the true negatives among negative cases, and because it is close to 100%, suggest that STRAT4 is essentially as good as IHC. Furthermore, Trevethan (2017) suggested that NPV may be used under certain circumstances such as in disease that require treatment early in course or particularly if the condition can be treated effectively and likely progresses quicker than expected such as in breast cancer cases. The overall percentage agreement (OPA) of PR in the present study was 90% which is similar to Wu *et al*. who recorded 90.4%. However, Denkert *et al*. reported a higher OPA of 97.8%. The results therefore suggest high levels of concordance between STRAT4 and IHC for PR, and was supported by ROC AUC value of 0.89.

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5.1.4 Human Epidermal Growth Factor Receptor 2 (HER2/*ERBB2)*

Our study showed that the sensitivity of STRAT4 compared to IHC for HER2 (including equivocal cases as positive) was 68%. However, when STRAT4 was compared with IHC, including FISH for resolution of equivocal cases to either negative or positive, our results were within the sensitivity range reported by Wu *et al*. (2018) of 86% – 98.4%. It is not surprising that less false positive cases were recorded when equivocal IHC results were categorized by FISH, resulting in a higher sensitivity of 86.96%. In context, several studies have demonstrated that FISH has more accurate results as compared to IHC, because with dual probe FISH testing, a chromosome count can be done while this is not possible on IHC. However, FISH does not necessarily reflect protein expression, but only demonstrates the amplification, or lack thereof, of the *ERBB2* gene (Bahreini *et al*., 2015; Wolff *et al*., 2007). Wasserman *et al*. (2017) reported a sensitivity of 87% between STRAT4 assay and IHC. In addition, Wasserman *et al*. also reported a sensitivity of 90% between STRAT4 assay and quantification of the immunofluorescent (QIF) staining performed using the method of Automated Quantitative Analysis (AQUA) which have been suggested by previous findings to be more analytically accurate than the IHC standard of care. However, our HER2 sensitivity was a little lower than that reported by Wu *et al*. and Denkert *et al*. of 94.3% and 97.3% respectively. Erber *et al*. reported a higher sensitivity of 100% in their study. Aberrant cytoplasmic IHC staining of tumor cells by HER2 may have hampered accurate interpretation of membrane staining (Allison, 2018). The HER2 antibody clone used to score these cases originally, at time of diagnosis, was detecting the intracytoplasmic domain of HER2. Since December 2019, the antibody clone was changed to one recognizing the external domain of HER2. This change in our laboratory appeared to have reduced the amount of cytoplasmic staining. Increased cytoplasmic staining, interpreted as negative, could have been responsible for false negative results. A lower sensitivity can also suggest reviewing the cuts-off for *ERBB2* mRNA transcripts on the STRAT4 assay. There was a moderate PPV of 76.92% which could be acceptable.

A specificity of 92.31% was recorded for HER2 in our study between STRAT4 and IHC/FISH, which was higher than the specificity between STRAT4 and IHC including equivocal as positives (86.96%). Erber *et al*. reported the highest specificity of 100% followed by 94% reported by both Wassermann *et al*. and Denkert *et al*., and lastly Wu *et al*. reported 93.1%. The results of our study suggest that STRAT4 is a very effective test in identifying biopsies with no overexpression of

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ERBB2 (NPV of 96%). The OPA recorded in this study between STRAT4 and IHC/FISH results was 91.09%.

5.1.5 Proliferation Index (KI-67/*MKI67)*

Concordance between STRAT4 and IHC for determination of the proliferation index (*MKI67*/KI-67), was variable depending on a cut-off of 10% and 20%. There has been considerable debate over the performance and interpretation of proliferative markers (Hashmi *et al.,* 2019). No optimal cut-off point for the KI-67 proliferative index has been standardized, and this may be responsible for the difficulty in choosing a standard threshold for daily practice (Ahmed *et al*., 2018). Furthermore, currently the ASCO guidelines do not recommend the use of KI-67 routinely on all breast carcinoma cases (Harris *et al.,* 2016). In our study, the cut-off points for KI-67 status were 10% and 20% of positively stained cells, which was similar to that of Wu *et al*. Our results showed that STRAT4 had the highest sensitivities of KI-67 status when compared to other biomarkers. *MKI67* at a cut-off of 20% recorded a higher sensitivity than the 10% cut-off, with a sensitivity of 96.05% and 91.00% respectively. Hence, raising the IHC cut-off for determination of the proliferation index has an impact on the sensitivity. For a 10% cut-off, there were more false negatives and less false positives than for the 20% cut-off. To put it another way, cases were over scored by STRAT4 using a cut-off of 10% on IHC scoring. Our study recorded one of the highest sensitives of *MKI67* at 20% cut-off after Denkert *et al*., who had a value of 97.6% at 20% cut-off. However, Wu *et al*. (2018) reported a lower sensitivity for both *MKI67* cut-off at 10% (80.7%) and 20% (88.6%) than our study. In addition, we recorded a PPV of 98.82% and 85.88% for 10% and 20% cut-off values, respectively.

We had a specificity of 80% for a 10% cut-off and 42.86% for a 20% cut-off in our study. Unlike the sensitivity, the specificity of the 20% cut-off was strikingly lower (42.85%) than that of the 10% cut-off (80.00%). This observation was similar to that of Denkert *et al*. who reported a far lesser specificity of 30.5% using a 20% cut-off. Wu *et al.* reported a higher specificity of 59.9%. It is evident from our study that even though the 20% cut-off recorded a lower specificity, it seemed the better cut-off due to the higher sensitivity which could infer that very few cases were missed (false negatives). Furthermore, in relation to the low specificity at 20% cut-off, a moderate negative predictive value (NPV) was recorded and would be tolerable if breast cancer was not a

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progressing disease or one which does benefit from early treatment. These findings suggest a re-adjustment of the STRAT4 assay cut-off for determining the *MKI67* status. Using 10% and 20% cut-offs resulted in OPAs of 90.72% and 84.54% respectively. This is the second highest OPA which has been recorded after Denkert *et al*. (86.7%) for detection of *MKI67* at a 20% cut-off.

5.1.6 Breast cancer subtypes

We recorded twenty cases that had one discordant result in one of the four targeted biomarkers between the STRAT4 assay and the IHC which resulted in a change of subtype. More than half of the discordant results was due to discordant ER results. According to Grant *et al*., 2015 there is a significant variation between mRNA expression and receptor protein levels on the ER gene. In the case of false positives, ER variation could be due to splicing, which may produce a non-functional variant of the *ESR1* gene that express epitopes recognised by IHC antibodies. Another factor to be taken into consideration, would be decreased antigens for antibodies to bind to, during the antigen retrieval stage of IHC leading to false negative results.

Tumors were re-classified as luminal A, luminal B and triple negative subtypes, with the majority changed to luminal B (17 cases). There was only one triple negative which had been changed to a HER2-enriched tumor, due to discordance in HER2 status. In this scenario, the patient may miss the benefits of trastuzumab, because it is not administered to triple negative breast carcinomas. On the other hand, the patient would be spared from the side effects of chemotherapy. Furthermore, three luminal A carcinomas were re-classified to luminal B, due to variability of the HER2 status (false positive cases). In these cases, the patients would not benefit from chemotherapy if classified as luminal A but would still benefit from hormonal therapy. These findings suggest that patient treatment may have been different, depending on the detection method. In terms of the luminal B cases, out of 17 changed subtypes, six were changed to luminal A therefore would not secure the benefits of chemotherapy; two into HER2–enriched and would respond better to trastuzumab, however, no evidence has been reported on the response of luminal A to trastuzumab. Lastly nine luminal A carcinomas were reclassified to TNBC; with both IHC and STRAT4 classification chemotherapy would be beneficial, however hormonal therapy would not be beneficial to TNBC.

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5.1.7 Ethics consideration

Ethical, legal and social implications were important considerations in the study. Only retrospective cases were used in this study, and according to Stellenbosch University "Guidance on applying for a waiver of consent when conducting retrospective record reviews", the following had to be included which were strictly adhered to during experimentation: justification regarding why participant consent cannot be obtained, whether the data will be anonymized at the point of data collection and whether the data will be aggregated and anonymized in the reporting of findings. These guidelines are in line with the University of Kansas, School of Medicine-Wichita campus (KUSM-W, USA) Human Subjects Committee 2 guidelines implemented from the 14 April 2003.

In addition to the University guidelines, the Protection of Personal Information Act (POPI Act) was published in the South Africa Government Gazette on 26 November 2013. The purpose of the POPI Act is to ensure all South African institutions conduct themselves in an accountable manner when collecting, processing, storing and sharing (local or international) another entity's personal information by holding them responsible.

In this study careful consideration was given to the POPI Act. Previously the South Africa's research ethics framework, allowed a broad consent model for secondary data purposes (National Department of Health. Ethics in Health Research Principles, Processes and Structures. 2015.). The use of biological material with clinical data and diagnostic results for secondary research purposes goes against POPI Act. Informed consent may be required especially since the biological material and data was not collected for research purposes initially. According to the POPI Act section (15), a waiver of consent was requested based on anonymization (de-identifying) of cases and that contacting all patients would not be feasible. Furthermore, the study was exempt from the POPI Act because performing the STRAT4 assay on the residual tissue, which is the same test done on IHC, but is on a different molecular level, would not have any implications for future treatment of the particular patient.

Previously samples could be shared with research institutions, both locally and internationally, while subjected to certain approvals (Staunton, 2019). In this study coded data was to be shared

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with the provider of the STRAT4 assay for in-house validation and calibration. According to the POPI Act, data can only be transferred to countries that have similar data protection, for example countries under the European General Data Protection Regulation (GDPR). In this study a Material Transfer Agreement (MTA) was signed between the university and the provider of the KIts. Staunton (2019) reported that the MTAs could include a legally binding agreement. However, it remains to be seen whether MTAs will be deemed to provide sufficient safeguards.

In context, LMIC and poorly resourced countries are prone to ethical challenges that are related to commercialization. Validation of high through-put technologies that are fully sponsored will have ethical concerns which may bring some inevitable association of commercial interest. One, highly likely scenario is that in order to protect commercial interests, some may seek to assert intellectual property rights over various aspects of translational research. Consenting for such research puts one at a risk of public expectation and may outpace the reality of the research. These complexies suggest a possible impact by the POPI Act on health research in South Africa.

5.2 Limitations

The sample size was small, which may lead in increasing the probability of defining a true positive result as false positive. Furthermore, this would pose the STRAT4 assay test to have no limitations as compared to the IHC. Furthermore, small sample size may not estimate the overall percentage agreements precisely.

5.3 Conclusion

Evaluation of the Xpert® STRAT4 breast cancer assay in the detection of breast cancer biomarkers (ESR, PGR, ERBB2 and MK167) indicated a significantly good performance. The overall percentage agreement between the STRAT4 and the gold standard was comparable. Furthermore, the STRAT4 assay showed a good turnaround time and confirmed its efficiency for rapid subtyping of breast cancers, which can be beneficial in countries with limited healthcare resources. Nonetheless, at this stage, it is advised to use STRAT4 as a supplementary method to the standard of care. In future the STRAT4 assay may facilitate breast cancer subtyping in resource poor settings.

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Chapter 6

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Chapter 7

Appendix

Table 7-1: Cases with discordant results between STRAT4 assay and IHC/FISH used to approximate breast cancer molecular subtype.

GX_No	IHC ER	IHC PR	IHC HER2	IHC KI67 (%)	Subtype	STRAT4 ER	STRAT4 PR	STRAT4 HER2	STRA4 KI67	Subtype
P5	0	0	0	60	LUMINAL B	0	0	1	0	LUMINAL A
P14	0	0	0	5	LUMINAL B	0	0	1	INDET	LUMINAL A
P29	0	0	0	20	LUMINAL B	0	0	1	0	LUMINAL A
P51	0	0	0	0	LUMINAL B	0	0	1	1	LUMINAL A
P67	0	0	0	14	LUMINAL B	0	0	1	0	LUMINAL A
P74	0	0	0	50	LUMINAL B	0	0	1	1	LUMINAL A
P1	0	0	1	15	LUMINAL A	0	0	0	0	LUMINAL B
P77	0	0	1	14	LUMINAL A	0	0	0	0	LUMINAL B
P84	0	0	1	15	LUMINAL A	0	0	0	0	LUMINAL B
P91	0	1	0	70	LUMINAL B	1	1	0	0	HER2RICH
P95	0	1	0	30	LUMINAL B	1	1	0	0	HER2RICH
P6	0	1	1	70	LUMINAL B	1	1	1	0	TRIPLE -

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P9	0	1	1	90	LUMINAL B	1	1	1	0	TRIPLE -
P26	0	1	1	25	LUMINAL B	1	1	1	0	TRIPLE -
P42	0	1	1	50	LUMINAL B	1	1	1	0	TRIPLE -
P45	0	1	1	60	LUMINAL B	1	1	1	0	TRIPLE -
P54	0	1	1	50	LUMINAL B	1	1	1	0	TRIPLE -
P59	0	1	1	70	LUMINAL B	1	1	1	0	TRIPLE -
P89	0	1	1	85	LUMINAL B	1	1	1	0	TRIPLE -
P90	0	1	1	60	LUMINAL B	1	1	1	0	TRIPLE -
P55	1	1	1	20	TRIPLE -	1	1	0	0	HER2RICH

0 = Positive   1 = Negative

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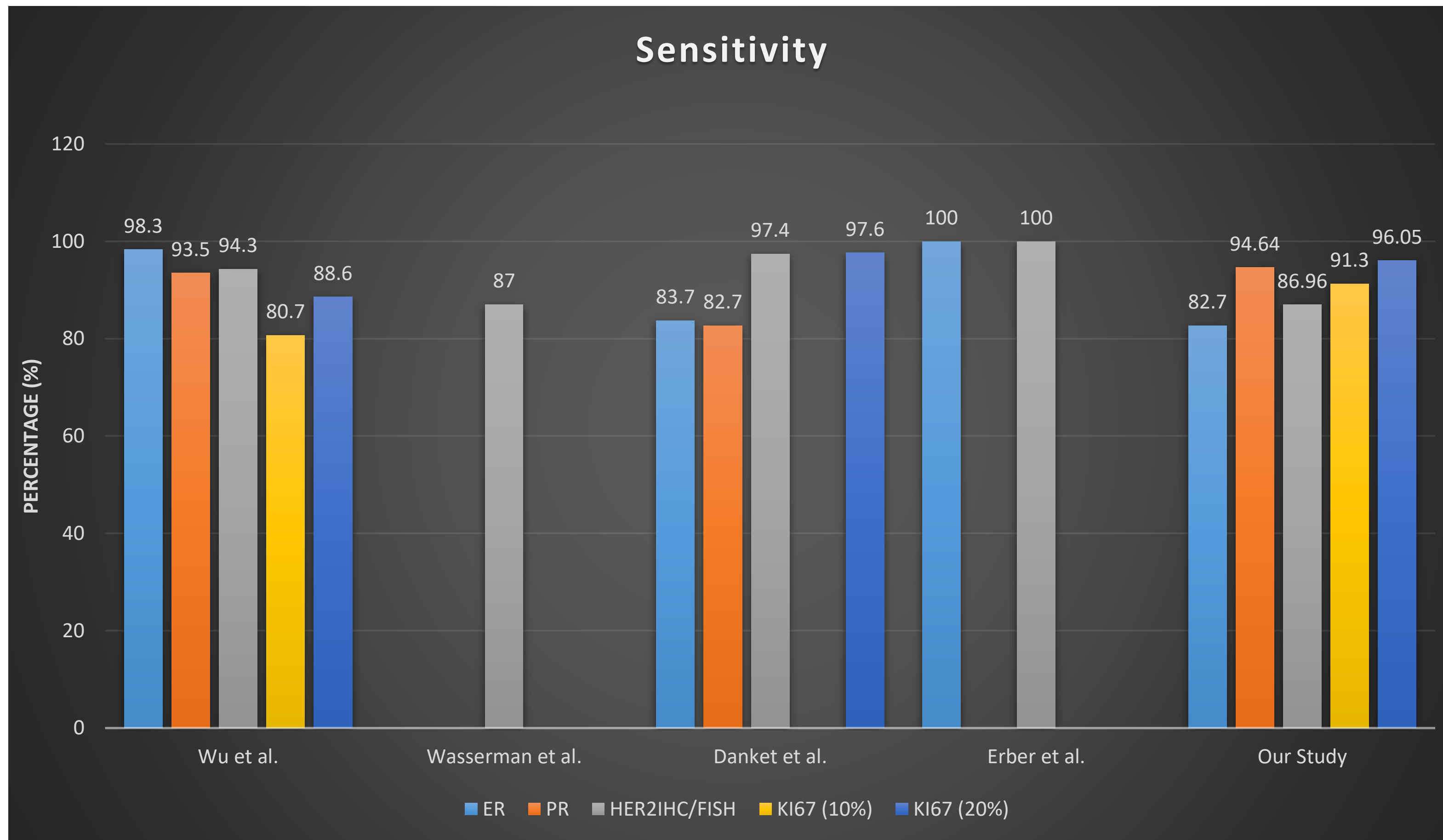

Figure-7-1: Comparison of sensitivity for all four biomakers (ER, PR, HER2 and KI-67) between STRAT4and IHC across different studies.

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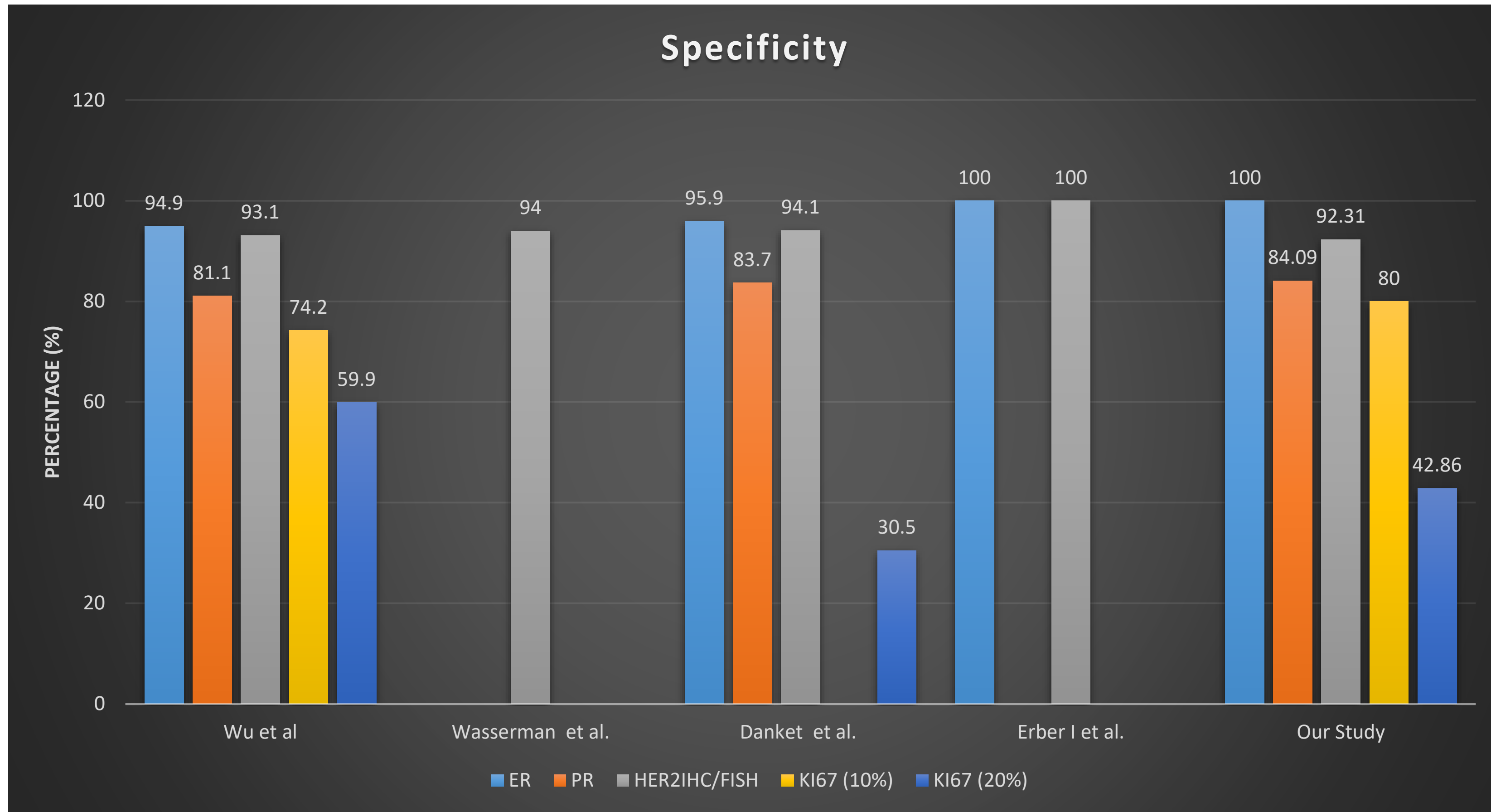

Figure 7-2: Specificity of all four biomakers (ER, PR, HER2 and KI-67) between STRAT4 assay and IHC across different studies.

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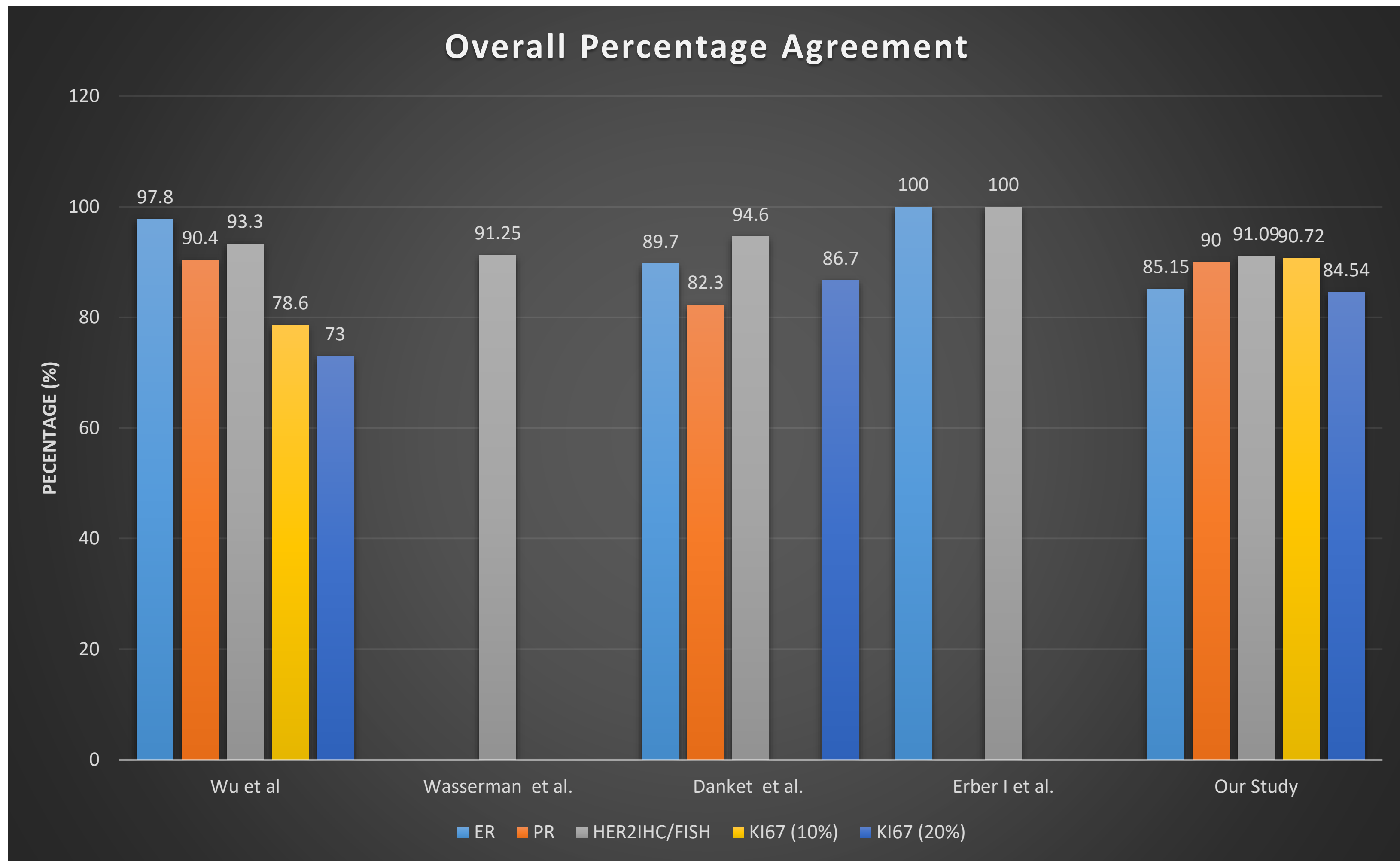

Figure 7-3: OPA of all four biomakers (ER, PR, HER2 and KI-67) between STRAT4 assay and IHC in different studies.

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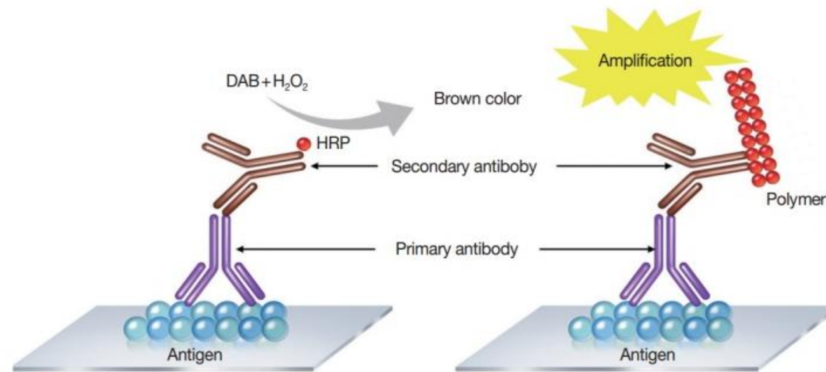

Illustration of polymeric amplification system. DAB, diaminobenzidine; HRP, horseradish peroxidase.

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