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Intrinsic subtype in hiv positive and negative patients with breast cancer

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INTRINSIC SUBTYPE IN HIV POSITIVE AND NEGATIVE PATIENTS WITH BREAST CANCER

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PhD THESIS

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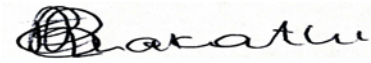
A THESIS SUBMITTED TO THE FACULTY OF HEALTH SCIENCES, UNIVERSITY OF THE WITWATERSRAND, IN FULFILMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Declaration

I, Boitumelo Precious Phakathi, declare that this thesis is my own unaided work. It is being submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at the Faculty of Health Sciences, University of Witwatersrand. It has not been submitted for any degree or examination in any other tertiary institutions.



14 September 2021

DEDICATION

Thus far has the Lord brought me. Indeed, had it not been for the Lord, I would not have reached this far. Thank you, Lord, for your sustaining, enabling power and sufficient grace that sustained me throughout this journey.

To my forever supportive and prayerful husband, Sibongiseni Phakathi. Thank you, my love, for believing in me and for always being there as my pillar of strength, courage and inspiration. The journey took longer than we had expected and there were more than enough reasons for me to quit but you always motivated me to keep pushing until the finish line. You are an amazing husband.

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To my family, friends and fellow bretherns Jesus Christ Ministries (JCM)

Finally, to every young person, especially those from rural areas. May this serve as a reminder that your place of birth does not determine your destiny. Your dreams are valid and you can achieve anything you set your mind on.

PRESENTATIONS

- 11 August 2018: Breast Interest group of Southern Africa (BIGOSA) Conference, Cape Town
    - **Oral presentation**: The clinicopathological characteristics of HIV positive and HIV negative women with breast cancer

- 28 November 2018: Department of Surgery Myburgh Research Forum
    - **Oral presentation**: The clinicopathological characteristics of HIV positive and HIV negative women with breast cancer

- 23 0ctober 2019: WITS Faculty of Health Sciences Research Day

    - **Oral presentation**: The clinicopathological characteristics of HIV positive and HIV negative women with breast cancer
    - **Poster presentation**: Survival of South African women with breast cancer receiving antiretroviral therapy for HIV

- 07 November 2019: African Organisation for Research and Training in Cancer (AORTIC) 12TH International Conference, Maputo, Mozambique

    - **Poster presentation**: The clinicopathological characteristics of HIV positive and HIV negative women with breast cancer

- 27 November 2019: Department of Surgery Myburgh Research Forum

    - **Oral presentation:** Survival of South African Women with Breast Cancer Receiving Antiretroviral Therapy for HIV

- 10 July 2021: 48th Surgical Research Society of Southern Africa (SRSSA) Annual Virtual conference

    - Oral presentation: PAM50 intrinsic subtype in HIV positive and HIV negative women with breast cancer

PUBLICATIONS

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- Phakathi B, Nietz S, Cubasch H et al. Survival of South African women with breast cancer receiving antiretroviral therapy for HIV. Breast. 2021 Jun 3; 59:27-36. DOI: 10.1016/j.breast.2021.05.014. Epub ahead of print. PMID: 34126376; PMCID: PMC8209274.
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ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome

AJCC: American Joint Committee on Cancer

ART: Antiretroviral therapy

ASCO: American Society of Clinical Oncology

ATAC trial: Anastrozole, Tamoxifen, Alone or in Combination trial

CBE: Clinical breast examination

cDNA: Complementary Deoxyribonucleic acid

CE: Conformite Europe (French for European Conformity)

CMJAH: Charlotte Maxeke Johannesburg Academic Hospital

CHBAH: Chris Hani Baragwanath Academic Hospital

CIs: Confidence intervals

COVID-19: Coronavirus disease-19

EBV: Epstein-Barr virus

EGAPP: Evaluation of genomic applications in practice and prevention

ER: Oestrogen receptor

ESBC: Early-stage breast cancer

ESMO: European Society for Medical Oncology

FDA: Food and Drug Administration

FFPE: Formalin-fixed paraffin-embedded

FISH: Fluorescence *in situ* hybridisation (FISH)

H & E: Haematoxylin and eosin

HER- 2: Human epidermal growth factor receptor-2

HIV: Human immunodeficiency virus

HPB virus: Hepatitis B virus

HPV: Human papilloma virus

IHC: Immunohistochemistry

IQRs: Inter-quartile ranges

NADM: Non-AIDS defining malignancies

NCCN: National Comprehensive Cancer Network

NHLS: National Health Laboratory Service

NNRTIs: Non-nucleoside reverse transcriptase inhibitors

NRTIs: Nucleoside reverse transcriptase inhibitors

PAM 50: Prediction analysis of micro-array

PHC: Primary Health Care

PHEIC: Public Health Emergency of International Concern

PLWHIV: People living with HIV

PR: Progesterone receptor

qRT-PCR: Quantitative real-time reverse transcriptase PCR

RNA: Ribonucleic acid

ROR: Risk of recurrence score

RUO: Research use only

SABCHO: South African Breast Cancer and HIV Outcome

SARS-CoV 2: Severe acute respiratory syndrome coronavirus 2

SISH: Silver-enhanced *in situ* hybridisation

TNBC: Triple-negative breast cancer

UNAIDS: Joint United Nations Programme on HIV/AIDS

UTT: Universal Test and Treat

VL: Viral load

WHO: World Health Organisation

ABSTRACT

Introduction

Breast cancer is the most common cancer in women and a leading cause of cancer-related mortality worldwide. In sub-Saharan Africa, breast cancer overall survival has been poor, with an estimated five-year survival of 50% compared to almost 90% in high-income countries. In South Africa, it remains the most common cancer among women. Moreover, South Africa has the largest global burden of HIV infection and the most extensive antiretroviral treatment (ART) programme. How HIV infection and ART use affect the clinicopathological characteristics and molecular-based intrinsic subtypes, and the overall survival of patients with breast cancer remains unknown. Our aim, therefore, was to investigate the association of HIV infection and ART use with the clinicopathological presentation of breast cancer, PAM 50 intrinsic subtypes and breast cancer survival in a South African urban female population known to have a high HIV prevalence.

Methodology

Study participants were females newly diagnosed with invasive breast cancer at two academic Surgical Breast Units in Johannesburg, South Africa, from 15 May 2015 to 30 September 2017. The two hospitals were the Charlotte Maxeke Johannesburg Academic Hospital and Chris Hani Baragwanath Academic Hospital. We compared demographic and clinical-pathological characteristics of HIV-positive and HIV-negative patients at the time of breast cancer diagnosis. We also analysed the overall and disease-free survival and compared HIV positive and negative patients. Among the HIV positive patients, the comparison was made between those on ART and not on ART at the time of breast cancer diagnosis. The effect of the duration of HIV seropositivity and ART use on overall and disease-free survival were also analysed. For the intrinsic subtyping, RNA was extracted from breast cancer confirmed formalin-fixed paraffin-embedded (FFPEs) samples, which were age-matched and equally split between HIV

positive and negative women with breast cancer. The RUO version of the NanoString RUO PAM50 algorithm was used to determine the intrinsic subtype of each sample.

Patients were categorised as HIV positive, HIV negative or unknown. Only 3.2% of the patients were HIV unknown and were excluded from further analysis. Demographic and clinicopathological data were categorised, and comparisons between HIV positive and negative patients performed using χ^2 tests. We obtained CD4 counts and HIV viral loads for the HIV-infected patients from the South African National Health Laboratory Service (NHLS) using results of tests performed closest to the date of a breast cancer diagnosis. Viral load was categorised as either detectable (>50 copies/ml) or below detectable limits (≤50 copies/ml). Age, CD4 count and viral load (when detectable) were non-normally distributed and were thus represented as medians and inter-quartile ranges (IQRs) and compared using a Kruskal Wallis test. Generalised linear models with binary outcomes and a log link function were used to determine prevalence ratios for non-missing variables to assess the relationship between stage at diagnosis, tumour grade, IHC 4 subtype and presence or absence of metastatic disease with HIV status controlling for age at diagnosis (continuous) and ethnicity (black vs non-black). The Kaplan-Meier survival curves were generated, assuming non-informative losses to follow-up; p-values were calculated using a log-rank test of equality and hazard ratios, and 95% confidence intervals (CIs) were estimated using Cox regression models. Associations between the PAM50 subtypes and categorical variables were evaluated by Fisher's exact test, and associations between the PAM50 subtypes and ordinal variables were assessed using the Kruskal Wallis test. Statistical analyses were conducted using STATA v12.4, and a $p<0.05$ was considered statistically significant.

Results

Of 1050 patients enrolled, 1016 (96.8%) had known HIV status, with 226 (22.2%) being HIV positive. HIV positive patients were younger (median (IQR) age 45 (40-52) years), than HIV-negative patients (median (IQR) age 57 (46-67)) ($p<0.001$). HIV positive patients were more likely to be diagnosed with late-stage breast cancer ($p=0.01$),

however after adjusting for age and ethnicity, the disease stage was not associated with the HIV status. However, HIV positive patients receiving ART at the time of breast cancer diagnosis were less likely to present with metastatic disease than those, not on ART (p=0.05).

The overall and disease-free survival were 53.5% and 55.8%, respectively after four years of follow up. HIV positive status was significantly associated with poorer overall and disease-free survival, [HR (95%CI: 1.50 (1.22-1.85). p< 0.001] and [HR (95% CI): 2.63 (1.71-4.03); p< 0.001], respectively. HIV positive patients on ART at the time of diagnosis had better overall and disease-free survival than those who were not on ART (p< 0.001). Advanced stage of disease, hormone receptor negative breast cancer subtypes were also associated with worse overall and disease-free survival. Of the 405 extracted RNAs, only 144 samples could be analysed with the Nanostring PAM50 assay due to a break-down of the Nanostring nCounter platform, which was further exacerbated by the Coronavirus outbreak. According to our preliminary results, HER-2 enriched intrinsic subtype was the commonest subtype, accounting for 32.6% of all subtypes, with Luminal A being the least common subtype. Neither the HIV status, ART use, nor the duration thereof had any association with the intrinsic subtypes.

C**onclusion**

HIV-positive patients present with breast cancer at a younger age and later-stage disease, when unadjusted for age and ethnicity, than HIV-negative patients. Neither the duration of HIV infection nor ART use was associated with clinicopathological characteristics and PAM50 intrinsic subtypes of breast cancer. However, HIV infection was significantly associated with worse overall and disease-free survival. Patients on ART had better overall and disease-free survival and with ART now being made accessible to everyone, the overall survival of women with HIV and breast cancer is expected to improve. According to PAM50 intrinsic subtypes, our cohort had aggressive intrinsic subtypes, known to be associated with poor breast cancer outcomes but this remains to be confirmed with further processing and completion of the study.

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CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

1.1.1. Epidemiology

The incidence of reported cancer has increased globally over recent years, with an estimated 18 million new cases in 2018[1]. According to Globocan, in 2018, there were over 2 million reported new breast cancer cases globally[1]. In general, breast cancer is the second commonest malignancy after lung cancer but the most common malignancy among women with an estimated incidence rate of 24.2%[1]. In South Africa, it is the commonest malignancy among females, with an estimated incidence rate of 22.58% of all cancers[2]. Regarding the cumulative lifetime risk, 1 in 25 women is reported to be at risk of developing in their lifetime, before the age of 74, however, this risk varies with race, with the lifetime risk being lower among black women (1 in 49)[2].

There is an interplay of various factors in the aetio-genesis of breast cancer, including genetics, patients, and environmental factors. It is estimated that almost 25% of breast cancer patients have a family history of breast cancer[3,4]. The likelihood of hereditary breast cancer increases when more family members are affected, especially at a younger age[3,4]. *BRCA 1/2* gene carriers are at higher risk of developing breast cancer, having a lifetime accumulative risk of about 65-81% and 45-80%, respectively[3]. Breast cancer development is also associated with other rare genetic disorders such as Li-Fraumeni syndrome, Peutz-Jeghers syndrome, Cowden Syndrome and hereditary diffuse gastric cancer [3,4].

In terms of patient factors, female gender and increasing age are known as non-modifiable risk factors for breast cancer. Concerning race, Ashkenazi Jewish descendants have been reported to have a higher risk of *BRCA1/ 2* gene mutations

compared to other ethnicities[4]. The patient's reproductive history also contributes to breast cancer development. For example, a woman having her first child at age 35 has a 5% greater risk to develop breast cancer than a woman who had her first baby in her 20s[3,4]. Moreover, nulliparous women are also at risk due to prolonged duration of uninterrupted oestrogen levels[3-5]. Similarly, women who had early menarche (< age 13) and late age of first birth or delayed menopause are a higher risk for breast cancer[3].

The oestrogen hormone plays a significant role in breast cancer development, through initiating and promoting the progression of breast carcinogenesis by its metabolites[6]. Exogenous oestrogen has a reversible effect on breast carcinogenesis, with the risk being higher among current users and those who have been on hormone replacement therapy for longer than five years[7]. Other hormones that may contribute to breast cancer development are high serum androstenedione and testosterone levels while progesterone has not been shown to play any role [5,8,9]. The body mass index directly correlates with the concentration of circulating oestradiol, oestrone and androgens among premenopausal women[5]. Breastfeeding has a protective effect against breast cancer development, with almost 4% reduction rate for every year of breast feeding[3]. Other risk factors include women with dense breast tissue on imaging and those who had chest wall irradiation at a younger age, previous history of breast cancer, proliferative breast disease with atypia[4].

1.1.2 Breast cancer intrinsic subtypes

Biologically, breast cancer is a heterogeneously distinct disease that arises from two distinct types of epithelial cells: the luminal and basal epithelial (non-luminal) cells[10]. In 1999, Perou and colleagues identified and described the pattern of breast epithelial cells genetic expression using the cDNA microarrays[11]. They observed that breast tumours vary greatly in their gene expression patterns and vary in behaviour patterns concerning their response to treatment and clinical outcome[11]. Tumours could be grouped according to genetic expression patterns using hierarchical clustering algorithms, to give forth the intrinsic breast cancer subtypes[11]. Four intrinsic subtypes of breast cancer have been identified and described as follows:

- Oestrogen positive/luminal-like
- ERBB-2 positive (HER-2 enriched)
- Basal-like
- Normal-like subtypes

The distinct intrinsic subtypes provide both prognostic and predictive information beyond that offered by clinico-pathological characteristics[12,13]. In early-stage, node-negative, ER+ and HER-2 negative breast cancer, the intrinsic subtype can risk-stratify patients, thereby assisting in selecting which patients should receive adjuvant chemotherapy and which ones can be adequately treated with adjuvant hormonal therapy alone[13,14]. Moreover, these intrinsic subtypes can also predict breast cancer recurrence risk, both loco-regionally and at distant sites, with each intrinsic subtype having a preferential distant site for metastasis[13,14].

1.1.2.1 *Oestrogen positive/luminal-like subtypes*

The oestrogen positive breast cancer subtype makes up 75% of all breast cancers and is predominant among post-menopausal and Caucasian women[14]. It is characterised by overexpression of genes from luminal epithelial cells and deletion of chromosomes 16q and 8p, 22 or both, and the gain of chromosome 1q and 8q, 16p or both[10, 15]. Furthermore, it is associated with fewer *TP 53* gene mutations when compared to the non-luminal subtypes[10]. Interestingly, it was noted that this subtype could further be sub-categorised into two distinct subgroups, the Luminal A and B subtypes, with the latter expressing low-moderate luminal specific genes and more proliferative genes compared to the former[10,12,15] leading to five intrinsic subtypes, namely:

- Luminal A intrinsic subtype
- Luminal B intrinsic subtype
- ERBB-2 positive intrinsic subtype
- Basal-like subtype
- Normal-like subtype

It was also demonstrated that Luminal B-subtypes could be further sub-divided into two clinically distinct subtypes with different clinical behaviour and outcome (Luminal B and C) and shares similar genetic expressions with the basal-like and ERBB-2 subtypes[12]. Concerning tumour behaviour and clinical outcome, the luminal-like (ER +) subtypes have favourable clinicopathological characteristics (small tumours, node-negative and low grade) when compared to ER-negative breast cancer subtypes[14]. It has a low recurrence rate with an estimated 2% rate of long-term recurrence and affecting bones rather than visceral organs[14].

1.1.2.2 *Oestrogen negative/non-luminal subtypes*

The non-luminal subtypes (ER-negative) are characterised by overexpression of genes from the basal epithelial cells[10] and are further sub-divided into basal-like and ERBB-2 positive subtypes. The ERRB-2 positive (HER-2 enriched) subtype accounts for about 20% of breast cancers and is characterised by high expression of erbb2/HER2-related genes and *TP53* mutations[10,11,15]. It is associated with aggressive biology and poorer clinicopathological outcome features, younger age, larger tumours with lymph node involvement, increased nuclear grade and negative hormone receptors[14]. It has an increased risk for loco-regional and distant site recurrence[16]. More than 50% of patients with this subtype will develop central nervous system metastases during their disease[16].

The basal-like/ triple-negative breast cancer subtype is characterised by ER-/PR-, HER 2- tumours with expressions of genes from myoepithelial cells (basal cytokeratins)[10, 15]. It is reported to have mutation in the *TP53* gene, a gene associated with poor breast cancer prognosis[10, 15]. It makes up15% of all breast cancers with a high prevalence among young patients, African-Americans and patients with *BRCA 1* gene mutations[14]. It is associated with worse disease-free and overall survival, with a higher risk for distant-site recurrence, with lungs and central nervous system being commonly affected18[14,16-18]. In the normal breast-like subtype, genes are usually expressed by adipose tissues and other non-epithelial cell types.[10]

Several predictive tools have been created to detect the biomarkers of breast cancer and determine its intrinsic subtypes. These tools may look at a specific protein

expressed on tumour cells (for example, protein-based immunohistochemistry) or at a molecular level by assessing the gene amplification (for example, fluorescence *in situ* hybridisation) or gene transcription (for example, reverse transcription-polymerase chain reaction)[19].

1.1.3 Intrinsic subtypes by immunohistochemistry

Due to the easy accessibility and availability of immunohistochemistry (IHC) compared to gene expression assays, immunohistochemical surrogates were defined using breast cancer hormone receptors (oestrogen and progesterone), HER-2 overexpression and the proliferative marker, Ki-67, as shown in Table 1.1 below[20].

Table 1.1: Breast cancer intrinsic subtypes by genetic/molecular assays and immunohistochemistry[20]

Intrinsic subtypes: Genetic/molecular assays	Immunohistochemical (IHC) surrogates
Luminal A	Luminal A ER/PR +, HER-2, Ki 67 < 14%,
Luminal B	Luminal B (HER-2 negative) ER +, PR +/-, HER-2 -, Ki 67 > 20% Luminal B (HER-2 positive) ER +, PR +/-, HER-2 +, any ki67
HER-2 enriched (ERRB-2)	HER-2 enriched ER-, PR-, HER-2 +
Basal-like	Triple-negative ER -, PR-, HER-2 -, any Ki 67
Normal-like	

The agreement between the intrinsic subtypes by genetic/molecular assays and immunohistochemical surrogates were described as follows:[21]

- Luminal A: 73-100%

- Luminal B: 73-100% (Ki 67 cut off >20%, as defined by St Gallens (2011)[20]
- HER-2 enriched: 41-69%
- Basal-like/TNBC: 80%

An Allred score is used to quantify the percentage and intensity of nuclear positivity of breast cancer cells positive for oestrogen and progesterone receptors to determine the status of the IHC surrogates[22]. The sum of the scores for both percentages of positive cells and intensity of nuclear positivity decides if the test is positive or negative, as shown below:

- Score 0-2: Negative
- Score 3-8: Positive

Table 1.2: Allred score: oestrogen and progesterone receptor analysis[22]

Percentage (%) of positive cells	Proportion score	Intensity	Intensity score	Percentages
0	0	None	0	< 1%
<1	1	Weak	1	1-25%
1-10	2	Intermediate	2	>25-75%
11-33	3	Strong	3	>75%
34-66	4			
>=67	5			

HER-2 testing can be done either by immunohistochemistry (IHC) where the percentage of positive breast cancer cells is observed and estimated[22], or by *in situ* hybridisation (ISH) where HER-2 genes copies are assessed to determine whether they are amplified or not. ISH can be done using fluorescent *in situ* hybridisation (FISH) or silver-enhanced *in situ* hybridisation (SISH)[22]. Both IHC and ISH may give negative, equivocal and positive HER-2 results.

- HER-2 testing results by IHC:
    - Negative: No staining or faint cell membrane staining in <10% of cancer cells.
    - Negative (score 1+): Minimal membrane staining in >10% of cancer cells.
    - Equivocal (score 2+):
        - Faint circumferential membrane staining in >10% of cancer cells or
        - Intense circumferential membrane staining of ≤10% of cancer cells.
        - Further tests are recommended using *in situ* hybridisation for results
    - Positive (score 3+): Intense membrane staining in >10% of cancer cells
- HER-2 testing results by *in situ* hybridisation (FISH/ SISH) depends on the average number of HER-2 signals seen:
    - Negative (no protein amplification): <4 copies
    - Equivocal: 4-6 copies detected.
        - In this case, further testing is recommended either by IHC or an alternative ISH test
    - Positive (protein amplification): ≥6 copies

Ki 67 is one of the proliferative markers first described in1983 by Gerdes and colleagues when they produced a monoclonal Ki 67 antibody[23]. It was found to only be present in proliferating cells and not in resting cells, which led to the conclusion that it may be useful as a tool to assess the proliferation status of malignant cells[23]. It is detected by the MIB 1 antibody. Based on the proportion and extent of nuclear staining, a Ki 67 score is given[24]. Currently, there is some controversy about the cut-off point for low and high Ki 67 due to high levels of inter-laboratory variation in the analysis. St Gallen's panel recommended a cut off for low Ki 67 of <14%, as correlated by PAM 50 molecular assays, and a high Ki 67 being >20%[25]. However, other panel members

advocated a lower cut off for high Ki 67 and proposed consideration of local laboratory-specific cut-offs or the use of multi-gene assays if available[25]. Bustreo and colleagues (2016) correlated the disease-free interval and disease-specific survival between breast cancers with Ki 67 <14%, 14-20% and >20% and found no difference between cancers with Ki 67 <14% and those with Ki 67 14-20%. They, therefore, suggested that a Ki 67of 20% should be the cut-off for a high Ki 67[26].

1.1.4. Intrinsic subtypes by genetic/molecular assays

Of the gene list described by Perou and colleagues (1999), 50 genes were found as a reasonable number to be used to identify the intrinsic subtypes accurately[11, 27]. A 50-gene molecular classifier, Prediction Analysis of Micro-array (PAM50), was developed and validated to determine the intrinsic subtypes of breast cancer[28]. This classifier was adapted and is currently performed using the nCounter Analysis System, Nanostring Prosigna assay, which has obtained a CE mark and FDA clearance as a breast cancer signature assay[24]. The Nanostring Prosigna assay is a quantitative real-time reverse transcriptase PCR (qRT-PCR)-based assay which measures the expression of 50 cancer-related genes and eight reference genes on RNAs extracted from formalin-fixed paraffin-embedded breast cancer specimens, thereby enabling it's use in a decentralised clinical laboratory setting[29-30].

The 50 genes assessed are dependent on the expression of oestrogen-related and proliferation-related genes. The genes are: *UBE2T, PTTG1, PGR, MKI67, MIA, MAPT, KRT17, KRT14, KIF2C, ESR1, CCNE1, ENPF, CEP55, FGFR4, MMP11, SFRP1, TMEM45B, TYMS, ERBB2, CDCA1, BCL2, CCNB1, CDC20, NAT1, ORC6L, RRM2, UBE2C, ACTR3B, ANLN, BAG1, BIRC5, BLVRA, CDC6, CDH3, CXXC5, EGFR, EXO1, FOXA1, FOXC1, GPR160, GRB7, KNTC2, KRT5, MDM2, MELK, MLPH, MYBL2, MYC, PHGDH* and *SLC39A6*. The eight reference genes are *TRFC, ACTB, MRPL19, PSMC4, PUM1, RPLP0, SF3A1, GUSB*[32]. The function of all the genes is listed in Appendix 5. Expressed genes are grouped based on the similarity in their expression pattern. Genes with similar expressions are grouped adjacent to each other using the hierarchical clustering algorithm to give the following intrinsic subtypes: Luminal A and

B, HER-2 enriched and basal-like (TNBC) subtypes[15]. Table 1.3 below shows which cluster of genes are for luminal and non-luminal breast cancer subtypes.

Table 1.3: Genes for various breast cancer subtypes[33]

	GENES
Luminal breast cancer	*ESR1* *TMEM 45B* *BAG1* *GPR160* *FOXA1* *BLVRA* *PGR* *MAPT* *BCL2* *ACTR3B* *MYC* *MLPH* *NAT1* *SLC3946* *MDM2* *CXXC5* *MMPII*
HER-2 / ERBB-2 enriched	*ERBB2* *GRB7* *FGFR4*
Basal-like/ TNBC	*SFRP1* *EGFR* *FOXC1* *CDH3* *KRT14* *KRT17* *PHGDH* *MIA* *KRT5*

Proliferative genes	*ANLN* *UBE27* *BIRC5* *ORC6L* *CDC6* *MYBL2* *CCNB1* *CEP55* *KIF2C* *MKI67* *PTTG1* *MELKEXO1* *TYM5* *UBE2C* *RRM2*

In line with the requirements of the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) initiative, before a molecular assay can be of clinical use, trials must be conducted to assess the analytical and clinical validity as well as the clinical utility of the molecular assay[34]. The analytical validity of the molecular assays describes the performance and reproducibility of the test, taking into consideration the sensitivity, specificity and robustness of the test[34, 35]. The robustness of the Nanostring Prosigna assay and the reproducibility of its results in various laboratories, including the clinical laboratory setting, were validated[28]. The assay was shown to be a simple and easy to follow laboratory diagnostic test[28]. The clinical validity was also assessed and validated on patients on the Anastrozole, Tamoxifen, Alone or in Combination (ATAC) trial and obtained Level 1 evidence for clinical validity[36]. The clinical utility of the PAM 50 assay was validated by several studies and proven to be effective and useful in the clinical setting[29, 31].

In addition to intrinsic subtypes, the PAM50 assay also gives a risk of recurrence score, which risk-stratifies patients according to the possibility for both loco-regional and

distant recurrence, thereby helping in adjuvant therapy decision-making[29, 37]. This aspect will be expanded on more in Chapter 4.

1.2. Human Immunodeficiency Virus (HIV)

HIV remains a worldwide epidemic, with almost 38 million people reported to be living with the disease globally in 2018, an increase from 2000 when almost 25 million people were living with HIV (PLWHIV)[38]. Worldwide some 23.3 million PLWHIV (62%) were reported to be receiving ART[38]. The accessibility and usefulness of ART are evidenced by a significant decrease in HIV-related mortality, with a reported 33% decline from the HIV-related mortality rate reported in 2010[38].

Sub-Saharan Africa carries the most significant burden of HIV, with 25.5 million (67.8% of the global population) people residing in the region[38]. South Africa carries the largest burden, with 7.52 million PLWHIV in the country[38]. However, this reported number could be explained by the significant progress made by South Africa in getting people to test for HIV[39]. In South Africa, the reported prevalence of HIV is estimated to be 13.1% and reported to be 19% in adults aged 15-49 years[39]. To date, South Africa has the most extensive ART programme in the world[38,39]. In 2018, 62% of PLWHIV in South Africa were on ART, and 52% of them were virally suppressed[38,39]. The number of PLWHIV on ART is expected to rise following the implementation of the Universal Test and Treat strategy (UTT) in September 2016, which allows all PLWHIV to receive treatment, regardless of their CD4 count and HIV clinical stage[40].

There are six classes of antiretroviral drugs for HIV that disrupt various life cycles of the virus. The drugs include nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), fusion inhibitors (FI), integrase inhibitors (II), protease inhibitors (PI) and chemokine receptor antagonists (CRA)[41,42]. The standard first-line regime currently in use consists of two NRTIs (tenofivir disoprostil fumarate (TDF), emtricitabine (FTC)/lamivudine (3TC) and an NNRTI (efavirenz (EFV)[41]. NRTIs were the first class of ART to be approved by the FDA and are competitive inhibitors of HIV-1 reverse transcriptase enzyme, which is involved in the

transcription of HIV RNA into cHIV DNA, the critical step needed for virus replication[42]. NNRTIs are a non-competitive inhibitor that directly binds HIV-1 reverse transcriptase enzyme and inhibiting its action[42]. After starting ART, the patient is monitored by measuring serum viral load (VL) at three months and then every six months[41]. The main treatment target is a suppressed and sustained viral load of fewer than 50 copies/ml within six months of initiating treatment[41].

1.3. Breast Cancer and HIV

HIV infection indirectly makes infected patients susceptible to develop certain malignancies by suppressing T-cell function[43,44]. It permits certain virally transmitted malignancies such as human papillomavirus-related (HPV) (anal, vaginal, cervical), hepatitis virus-related (hepatocellular carcinoma) and Epstein-Barr virus-related (EBV) (Hodgkin's and non-Hodgkin's lymphoma)[45]. About 30 – 40% of people living with HIV are at risk of having cancer in their lifetimes, however ARTs mitigate this risk[46]. Moreover, malignancies in people living with HIV are responsible for more than a third of mortality among these patients[47].The availability and use of ARTs have led to an improved longevity of PLWHIV[45]. While there has been an observed decrease in the prevalence of AIDS-defining malignancies, there has been a slow rise in the prevalence of non-AIDS defining malignancies (NADM), with a two-fold increased risk over the general population[45]. The risk is even greater with carcinomas associated with oncogenic viruses (HPV, EBV, HPB) and smoking (lung cancer)[45].

The association between breast cancer, a non-AIDS defining malignancy and HIV is still not fully understood. Molecular and genetic studies suggest a possible interaction between these two diseases when they occur concurrently; however, a causative link is still unknown[48]. Breast cancer and HIV share about 17 genes, ten of which were found to be overexpressed and seven under-expressed in both diseases, which may suggest that HIV infection may somehow alter breast cancer disease progression[48]. Some studies have postulated that HIV may promote breast cancer development and progression[48,49]. Some studies propose that HIV infection may protect against breast cancer development[50]. HIV infection of human breast cells affects the growth factors of breast cancer cells, thereby hindering their growth and multiplication[51]. HIV infection has

also been found to induce apoptosis of human breast cancer cells[52, 53]. This theory was supported by a mouse model, where mice were infected with a murine mammary tumour virus. Following kidney transplantation and administration of an immunosuppressant to these mice, breast cancer incidence was found to be reduced[54]. There is, however, still no conclusive direct link as to whether HIV infection protects, predisposes or alters the natural progression of breast cancer.

The true incidence of breast cancer among HIV-infected patients is currently unknown. Previous epidemiological studies reported a low incidence of breast cancer in HIV infected population[55]. In Tanzania, there was a reported decline in the incidence of breast cancer during the AIDS epidemic than previously[56]. Due to the co-incidence of the AIDS era and the decline in breast cancer incidence, it was suspected that the two might have an association; however, further studies were recommended to explore the relationship between these two diseases[56]. In France, Spano and colleagues (2012) demonstrated a low incidence of breast cancer compared to the general population and a much lower incidence than other NADMs[57]. In an epidemiological study in South Africa in the 2000s, Sitas and colleagues (2000) demonstrated that only 6.3% of patients with breast cancer were HIV positive, a lower incidence than in the general population[58]. As an NADM, the breast cancer incidence expected to rise among the PLWHIV[45]. Compared to other non-AIDS-defining malignancies, its incidence is reported to be lower than other NADMS but still higher than in the pre-ART era[45]. In sub-Saharan countries, the incidence varies between regions. In Southern Africa, it is estimated to be 11-32%, while in East and West Africa, it is estimated to be 11-14% and < 5%, respectively[59]. In South Africa, the studies done in the ART era showed no rise in the incidence of breast cancer among PLWHIV, with the reported incidence of 18%, which is similar to that of the general population[60,61]. There were similar findings in Brazil and Italy[62, 63]. However, two other South African studies done in the ART era reported an increased incidence of breast cancer, 32.3%(Durban, Kwa-Zulu Natal) and 37.98%(Ga-Rankuwa, Gauteng), respectively[64, 65]. It should be noted that the latter study had about 50% of their patients with unknown HIV status[65].

Surgical treatment among HIV positive with breast cancer women is the same as for HIV negative women with breast cancer, as studies have shown similar surgical outcomes between the two groups[61, 66]. Caution is required concerning chemotherapeutic agents among HIV positive patients due to known interactions between them and ART. In a case-matched study, HIV-infected patients with breast cancer treated with chemotherapy experienced adverse effects when HIV negative patients did not (55% vs 30%), mainly due to myelosuppression[53]. El-Rayes and colleagues (2002) showed that the incidence of myelosuppression with chemotherapy is increased in the HIV-infected population, probably due to inadequate bone marrow reserves and ARV-induced myelosuppression such as that induced by Zidovudine[67]. Doxorubicin-based and low-dose weekly taxane regimens are known to be associated with myelosuppression[64, 67]. It has also been demonstrated that HIV infection is an independent predictor for chemotherapy-induced neutropaenia[64]. HIV positive patients with breast cancer had an almost 2-fold increase in neutropenia development than the HIV negative patients[64]. Moreover, the cytotoxic chemotherapeutic agents may also lead to further immunosuppression, facilitating progression and worsening HIV infection[68].

Several studies have shown that HIV does not have any impact on the pathological characteristics of breast cancer[60,61,64,65]. Moreover, there was no difference found in the immune-histochemical surrogates among HIV positive negative patients with breast cancer[60,65]. The effects of HIV and HIV treatment on the molecular-based intrinsic subtypes have not yet been studied. This study hypothesised that HIV positive women with breast cancer would have a more aggressive tumour phenotype than HIV negative patients when the molecular assay, and not just immunohistochemical surrogates, is used. Our aim was to determine whether HIV infection is associated with aggressive tumour biology on a cohort of female breast cancer patients managed at Charlotte Maxeke Johannesburg Academic Hospital and Chris Hani Baragwanath Academic Hospital. Our objectives were as follows:

- **Primary Objectives**
  - To compare tumour biology and clinical profile between the HIV negative and positive patients
  - To compare gene expression profiles of HIV negative and positive patients using the PAM50 intrinsic subtyping assay.
  - To compare the survival outcomes between HIV positive & HIV negative patients with breast cancer.

- **Secondary Objective**
  - To determine if there is an association of intrinsic tumour subtype with any local or distant recurrence and site of recurrence.

CHAPTER 2

METHODS and MATERIALS

2.1. Methodology

2.1.1. Study design

This was a prospective, observational & descriptive study and a sub-study of the South African Breast Cancer and HIV Outcome (SABCHO) study[69]. Our study participants were managed at two Gauteng based breast units, the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) Surgical Breast Unit and Batho Pele Breast Unit of the Chris Hani Baragwanath Academic Hospital (CHBAH), from 15 May 2015 to 31 September 2017. These two breast units diagnoses and manage about 250 & 350 patients, respectively, of diagnosed with breast cancer yearly[69]. Moreover, these two study sites utilize the electronic breast cancer database to capture the clinical, radiological, histo-pathological information and management of all patients newly diagnosed with breast cancer.

2.1.2 Patient selection and data collection

All female patients newly diagnosed with invasive breast cancer and were 18 years and older, were asked to take part in the study and consent was obtained for their participation. Patients with unknown HIV status were counselled about HIV and were tested for HIV after they have given an informed consent for HIV testing. Patients who refused to consent to participate in the study or test for HIV were excluded from the study. All patients newly diagnosed with HIV were counselled and had blood samples drawn for CD4 counts and HIV viral load analysis. These patients were then referred to HIV clinic to start ARTs before the treatment of breast cancer, in line with South Africa's

universal test and treat policy[40]. Both the year of HIV diagnosis and ART initiation were documented.

The American Joint Committee on Cancer (AJCC) system was used for clinical staging of the breast cancer[70]. Patients were further classified into early (Stage I/II) and late (Stage III/IV) stage disease. The following histo-pathological characteristics were documented: histological diagnosis, tumour grade, oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the proliferative marker, Ki 67. To determine the positivity of both oestrogen and progesterone receptors, the Allred score was used [22]. The breast tumours were classified through immunohistochemistry into four intrinsic subtypes: Luminal A (ER +, PR+, HER2 -, Ki 67 <14%); Luminal B (ER+ PR +/- and HER2 negative with Ki 67% ≥14% or HER2 positive with any Ki 67); HER2 positive subtype (ER and PR negative, HER2 positive); and triple-negative breast cancer (TNBC) (ER, PR, and HER2 negative)[20]. The tumour grading was determined through the modified Bloom and Richardson grading system, which assesses tubule formation, mitosis and nuclear pleomorphism to give the score, as follows:[71]

- A score of ≤5: Grade 1 (well-differentiated) tumour,
- A score of 6-7: Grade 2 (moderately differentiated) tumour,
- A Score of 8-9: Grade 3 (poorly differentiated) tumour[71]

Following the completion of prescribed breast cancer treatment, patients were followed up at three- to six- month intervals. If follow-up was missed, patients were called by the research navigators. Patients who could not follow up for more than 9 months or their status could not be ascertained via the 'Verify ID' portal were regarded as being lost-to-follow up. Completion of chemotherapy and radiation treatment was defined as patients who received the full prescribed number of doses. The first line chemotherapy regime included: Adriamycin (Doxorubicin), Cyclophosphamide and Taxanes (AC-T). At the time of the study, Trastuzumab was not yet available at these two study sites. Overall survival was defined as survival from the date of breast cancer diagnosis to the date of death for any cause while disease free survival was defined as survival from the date of breast cancer diagnosis to the date of disease recurrence or death for any cause.

Patients with disease recurrence included all patients with radiologically and histologically confirmed breast cancer recurrence. Patients with stage IV disease at presentation were excluded in the analysis of disease-free survival. The site of disease recurrence was sub-classified into loco-regional (ipsilateral chest wall, ipsilateral breast following breast conserving surgery and/ or regional lymph nodes) and distant metastases (visceral and non-visceral sites). The time to and site of disease recurrence were documented. For patients who died, the date of death, as documented in the medical record or as provided by the next-of-kin, was also documented.

An electronic breast cancer database was used to extract the following data: demographics, clinical stage at presentation, co-morbidities, level of education and the status of the patient when last seen (alive with no evidence of disease, alive with stable disease, alive with disease progression or dead).

2.1.3 Tissue processing, RNA extraction and PAM 50 analysis

In preparation for the RNA isolation and PAM 50 intrinsic subtype analysis, 400, age matched, FFPE specimens of histologically confirmed invasive carcinoma were selected, 200 each from HIV positive and negative patients. An excision specimen (mastectomy or wide local excision specimens) was used for patients who had primary surgery, but a core biopsy specimen was used for patients who received primary chemotherapy. The methodology for tissue processing, RNA extraction and PAM 50 analysis is described in full in Chapter 4.

2.2 Data Analysis

HIV status was used to categorise patients as HIV positive, negative or unknown. Demographic and clinicopathological data were categorised with comparisons done between HIV positive and negative patients using χ^2 tests. Viral load was categorised as either detectable (>50 copies/ml) or below detectable limits (≤50 copies/ml). Age, CD4 count and viral load (when detectable) were non-normally distributed and were thus represented as medians and inter-quartile ranges (IQRs) and compared using a Kruskal-Wallis test. Generalised linear models with binary outcomes and a log link function were used to determine prevalence ratios for non-missing variables to assess

the relationship between stage at diagnosis, tumour grade, IHC4 subtype and the presence or absence of metastatic disease with HIV status controlling for age at diagnosis (continuous) and ethnicity (black vs non-black). Collected data were analysed using STATA v12.1.

For survival analysis, demographic and clinico-pathological data were represented as medians and interquartile ranges for continuous data or as frequencies and percentages for categorical data. Categorical comparisons between HIV positive and HIV negative patients were performed using a χ^2 test; age and body mass index (BMI) were compared using a Mann-Whitney test. Patients were censored on the last date they were known to be alive with an administrative censoring date of 31 July 2020 for those still alive at the time of data extraction. Patients censored before the 31 July 2020 were lost to follow up if they could not follow up for more than 9 months and their status could not be ascertained via the 'Verify ID' portal. Kaplan-Meier survival curves were generated assuming non-informative losses to follow-up; p-values were calculated using a log-rank test of equality. Hazard ratios (HRs) and their 95% confidence intervals (CIs) were estimated using Cox regression models. Both unadjusted (crude) HRs and manually adjusted HRs (adjusted for age at diagnosis (continuous), BMI (continuous), stage at diagnosis (2 categories), receptor subtype (4 categories), employment status (2 categories) and education (3 categories)) were calculated. Logistic regression analyses, adjusting for age at diagnosis (2 categories), stage at diagnosis (2 categories) and receptor subtype (4 categories), was used to calculate odds ratios for disease recurrence or death. Survival data was analysed using STATA v14.2 and the stset suite of commands

The RNA dataset was analysed using the RUO version of the NanoString RUO PAM50 algorithm to determine each sample's molecular subtype. The RUO PAM50 algorithm measures the geometric mean of eight housekeeping genes (HK geomean) to ensure RNA quality. Each sample meeting the QC threshold was reported as one of the four molecular subtypes, Luminal A, Luminal B, Her2 Enriched and Basal-like. Subtypes were determined using the PAM50 classification algorithm, which measures the

expressed 50 genes, eight housekeeping genes used for signal normalisation, six positive control and eight negative controls. Fisher's exact test evaluated an association between the PAM50 subtypes and categorical variables. Statistical analyses were conducted using the SAS System, and a p-value <0.05 was considered statistically significant. The study obtained ethics clearance from the Human Research Ethics Committee (Medical) at the University of Witwatersrand, with the clearance number: M161130.

CHAPTER 3: PAPER 1

CLINICO-PATHOLOGICAL CHARACTERISTICS AMONG SOUTH AFRICAN WOMEN WITH BREAST CANCER RECEIVING ANTI-RETROVIRAL THERAPY FOR HIV

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ABSTRACT

PURPOSE

Breast cancer is the most common cancer in women and a leading cause of cancer-related mortality worldwide. South Africa has the largest global burden of HIV infection and the largest anti-retroviral treatment (ART) program. This study aimed to analyse the association of HIV and ART use with breast cancer clinico-pathological characteristics.

METHODS

Study participants were females, newly diagnosed from May 2015 through September 2017 with invasive breast cancer at two academic Surgical Breast Units in Johannesburg, South Africa at the Charlotte Maxeke Johannesburg Academic Hospital and Chris Hani Baragwanath Academic Hospital. We compared HIV-positive and HIV negative patients' demographic and clinical-pathological characteristics at the time of breast cancer diagnosis.

RESULTS

Of 1050 patients enrolled, 1016 (96.8%) had known HIV status, with 226 (22.2%) being HIV positive. HIV positive patients were younger (median (IQR) age 45 (40-52) years), than HIV-negative patients (median (IQR) age 57 (46-67)) ($p<0.001$). HIV positive patients were more likely to be diagnosed with late-stage breast cancer($p=0.01$). However, HIV positive patients receiving ART at the time of breast cancer diagnosis were less likely to present with metastatic disease than those not on ART ($p=0.05$).

CONCLUSION

HIV-positive patients present with breast cancer at a younger age and later stage disease, when undajusted for age and ethnicity, than HIV-negative patients. Neither the duration of HIV infection nor ART use was associated with clinico-pathological characteristics of breast cancer.

KEYWORDS: BREAST CANCER HUMAN IMMUNODEFICIENCY VIRUS (HIV) ANTI-RETROVIRAL THERAPY (ART)

INTRODUCTION

Breast cancer is the most common malignancy and the leading cause of cancer-related deaths among women worldwide[1]. For at least two decades, reported incidence rates of breast cancer have increased worldwide and now account for 24.2% of all cancers and 15% of cancer-related deaths among women[1]. In South Africa, breast cancer is the most common female malignancy, accounting for about 22% of all malignancies[2]. It has an age-standardised rate of 33.35 per 100 000 population with a lifetime risk (before the age of 74) of 1 in 27 women[2].

South Africans carry 20% of the global HIV burden[3], with 15% of new HIV infections and 11% of AIDS related deaths[3]. From 2002 through 2016, the total number of persons living with HIV in South Africa increased from 4 million to 7.03 million[4]. The prevalence of HIV in the entire population is 12.8%, which is even higher in adults aged 15-49 years (19.1%)[5]. The prevalence of HIV among people above 50 years of age has also increased over the years, with 80% of them residing in low-middle income countries[6,7].

In 2016, South Africa was reported to have the largest anti-retroviral treatment (ART) program in the world. More than 50% of people living with HIV (PLWH) are receiving treatment and about 45% of those on treatment have a viral load below detectable levels[3]. The number of those on treatment is expected to increase due to the change of the treatment guidelines, recommending ART in all patients diagnosed with HIV regardless of the CD 4 count or clinical stage of the disease[8]. The availability and effectiveness of ART has led to a decrease in HIV-associated mortality and has prolonged the life-expectancy of people living with HIV[5]. A standard fixed dose combination (FDC) is currently being used as a first line regime and consists of 2 nucleoside reverse transcriptase inhibitors (NRTI), tenofivir disoprostil fumarate (TDF) & emtricitabine (FTC)/lamivudine(3TC) and a non-nucleoside reverse transcriptase inhibitor (NNRTI), efavirenz (EFV)[8].

HIV is not an oncogenic virus but rather a permissive virus which indirectly predisposes infected patients to the development of certain malignancies through suppression of T-cell function [9,10]. An estimated 30-40% of HIV infected patients are expected to have cancer in their lifetimes[11], although the risk is mitigated by the use of ART. Malignancies may account for more than one-third of deaths among patients living with HIV[12].

In the past few years, several studies have explored associations between HIV infection and breast cancer. Molecular and genetic studies have demonstrated possible interaction between HIV and breast cancer, however, there is no proven direct link between them[13]. Some studies suggest HIV infection may modify breast cancer growth and progression while other studies have postulated that HIV may be protective against the development and growth of breast cancer[13].

This study aims to investigate the association of HIV infection and ART, with the clinico-pathological presentation of breast cancer in a South African urban female population with known high HIV prevalence.

PATIENTS AND METHODS

This observational, descriptive study draws on data from the South African Breast Cancer and HIV Outcome (SABCHO), a cohort of breast cancer patients diagnosed and treated at five hospitals in Gauteng and KwaZulu Natal, South Africa. Our study participants were diagnosed at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) Surgical Breast Unit and the Batho Pele Breast Unit of the Chris Hani Baragwanath Academic Hospital (CHBAH), from 15 May 2015 through 31 September 2017. Both are public hospitals based in an urban setting that serve the socio-economically disadvantaged majority of the population. The CMJAH Surgical Breast Unit is based in central Johannesburg and diagnoses about 250 patients with breast cancer yearly. The Batho Pele Breast Unit serves patients from Soweto and surrounding areas and diagnoses about 350 patients with breast cancer yearly[14].

All consenting female patients aged 18 years and older, newly diagnosed with invasive breast cancer, were enrolled on the study. Patients who were HIV-unknown were counselled about HIV and were asked to give informed consent for testing. Patients newly diagnosed with HIV were offered post-test counselling. All HIV positive were asked to provide blood samples for CD 4 counts and HIV viral load testing. We recorded the time span since HIV diagnosis, as per the first positive HIV serological test recorded on the National Health Laboratory system, and initiation of ART prior to histologically confirmed breast cancer diagnosis. HIV positive patients not yet on ART were sent to the HIV/ART clinic for initiation of ART prior to cancer treatment.

Our clinical staging of breast cancer followed the American Joint Committee on Cancer (AJCC) system[15]. We also categorised patients into early (Stage I/II) and late (Stage III/IV) stage disease. Pre-treatment pathology reporting included the histological diagnosis, tumour subtype, grade, oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki 67. The Allred score was calculated from the intensity and proportion scores for oestrogen and progesterone receptors; specimens scored 3-8 were regarded as hormone receptor positive[16]. HER2 was regarded as positive when the test showed 3+ and negative when it was 1+. A HER2 score of 2+ was regarded as equivocal, and HER2 positive result was confirmed using in situ hybridization (FISH or SISH) [16]. Ki 67 is a nuclear antigen used as a marker of cell proliferation; we categorized specimens in which < 14% of cells expressed Ki67 as having low expression, as per the St Gallen 2011 guidelines[17,18]. The breast tumours were categorized based on IHC 4 subtype into: Luminal A [ER and/or PR positive, HER2 negative, Ki 67 < 14%]; Luminal B [ER and/or PR positive and HER2 negative

with Ki 67% ≥ 14% OR HER2 positive with any Ki 67]; HER2 positive subtype [ER and PR negative, HER2 positive]; and triple negative breast cancer (TNBC) [ER, PR, and HER2 negative][18].The Modified Bloom & Richardson grading system was used which is based on tubule formation, mitosis, and nuclear pleomorphism; a score of ≤ 5 denoted a Grade 1 (well differentiated) tumour, 6-7 a Grade 2 (moderately differentiated) tumour, and 8-9 a Grade 3 (poorly differentiated) tumor[19].

Patients were categorised as HIV positive, HIV negative or HIV unknown. Only 3.2% of the patients were HIV unknown and were excluded from further analysis. Demographic and clinico-pathological data were categorised and comparisons between HIV positive and HIV negative patients performed using χ^2 tests. We obtained CD 4 counts and HIV viral loads for the HIV-infected patients from the South African National Health Laboratory Service (NHLS) (www.nhls.ac.za) using results of tests performed closest to the date of breast cancer diagnosis. Viral load was categorised as either detectable (>50 copies/ml) or below detectable limits (≤50 copies/ml). Age, CD 4 count, and viral load (when detectable) were non-normally distributed and were thus represented as medians and inter-quartile ranges (IQRs) and compared using a Kruskal-Wallis test. Generalised linear models with binary outcomes and a log link function were used to determine prevalence ratios for non-missing variables to assess the relationship between stage at diagnosis, tumour grade, IHC4 subtype and presence or absence of metastatic disease with HIV status controlling for age at diagnosis (continuous) and ethnicity (black vs non-black). Collected data were analysed using STATA v12.1.

RESULTS

Of the 1050 patients newly diagnosed with invasive breast cancer enrolled in the study, 34 had unknown HIV status and were excluded from the analysis. The demographic and clinico-pathological characteristics of 1016 patients with and without HIV are shown in Table 1. Of these, 226 (22.2%) were HIV positive, 855 (84.2%) patients were self-reported as black. The median (interquartile range, IQR) age at diagnosis of those analysed was 54 (IQR 44-64) years, and 560 (55.1%) were diagnosed at late-stage disease (stage III/IV).

HIV positive patients were younger at diagnosis (median age 45 (IQR 40-52) years) than HIV negative ones (57 (IQR 46-67) years, $p<0.001$). They were also more likely to be self-reported as being black ($p<0.001$) and to be diagnosed at a late stage of breast cancer ($p=0.02$) than HIV negative patients (Table 1). However, in a model adjusted for age and ethnicity, tumour stage was not associated with HIV status (Table 2). IHC 4 breast cancer subtype and tumour grade did not differ between HIV positive and HIV negative patients, with or without adjustment for age and ethnicity, except in the unadjusted comparison within subgroups of Luminal B (Table 2).

Table 1 –Demographic and clinico-pathological characteristics of patients with known HIV status* at breast cancer diagnosis.

	Total n (%)	**HIV positive** n (%)	**HIV negative** n (%)	**p value****
Total***	1016 (100%)	226 (22.2%)	790 (77.8%)	
Age at Breast Cancer Diagnosis				
20-39	145 (14.3%)	56 (24.8%)	89 (11.3%)	
40-49	258 (25.4%)	103 (45.6%)	155 (19.6%)	
50-59	236 (23.2%)	43 (19.0%)	193 (24.4%)	
60-69	202 (19.9%)	19 (8.4%)	183 (23.2%)	
70-79	127 (12.5%)	4 (1.8%)	123 (15.6%)	
≥80	48 (4.7%)	1 (0.4%)	47 (6.0%)	
Age at Breast Cancer Diagnosis (median (IQR))	54 (44-64)	45 (40-52)	57 (46-67)	<0.001‡
Ethnicity				
Black	855 (84.2%)	219 (97.3%)	636 (80.6%)	<0.00001
White	82 (8.1%)	1 (0.4%)	81 (10.3%)	
Coloured	60 (5.9%)	5 (2.2%)	55 (7.0%)	
Asian	17 (1.7%)	0	17 (2.2%)	
Stage at Diagnosis				
Stage I	62 (6.2%)	6 (2.7%)	56 (7.2%)	
Stage II	384 (38.2%)	77 (34.2%)	307 (39.3%)	
Stage III	401 (40.1%)	103 (45.8%)	298 (38.2%)	
Stage IV	159 (15.5%)	39 (17.3%)	120 (15.4%)	
Early Stage	446 (43.9%)	83 (36.9%)	363 (46.5%)	0.01
Late Stage	560 (55.1%)	142 (63.1%)	418 (53.5%)	
Metastasis				
No known metastasis	887 (84.5%)	186 (82.3%)	669 (84.7%)	0.39
Metastasis	163 (15.5%)	40 (17.7%)	121 (15.3%)	
Site of Metastasis				
Visceral	70 (45.5%)	20 (54.1%)	49 (42.6%)	0.17
Non-visceral	43 (27.9%)	6 (16.2%)	37 (32.2%)	
Visceral and non-visceral	41 (26.6%)	11 (29.7%)	29 (25.2%)	
Tumour grade at diagnosis				

1	57 (5.8%)	11 (5.2%)	44 (5.9%)	0.34
2	496 (50.2%)	116 (54.5%)	363 (48.8%)	
3	435 (44.0%)	86 (40.4%)	337 (45.3%)	
ER status at diagnosis				
Positive	769 (75.6%)	162 (74.0%)	580 (75.9%)	0.56
Negative	248 (24.4%)	57 (26.0%)	184 (24.1%)	
PR status at diagnosis				
Positive	661 (65.1%)	137 (62.8%)	500 (65.5%)	0.46
Negative	354 (34.8%)	81 (37.2%)	263 (34.5%)	
Her2 status at diagnosis				
Positive	257 (25.6%)	65 (30.1%)	179 (23.8%)	0.06†
Negative	738 (73.6%)	149 (69.0%)	570 (75.7%)	
Ki67 status at diagnosis				
<14%	164 (16.4%)	30 (14.0%)	127 (16.8%)	0.31
≥14%	838 (83.6%)	185 (86.1%)	627 (83.2%)	
IHC4 subtype at diagnosis				
Luminal A	136 (13.8%)	23 (10.9%)	107 (14.4%)	0.47
Luminal B	639 (64.9%)	138 (65.1%)	481 (64.8%)	
Her2-positive	59 (6.0%)	13 (6.1%)	43 (5.8%)	
Triple Negative	151 (15.3%)	38 (17.9%)	111 (15.0%)	
Luminal B (ER+/PR+; Her2-; Ki67≥14%)	432 (69.8%)	86 (62.3%)	346 (71.9%)	
Luminal B (ER+/PR+; Her2+)	187 (30.2%)	52 (37.7%)	135 (28.1%)	

*34 patients (3.2%) had unknown HIV status and were excluded from the analysis.
**Chi square test or ‡Mann Whitney test comparing HIV positive to HIV negative proportions for non-missing values.
***Of 1016 patients, 10 (1.1%) were missing stage at diagnosis; 2 (0.2% were missing ethnicity; 62 (6.1%) (n=62) were missing tumour grade; 33 (3.2%) were missing ER status; 35 (3.4%) were missing PR status; 47 (4.6%) were missing Her2 status; 48 (4.7%) were missing Ki67 status; 65 (6.4%) were missing IHC4 subtype. Nine of 163 patients with known metastatic disease (5.5%) were missing the site of metastasis.
†Comparison for positive and negative HER2 values only.

Table 2 - Prevalence ratios (PRs) of HIV by breast cancer stage, grade, metastatic disease status, and IHC subtype.

	Unadjusted PR	p-value	Adjusted PR*	p-value
Stage at Diagnosis				
Early Stage	1 (Ref)		1 (Ref)	
Late Stage	1.36 (1.07-1.73)	0.01	1.12 (0.90-1.39)	0.31
Metastatic disease				
No metastasis	1 (Ref)		1 (Ref)	
At least 1 known site of metastasis	1.14 (0.85 - 1.54)	0.38	1.26 (0.96 - 1.65)	0.10
Tumour grade at diagnosis				
1	1 (Ref)		1 (Ref)	
2	1.21 (0.70 - 2.11)	0.49	1.27 (0.79 - 2.04)	0.33
3	1.01 (0.58 - 1.78)	0.96	1.00 (0.61 - 1.63)	0.99
IHC4 subtypes at diagnosis				
Luminal A	1 (Ref)		1 (Ref)	
Luminal B	1.26 (0.85 - 1.88)	0.26	0.88 (0.61 - 1.27)	0.49
Her2-positive	1.31 (0.72 - 2.40)	0.38	1.00 (0.58 - 1.70)	0.99
Triple Negative	1.44 (0.91 - 2.29)	0.12	1.10 (0.73 - 1.66)	0.64
Luminal B (ER+/PR+; Her2-; Ki67≥14%)	1 (Ref)		1 (Ref)	
Luminal B (ER+/PR+; Her2+)	1.40 (1.04 - 1.88)	0.03	1.19 (0.91 - 1.57)	0.21

*Adjusted for age (linear) and ethnicity (black vs non-black)

Patients with HIV

Of the 226 patients with HIV infection, 129 (57.1%) knew their HIV status at the time of their breast cancer diagnosis. The duration (median (IQR) of known HIV status prior to breast cancer diagnosis was 4 years. (0-9) years).

The HIV-infected patients had a median (IQR) CD 4 count of 477 (287-670) cells/mm^3 and 59.4% of patients on ARTs had a viral load below detectable levels (i.e. viral load below 50 copies/ml). Among patients with detectable viral loads, the median (IQR) viral load was 4757.5 (268.5-58609.5) copies/ml. The duration of seropositivity and ART use, CD 4 cell count and viral load were not associated with the clinical stage and pathological characteristics of the breast cancer (Tables 3 & 5; Figure 1). Patients not on ARTs were diagnosed at a later stage than those on ARTs; the difference was of marginal statistical significance (Table 4). Patients who had been diagnosed with HIV more than a year prior to their breast cancer diagnosis, and those on ART had lower viral loads and higher CD4 cell counts than other patients.

Table 3 - Clinical characteristics of HIV positive patients by time since HIV diagnosis.

	Less than or equal to 1 year (n=68)	More than 1 year (n=158)	P-value*
Stage at BC diagnosis			
Early Stage	26 (38.2%)	57 (36.3%)	0.78
Late Stage	42 (61.8%)	100 (63.7%)	
Metastatic disease			
No metastasis	51 (75%)	135 (85.4%)	0.06
Metastasis	17 (25%)	23 (14.6%)	
IHC4 subtypes at diagnosis			
Luminal A	8 (12.5%)	15 (10.1%)	0.51‡
Luminal B	45 (70.3%)	93 (62.8%)	
Her2-positive	3 (4.7%)	10 (6.8%)	
Triple Negative	8 (12.5%)	30 (20.3%)	
Luminal B (ER+/PR+; Her2-; Ki67≥14%)	29 (64.4%)	57 (61.3%)	0.72
Luminal B (ER+/PR+; Her2+)	16 (35.6%)	36 (38.7%)	
Viral Load			
Detectable	46 (74.2%)	44 (33.6%)	<0.001
Below detectable levels	16 (25.8%)	87 (66.4%)	
CD 4 count (Median (IQR))	362 (236 - 616)	495.5 (337.5 - 687.5)	0.003#
Viral load when detectable (median (IQR))	32500 (2000-84000)	738 (135.5 - 6755)	<0.001#

* Chi square test, ‡Fisher's exact test and #Mann Whitney test comparing duration of HIV positivity.

Table 4- Clinical characteristics of HIV positive patients by anti-retroviral (ART) use at breast cancer diagnosis

	Not on ART (n=63) **	On ART (n=160) **	P-value*
Stage at BC diagnosis			
Early Stage	18 (28.6%)	65 (40.9%)	0.09
Late Stage	45 (71.4%)	94 (59.1%)	
Metastatic disease			
No metastasis	47 (74.6%)	137 (85.6%)	0.05
Metastasis	16 (25.4%)	23 (14.4%)	
IHC4 subtypes at diagnosis			
Luminal A	5 (8.3%)	18 (12.0%)	0.74‡
Luminal B	42 (70.0%)	94 (62.7%)	
Her2-positive	4 (6.7%)	9 (6.0%)	
Triple Negative	9 (15.0%)	29 (19.3%)	
Luminal B (ER+/PR+; Her2-; Ki67≥14%)	28 (66.7%)	57 (60.6%)	0.50
Luminal B (ER+/PR+; Her2+)	14 (33.3%)	37 (39.4%)	

* Chi square test, ‡Fisher's exact test and #Mann Whitney test comparing use of ART.

** ART use missing for 3 HIV positive patients (1.3%).

Table 5- Clinical characteristics of HIV positive patients by duration of anti-retroviral therapy (ART) at breast cancer diagnosis

	Less than or equal to 1 year (n=30) **	More than 1 year (n=130) **	P-value*
Stage at BC diagnosis			
Early Stage	13 (43.3%)	52 (40.3%)	0.76
Late Stage	17 (56.7%)	77 (59.7%)	
Metastatic disease			
No metastasis	23 (76.7%)	114 (87.7%)	0.12
Metastasis	7 (23.3%)	16 (12.3%)	
IHC4 subtypes at diagnosis			
Luminal A	5 (17.2%)	13 (10.7%)	0.19‡
Luminal B	21 (72.4%)	73 (60.3%)	
Her2-positive	1 (3.5%)	8 (6.6%)	
Triple Negative	2 (6.9%)	27 (22.3%)	
Luminal B (ER+/PR+; Her2-; Ki67≥14%)	15 (71.4%)	42 (57.5%)	0.25
Luminal B (ER+/PR+; Her2+)	6 (28.6%)	31 (42.5%)	

* Chi square test, ‡Fisher's exact test and #Mann Whitney test comparing duration of ART.

** Duration of ART missing for 66 HIV positive patients (29.2%).

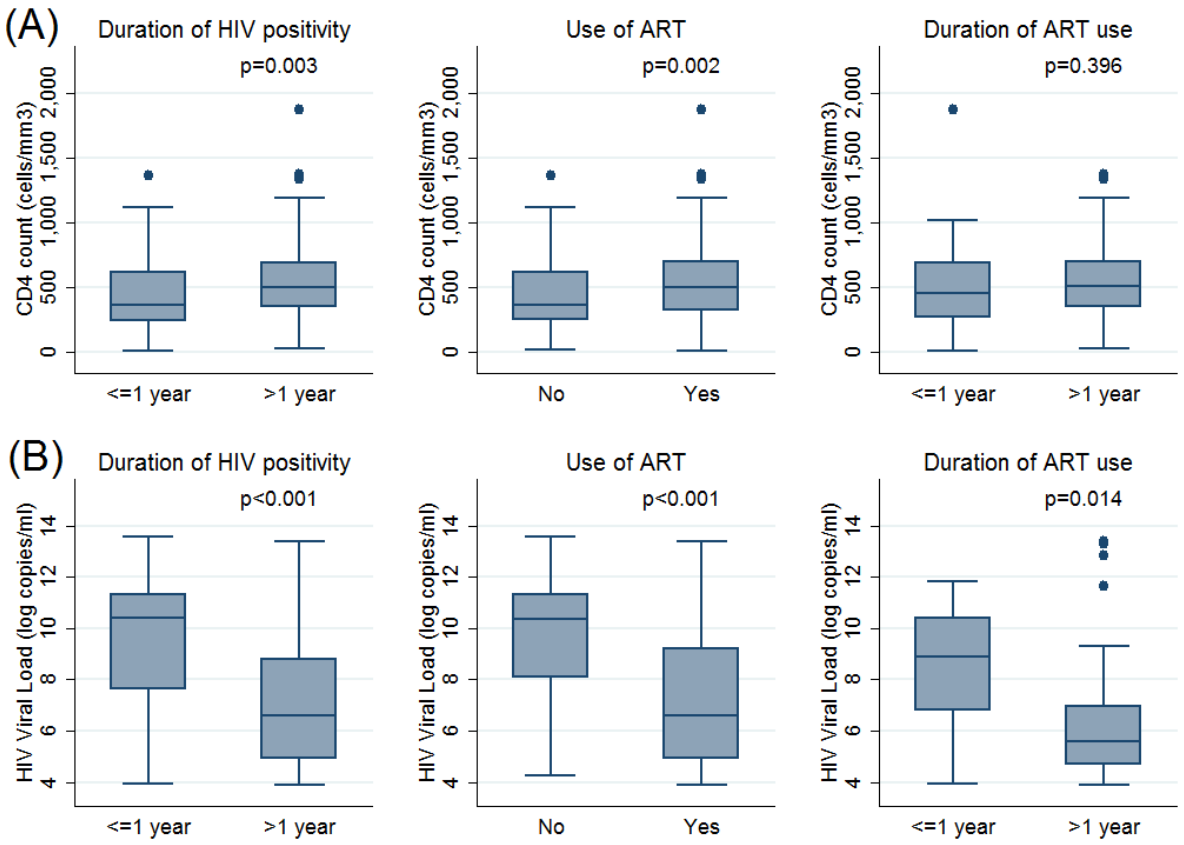

Figure 1: CD 4 count and HIV viral load. Box and whisker plots of CD 4 cell counts (cells/mm$^{3)}$, and HIV viral loads (log copies/ml) categorized by duration of HIV seropositivity, use of ARTs and the duration of ART use. Boxes represent interquartile ranges with the median line drawn. Outliers are shown as dots. p-values were calculated using the Mann Whitney test.

DISCUSSION

Patients in low-and middle-income countries diagnosed with breast cancer have been reported to present with more advanced disease (Stage III/IV) than patients in high income countries[20-22]. Both patient-dependent (low socio-economic status, lack of awareness of breast cancer, patients' belief system) and health care system-dependent (travelling distance to the treating hospital, number of health facilities visited prior to the treating hospital) factors have been found to play a significant role in late presentation[20-22]. Moreover, low level of education and visiting more than 2 health care facilities before breast cancer diagnosis were shown to contribute to the advanced disease stage at the time of diagnosis[22]. In several countries of sub-Saharan Africa, more than 75% of patients have stage III/IV disease at diagnosis[20,23], compared to less than 20% in high income countries[24]. In this study, 55.1% of patients presented with stage III/IV disease,

which is lower than other countries in sub-Saharan Africa. This finding could be explained by the fact that the study was done on an urban population which has good access to health care. Moreover, the study sites operate open-access clinics, whereby not only patients referred from other health professionals are seen, but self-referred patients and 'walk-in' patients are also seen on the day of presentation. It has been shown that a system that only allows referral of patients from another health professional and referral secondary hospitals may create a barrier to time to diagnose and treat as there may be delay in getting an appointment, patients may need to be seen by multiple health professionals before the diagnosis is made and the patient may thus incur added costs[22,25]. This may lead to some patients being discouraged along the process, even reaching the breast specialists for diagnosis and treatment. Though at the time of the study, there were no formal national screening programmes in place, but an extensive breast cancer awareness programmes offered by various non-governmental organisations. The South African National Department of Health has recently instituted a Breast Cancer Policy recommending routine Clinical Breast Examination (CBE) at Primary Health Care (PHC) level[26].

More than 75% of breast tumours in the current study were oestrogen receptor positive, the histo-pathological parameter associated with a favourable clinical outcome and responsiveness to adjuvant hormonal therapy[27]. The Luminal B subtype was predominant. The human epidermal growth factor (HER2) oncogene was positive in 24.4% of cases, a larger proportion than reported in other populations (18-20%)[27]. HER2 positive result in breast cancer is associated with aggressive clinico-pathological outcome[27]. In areas whereby HER2 targeted therapy is not available, as was the case in this cohort, the overall survival is worse than for other cancer subtypes[276]. However, the HER2 targeted therapy has recently become available in the adjuvant setting for patients with HER2 positive breast cancer, in the Public Hospitals in South Africa

As a non-AIDS defining malignancy, the incidence of breast cancer in people living with HIV is expected to rise in the era of anti-retroviral therapy[28]. The accessibility and effectiveness of ART has led to an improved life expectancy of people living with HIV, a decline in the incidence of AIDS-defining malignancies but a steady increase in the incidence of non-AIDS defining malignancies[28-29]. There have been conflicting findings in the literature about the true incidence of breast cancer in HIV positive patients. In the current study, the prevalence of breast cancer in HIV positive patients was similar to the general population. Previous studies have shown similar findings[30,31]. On the contrary, two South African studies conducted in the era of ART have demonstrated a higher incidence of breast cancer in HIV positive patients[31,32], however, one of these studies indicated that about 50% of the population studied had unknown HIV status[32].

Among the HIV positive patients in the current study, breast cancer was diagnosed at a younger age than among HIV negative patients. This finding was demonstrated by

several other studies as well[29-35]. However, when age-stratified, in patients younger than 50 years of age there was no difference in the median age of presentation between the HIV positive and HIV negative patients. Recent studies show that HIV prevalence in women younger than 50 is declining but steadily increasing among women older than 50 [6,36], with one study showing more than half of adults tested sero-converting after the age of 50[36]. This finding indicates the need for prospective studies to investigate the association of HIV and breast cancer specifically in women older than 50 where central adiposity associated both with obesity and prolonged ART may influence breast cancer treatment outcomes.

In this study, immunosuppression was not severe among our HIV infected patients; their median CD 4 cell count was 477 cells/mm^3. Similar counts have been reported in other studies, such as that by Shaaban *et al.,* who found a median CD4 count of 410 cells/mm^3 [29-38]. The level of CD 4 count was not associated with the stage at the diagnosis, tumour grade or the tumour subtype found in other studies [29,30].

The duration of HIV sero-positivity was not associated with the pathological characteristics of breast cancer. Patients diagnosed with breast cancer within 1 year of HIV diagnosis were more likely to have metastases than those diagnosed more than a year previously, but the difference was only marginally statistically significant. In a model adjusted for age and ethnicity, HIV positive and HIV negative patients did not differ in tumour stage at presentation, IHC 4 breast cancer subtype, or tumour grade. Several other studies have also demonstrated no difference between the two groups in terms of staging and pathological characteristics of the tumour[29,30,39]. In Uganda, HIV positive patients were found to be diagnosed with cancer at an earlier stage than HIV negative patients[40], perhaps because they were already participating in the health care system for HIV treatment providing opportunity for incidental identification of their breast cancer symptoms.

15.5% of our patients had metastatic disease at diagnosis. We may have missed other patients with metastatic breast cancer because not all patients with metastatic disease present to the Surgical Breast Units. Moreover, our patients with breast cancer have a staging CT scan only if they have a suspicious chest x-ray and/or liver ultrasound. However, we have no reason to think that the missed patients were more or less likely to be infected with HIV than those included in our sample.

More than 70% of HIV positive patients were on ART at the time of diagnosis, and more than 50% had a viral load below the detectable level. Patients who were not on ART at the time of breast cancer diagnosis were referred to the HIV/ART clinic to be initiated on ART before starting cancer treatment. The use of ART did not affect the clinico-pathological characteristics of breast cancer even though there was a trend shown among those not on ARTs to present with metastatic disease.

The limitations of this study include the possible bias in detecting metastasis because not all patients diagnosed with breast cancer receive a staging CT scan at the study sites and some patients with metastatic disease may be referred directly to Medical Oncology Unit, bypassing the Surgical Breast Units completely. Future studies might recruit patients with metastatic breast cancer diagnosed in Medical Oncology Unit or detected in other units of the participating hospitals, including the HIV clinics. Finally, although the duration of HIV seropositivity and ART use within 1 year of breast cancer diagnosis were not associated with the clinical-pathological characteristics of breast cancer, it would be interesting to see if there is any effect within 6 months of HIV diagnosis and initiation of ART on the outcome of breast cancer. We did not have information regarding the month, only year, of HIV diagnosis or ART initiation in our patient cohort. Furthermore, we did not have the data on the co-existence of the co-morbidities and co-infection as well as the treatment outcome of our patients. However, the latter has been considered for future research projects.

CONCLUSION

HIV-positive patients present with breast cancer at a younger age and later stage disease, when unadjusted for age and ethnicity, than HIV-negative patients. The use of ART did not affect the clinico-pathological characteristics of breast cancer even though there was a trend shown among those not on ARTs to present with metastatic disease.

DECLARATION OF INTEREST: NONE

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ETHICS CLEARANCE

Ethics clearance for this study was obtained from the Human Research Ethics Committee (Medical) at the University of Witwatersrand (clearance number: M161130 and M150351).

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CHAPTER 4:

PAM50 INTRINSIC SUBTYPES IN HIV POSITIVE AND NEGATIVE PATIENTS WITH BREAST CANCER - PRELIMINARY RESULTS AND OTHER MOLECULAR ASSAYS USED FOR BREAST CANCER

4.1 Introduction

About 80% of breast cancers have positive oestrogen receptors and only some of them will require chemotherapy as part of their treatment. Some of these patients may be adequately managed with adjuvant hormonal therapy without the added risk for distant recurrence in the first ten years of breast cancer diagnosis[72]. The risk for distant site recurrence, therefore, determines if adjuvant chemotherapy should be given or not[73].As a result, risk-stratifying these patients by their likelihood to develop distant recurrence is critical in treatment decision-making. Traditionally, clinicopathological characteristics such as the patient's age, tumour size, nodal status, tumour grading and presence of lympho-vascular invasion were used[74]. However, with a continued understanding of the complexity and heterogeneity of breast cancer, it was later noticed that the intrinsic subtypes of breast cancer are more prognostic and predictive than the traditional methods[74]. Several molecular assays have been identified and validated to help with the risk stratification of patients with ESBC, oestrogen receptor-positive, HER-2 negative and node-negative breast cancer. The immunohistochemical surrogates were also described to provide the immunohistochemical-based intrinsic subtyping, as discussed extensively in Chapter 1.

The breast cancer molecular assays depend on the expression of oestrogen, and proliferation-related genes and their roles may, therefore, be limited in oestrogen negative (non-luminal) breast cancer subtypes[75]. As mentioned previously, the assays provide more prognostic and predictive information beyond that given by traditional methods, thereby playing a critical role in managing early-stage breast cancer[74]. They do so by providing a distinct intrinsic subtype of breast cancer and risk-stratifying

patients based on the degree of breast cancer recurrence at distant sites[74,75]. Moreover, they can predict which patients will benefit from adjuvant chemotherapy thereby sparing patients who would not benefit from it the unnecessary side-effects associated with chemotherapy[74].

Before the molecular assay can be of clinical use, it must be critically evaluated in line with the evaluation components described by the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group, an independent panel up of a multidisciplinary team of experts in cancer management[34]. These evaluation components were established to guide all aspects of molecular assay evaluation, thereby minimising conflicts of interests and enhancing transparency and accountability as far as molecular assays and their use are concerned[34]. The components are listed in Figure 4.1.

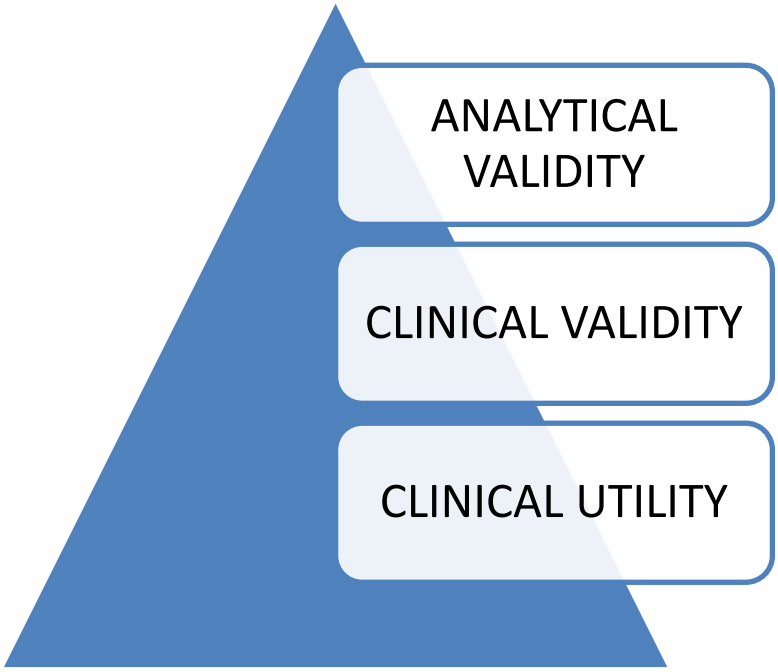


Figure 4.1: Components of evaluation for the clinical use of molecular assays[34].

- Analytical validity
    - The analytical validity of the molecular assays describes the performance and reproducibility of the test. It also takes into consideration the sensitivity, specificity and robustness of the test[34,35].
- Clinical validity
    - The clinical validity refers to the assay's ability to predict the outcome of the disease concerned accurately[34].
- Clinical utility
    - The clinical utility of the assay refers to the effectiveness and usefulness of the test for clinical use.

In 2011, it was recommended that ethical and legal implications of the assay of interest needed to be incorporated in the criteria mentioned above[35].

Of the many molecular assays available, there are four commercial and FDA-approved in clinical use. These are the PAM50 assay, Oncotype DX, MammaPrint, EndoPredict and Breast Cancer Index (BCI). Several trials have been undertaken to validate their robustness and confirm clinical validity and utility. They have now been endorsed and incorporated in AJCC 8th TNM staging[70], ASCO therapy guidelines for early-stage breast cancer[76], NCCN clinical practice guidelines[4], ESMO clinical practice guidelines[77] and the St Gallens' consensus panel guidelines[25]. In addition to the evaluation components described above, the economic value of these tests was also determined.

4.2. Nanostring Prosigna Assay (PAM50 assay)

The ability of the PAM50 assay to prognosticate and predict the possibility of recurrence exceeds that provided by the traditional clinicopathological characteristics of breast cancer[37]. Through this assay, the following information is obtained: intrinsic subtypes, risk of recurrence (ROR) score, which can risk-stratify patients into low, intermediate and high risk of distant recurrence[31]. The ROR score is a by-product of the 50 genes

assessed by the PAM50 assay with the addition of the clinical tumour size[73]. The risk of recurrence cut-offs in risk-stratifying patients are as follows[37]:

- Low risk: ROR ≤40
- Intermediate risk: ROR 41 – 60, pN0
- High risk: ROR 41 – 60, pN1 or ROR >60

The technical performance, robustness and reproducibility of the results offered by the Nanostring Prosigna assay on FFPE breast cancer samples were assessed and found to be more prognostic in both node-negative and positive disease when compared to clinicopathological prognostic factors[29]. It was shown to be a simple, easy to follow assay, which can be conducted in various laboratories, including the clinical laboratory setting[29]. For first time users, it is strongly recommended for them to undergo training before use[28]. The clinical validity of the Nanostring Prosigna assay was also assessed and validated on patients who were on the ATAC trial. This trial compared the safety and effectiveness of anastrozole with tamoxifen given daily for five years in post-menopausal women with early-stage breast cancer (ESBC)[78]. The prognostic ability and value of the ROR score were evaluated in this population. The ability of the ROR score to predict the risk of distant recurrence after adjuvant hormonal therapy was compared with that of the recurrence score (RS) by Oncotype DX and Immunohistochemistry IHC-4[73]. It was found that the ROR score added more prognostic information than that given by the clinical treatment score, RS and IHC-4 in both node-negative and positive, HER-2 negative EBC disease[73]. It was further shown that the ROR score could risk-stratify patients into intermediate and high-risk for recurrence much better than the RS, the valuable information which may assist in selecting patients who may benefit from systematic cytotoxic chemotherapy.[73] Gnant and colleagues (2013) reported similar findings and provided Level 1 evidence for the clinical validity of PAM50 ROR in predicting the risk of recurrence in post-menopausal women[79]. The validity was assessed on post-menopausal women enrolled for the ADCSG-8 trial. They found that the addition of the ROR score and the ROR-based risk groups added significant prognostic information to the clinical and pathological parameters[79]. Using

ROR, the estimated ten years distant-recurrence free survival per risk group was found to be:

- Low risk: 96.7%
- Intermediate risk: 91.3%
- High risk: 79.9%

Several studies have proved and validated the PAM50 assay to be effective and useful in the clinical setting. Among the EBC, node-negative, ER +/HER-2 breast cancer patients, ROR was able to identify accurately the low-risk patients who could be adequately treated with adjuvant hormonal therapy only[29]. Among the high-risk group, PAM50 was able to discriminate between those who would and would not respond well to chemotherapy by intrinsic subtype[29]. Similarly, among the low-risk group, the PAM50 assay could predict those who may be observed only without any adjuvant hormonal therapy. This group of patients still had an excellent breast-cancer specific survival of 96.3% after 15 years of follow-up[37]. It further showed some patients in the intermediate risk group who may be treated with adjuvant hormonal therapy only without any impact in survival compared to the low-risk group[37]. Not only can the PAM50 assay predict the effectiveness of the adjuvant chemotherapy but also predict the efficacy of neoadjuvant chemotherapy with an estimated negative predictive value for a complete pathological response of more than 97%[31]. Moreover, the PAM50 ROR was able to predict which patients with EBC, ER +/ HER-2-, node-positive breast cancer may be safely treated with adjuvant hormonal therapy only as well as those who may benefit from chemotherapy[30]. Lastly, the Nanostring Prosigna PAM50 assay was cost-effective when compared to current clinical practice and other molecular assays[80].

4.2.1. PAM50 intrinsic subtypes in HIV positive and negative patients with breast cancer: Preliminary results

4.2.1.1 *Background*

Even though the Nanostring Prosigna assay (PAM50) has been validated in western countries, it has not yet been tested in our setting. No studies have been done to looking at the impact of HIV infection on the molecular assay-based intrinsic subtypes of

breast cancer. As a result, we conducted an observational, descriptive study to compare gene expression profiles of HIV negative and positive patients using the PAM50 intrinsic subtyping assay to determine if there is an association of PAM50 intrinsic subtypes with any local or distant recurrence and site of recurrence. As far as we can determine, the present study is the first to report on the impact of HIV on the PAM50-based intrinsic subtypes. We hypothesised that HIV positive women with breast cancer would have a more aggressive tumour phenotype than HIV negative patients.

4.2.1.2 Methodology

4.2.1.2.1 Tissue selection

Four hundred (400), age matched patients newly diagnosed with breast cancer were selected, 200 HIV positive & 200 HIV negative patients. Their personal details & hospital numbers were submitted to the histo-pathologist to help with the retrieval of the FFPE breast tissue blocks. For patients who had primary surgery, excision specimens (mastectomy or wide local excision specimen) were requested while for those who had primary chemotherapy, core-biopsy specimens were requested.

4.2.1.2.2 Tissue processing

FFPE breast tissue blocks, confirmed by histo-pathologist to be invasive breast cancer, were retrieved from the NHLS archives. The histo-pathologist examined a haematoxylin and eosin (H&E) stained slide and marked the area of invasive breast cancer suitable for the test. The tumour surface area (TSA) (mm^2) was measured on the slide, and this guided the number of unmarked slides to be used to get adequate extracted RNA concentrations (RNA >12.5ng/ul) and an acceptable A260/A280 purity ratio (1.7- 2.5), Table 4.1. As per the protocol, the minimum acceptable tumour surface area must be ≥4mm^2 with a tumour cellularity percentage of ≥10% to ensure sufficient tumour tissue for the test.

Table 4.1: Tumour surface area and the required number of unmarked slides

Measured tumour surface area (TSA) on H&E stained slide (mm^2)	**Number of unmarked slides (10 µm)**	**Number of unmarked slides (4 µm)**
4-19	6	
20-49	3	6
50-99	3	5
≥100	1	3

The selected unmarked slides underwent a deparaffinisation process under a fume hood, using xylene and absolute ethanol (Figure 4.2).

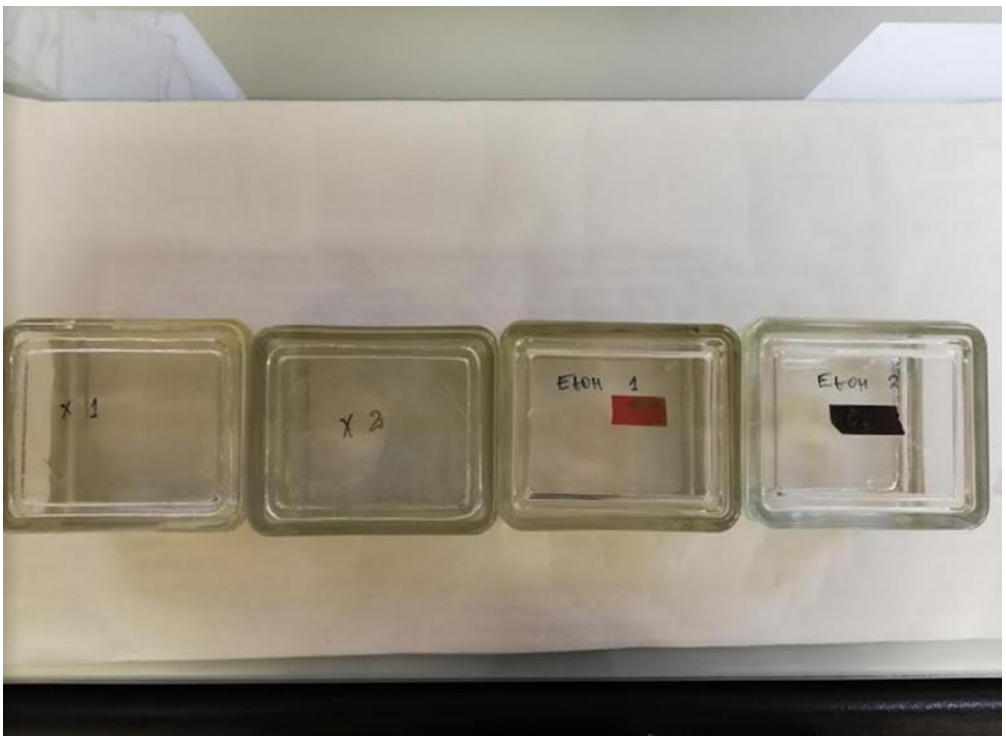


Figure 4.2: Deparaffinisation of formalin-fixed, paraffin embedded samples. Glass boxes containing xylene (X1 and X2) and absolute alcohol (EtOH 1 and 2).

The deparaffinised slides were removed from the alcohol and allowed to dry. The tumour was outlined and marked, corresponding to the tumour area marked by the pathologist (Figure 4.3).

Figure 4.3: Glass slides illustrating the outlining of the tumour, as guided by the slide marked by the pathologist (slide 8) No non-tumour area is shown on this slide

A minimum of 10µm thick slide-mounted tissue section was required. After outlining the tumour on the slides, they were briefly immersed in 3% glycerol for two minutes to rehydrate, after which they were removed, and excess glycerol drained off. For slides with both tumour and non-tumour areas, the non-tumour area was scraped off the slide first and transferred into a 1.5 ml microcentrifuge tube labelled ‘non-tumour’, followed by the scraping of the tumour area. For slides without a non-tumour area, the tumour area was scraped off and transferred into an appropriately labelled tube (Figure 4.3). Following macro-dissection, all microcentrifuge tubes were stored at -70°C.

Figure 4.4: Macro-dissection of tumour and non-tumour areas from the slides.

4.2.1.2.3 RNA extraction

We used the AllPrep® DNA/RNA FFPE kit, Qiagen (Germany) for extraction and purification of RNA from the FFPE. The yield and purity of the extracted RNA were assessed using the NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Inqaba Biotechnology). Only RNA samples with a concentration of RNA >12.5ng/ul and an A260/A280 purity ratio between 1.7- 2.5 were included in the downstream analysis, as required by the Prosigna protocol. The extracted RNA was stored at -80^{0}C until needed for analysis with the PAM 50 assay.

4.2.1.2.3 *PAM 50 assay analysis*

The extracted RNA was measured on the Nanostring nCounter Analysis System (Nanostring Technologies). This system measures the adequacy of each mRNA transcript of interest using a multiplexed hybridisation reaction and a digital readout of fluorescent barcodes hybridised to each transcript. A code set with probes specific for each of the genes in the PAM50 assay was hybridised overnight with 250ng RNA at 65^{0}C. The samples were further processed using the NanoString nCounter Prep Station and digital analyser. The dataset was analysed using the RUO version of the

NanoString RUO PAM50 algorithm to determine the molecular subtype of each sample. The RUO PAM50 algorithm measures the geometric mean of eight housekeeping genes (HK geomean) to ensure RNA quality. Each sample meeting the QC threshold was reported as one of the four molecular subtypes, Luminal A, Luminal B, Her2-enriched and Basal-like. Subtypes were determined using the PAM50 classification algorithm, which measures the level of expressed 50 genes, eight housekeeping genes used for signal normalisation, six positive and eight negative controls (Supplementary Table-Appendix 4).

The demographics, clinical stage at presentation, PAM 50 intrinsic subtypes, HIV status, and the patients' status (alive or dead) at last contact, were extracted from the electronic breast cancer data base. For HIV positive patients, the duration of HIV seropositivity, CD4 count and viral load and whether they were on ART or not were captured on the electronic breast database. The Fisher's exact test evaluated associations between the PAM50 subtypes and categorical variables, and associations between the PAM50 subtypes and ordinal variables were assessed using the Kruskal Wallis test. Statistical analyses were conducted using the SAS System, and a $p<0.05$ was considered statistically significant.

4.2.1.3 Results

A total of 400 samples were retrieved from the NHLS archives and underwent deparaffinisation and macro-dissection. RNA was extracted and 97.85% (365/373) of the extracted RNA met the acceptable concentration of >12.5 ng/ul and A260/A280 purity ratio of 1.7 – 2.5. However, while analysing the extracted RNAs, the nCounter Sprint Nanostring instrument broke down and could not be repaired. The Nanostring-trained technicians are based in the United States and could not come to South Africa due to the prohibition on international travel during the Covid-19 pandemic. As a result, only 144 samples, age-matched and equally split between the HIV positive and negative patients, could be analysed using the PAM50 assay.

The median age of this cohort was 46 years (41-53). At the time of breast cancer diagnosis, 57.6 % of them had advanced disease. Although it appeared as though HIV

positive patients were more likely to present with advanced disease (62.5% vs 52.8%), this difference was not statistically significant (p=0.238). The HER-2 enriched intrinsic subtype was the commonest, accounting for 32.6% of all subtypes and was associated with poor survival, along with basal-like subtype (Table 4.3 & figure 4.8). HIV status did not have an impact on the intrinsic subtypes (p=0.570; Table 4.2).

Table 4.2: Demographics, clinical characteristics and PAM50 intrinsic subtypes

		Overall	HIV Positive	HIV Negative	p-value
Age (n=144)		46 (41-53)	44 (39-50)	47.5 (42-54.5)	p=0.083
Stage (n=144)	**Stage 1**	5 (3.5%)	3 (4.2%)	2 (2.8%)	
	Stage 2	56 (38.9%)	24 (33.3%)	32 (44.4%)	
	Stage 3	67 (46.5%)	35 (48.6%)	32 (44.4%)	
	Stage 4	16 (11.1%)	10 (13.9%)	6 (8.3%)	p=0.479
	Early stage (I/II)	61 (42.4%)	27 (37.5%)	34 (47.2%)	
	Advanced stage (III/IV)	83 (57.6%)	45 (62.5%)	38 (52.8%)	p=0.238
PAM 50 Subtype (n=144)	**Luminal A**	24 (16.7%)	12 (16.7%)	12 (16.7%)	
	Luminal B	38 (26.4%)	17 (23.6%)	21 (29.2%)	
	Her2-enriched	47 (32.6%)	22 (30.6%)	25 (34.7%)	
	Basal-like	35 (24.3%)	21 (29.2%)	14 (19.4%)	p=0.570
#Status (n=142)	**Alive**	92 (64.8%)	41 (56.9%)	51 (72.9%)	
	Dead	50 (35.2%)	31 (43.1%)	19 (27.1%)	p=0.047
Disease progression (n=95)	**Yes**	32 (33.7%)	15 (34.1%)	17 (33.3%)	
	No	63 (66.3%)	29 (65.9%)	34 (66.7%)	p=0.938

Status as documented at the last known contact

All 144 samples met the required RNA quality for the test, with 143 samples meeting the specified QC threshold. The 50 genes analysed include *UBE2T, PTTG1, PGR, MKI67, MIA, MAPT, KRT17, KRT14, KIF2C, ESR1, CCNE1, ENPF, CEP55, FGFR4, MMP11, SFRP1, TMEM45B, TYMS, ERBB2, CDCA1, BCL2, CCNB1, CDC20, NAT1, ORC6L,*

RRM2, UBE2C, ACTR3B, ANLN, BAG1, BIRC5, BLVRA, CDC6, CDH3, CXXC5, EGFR, EXO1, FOXA1, FOXC1, GPR160, GRB7, KNTC2, KRT5, MDM2, MELK, MLPH, MYBL2, MYC, PHGDH and SLC39A6 (Supplementary Figure 1). Eight reference genes were also used and included *TRFC, ACTB, MRPL19, PSMC4, PUM1, RPLP0, SF3A1, GUSB* (Supplementary Figure 1). The hierarchical clustering algorithm (PAM 50 clustering algorithm) was used with genes grouped based on the similarity in the pattern of their expressions, with genes with similar expressions being grouped adjacent to each other (Figure 4.5, vertical axis). Similarly, the breast cancer samples analysed were grouped with samples showing similar expression patterns across all analysed genes placed next to each other by the hierarchical clustering algorithm (Figure 4.5, horizontal axis). By expressed genes, the intrinsic subtype was produced as follows: Luminal A, Luminal B, Her2-enriched and Basal-like (Figure 4.).

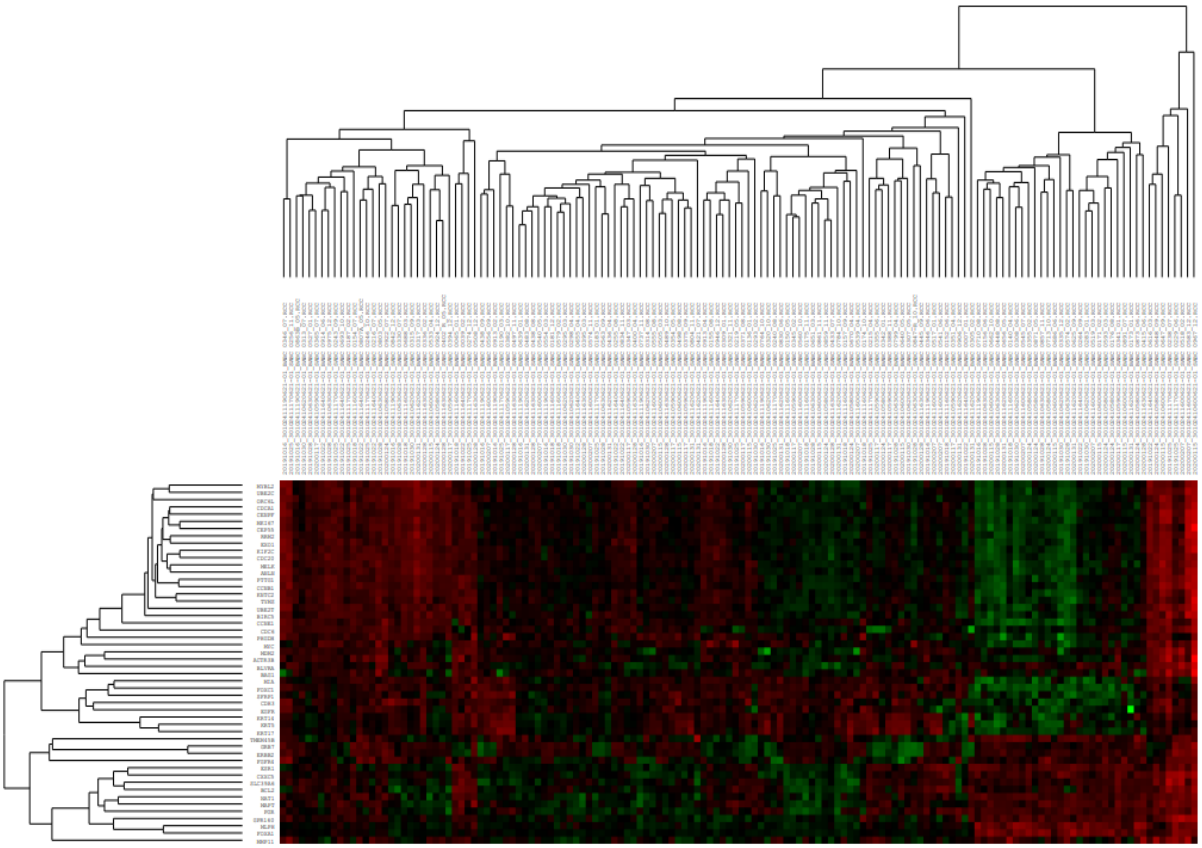
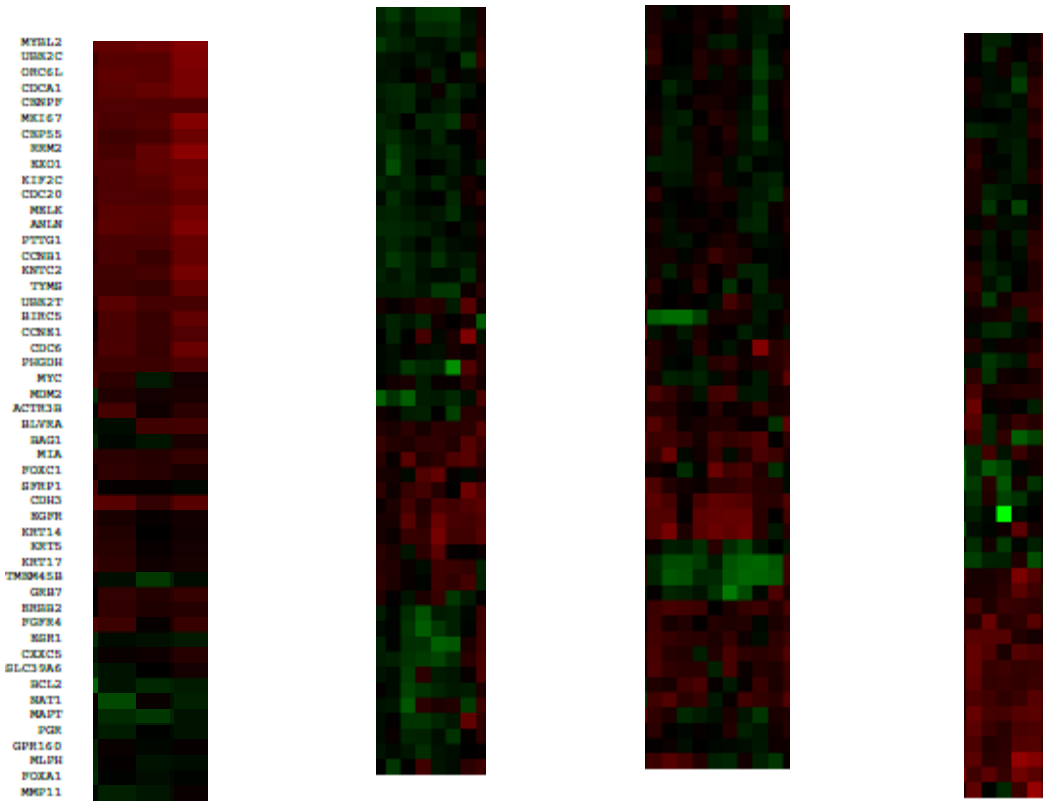


Figure 4.5: Hierarchical clustering of 50 genes analysed by the PAM50 Nanostring assay. Vertical axis: 50 genes analysed. Horizontal axis: Breast cancer samples analysed. Each colour represents the measured expression ratio, with the colour intensity being directly proportional to the magnitude of the gene expressed, as was initially described by Perou and colleagues (1999) [11]. Supplementary results for the results of all samples analysed in this cohort are attached as an appendix. (Supplementary Figure-Appendix 4).



A: Luminal A B: Luminal B C: HER-2 enriched D: Basal-like

Figure 4.7: PAM50 intrinsic subtypes

Among the HIV positive patients, the median CD4 count was 477(253-656) and 43.8% had a detectable viral load. HER-2 enriched, and basal-like intrinsic subtypes had an association with death that trended towards significance (p=0.08). However, the intrinsic subtype did not have any influence on the disease progression.

At the time of breast cancer diagnosis, 78.9% of the patients were already on ART, and 32.3% still had a detectable viral load. Patients not on ART were associated with an advanced stage at presentation (p=0.011). There was no association between ART use and the intrinsic subtype, progression disease or death (Table 4.3).

Table 4.3: PAM50 intrinsic subtypes in HIV positive and HIV negative patients

		Overall	Luminal A	Luminal B	Her2-enriched	Basal like	p-value
HIV-positive participants		**(n=72)**	**(n=12)**	**(n=17)**	**(n=22)**	**(n=21)**	
CD4 count		477 (253-656)	494 (270 - 656)	611 (393 - 713)	385.5 (163 - 584.5)	463 (244 - 656)	p=0.514
Viral Load Detectable		28 (43.8%)	4 (36.4%)	9 (52.9%)	8 (44.4%)	7 (38.9%)	p=0.807
Viral Load if detectable		2971.5 (197 - 46833)	2220.5 (168.5 - 18335)	1520 (302 - 36466)	3629 (907 - 77590.5)	9230 (116 - 60019)	p=0.794
On ARTs		56 (78.9%)	9 (75.0%)	14 (82.4%)	14 (66.7%)	19 (90.5%)	p=0.270
Time since HIV diagnosis (years)		6.5 (1 - 10)	5.5 (1.5 - 10.5)	5 (0 - 9)	6.5 (0 - 12.5)	7 (4 - 10)	p=0.742
Duration HIV positivity:	≤1 year	19 (26.4%)	3 (25.0%)	6 (35.3%)	7 (31.8%)	3 (14.3%)	p=0.452
	> 1 year	53 (73.6%)	9 (75.0%)	11 (64.7%)	15 (68.2%)	18 (85.7%)	
Status:	Alive	41 (56.9%)	8 (66.7%)	13 (76.5%)	8 (36.4%)	12 (57.1%)	p=0.081
	Dead	31 (43.1%)	4 (33.3%)	4 (23.5%)	14 (63.6%)	9 (42.9%)	
Disease progression:	Yes	15 (34.1%)	4 (40.0%)	2 (20.0%)	3 (30.0%)	6 (42.9%)	p=0.730
	No	29 (65.9%)	6 (60.0%)	8 (80.0%)	7 (70.0%)	8 (57.1%)	
HIV-negative participants		**(n=72)**	**(n=12)**	**(n=21)**	**(n=25)**	**(n=14)**	
Status:	Alive	51 (72.9%)	10 (83.3%)	16 (84.2%)	17 (68.0%)	8 (57.1%)	p=0.286
	Dead	19 (27.1%)	2 (16.7%)	3 (15.8%)	8 (32.0%)	6 (42.9%)	
Disease progression:	Yes	17 (33.3%)	1 (10.0%)	2 (13.3%)	9 (50.0%)	5 (62.5%)	p=0.014
	No	34 (66.7%)	9 (90.0%)	13 (86.7%)	9 (50.0%)	3 (37.5%)	

As expected, patients on ART had a higher CD4 count, 481 (282 – 713), and only 32.7% still had a detectable viral load compared to those not on ART. Patients not on ART were associated with a more advanced disease stage at presentation than patients

who were on ART (Table 4.4). The use of ART did not influence the intrinsic subtypes, death of patients or progression of disease.

Table 4.4: Clinical characteristics and PAM50 subtypes of HIV positive patients on ART

		Overall	**On ART**	**Not on ART**	**p-value**
		(n=71)	**(n=56)**	**(n=15)**	
Duration ART use (years)		6 (3-9.5)			
CD4 count		477 (253-656)	481 (282-713)	257 (117.5-512.5)	p=0.030
Viral load detectable		28 (43.8%)	17 (32.7%)	11 (91.7%)	p<0.001
Viral load if detectable		2971.5 (197-46833)	302 (114-32500)	16900 (1700-96085)	p=0.041
Stage	Stage 1	3 (4.2%)	3 (5.4%)	0	
	Stage 2	24 (33.8%)	21 (37.5%)	3 (20.0%)	
	Stage 3	34 (47.9%)	28 (50.0%)	6 (40.0%)	
	Stage 4	10 (14.1%)	4 (7.1%)	6 (40.0%)	p=0.011
	Early stage	27 (38.0%)	24 (42.9%)	3 (20.0%)	
	Late stage	44(62.0%)	32 (57.1%)	12 (80.0%)	p=0.105
PAM50 subtype	Luminal A	12 (16.9%)	9 (16.1%)	3 (20.0%)	
	Luminal B	17 (23.9%)	14 (25.0%)	3 (20.0%)	
	Her2-enriched	21 (29.6%)	14 (25.0%)	7 (46.7%)	
	Basal-like	21 (29.6%)	19 (33.9%)	2 (13.3%)	p=0.283
Status	Alive	41 (57.8%)	35 (62.5%)	6 (40.0%)	
	Dead	30 (42.3%)	21 (375%)	9 (6.0%)	p=0.147
Disease progression	Yes	14 (32.6%)	13 (33.3%)	1 (25.0%)	
	No	29 (67.4%)	26 (66.7%)	3 (75.0%)	p=1.000

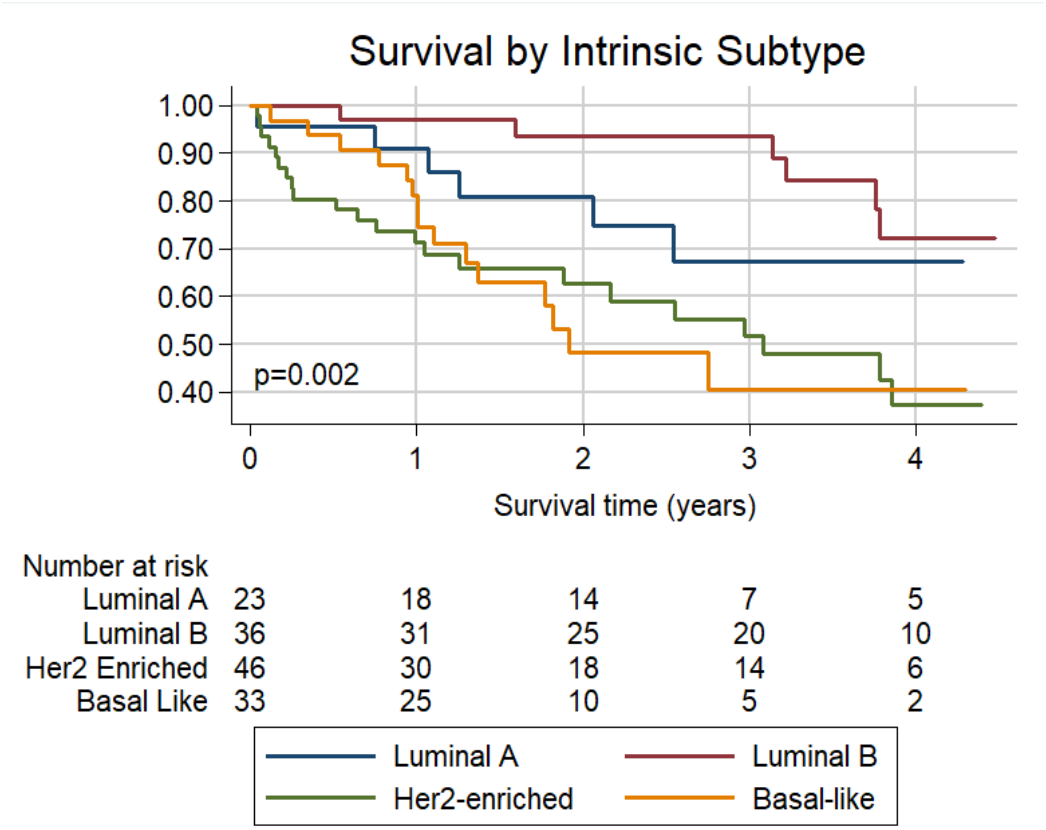


Figure 4.8: Survival by PAM50 intrinsic subtypes

4.2.1.4 Discussion

Even though our results are preliminary, the HER-2 enriched subtype was the most predominant subtype accounting for 32.6% of all intrinsic subtypes. This is significantly greater than has previously been published. A study done in the United States PAM50-based HER-2 enriched subtype only accounted for 11% of all intrinsic subtypes[81]. Other studies only had the PAM50 based HER-2 enriched subtype accounting for 4% and 8%, respectively[73]. In the US, it was estimated that HER-2 is overexpressed in about 18-20% of breast cancers[14]. The HER-2 overexpression is associated with an aggressive biology and poorer clinicopathological outcome with a high risk for local recurrence and metastases, with liver and lungs predominantly involved[14]. In our study, HER-2 intrinsic subtype was associated with poor survival, along with the basal-like subtype. Luminal A subtype, a less aggressive subtype, was the least of all subtypes, accounting for only 16.7%. This finding contrasted findings in the US, where Luminal A was predominant,

accounting for 45% of all intrinsic subtypes[81]. Similarly, other studies found the Luminal A subtypes to be predominant, accounting for 47.3% and 71.6%, respectively[29,73]. Other subtypes associated with aggressive tumour biology and poor clinical outcome were also noted to be dominant compared to what has been published previously. Luminal B and Basal-like subtypes, 26.4% and 24.3% respectively, compared to 23% and 18%, respectively seen in the US[81]. A conclusion cannot be made at this point as to whether patients seen in our setting have aggressive tumour biology based on molecular assay intrinsic subtypes because of the small number of our sample.

So far, no association has been found between HIV status and the IHC-4 breast cancer subtypes[60, 61, 64, 65]. In our study, HIV status did not affect the intrinsic subtypes of breast cancer by PAM 50 (p=0.570). There was also no association found between the use of ART, the PAM 50 intrinsic subtypes (p=0.283) and the duration thereof.

4.3. Other Molecular Assays Used in Early-stage Breast Cancer

4.3.1: Oncotype DX

The Oncotype DX is the first multi-gene test to be developed to help predict the risk for recurrence in postmenopausal women with early-stage breast cancer, ER +/ HER-2-, node-negative breast cancer managed with adjuvant hormonal therapy[82]. It also provides prognostic information even among women with involved, metastatic nodes[83]. It is an FDA-approved, RNA-based test done by a reverse-transcriptase polymerase chain reaction (RT-PCR) on formalin-fixed paraffin-embedded (FFPEs) breast cancer specimens[84]. The test is based on the expression of 21 genes, 16 cancer-related genes and five reference genes, namely: *ACTB, BAG1, BCL2, BIRC5, CCNB1, CD68, CTSL2, ESR1, GAPDH, GRB7, GSTM1, GUS, HER2, Ki67, MMP11, MYBL2, PGR, RPLPO, SCUBE2, STK15 and TRFC*[85]. The molecular information obtained from the genes provides a Recurrence Score (RS) which can risk-stratify patients into three categories, based on the patient-specific ten-year risk for recurrence at distant sites[84]. Categories include:

- Low risk: score <18
- Intermediate risk: 18-30
- High risk: >30

The cut-offs for the RS were later amended to minimise the risk of under-treatment, with the new cut-offs being[87]:

- Low risk: < 11
- Intermediate risk: 11-25
- High risk: > 25

It has been validated by several trials for clinical usefulness in early-stage breast cancer management, in accordance with the EGAPP recommendations[34]. As Oncotype DX was the first multi-gene test developed and used, there was no other test to be used for comparison to determine its analytical sensitiveness and specificity[34]. Several clinical trials validated the strong correlation between the RS and the clinical outcome of early-stage breast cancer, ER +/HER 2- subtypes, confirming the clinical validity of the RS by Oncotype DX[86]. In the NSABP B-14 trial, the RS could significantly predict the ten-year risk of recurrence and showed it to be higher among the intermediate and high-risk groups. It was also able to demonstrate the added benefit of adjuvant hormonal therapy (Tamoxifen) in decreasing the risk of recurrence in all risk groups compared to those who did not receive any adjuvant therapy. The NASBP B-20 trial demonstrated the usefulness of chemotherapy use in decreasing the recurrence risk in all risk groups[86]. These two trials also demonstrated how the prognostic ability of RS was independent of clinical and pathological parameters[86]. In addition to its predictive value for distant risk of recurrence, RS could also predict the ten-year overall survival[84]. The clinical effectiveness and usefulness of RS were also validated. In the TAILORx trial (Trial assigning individualised options for treatment), the low-risk group patients (RS 0-10), node-negative, ER +/ HER2- early-stage breast cancer were treated with adjuvant tamoxifen for only five years[87]. At the end of five years, the overall disease-free survival and distant recurrence-free survival were 98% and 99.3%, respectively[87]. Due to this

favourable outcome, it was recommended that chemotherapy be avoided in the low-risk group of patients with ER +/HER 2-, node-negative disease as adjuvant hormonal therapy was adequate treatment for this group. In the intermediate-risk group (RS: 11-25), the RS identified the sub-group of patients who should not receive chemotherapy[87]. This group includes women older than 50 years with RS 11-25 and all women younger than50 years with RS 11-15. In the high-risk groups (RS > 25), it is recommended that chemotherapy be given, followed by adjuvant hormonal therapy[87]. The Oncotype DX has been shown to be cost-effective in the UK as it could change the treatment decision for 27% of patients, with 45.6% of them being spared the cytotoxic effects of chemotherapy prescribed before the test[88]. So far, it remains the most widely used molecular assay in the United States of America[89]. In Israel, Oncotype DX changed the treatment plan of about 40% of patients successfully[90]. In this sub-group of patients, 84% were spared chemotherapy and treated with adjuvant hormonal therapy only. Among the high-risk group of patients, 8% were given chemotherapy in addition to hormonal therapy[90].

4.3.2: MammaPrint

The MammaPrint is an FDA-approved molecular assay designed to predict the risk of distant recurrence in ER+/HER 2, node-negative ESBC[91]. It risk-stratifies into those with a low clinical risk score (good prognosis group) and those with a high clinical risk score (poor prognosis group), based on their risk for distant recurrence at both five and ten years[92]. The patients assigned to the low-risk group were those whose five-year distant metastases-free survival probability is more than 90%[91].

It assesses 70 cancer-related genes namely, *AA555029_RC, ALDH4A1, AP2B1, AYTL2, BBC3, C16orf61, C20orf46, C9orf30, CCNE2, CDC42BPA, CDCA7, CENPA, COL4A2, DCK, DIAPH3, DTL, EBF4, ECT2, EGLN1, ESM1, EXT1, FGF18, FLT1, GMPS, GNAZ, GPR126, GPR180, GSTM3, HRASLS, IGFBP5, JHDM1D, KNTC2, LGP2, LIN9, LOC100131053, LOC100288906, LOC730018, MCM6, MELK, MMP9, MS4A7, MTDH, NMU, NUSAP1, ORC6L, OXCT1, PALM2, PECI, PITRM1, PRC1, QSCN6L1, RAB6B, RASSF7, RECQL5, RFC4, RTN4RL1, RUNDC1, SCUBE2,*

SERF1A, SLC2A3, STK32B, TGFB3, TSPYL5, UCHL5, WISP1 and ZNF533[93]. The genes may be extracted from either fresh or formalin-fixed, paraffin-embedded breast cancer specimens and analysed using DNA microarray technology[21].

The MammaPrint was analytically validated by the TRANSBIG Consortium and proved to be a prognostic factor for women with early-stage breast cancer. Its predictive value was beyond that provided by clinicopathological characteristics[91]. It has also demonstrated high inter- and intra-laboratory reproducibility when tested in laboratories across three countries[94]. It has also been clinically validated to be superior to traditional clinicopathological prognostic factors in patients with node-negative and positive early breast cancer[92, 95]. It predicted patients based on their clinical risk score, with patients with high clinical risk score having a significantly poor ten-year overall survival (54.6% vs 94.5%). The hazard ratio for distant-free recurrence was also poor among the high clinical risk score (50.6% vs 85.2%, 5.1 95% CI: 2.9-9, $P < 0.001$)[92]. MammaPrint could identify node-positive patients withfavourable clinical outcome, even without the administration of chemotherapy[95]. The MINDACT trial[96] validated the clinical usefulness and effectiveness of the MammaPrint. In this trial, patients regarded as high risk on clinical grounds but low genomic risk by MammaPrint had a favourable clinical outcome, despite not receiving chemotherapy. The five-year distant-free survival rate was 94.7%, which was only slightly lower than those in this risk group who received chemotherapy. The outcome was the same among node-negative and positive ESBC, ER +/ HER 2-disease[96]. With these findings, about 46% of patients regarded as high risk on clinicopathological grounds could have been spared the cytotoxic effects of chemotherapy had the genomic risk profile been determined[96].

4.3.3: EndoPredict

EndoPredict is one of the novel molecular assays developed to predict the risk of distant recurrence among postmenopausal women with early-stage breast cancer, ER +/HER-2-, node-negative disease who received adjuvant hormonal therapy[97,98]. It is an RNA-based assay, assessed by qT-PCR from FFPEs[97]. It assesses eight cancer-related

genes, namely, *BIRC5, UBE2C, DHCRT, RBBP8, IL6ST, AZGP1, MGP& STC 2,* and three reference genes namely*, CALM 2, OAZ 1 and RPL37A*[97]. The test provides a risk score, EP, using the molecular information from the genes mentioned above. When the clinical parameters (tumour size and the nodal involvement) are added to the risk score EP, the test will provide a comprehensive risk score named EPclin[97]. The EP and EPclin risk score patients are risk-stratified into low and high risk for distant recurrence. The following cut-off points are used for the risk-stratification, as they correlated with a 10% disease recurrence rate ten years after breast cancer diagnosis[97].

- EP low: <5 EP high ≥5
- EPclin low: <3.3 EPclin high ≥3.3

Like other molecular assays, the EndoPredict test was analytically validated to predict the risk of distant recurrence[99, 100]. The test proved to be suitable and reliable for a specialised molecular pathology laboratory and showed reproducibility, accuracy and robustness. It also showed non-significant inter- or intra-laboratory variations[99,100]. The assay was also clinically validated by the ABCSG-6 and ABCSG-8 clinical cohorts[98,101]. The EP risk score provided a prognostic value beyond traditional clinical and pathological parameters[97, 98,101]. It also proved to predict the ten-years risk of distant recurrence for patients with EPclin low and high risk to be 4% and 28%, respectively[97]. Over a median follow up of 72 months, Fitzal and colleagues (2015) demonstrated a 2.6% overall incidence of local recurrence, which is approximately 0.4% risk per year[98]. Over ten years, the low-risk patients, as per the EP risk score, had a higher rate of local recurrence-free survival than the high-risk group (97.5% vs 91%, P< 0.005)[98]. Compared to the recurrence score provided by Oncotype DX, the EP and EPclin risk scores provide more prognostic information regarding the risk of distant recurrence[102]. Concerning the clinical usefulness of the MammaPrint, the risk score provided by the EP and EPclin was shown to be particularly useful in assisting with individualised, clinical decisions about the treatment of patients in the adjuvant setting. Muller and colleagues (2013) showed that the test could be done routinely in the molecular laboratory, and results should be out within three days (0-11 days)[103]. After the test, the

decision regarding adjuvant treatment to be given was changed in 37.7% of patients. Of these, 12.3 % required more chemotherapy and 25.4% were moved adjuvant hormonal therapy only[103]. About a quarter of patients were spared the cytotoxic effects of chemotherapy after the EndoPredict test was done. This shows that not only is EndoPredict clinically useful but cost-effective as well.

4.3.4: Breast cancer index

The Breast Cancer Index (BCI) assesses the ratio of this expressed genes, *HOXB13* and *IL17BR*, which when combined with the five genes molecular grade index, gives a risk of distant recurrence score[104]. It is a qRT-PCR assay that can be done on FFPEs of ER+, lymph node negative- early-stage breast cancer specimens[104]. The score ranges between 0 – 10, and using it, patients can be risk-stratified into three risk categories, as follows:

- Low-risk group: <5
- Intermediate-risk group: 5 – 6.3
- High-risk group: ≥6.4

At present, there is no evidence on the analytical validity and clinical utility of BCI. The clinical validity of BCI was analysed and validated by a retrospective study conducted on 588 postmenopausal women with ER+, LN- breast cancer, which found that the BCI was prognostic and independent of the clinicopathological characteristics of breast cancer[105]. Its prognostic ability was shown to be comparable with that of the Adjuvant! Online, in predicting for distant site recurrence[105]. However, no definitive conclusions can be made regarding its use until the analytical validity and clinical utility has been validated by trials[104].

Table 4.5: Summary of molecular assays[104]

MOLECULAR ASSAY	PAM 50(Nanostring Technologies, Seattle WA)	ONCOTYPE DX (Genomic Health Inc, Redwood City, CA, USA)	MAMMAPRINT (Agendia, Amsterdam, the Netherlands)	ENDOPREDICT (Myriad genetics Inc, Salt Lake City, US)	BREAST CANCER INDEX (bioTheranostics Inc, San Diego, CA, USA)	BLUEPRINT (Agendia Amsterdam, the Netherlands)
Function	Intrinsic subtyping Risk of recurrence	Recurrence score	Risk of recurrence	Risk of recurrence	Risk of recurrence	Intrinsic subtyping
Number of genes	50	21	70	11	7	80
Name of genes	*UBE2T, PTTG1, PGR, MKI67, MIA, MAPT, KRT17, KRT14, KIF2C, ESR1, CCNE1, ENPF, CEP55, FGFR4, MMP11, SFRP1, TMEM45B, TYMS, ERBB2, CDCA1, BCL2, CCNB1, CDC20,*	*ACTB, BAG1, BCL2, BIRC5, CCNB1, CD68, CTSL2, ESR1, GAPDH, GRB7, GSTM1, GUS, HER2, Ki67, MMP11, MYBL2, PGR, RPLPO, SCUBE2, STK15 and TRFC*[85]	*AA555029_RC, ALDH4A1, AP2B1, AYTL2, BBC3, C16orf61, C20orf46, C9orf30, CCNE2, CDC42BPA, CDCA7, CENPA, COL4A2, DCK, DIAPH3, DTL, EBF4, ECT2, EGLN1, ESM1, EXT1, FGF18, FLT1, GMPS, GNAZ, GPR126,*	BIRC5, UBE2C, DHCRT, RBBP8, IL6ST, AZGP1, MGP& STC 2, and three reference genes namely, CALM 2, OAZ 1 and RPL37A [97]		

	NAT1, ORC6L, RRM2, UBE2C, ACTR3B, ANLN, BAG1, BIRC5, BLVRA, CDC6, CDH3, CXXC5, EGFR, EXO1, FOXA1, FOXC1, GPR160, GRB7, KNTC2, KRT5, MDM2, MELK, MLPH, MYBL2, MYC, PHGDH and SLC39A6		*GPR180, GSTM3, HRASLS, IGFBP5, JHDM1D, KNTC2, LGP2, LIN9, LOC100131053, LOC100288906, LOC730018, MCM6, MELK, MMP9, MS4A7, MTDH, NMU, NUSAP1, ORC6L, OXCT1, PALM2, PECI, PITRM1, PRC1, QSCN6L1, RAB6B, RASSF7, RECQL5, RFC4, RTN4RL1, RUNDC1, SCUBE2, SERF1A, SLC2A3, STK32B, TGFB3, TSPYL5,*			

			UCHL5, WISP1 and ZNF533[93]			
Type of specimen	FFPE	FFPE	FFPE	FFPE	FFPE	Fresh
'technology'	qT-PCR	qT-PCR	qT-PCR	qT-PCR	qT-PCR)	qT-PCR
FDA approved/ CE certified?	Yes	Yes	Yes	Yes		
Population	ESBC, ER +/ HER 2-, Node -/+	ESBC, ER +/ HER 2-, Node -/+	ESBC, ER +/ HER 2-, Node -/+	ESBC, ER +/ HER 2-, Node -/+	ER+/ Node negative	ESBC, ER +/ HER 2-, Node -/+
Analytical validity	Yes[28]	First multi-gene test developed, thus no other test to be used for comparison[34]	Yes[91]	Yes[99,100]	No available evidence[104]	
Clinical validity	Yes[73,79] Level 1 evidence	Yes[86]	Yes[92,95]	Yes[98,101]	Yes[104]	
Clinical utility	Yes[29,31]	Yes[86]	Yes[96]	Yes[103]	No available data[104]	
Cost-effectiveness						
Cost	US$3200	£2580	£2675		US$3200	No additional cost MammaPrint)

Location of testing						
Commercially available	Yes	Yes	Yes	Yes	Yes	
Results	**Intrinsic subtypes**: Luminal A, Luminal B, HER-2 enriched, Basal-like, Normal like **Risk of recurrence score**: Low risk: ROR <= 40 Intermediate risk: ROR 41-60, pN0 High risk: ROR 41-60, pN1 or ROR > 60	**Recurrence score**: Low risk: < 11 Intermediate risk: 11-25 High risk: > 25	Low clinical risk score High clinical risk score	**EndoPredict score** EP low: <5 EP high >= 5 **EndoPredict clinical score** EPclin low: < 3.3 EPclin high >=3.3	**Recurrence score**: 0 to 10 **Low-risk:** < 5 **Intermediate-risk:** 5–6.3 **High-risk:** ≥ 6.4.	**Intrinsic subtypes: 3 categories -** Basal-type Luminal-type ERBB2-type cancers

4.3.5: Protein expression profiling

The immunohistochemical biomarkers may also be used to predict the risk of distant site breast cancer recurrence. There are currently three protein expression profiling assays available[104]:

- IHC-4
  - This test uses the four immuno-histochemical biomarkers, ER, PR, HER-2 and Ki 67. When combined with clinical and other

pathological parameters, a risk for distant recurrence score can be obtained[104]. It is less costly than gene expression assays, being estimated to be approximately £100–200[104]. However, other quality assurance measures still need to be assessed and validated[104].

- Mammostrat test
  - This test uses the following five immuno-histochemical markers, independent of one another, to give the risk score for distant site breast cancer recurrence[104].
    - P53
    - N-myc downstream-regulated (NDRG1)
    - HpaII tiny fragments locus 9c protein (HTF9C)
    - Member 5 (SLCTA5)
    - Solute carrier family 7 (amino acid transporter light chain, L system)
  - Patients can be risk-stratified into three risk categories using the risk score:
    - Low-risk group: Prognostic index <0
    - Moderate-risk group: Prognostic index >0 but <0.7
    - High-risk group: Prognostic index >0.7
  - The cost is estimated to be about £1120 – £1620.
- NPI plus (NPI +)
  - NPI + uses the following ten immuno-histochemical biomarkers to predict the risk of distant site breast cancer recurrence:
    - ER, PR, HER 2, HER 3, HER 4, cytokeratin 5/6, CK 7/8, EGFR, p53, mucin 1.

CHAPTER 5: PAPER 2

SURVIVAL OF SOUTH AFRICAN WOMEN WITH BREAST CANCER RECEIVING ANTI-RETROVIRAL THERAPY FOR HIV

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ABSTRACT

PURPOSE

Breast cancer outcomes in sub-Saharan Africa is reported to be poor, with an estimated five-year survival of 50% when compared to almost 90% in high-income countries. Although several studies have looked at the effect of HIV in breast cancer survival, the effect of ARTs has not been well elucidated.

METHODS

All females newly diagnosed with invasive breast cancer from May 2015- September 2017 at Charlotte Maxeke Johannesburg Academic and Chris Hani Baragwanath Academic Hospital were enrolled. We analysed overall survival and disease-free survival, comparing HIV positive and negative patients. Kaplan-Meier survival curves were generated and p-values were calculated using a log-rank test of equality. Hazard ratios, with 95% confidence intervals (CI), were estimated using Cox regression models."

RESULTS

Of 1019 patients enrolled, 22% were HIV positive. The overall survival (95% CI) was 53.5% (50.1 – 56.7%) with a disease-free survival of 55.8% (52.1 – 59.3) after 4 years of follow up. HIV infection was associated with worse overall survival (HR (95% CI): 1.50 (1.22-1.85), $p < 0.001$) and disease-free survival (OR (95% CI):2.63 (1.71-4.03), $p < 0.001$), especially among those not on ART at the time of breast cancer diagnosis. Advanced stage of the disease and hormone-receptor negative breast cancer subtypes were also associated with poor survival.

CONCLUSION

HIV infection was associated with worse overall and disease-free survival. HIV patients on ARTs had favourable overall and disease-free survival and with ARTs now being made accessible to all the outcome of women with HIV and breast cancer is expected to improve.

KEYWORDS: SURVIVAL, BREAST CANCER, HIV, ART

INTRODUCTION

Breast cancer is the commonest cancer and cause of cancer-related mortality among women globally.[1] In South Africa, it remains the commonest malignancy among women, accounting for approximately 22% of all malignancies.[2] The lifetime risk (LR) for developing breast cancer in South Africa is estimated to be 1 in 25 women, however, this varies with self-reported race, which is lower than what is reported in the USA (1 in 8 women).[2,3]

In 2018, about 38 million people were reported to be living with HIV/AIDS (PLWHIV) worldwide with 68% of them receiving anti-retroviral therapy (ART).[4] In South Africa, 7.52 million PLWHIV were reported with 62% of them being on ARTs in 2018.[4,5] Due to the implementation of the universal "test and treat" strategy for all PLWHIV in 2016, the number of patients on ART is expected to rise.[6] So far, the success of the ART program is evidenced by a significant decrease in AIDS-related mortality from 37.2% reported in 2002 to 22.06% reported in 2018.[5] The ART regime previously used included, tenofivir disoprostil fumarate & emtricitabine /lamivudine and efavirenz , but has recently been changed to a new regime: tenofovir, lamivudine and dolutegravir.[7,8]

Breast cancer survival rates vary globally with high income countries (HICs) having better survivals than low-middle income countries (LMICs). In the United States, the reported 5-year overall survival is 89.9%, compared to 52.3% reported in the sub-Saharan Africa.[3,9] Some of the contributing factors to the poor overall survival in sub-Saharan Africa include young age at breast cancer diagnosis, advanced clinical stage at presentation, co-morbid disease, black race and aggressive pathological characteristics of breast cancer.[9-13]

Multiple studies were conducted looking at the effect of HIV on breast cancer survival. In a study done in Soweto, South Africa, HIV did not affect the overall survival of breast cancer after 2 years although 42% of patients were lost to follow up.[14] In Botswana, the 2 years overall survival was very low among HIV positive patients than the HIV negative ones (57% vs 73%, $P<0.001$).[15] Similarly, in the USA, a high cancer-specific mortality among HIV positive patients with breast cancer [HR 2.64; 96% CI 1.86-3.73] was reported.[16] A recently published meta-analysis also demonstrated that HIV positive patients had the worst overall survival when compared to HIV negative patients.[17] But none of the published studies specifically looked at the effect of ART use on the survival of breast cancer in HIV positive patients. This study, therefore, aims to describe the effect of HIV infection, duration of HIV positivity, ART use, as well as the duration of ART use on the outcomes of breast cancer in South Africa.

METHODOLOGY

This descriptive study is a sub-study of the South African Breast Cancer and HIV Outcome (SABCHO) study[18], which was conducted at two public hospitals, Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) Surgical Breast Unit and the Batho Pele Breast Unit of the Chris Hani Baragwanath Academic Hospital (CHBAH). Both these surgical breast units have an estimated HIV prevalence of 22% and diagnose between 250 and 350 new patients with breast cancer every year [18, 19]. The study participants were all consenting women newly diagnosed with breast cancer from 15 May 2015 through 31 September 2017.

An electronic breast cancer database was used to extract the following data: demographics, clinical stage at presentation, co-morbidities, level of education and the status of the patient when last seen (alive with no evidence of disease, alive with stable disease, alive with disease progression or dead). Histopathological characteristics (histological type, tumor grade, estrogen (ER) and progesterone receptor (PR) statuses, human epidermal growth factor receptor 2 (HER-2) and Ki67 expression) was obtained from the National Health Laboratory Service (NHLS). An Allred score was used to determine the positivity of both oestrogen and progesterone receptors, with the score of more than 3 regarded as positive.[20] HER2 was regarded positive when the test showed 3+, with the use of fluorescence in situ hybridization (FISH) reserved for tests with equivocal results(HER2 2+).[20] Receptor subtypes were defined as: Luminal A (ER +, PR +, HER2 -, Ki 67 <14%), Luminal B (ER +, PR +/-, HER2 -, Ki 67 >14% or ER +, PR +/-, HER-2 +, any Ki 67), HER2-enriched (ER/PR -, HER2 +, any Ki 67) and Triple negative (ER/PR/HER2 -, any Ki 67).[21] The American Joint Committee on Cancer (AJCC) system, 7th edition was used for clinical TNM staging at diagnosis. [22]

Patients with unknown HIV status were counselled and an informed consent for testing was obtained. Post-test counselling was offered to all those newly diagnosed with HIV and blood samples for CD 4 counts and HIV viral load testing were drawn. We recorded the year of HIV diagnosis and when ARTs were initiated, with a cut-off period of one year used to compare those who were on ARTs for a longer period with those who have recently started ARTs (less than a year). Those newly diagnosed with HIV and not on ART were referred to the ART clinic for commencement of ARTs (tenofivir disoprostil fumarate& emtricitabine /lamivudine and efavirenz. For HIV positive patients, CD 4 counts and HIV viral loads were obtained from the NHLS. The viral load below detectable limit was defined as a viral load ≤50 copies/ml.

Following the completion of prescribed breast cancer treatment, patients were followed up at three- to six- month intervals. If follow-up was missed, patients were called by the research navigators. Completion of chemotherapy and radiation treatment was defined as patients who received the full prescribed number of doses. The first line

chemotherapy regime included: Adriamycin (Doxorubicin), Cyclophosphamide and Taxanes (AC-T). At the time of the study, Trastuzumab was not yet available at these two study sites. Overall survival was defined as survival from the date of breast cancer diagnosis to the date of death for any cause while disease free survival was defined as survival from the date of breast cancer diagnosis to the date of disease recurrence or death for any cause. Patients with disease recurrence included all patients with radiologically and histologically confirmed breast cancer recurrence. Patients with stage IV disease at presentation were excluded in the analysis of disease-free survival. The site of disease recurrence was sub-classified into loco-regional (ipsilateral chest wall, ipsilateral breast following breast conserving surgery and/ or regional lymph nodes) and distant metastases (visceral and non-visceral sites). The time to and site of disease recurrence were documented. For patients who died, the date of death, as documented in the medical record or as provided by the next-of-kin, was also documented.

STATISTICAL ANALYSIS

Demographic and clinico-pathological data were represented as medians and interquartile ranges for continuous data or as frequencies and percentages for categorical data. Categorical comparisons between HIV positive and HIV negative patients were performed using a χ^2 test; age and body mass index (BMI) were compared using a Mann-Whitney test. Patients were censored on the last date they were known to be alive with an administrative censoring date of 31 July 2020 for those still alive at the time of data extraction. Patients censored before the 31 July 2020 were lost to follow up if they could not follow up for more than 9 months and their status could not be ascertained via the 'Verify ID' portal. Kaplan-Meier survival curves were generated assuming non-informative losses to follow-up; p-values were calculated using a log-rank test of equality. Hazard ratios (HRs) and their 95% confidence intervals (CIs) were estimated using Cox regression models. Both unadjusted (crude) HRs and mutually adjusted HRs (adjusted for age at diagnosis (continuous), BMI (continuous), stage at diagnosis (2 categories), receptor subtype (4 categories), employment status (2 categories) and education (3 categories)) were calculated. Logistic regression analyses, adjusting for age at diagnosis (2 categories), stage at diagnosis (2 categories) and receptor subtype (4 categories), was used to calculate odds ratios for disease recurrence or death. Data were analysed using STATA v14.2 and the stset suite of commands.

RESULTS

1019 women newly diagnosed with breast cancer were followed up with a median follow up time of 36.9 months (Interquartile range (IQR) 16.8 – 46.5 months). The HIV status was unknown in 3.33% of patients and 36 (3.5%) were lost to follow up, with a median time to loss to follow up of 16.5 months (IQR 8.3 - 28.9 months) (Figure 1). Many of our

patients (55.8%) presented in stage III & IV of the disease. Basic demographics, clinical and pathological characteristics of all patients are shown in Table 1.

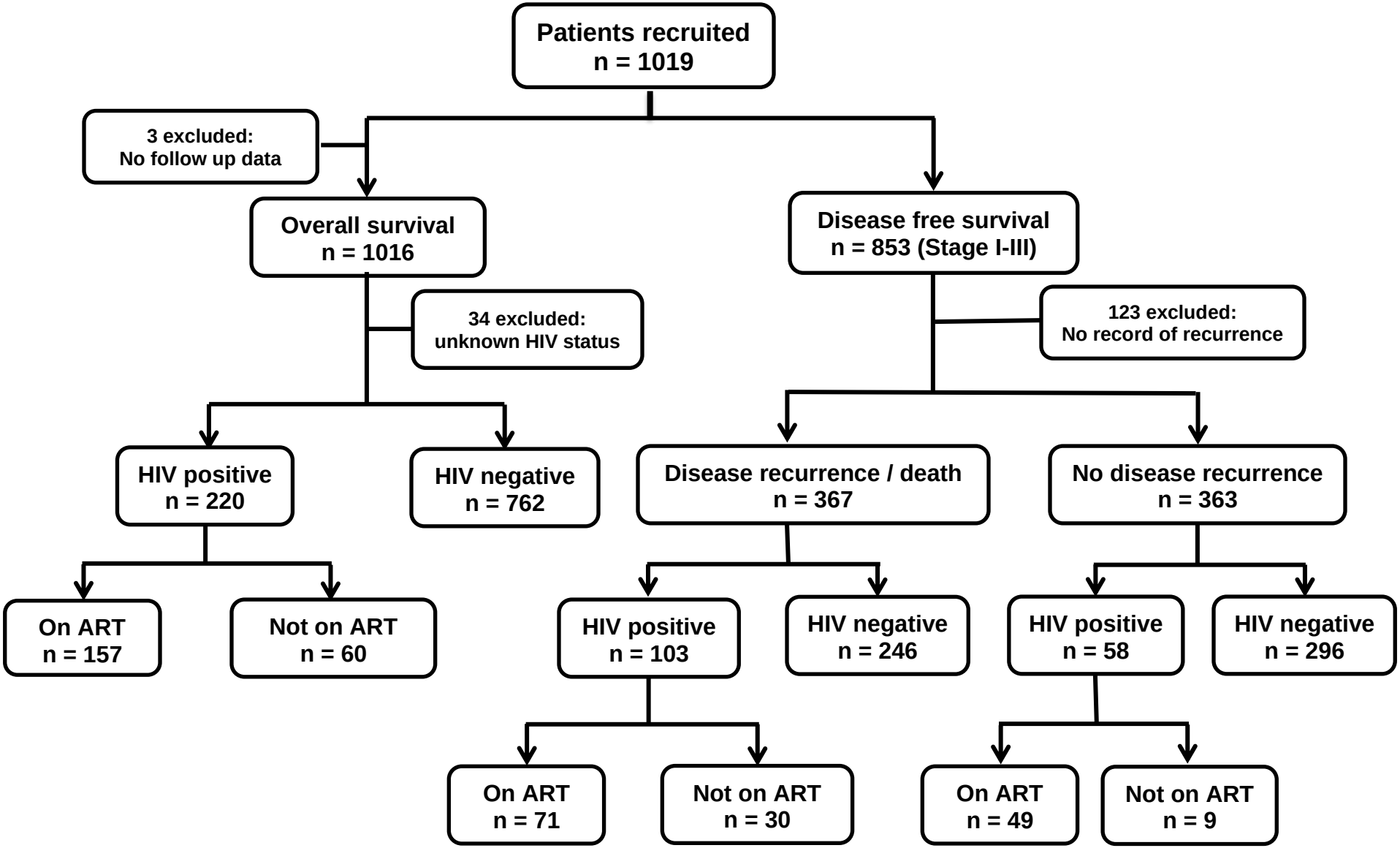


Figure 1: Flowchart showing the total number on recruited patients, included and excluded from the analysis.

Table 1: Demographics, clinical and pathological characteristics of 1019 HIV positive and negative women diagnosed with breast cancer at Chris Hani Baragwanath and Charlotte Maxeke Johannesburg Academic Hospitals between May 2015 and September 2017.

	Overall (n=1019)	HIV-positive (n=221)	HIV-negative (n=764)	p-value[a]
Patient Demographics				
Age at diagnosis (median (IQR))	54 (44-64)	44 (39-52)	57 (46-67)	**<0.001**
Self-Reported Ethnicity				
Black	854 (84.0%)	214 (97.3%)	614 (80.5%)	
White	85 (8.4%)	1 (0.5%)	80 (10.5%)	
Mixed Ancestry	58 (5.7%)	5 (2.3%)	52 (6.8%)	
Asian	20 (2.0%)	0 (0%)	17 (2.2%)	
BMI (median (IQR))	30.5 (25.5-35.8)	27.3 (22.9-33.9)	31.6 (26.7-36.2)	**<0.001**
Co-morbidities [b]				
Yes	484 (47.9%)	78 (35.8%)	387 (51.0%)	
No	527 (52.1%)	140 (64.2%)	372 (49.0%)	**<0.001**
Losses to follow-up	36 (3.5%)	7 (3.2%)	28 (3.7%)	0.725
Education [c]				
Primary or lower	238 (23.8%)	42 (19.3%)	186 (24.8%)	
Secondary	669 (66.8%)	156 (71.6%)	491 (65.6%)	
Tertiary	94 (9.4%)	20 (9.6%)	72 (9.6%)	0.206
Employment				
Employed	553 (55.0%)	109 (49.8%)	428 (56.9%)	
Unemployed	452 (45.0%)	110 (50.2%)	324 (43.1%)	0.061
Tumor characteristics				
Clinical stage at diagnosis				
Stage 1	60 (6.1%)	6 (2.7%)	54 (7.1%)	
Stage 2	372 (38.1%)	77 (35.0%)	295 (39.0%)	
Stage 3	390 (39.9%)	99 (45.0%)	291 (38.4%)	
Stage 4	155 (15.9%)	38 (17.3%)	117 (15.5%)	**0.040**
Early Stage (1 and 2)	447 (44.3%)	83 (37.7%)	349 (46.1%)	
Advanced Stage (3 and 4)	563 (55.7%)	137 (62.3%)	408 (53.9%)	**0.028**
Tumour grade				
Grade 1	55 (5.7%)	11 (5.3%)	42 (5.8%)	
Grade 2	486 (50.6%)	113 (54.1%)	356 (49.4%)	
Grade 3	420 (43.7%)	85 (40.7%)	323 (44.8%)	0.490
Receptor subtype [d]				

Luminal A	135 (14.1%)	23 (11.1%)	106 (14.8%)	
Luminal B	615 (64.3%)	135 (64.9%)	460 (64.1%)	
Her2-enriched	58 (6.1%)	12 (5.8%)	43 (6.0%)	
Triple negative	149 (15.6%)	38 (18.3%)	109 (15.2%)	0.459
Treatments				
Completed Chemotherapy	655 (90.9%)	164 (92.1%)	478 (90.9%)	0.608
Completed Radiation	413 (99.8%)	83 (100%)	318 (99.7%)	0.610
HIV positive patients:				
Detectable viral load [e]		89 (47.1%)		
CD4 count (median (IQR))		477 (295 – 677)		
Duration HIV positivity				
≤1 year		66 (29.9%)		
>1 year		155 (70.1%)		
On ART		158 (72.5%)		
On ART at diagnosis		131 (60.1%)		
Duration ART usage				
≤1 year		30 (19.0%)		
>1 year		128 (81.0%)		

Note: 34 patients had unknown HIV status. Missing values as follows: ethnicity n=2, BMI n=78, co-morbidities n=8, education n=18, employment n=14, stage n=9, grade n=58, receptor subtype n=62, viral load n=32, CD4 count n=12, art n=3, duration of ART n=63. 298 participants did not receive chemotherapy and 605 participants did not receive radiation therapy.

[a] P-values compare HIV-positive to HIV-negative patients using a chi-squared test except for age, BMI and follow-up time which use a Mann-Whitney test.

[b] Co-morbidities included hypertension (77.7%), diabetes (18.8%), arthritis (17.6%), tuberculosis (11.6%), asthma/COPD (11.2%), heart disease (5.0%), stroke (3.9%).

[c] Primary education refers to the first 7 years of school education and secondary as the first 12 years.

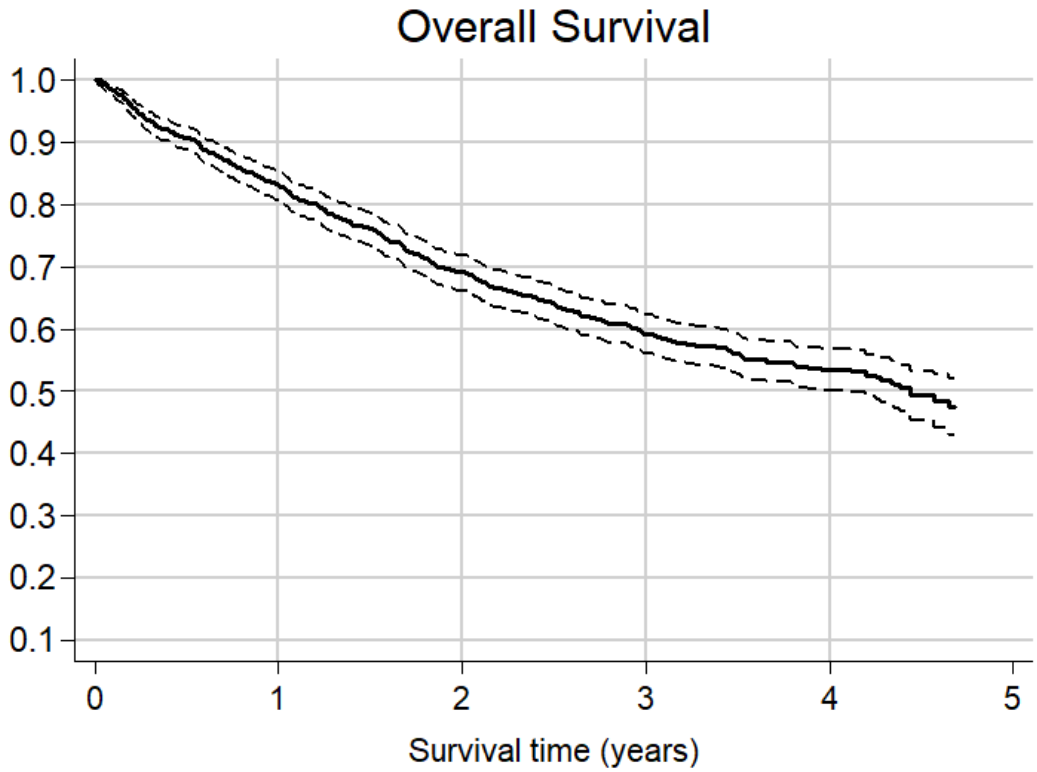
[d] Receptor subtypes were defined by immunohistochemistry as follows: Luminal A (ER +, PR +, HER2 -, Ki 67 <14%), Luminal B (ER +, PR +/-, HER2 -, Ki 67 >14% or ER +, PR +/-, HER-2 +, any Ki 67), HER2-enriched (ER/PR -, HER2 +, any Ki 67) and Triple negative breast cancer (ER/PR/HER2 -, any Ki 67).

[e] Viral loads were detectable when >50 copies/ml

OVERALL SURVIVAL

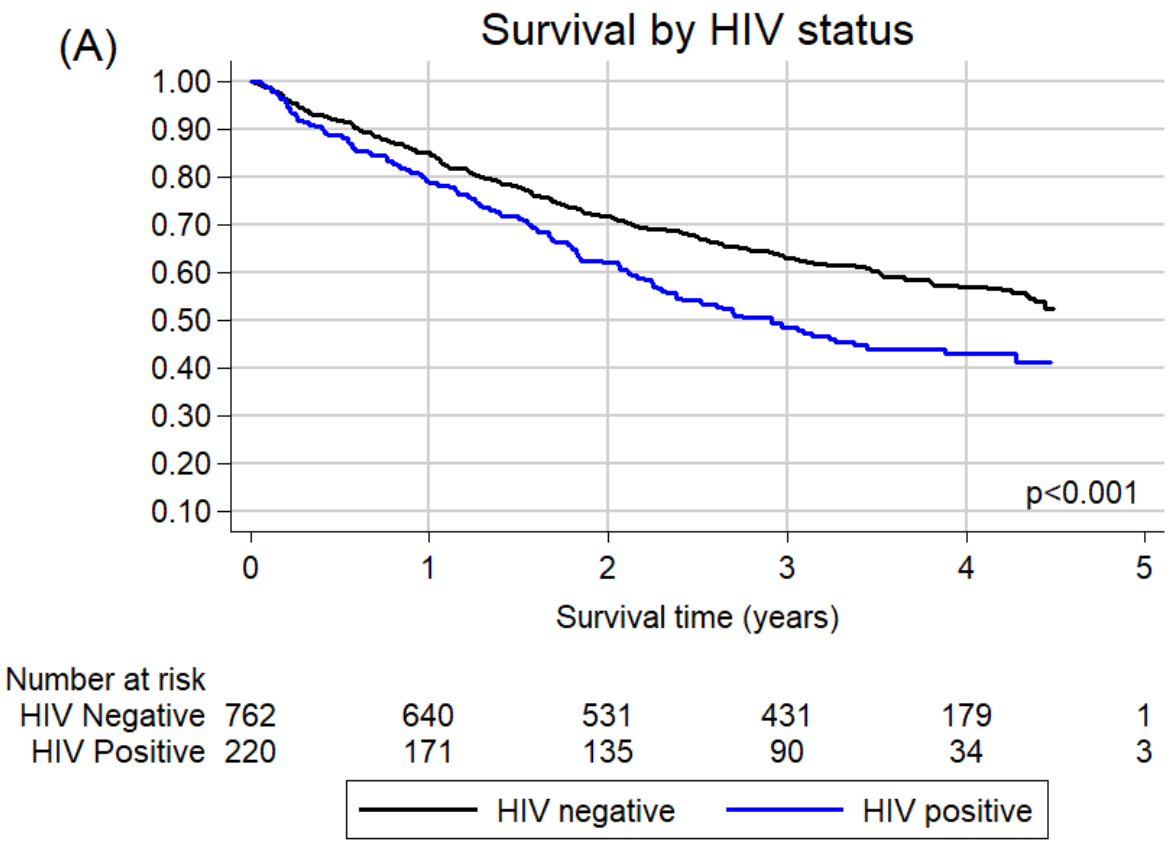
After 4 years, there were documented 446 deaths (43.8%) with a median (IQR) time to death of 15.7 months (6.9 – 25.9). The source of deaths was predominantly next of kin (72.0%), followed by the 'Verify ID' portal (16.4%) and hospital records (9.5%). Two percent of patients (n=11) had no record of the source of death. The overall survival

rate (95% CI) was 53.5% (50.1 – 56.7%) (Figure 2) after 4 years. Age at breast cancer diagnosis did not affect the overall survival (p=0.326) while HIV positive status, and those not on ART, was associated with poor overall survival (p<0.001). (Figure 3, Table 2). Early stage of the disease at diagnosis, hormone receptor positive tumours and a luminal A breast cancer subtype were associated with more favorable outcomes (Table 2).



Number at risk	1016	834	685	533	221	4
Number of deaths	172	139	96	39	12	0
Lost to follow-up	13	10	12	1	0	0

Figure 2: Overall survival and 95% confidence interval for 1016 patients. At 1 year, survival (95% CI) was 83% (81-85%), at 2 years it was 69% (66-72%), at 3 years it was 59% (56-62%) and at 4 years it was 53% (50-57%).


(A)
Survival by HIV status
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
p<0.001
0
1
2
3
4
5
Survival time (years)
Number at risk
HIV Negative 762 640 531 431 179 1
HIV Positive 220 171 135 90 34 3
HIV negative
HIV positive

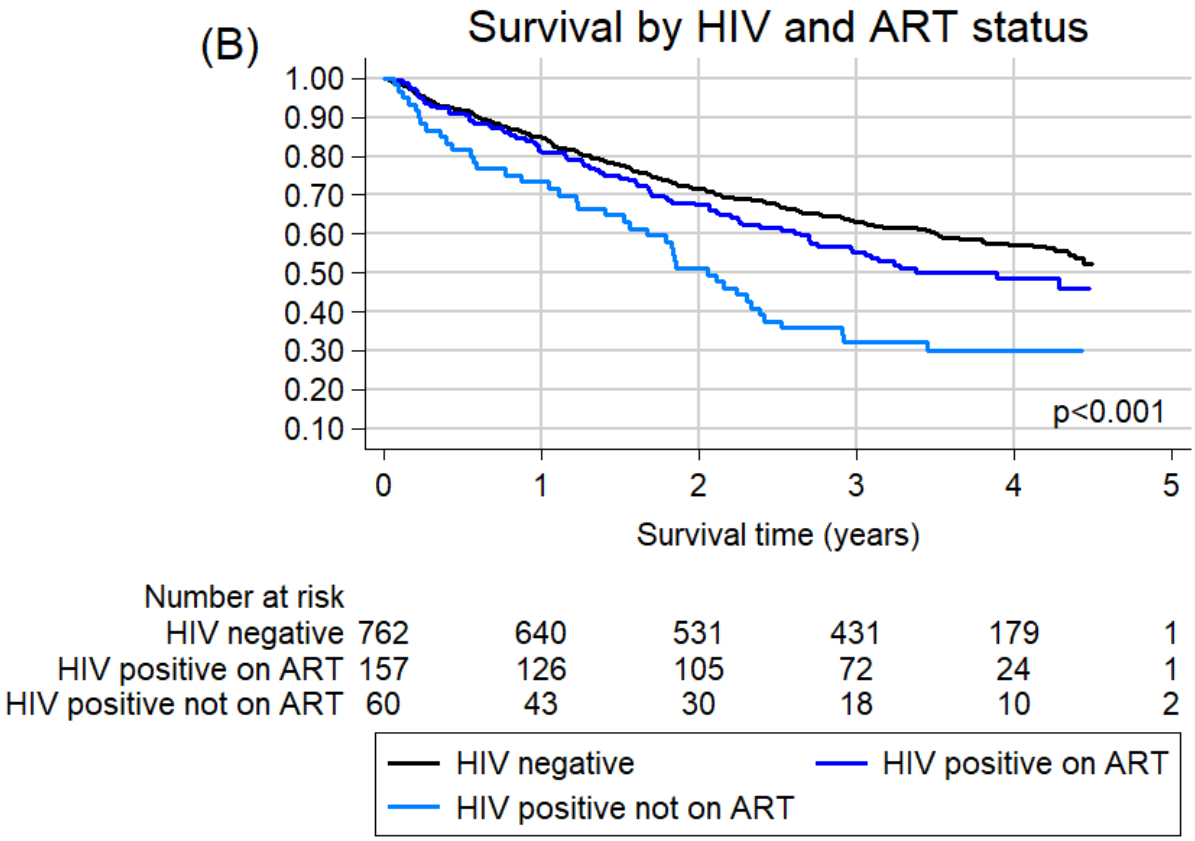


Figure 3: (A) Overall survival by HIV status for 982 patients with known HIV status. Unadjusted HR (95% CI): 1.50 (1.22 – 1.85), p<0.001. (B) Overall survival by HIV status for 979 patients with known HIV and ART status diagnosed with breast cancer. Unadjusted HR (95% CI) compared to HIV negative: HIV positive on ART 1.28 (1.00 – 1.63), p=0.054; HIV positive not on ART 2.10 (1.52 – 2.91), p<0.001.

Table 2: Unadjusted and adjusted Hazard Ratios for overall survival in patients diagnosed with breast cancer

			Unadjusted		Adjusted*	
	Number at risk	Number of deaths	Hazard Ratio (95% CI)	p-value	Hazard Ratio (95% CI)	p-value
Age at diagnosis (years)	1016	457				
<50			1.00 (Reference)		1.00 (Reference)	
≥50			1.10 (0.91-1.33)	0.326	1.09 (0.87-1.36)	0.450
Linear trend			1.01 (1.00-1.01)	0.067	1.01 (1.00-1.02)	0.015
HIV status	982	437				
Negative			1.00 (Reference)		1.00 (Reference)	
Positive			1.50 (1.22-1.85)	**<0.001**	1.77 (1.37-2.28)	**<0.001**
Stage at Diagnosis	1007	456				
Early stage (Stage I/II)			1.00 (Reference)		1.00 (Reference)	
Advanced Stage (Stage (III/IV)			3.85 (3.09-4.79)	**<0.001**	3.71 (2.92-4.72)	**<0.001**
Receptor Status [a]						
ER positive	986	449	0.68 (0.55-0.83)	**<0.001**	0.71 (0.57-0.88)	**0.002**
PR positive	984	448	0.65 (0.54-0.78)	**<0.001**	0.74 (0.60-0.90)	**0.003**
Her2-positive	964	439	1.16 (0.94-1.43)	0.166	0.94 (0.75-1.18)	0.601
Receptor Subtype [b]	954	436				
Luminal A			1.00 (Reference)		1.00 (Reference)	
Luminal B			1.84 (1.32-2.58)	**<0.001**	1.88 (1.30-2.72)	**0.001**
Her2-enriched			1.76 (1.06-2.93)	**0.029**	1.46 (0.84-2.56)	0.182

Triple negative			2.52 (1.72-3.69)	**<0.001**	2.65 (1.75-4.01)	**<0.001**
BMI (kg/m^2)	939	411	0.99 (0.98-1.00)	0.206	0.99 (0.98-1.00)	0.178
Employment status	1002	447				
Employed			1.00 (Reference)		1.00 (Reference)	
Unemployed			1.13 (0.94-1.37)	0.182	1.17 (0.96-1.44)	0.129
Education [c]	998	445				
Primary or lower			1.00 (Reference)		1.00 (Reference)	
Secondary			0.65 (0.53-0.81)	**<0.001**	0.75 (0.59-0.95)	**0.017**
Tertiary			0.49 (0.34-0.73)	**<0.001**	0.78 (0.51-1.20)	0.256

* Adjusted for age at diagnosis (continuous), BMI (continuous), stage at diagnosis (early vs advanced), receptor subtype (luminal A vs luminal B, Her2-enriched, triple negative), employment status (employed vs unemployed) and education (primary vs secondary, tertiary). Receptor Status adjusted for age at diagnosis (continuous), BMI (continuous), stage at diagnosis (early vs late), employment status (employed vs unemployed) and education (primary vs secondary, tertiary).

[a] Reference for each receptor is the receptor negative

[b] Receptor subtypes were defined by immunohistochemistry as follows: Luminal A (ER +, PR +, HER2 -, Ki 67 <14%), Luminal B (ER +, PR +/-, HER2 -, Ki 67 >14% or ER +, PR +/-, HER-2 +, any Ki 67), HER2-enriched (ER/PR -, HER2 +, any Ki 67) and Triple negative breast cancer (ER/PR/HER2 -, any Ki 67).

[c] Primary education refers to the first 7 years of school education and secondary as the first 12 years.

DISEASE FREE SURVIVAL

Out of 730 patients diagnosed with stage I-III, 160 (21.9%) patients had disease recurrence and 207 (28.4%) had died. One hundred and twenty-three stage 1-3 patients had no record of whether they had disease recurrence. The overall median (IQR) time to disease recurrence was 18.9 (12.0 – 27.1) months with the time being shorter among those who presented with recurrence at distant sites (17.2 (10.1 – 26.2) months) compared to loco-regional sites (22.4 (14.5 – 29.7) months). Majority presented with recurrence at distant sites (55.7%) with lung and pleura combined (35.4%) being the commonest site followed by bone (33.5%), liver (25.3%) and brain (12.0%). Thirty-seven (23.4%) patients presented with disease recurrence loco-regionally while 20.9% had both loco-regional and distant sites of recurrence on follow up. One hundred and seven (66.9%) patients with disease recurrence subsequently died.

HIV positive status, an advanced stage at diagnosis, hormone receptor negative tumours and patients who did not completed their prescribed course of chemotherapy had greater odds of disease recurrence or death (Table 3). Disease free survival (95% CI) was 87.7% (85.3% - 89.7%) after 1 year, 72.8% (69.7% - 75.7%) after 2 years, 61.5% (58.0% - 64.7%) after 3 years and 55.8 (52.1% - 59.3%) after 4 years.

Table 3: Characteristics of those with disease recurrence or death compared to those with no evidence of disease recurrence.

	Disease Recurrence or death	No disease recurrence	Odds Ratio (95% CI) [a]	p-value
Total	367 (50.3%)	363 (49.7%)		
Age at Presentation				
<50	149 (40.6%)	139 (38.3%)	1.00 (Ref)	
≥50	218 (59.4%)	224 (61.7%)	1.00 (0.72 – 1.38)	0.982
HIV status				
Negative	246 (70.5%)	296 (83.6%)	1.00 (Ref)	
Positive	103 (29.5%)	58 (16.4%)	2.63 (1.71 – 4.03)	**<0.001**
Stage at diagnosis				
Early Stage (Stage I/II)	133 (36.2%)	246 (67.8%)	1.00 (Ref)	
Advanced Stage (Stage III)	234 (63.8%)	117 (32.2%)	3.88 (2.80 – 5.36)	**<0.001**
Receptor Status [b]				
ER positive	254 (70.8%)	282 (80.6%)	0.66 (0.46 – 0.95)	**0.027**
PR positive	205 (57.1%)	253 (72.3%)	0.58 (0.42 – 0.81)	**0.001**
Her2 positive	94 (26.6%)	79 (23.2%)	1.03 (0.71 – 1.50)	0.881
Receptor subtype [c]				
Luminal A	37 (10.5%)	60 (17.8%)	1.00 (Ref)	
Luminal B	222 (63.3%)	220 (65.3%)	1.43 (0.89 – 2.30)	0.141
Her2-enriched	22 (6.3%)	13 (3.9%)	2.23 (0.96 – 5.21)	0.063
Triple negative	70 (19.9%)	44 (13.1%)	2.13 (1.19 – 3.84)	**0.011**
Completed Chemotherapy	234 (85.4%)	273 (97.7%)	0.13 (0.05 – 0.31)	**<0.001**
Completed Radiation	123 (100%)	219 (99.6%)		

HIV positive patients:				
Duration HIV positivity				
≤1 year	29 (28.2%)	15 (25.9%)	1.00 (Ref)	
>1 year	74 (71.8%)	43 (74.1%)	1.03 (0.46 – 2.32)	0.936
On ART at diagnosis	62 (61.4%)	38 (65.5%)	0.96 (0.45 – 2.02)	0.905
Duration ART usage				
≤1 year	10 (14.1%)	11 (22.5%)	1.00 (Ref)	
>1 year	61 (85.9%)	38 (77.6%)	1.87 (0.65 – 5.36)	0.243

[a] Odds ratios were adjusted for age at diagnosis (continuous), stage at diagnosis (early (I/II) vs advanced (III)) and receptor subtype (luminal a vs luminal B, Her2-enriched, triple negative)

[b] Reference for each receptor is the receptor negative

[c] Receptor subtypes were defined by immunohistochemistry as follows: Luminal A (ER +, PR +, HER2 -, Ki 67 <14%), Luminal B (ER +, PR +/-, HER2 -, Ki 67 >14% or ER +, PR +/-, HER-2 +, any Ki 67), HER2-enriched (ER/PR -, HER2 +, any Ki 67) and Triple negative breast cancer (ER/PR/HER2 -, any Ki 67).

BREAST CANCER IN HIV POSITIVE PATIENTS

Of the 985 patients with known HIV status, 22% were HIV positive with 71.4% of them already diagnosed with HIV before the time of breast cancer diagnosis. Moreover, 60.1% of HIV positive patients were already on ARTs at the time of breast cancer diagnosis. (Table 1). HIV positive patients were younger than the HIV negative patients with a median (IQR) age of 44 (39-52), when compared to the HIV negative patients with the median age of 57 years (IQR: 46-67, p <0.001). Moreover, the HIV positive patients presented more often with an advanced stage of the disease at the time of breast cancer diagnosis than the HIV negative patients (62.3% vs 53.9%, p=0.028).

Regarding overall survival, HIV infection was strongly associated with a worse overall survival (p<0.001, Figure 3, Table 2). Adjusted hazard ratios (95% CI) for overall survival were 1.77 (1.37 – 2.28) for HIV positive versus HIV negative patients, with age and stage at diagnosis accounting for many of the differences between the unadjusted and adjusted ratios. Among the HIV positive patients, patients who were on ARTs had a better survival when compared to those who were not on ARTs. [HR 0.60 (0.41-0.88), p=0.008; Figure 3B]. Duration of ART use did not however affect overall survival or disease recurrence or death. (Table 4).

HIV infection increased the odds of disease recurrence or death (29.5% vs 16.4%, OR (95% CI): 2.63 (1.71 – 4.03), p<0.001; Table 3). Disease free survival by HIV status for

those on and not on ART is shown in Figure 4. After adjusting for age, stage and receptor subtypes, compared to HIV negative patients, hazard ratios (95% CI) in HIV positive patients on ART were 1.93 (1.44 – 2.58) and HIV positive patients not on ART they were 2.33 (1.57 – 3.48).

Table 4: Hazard ratios for survival from breast cancer in HIV positive patients

			Unadjusted		Adjusted[a]	
	Number at risk	Number of deaths	Hazard Ratio (95% CI)	p-value	Hazard Ratio (95% CI)	p-value
Duration of known HIV positivity	220	122				
Less than or equal to 1 year			1.00 (Reference)		1.00 (Reference)	
More than 1 year			0.76 (0.53-1.12)	0.169	0.80 (0.54 – 1.18)	0.262
Taking ART	217	119				
No			1.00 (Reference)		1.00 (Reference)	
Yes			0.60 (0.41-0.88)	**0.008**	0.74 (0.50 – 1.10)	0.137
Duration of ART use	157	78				
Less than or equal to 1 year			1.00 (Reference)		1.00 (Reference)	
More than 1 year			1.05 (0.59-1.87)	0.880	0.94 (0.51 – 1.71)	0.833
Stage at diagnosis	219	122				
Early stage (Stage I/II)			1.00 (Reference)		1.00 (Reference)	
Advanced Stage (Stage (III/IV)			3.16 (2.06-4.82)	**<0.001**	3.50 (2.24-5.46)	**<0.001**
Receptor Subtype [b]	207	119				
Luminal A			1.00 (Reference)		1.00 (Reference)	
Luminal B			2.27 (1.05 – 4.92)	**0.038**	1.95 (0.90 – 4.24)	0.091
Her2-enriched			3.08 (1.12 – 8.50)	**0.030**	2.10 (0.75 – 5.86)	0.158
Triple negative			2.96 (1.28 – 6.83)	**0.011**	2.88 (1.25 – 6.66)	**0.013**

[a] Adjusted for age at diagnosis (continuous), stage at diagnosis (early vs advanced) and receptor subtype (luminal A vs luminal B, Her2-enriched, triple negative)

[b] Receptor subtypes were defined by immunohistochemistry as follows: Luminal A (ER +, PR +, HER2 -, Ki 67 <14%), Luminal B (ER +, PR +/-, HER2 -, Ki 67 >14% or ER +, PR +/-, HER-2 +, any Ki 67), HER2-enriched (ER/PR -, HER2 +, any Ki 67) and Triple negative breast cancer (ER/PR/HER2 -, any Ki 67).

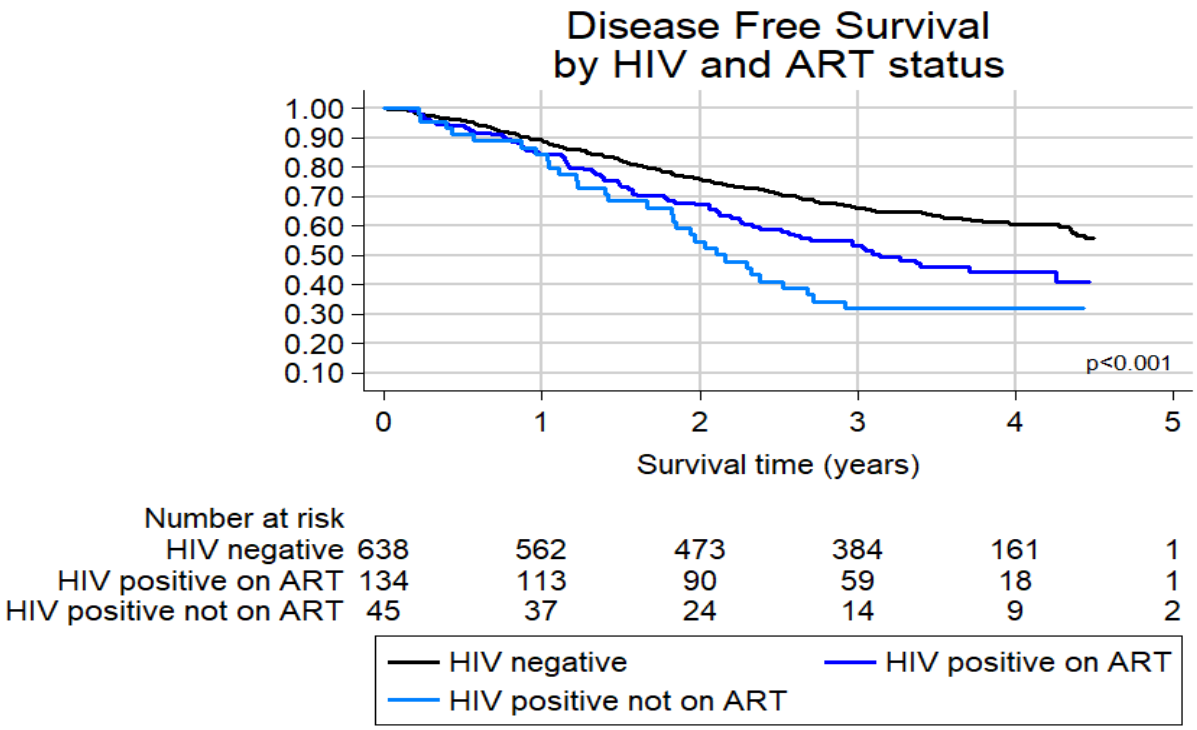


Figure 4: Disease free survival by HIV and ART status for 817 patients diagnosed with stage I – III breast cancer. Unadjusted HR (95% CI) compared to HIV negative: HIV positive on ART 1.60 (1.23 – 2.08), p<0.001; HIV positive not on ART 2.18 (1.49 – 3.19), p<0.001.

DISCUSSION

HIV infection was associated with poorer breast cancer overall survival and greater odds for disease recurrence. A study from Botswana showed that women living with HIV had a lower breast cancer survival than those who were HIV negative.[15] Other studies have also shown that cancer-associated mortality is increased among HIV-positive patients when compared to HIV-negative patients.[16,23,24] In the United States, a double-fold increase in mortality among HIV positive patients with breast cancer was reported, with the hazard ratio range between 1.20 (95% CI, 1.14-1.26) and 2.93 (95% CI, 2.08-4.13).[25] The recently published meta-analysis also revealed a poor overall survival among HIV positive women with breast cancer.[17]

Possible causes for poor breast cancer survival among HIV positive patients remain unclear. One contributory factor is the advanced stage at presentation, which correlates with poorer overall survival. [10,11,26-28] In this study, an advanced disease stage at presentation was strongly associated with a poorer survival compared to an early stage of the disease ($p < 0.001$), even after adjusting for other known risk factors for poor survival. More of HIV positive patients presented with an advanced stage (Stage III/IV) of the disease at the time of breast cancer diagnosis compared to the HIV negative patients (62.3% vs 53.9%, $p = 0.028$). Similarly, other studies have shown that PLWHIV are more likely to present with an advanced stage of disease at the time of cancer diagnosis.[14,19,24] These findings were, however, contrasted by the findings of a study conducted in Uganda, which showed that HIV positive patients were less likely to present at an advanced stage of disease at the time of cancer diagnosis possibly due to an indirect benefit of being part of the healthcare system for ART.[29]

Another contributory factor is age at breast cancer diagnosis. Women younger than 40 years of age and elderly patients (70-89 years old) are reported to have worse outcomes.[10-12] The mortality rate of breast cancer in young women is reported to be twice the rate of women aged 50-59.[11] Factors associated with worse prognosis among young women with breast cancer include triple negative breast cancer, luminal B HER-2 enriched and HER-2 enriched breast cancer subtypes and advanced disease stage at the time of diagnosis.[10,11,30] Furthermore, it has also been shown that despite adequate treatment, the risk of young women dying of breast cancer remains higher compared to the middle-aged women.[31] However, in this study, there was no association between age and worse overall or disease-free survival.

Among HIV positive patients, studies have shown improved survival of patients with both AIDS-defining and non-AIDS defining malignancies with the use of ART.[32,34] However, there was no reported association between the use of ART and survival of patients with breast and lung cancers.[24] In this study, we observed an improved breast cancer overall and disease-free survival among HIV positive patients who were on ART compared to those who were not on ART. This could be explained by the effectiveness of ART use in decreasing HIV-associated deaths and not necessarily the breast-cancer specific mortality. Moreover, ART use is also associated with improved immune system and level of CD4 cells lymphocytes, thus improved level of tumour infiltrating lymphocytes which are associated with favourable breast cancer survival.[35] However, the actual effect of ART use on breast cancer specific mortality still needs to be further explored.

Other contributory factors include triple negative breast cancer and HER-2 enriched subtypes.[10-13] A study conducted in Soweto, South Africa showed a 2.5 and 3-fold increase in mortality among patients with HER- 2 enriched and triple negative breast cancer subtypes, respectively.[14] Similarly, in this study, triple negative, luminal B and

HER-2 enriched breast cancer subtypes were significantly associated with worse breast cancer outcomes.

The overall survival was poor at 55.6% after 4 years of follow-up in this study, an outcome which remains low compared to other geographical regions. In China, an overall survival of 88.64% after 3 years of follow up was reported.[12] In HICs, the overall survival is reported to be higher with the 5-year overall survival of more than 90%.[26] An advanced stage at breast cancer diagnosis is one of the contributing factors for poor survival in LMICs. Studies have shown that more than 75% of patients from several countries in the sub-Saharan Africa have an advanced stage of diseases (stage III/IV) at the time of breast cancer diagnosis.[34,36-38] This is in comparison with patients from HICs, from which less than 20% of breast cancer patients present with an advanced stage of breast cancer.[39] Several other patient-dependent and health care system-dependent factors have been shown to contribute significantly towards late presentation.[34,36,37]

The limitations of this study include short follow up of patients and lack of information to determine the breast cancer-specific mortality rate. Routine staging CT scan is not done therefore, it is possible that we might have under-staged our patients. Moreover, we did not adjust for history of AIDS-defining diseases and CD4+ count at the time of diagnosis, which may play a role in the overall outcome. The severe acute respiratory syndrome coronavirus-2 outbreak disrupted both the clinical care and research not related to COVID-19 globally.[40] In South Africa, the country was put on lockdown from the 26 March 2020, entailing strict movement of individuals except those rendering essential services. [41] During this period, most patients could not come for physical follow-ups mainly due to the increased fear of contracting the virus at the hospital, however telephonic follow-up of these patients was made. Therefore, it is possible that the number of patients with disease recurrence may be underestimated.

CONCLUSION

HIV infection was associated with poor overall and disease-free survival. Patients on ARTs had favourable overall and disease-free survival and with ARTs now being made accessible to all people living with HIV, the overall survival of women with HIV and breast cancer is expected to improve beyond what it is now.

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ETHICS CLEARANCE

Ethics clearance for this study was obtained from the Human Research Ethics Committee (Medical) at the University of Witwatersrand (clearance number: M161130 and M150351).

DISCLAIMER

The authors declare no conflicts of interest.

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CHAPTER 6:

GENERAL DISCUSSION

The true incidence of breast cancer among PLWHIV is unknown due to various and conflicting published findings. In the pre-ART era, it was reported to be lower than in the period before the AIDS epidemic[56]. Amir and colleagues reported a significant decline in the number of reported breast cancer cases in the era of AIDS than was reported before 1983[56]. Several factors may account for the decline observed, including under-reporting, failure of patients to seek medical help, resorting to alternative medicine and the high AIDS-associated mortality rate observed in the pre-ART era[56]. It was also initially thought that HIV-associated immunosuppression offered some protection against breast cancer, thus the decrease in breast cancer cases.[50,54].

Studies have shown a significant decrease in the incidence of AIDS-defining malignancies due to a decline in AIDS-associated mortalities because of the availability and effectiveness of ART[45,60]. However, there was an observed steady increase in the incidence of NADMs due to improved and prolonged life expectancy[45, 60]. The incidence of breast cancer, a non-AIDS defining malignancy, was also expected to increase[45]. Interestingly, it was observed that ART might, directly and indirectly, predispose patients to breast cancer development. PLWHIV on ART were reported to have a high prevalence of metabolic syndrome which is an independent risk factor for breast cancer compared to those not on ART[106,107]. Efavirenz, one of the ART drugs previously used, was reported to stimulate oestrogen receptors in breast tissue, thereby predisposing patients to develop breast cancer. However, its carcinogenic potential remains to be confirmed[108]. If HIV-associated immunosuppression offered protection against breast cancer as was thought, then ART-associated immune restoration may reduce protection against breast cancer development[106].

Contrary to what has been predicted regarding the true incidence of breast cancer among PLWHIV, the current study found the incidence of breast cancer in HIV positive patients being similar to that of the general population. Several studies have reported similar findings[60,61,] but two other South African studies conducted in the ART era in

Durban and Ga-Rankuwa demonstrated a higher incidence of breast cancer in HIV positive patients[64,65]

Among HIV positive patients, several published studies have reported breast cancer being diagnosed at a younger age [56, 60, 61, 64, 65,110,111]. In the current study, we found similar findings with breast cancer diagnosed at a younger age, with a median age of 45 (40-52) years in HIV positive patients and a median age of 57 (46-67) years in HIV negative patients ($p<0.001$). However, when age-stratified, there was no difference in the age of presentation between the two groups.

In this study, 55.1% of patients under 50 years of age presented with an advanced stage of disease (Stage III/IV), which is significantly higher than that found in high-income countries (<20%)[109]. However, this is better than that reported in other sub-Saharan African countries, in which >75% of patients presented with an advanced stage of disease at the time of breast cancer diagnosis[56,64,65,110,111]. Several patient-dependent and health system-dependent factors have been identified, which may explain the reasons for the advanced stage of disease at the time of diagnosis[126-128]. Delayed presentation is one of the contributory factors. It may be due to lack of breast cancer awareness resulting in symptoms being taken lightly, a low level of education, low-income status and patients having to attend several health facilities before a breast cancer diagnosis is made[128,131]. In a local study done by Joffe and colleagues (2018), it was found that limited patient education, lack of breast cancer awareness and health facility factors were barriers to patients presenting early for breast cancer diagnosis[128]. Patients who had breast symptoms for longer than three months when they first present to any health facility were strongly associated with an advanced stage of breast cancer at the time of diagnosis [128]. Among these patients, most of them revealed a fear of breast cancer diagnosis, not taking breast symptoms seriously and varying beliefs as reasons for seeking health care services late[128]. Most patients reported having no knowledge or awareness of breast cancer[128]. Patients who did not complete high school and those from households with low-income levels were also more likely to seek healthcare services late than those who completed high school or middle- to high-income households[128]. In Cape Town, South Africa, Hoffman and colleagues (2000)

also demonstrated that patients with a higher education level, those who could afford private healthcare facilities as they had medical aids and those from urban areas, which is commonly those from middle- to high-income households, were associated with early presentation to health care facilities and an early diagnosis of breast cancer[132].

Moreover, the distance between where patients lived and the healthcare facility also contributes to patients seeking healthcare services early. In a study done by Murugan and colleagues (2014), it was found that patients who lived further than 20 km from the healthcare facility were associated with delayed presentation and an advanced stage of disease at the time of breast cancer diagnosis[133]. Patients who had to visit more than two health care facilities before diagnosis were also more likely to have an advanced stage of disease[125]. Some patients had to have repeated follow-up visits in the same facility before being referred to a breast unit for diagnostic investigation[128]. In our study, the possible explanation for the findings could be that the study was done on an urban population with easy access to health care. Various breast cancer awareness campaigns are conducted by different non-governmental organisations in the region, increasing awareness and education about breast cancer. Majority of HIV positive patients presented with an advanced stage (Stage III/IV) of disease at the time of breast cancer diagnosis compared to HIV negative patients (62.3% vs 53.9%; p =0.028), when unadjusted for age and ethnicity. Several previous studies have also shown that people living with HIV are likely to present with an advanced stage of disease at the time of cancer diagnosis[60,112,114]. However, in contrast, findings of a study conducted in Uganda showed that HIV positive patients were less likely to present at an advanced stage of disease at the time of cancer diagnosis, possibly due to an indirect benefit of being part of the healthcare system for ART[114].

More than 75% of breast cancers were shown to be oestrogen receptor-positive in this study using immunohistochemistry, which correlates with published data in the US[14]. However, Luminal A subtype only accounted for 13.8% of all immunohistochemistry-based intrinsic subtypes, however, with the PAM50 assay, it accounted for only 16.7% of all the intrinsic subtypes, the value which is significantly lower than that reported in the US (45% (US) vs 16.7% (current study)[81] n Other PAM50-based studies had similar

findings, showing Luminal A as the predominant subtype[24,29]. The Luminal B intrinsic subtype was predominant in the current study, accounting for 64.9% of subtypes by immunohistochemistry. However, using PAM50, the HER-2 enriched subtype was the most predominant, accounting for 32.6% of all intrinsic subtypes. This number is significantly greater than previously published, with PAM50-based HER-2 enriched subtype accounting for less than 4-20% of all intrinsic subtypes in studies done in the US[14,29,73,81].

The HER-2 enriched subtype is associated with poorer clinicopathological outcomes, with a high risk for loco-regional and distant site recurrence, with liver and lungs predominantly involved[14]. In areas where HER-2 targeted therapy is not available, the overall breast cancer survival is poor[14]. At the time of this study, HER-2 targeted therapy was not available but, it has recently become available in Public Hospitals to selected patients with a better overall prognosis and it is given in an adjuvant setting only. In the current study, the HIV status did not impact the immunohistochemistry-based nor PAM50-based intrinsic subtypes. Several other studies have also demonstrated that HIV infection had no impact on the pathological characteristics of breast cancer[60,61,64,65]. Unfortunately, there is no other available published literature on the impact of HIV on the PAM50-based intrinsic subtypes with which to compare our findings.

In this cohort, the overall survival was poor at 55.6% after 4 years of follow-up, an outcome which remains low compared to other geographical regions. A retrospective study done in China showed an overall survival of 88.64% after three years of follow-up[130]. In HICs, the overall survival is reported to be higher, with the five-year overall survival estimated to be more than 90%[114]. Other studies have also shown that cancer-associated mortality is high among HIV-positive compared to HIV-negative patients[112,115,117]. In the United States, Coghill and colleagues (2019) found a two-fold increase in mortality among HIV positive patients with breast cancer, with the hazard ratio range between 1.20 (95% CI, 1.14-1.26) and 2.93 (95% CI, 2.08-4.13)[117]. Even though overall survival might be poorer, breast cancer survivals were shown to be better compared to other non-AIDS defining malignancies[115,117].

Possible causes for poor breast cancer survival among HIV positive patients remain unclear. One contributory factor is the advanced stage at presentation, which correlates with poorer overall survival[114, 118-120]. In this study, an advanced disease stage at presentation was strongly associated with a poorer survival compared to an early stage of the disease ($p < 0.001$), even after adjusting for other known risk factors for poor survival. More of HIV positive patients presented with an advanced stage (Stage III/IV) of the disease at the time of breast cancer diagnosis compared to the HIV negative patients (62.3% vs 53.9%, $p = 0.028$). Similarly, Gotti D et al (2014) also found that PLWHIV are more likely to present with an advanced stage of disease at the time of cancer diagnosis[116].

Age is also one of the prognostic factors for breast cancer survival, with women younger than 40 years of age and elderly patients (70-89 years old) are reported to have worse outcomes[118,119,125]. The mortality rate from breast cancer in young women has been shown to be twice the rate of women aged 50-59, with a hazard ratio of 2.26 (95% CI 1.81-2.82)[119]. The postulated reasons for this observation are the strong association with young age and poor prognostic factors such as advanced stage at presentation and aggressive tumour biology such as triple-negative breast cancer, Luminal B, HER-2 enriched and HER-2 enriched breast cancer subtypes and advanced disease stage at the time of diagnosis[118,129,121]. It was also shown that, despite adequate treatment, the risk of young women dying of breast cancer remains high compared to the middle-aged women[122]. Several local studies have, however, shown contrasting finding concerning young women presenting at an advanced stage of disease at the time of diagnosis and reported no association between the age and stage of disease at breast cancer diagnosis[128, 133]. In the current study, we found no association between age at breast cancer diagnosis and an advanced stage at presentation or between age and overall poor survival ($p=0.230$).

Other factors known to be associated with worse overall breast cancer survival and disease progression include triple-negative breast cancer and HER-2 enriched subtypes[118, 119,125,134]. A retrospective study conducted in Soweto, South Africa, showed a 2.5 and 3-fold increase in mortality among patients with HER- 2 enriched and triple-

negative breast cancer subtypes, respectively[112]. In our study, IHC-4 receptor subtypes did affect the overall survival and disease progression , with triple-negative and HER-2 enriched breast cancer subtypes being significantly associated with worse outcomes. Moreover, our preliminary PAM50- based intrinsic subtypes results also demonstrated an association between PAM50 HER-2 enriched & basal-like (triple negative) breast cancer subtypes, the findings which will be confirmed with a larger sample size.

Patients who were not on ART at the time of breast cancer diagnosis appeared to be more likely to present at an advanced stage (Stage III/IV) of disease than those who were on ART (59.1% vs 71.4%; p=0.09). These patients were also more likely to present with evidence of distant metastases than those on ART (25.4% vs 14.4%; p=0.05). However, ART use had no impact on the pathological characteristics of breast cancer and the PAM50-based intrinsic subtypes. We further observed an improved overall and disease-free survival among HIV positive patients on ART at the time of breast cancer diagnosis than those who were not. Several studies have shown improved survival of patients with AIDS- and non-AIDS defining malignancies with the use of ART[123,124]. However, the impact of ART use on the survival of cancer patients was shown to be greater among patients with AIDS- rather than non-AIDs defining malignancies[116]. Whether ART directly or indirectly affects breast cancer progression is an area that still needs to be explored further. The use of ART did not have a significant impact on disease progression (p=0.158).

The use of molecular assays (Oncotype DX, MammaPrint, PAM50, EndoPredict and so on) to predict the risk of breast cancer recurrence and likelihood of an early stage, ER-positive breast cancer to respond or benefit from the use of adjuvant hormonal therapy with or with the addition of adjuvant chemotherapy is common in high-income countries. However, in low- middle-income countries, immunohistochemistry is the mainstay of determining the intrinsic subtypes due to easy accessibility and lower cost than molecular assays. MammaPrint has been available in South Africa since 2007 and has significantly changed and positively impacted the decision making in the treatment of patients with early-stage breast cancer[135]. Several studies conducted in Cape Town, South Africa, to determine the impact of MammaPrint on treatment decision-making in

patients with early-stage breast cancer found that it could provide predictive information beyond that given by the clinicopathological characteristics[136]. It was also shown that the use of MammaPrint could reduce the use of adjuvant chemotherapy by more than 15% as it could reclassify patients deemed high risk for recurrence when clinicopathological parameters were used to being low risk[136, 137]. In the same studies, it was also shown that using MammaPrint in combination with immunohistochemistry could identify endocrine treatment-resistant hormone-positive breast cancer; something that immunohistochemistry alone cannot achieve[135]. This information is particularly useful as these groups of patients may be spared hormonal therapy and be treated accordingly. With the local data available, there is proof of the clinical usefulness and utility of molecular assays that may significantly contribute to minimising chemotherapy use.

Oncotype Dx is also available in South Africa but is used predominantly in private healthcare institutions due to cost (estimated to be >R30000 per patient). The University of the Witwatersrand Molecular Biology Laboratory is the first centre in the country to use the Prosigna Nanostring PAM50 assay. Its use is currently limited to research, with this study being the first to use the assay. In addition to the cost-related issues associated with the use of molecular assays in low and middle-income countries is the shortage of trained technical expertise for the equipment needed to use the assays. In the current study, the Prosigna Nanostring PAM50 assay was used, and, unfortunately, the Nanostring machine broke down while busy with the analysis of the extracted RNAs. There were no local Nanostring-trained technicians, and we had to rely on the technicians from the US. Unfortunately, this incident coincided with the beginning of the Covid-19 pandemic.

Following the rapid rise of confirmed Covid-19 infections in South Africa, the country was placed on a Level 5 national lockdown in March 2020 as one of the ways to curb the further spread of the virus[138]. The regulations strictly curtailed the movement of people, and international travel was not permitted, prohibiting the US-based trained Nanostring technician from assisting with repairing the Nanostring machine. Almost a

year later, the machine has still not been repaired, something that could have been avoided if we had trained technicians in the country. However, with more training of technical expertise and medical and clinician-scientists being made more available in low- middle-income countries, there is still hope for the wide use of breast cancer molecular assays extending their use to influence clinical decision making.

The limitations of this study include a possible underestimation of the number of patients with metastatic disease as the staging CT scan is not routinely done for all patients diagnosed with breast cancer. Moreover, we only had a year when ARTs were started, as a result we could assess if there was any change in the presentation and outcome of breast cancer within 6 months of ARTs initiation. Furthermore, we also had a short patient follow-up which made it difficult to determine a reasonable overall survival of patients. At least five years of follow-up is reasonable to determine the survival of breast cancer patients. Cancer-specific mortality could also not be ascertained as, in most cases, the information regarding the death of patients was obtained from relatives with breast cancer being mentioned as the cause of death, making it difficult to determine the cancer-specific mortality. Moreover, patients presenting with locally advanced disease did not get routine staging CT scans; therefore, it is possible that we might have under-staged this group of patients. There was also bias in the selection of patients' who sample were selected for the PAM50 analysis.

Lastly, the COVID-19 pandemic also had a significant adverse effect on our study. Since the implementation of alert Level 5 lockdown in our country, patients' physical follow-ups were interrupted, making it possible that the true number of patients with disease progression may be underestimated. These patients mainly were followed up telephonically. With the technical difficulty we had with the Nanostring machine, only 144 RNA samples could be analysed, a small sample to make any definitive conclusion on the findings or determine the risk of recurrence (ROR) score. Moreover, the strength of the study is that this was the first study we know of to look at the impact of HIV on the PAM50-based intrinsic subtypes and when all samples have been analysed, the findings of which will open many research opportunities in this area, globally.

CHAPTER 7:

CONCLUSION

HIV-positive patients were younger and presented with a late stage of disease than HIV-negative ones, the latter being significant when unadjusted for age & ethnicity. Moreover, HIV infection was associated with a worse overall and disease-free survival. HIV positive patients who were not on ARTs at the time of breast cancer diagnosis were more likely to present at an advanced stage of the disease at the time of presentation, worse overall and disease-free survival. Neither did HIV infection nor the use of ARTs had an impact on the histopathological characteristics & PAM50 intrinsic subtypes. PAM 50 intrinsic subtypes demonstrate that our cohort had aggressive intrinsic subtypes, however, this finding needs to be confirmed with a larger sample size. We recommend completion of the PAM50 analysis to enable us to make a conclusive conclusion on the impact of HIV on the PAM50 intrinsic subtypes as well as to determine the risk of recurrence (ROR) score for our cohort. The findings have a great potential to add to the body of knowledge in this area as well as to influence future research projects.

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APPENDIX

1. PAM50 GENES WITH THEIR FUNCTIONS

GENES	FULL NAME	FUNCTION
UBE2T	Homo sapiens ubiquitin-conjugating enzyme E2T (putative) (UBE2T)	-It's overexpression is associated with worse outcome in breast cancer, especially in Luminal and basal subtype tumours[139] -Plays a role in the mutation of BRCA 1[139]
PTTG1	Homo sapiens pituitary tumor-transforming 1 (PTTG1)	-An oncogenic gene -Promotes the development of breast cancer through the regulation of p27 gene, which partakes in the cell division[140]
PGR	Homo sapiens progesterone receptor (PGR)	- Influences ER transcription hereby promoting breast cancer differentiation and Growth[141]
MKI67	Homo sapiens antigen identified by monoclonal antibody Ki-67 (MKI67)	-Proliferative marker -Associated with worse tumour biology and worse outcome[142]
MIA	Homo sapiens melanoma inhibitory activity (MIA)	-Plays a significant role in the development of melanoma[143]
MAPT	Homo sapiens microtubule-associated protein tau (MAPT)	-It's overexpression is associated with better overall outcome in breast cancer patients[144]
KRT17	Homo sapiens keratin 17 (KRT17)	-Involved in the development of breast cancer and other tumours, including gastric cancer -Overexpressed in basal-like breast cancers[145]
KRT14	Homo sapiens keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner) (KRT14)	-Prognostic gene associated with worse overall survival (KRT14 word) -Overexpressed in tipple negative breast cancers[146]
KIF2C	Homo sapiens kinesin family member 2C (KIF2C)	-Involved in the development and progression of breast cancer It's overexpression is associated with worse tumour biology and overall outcome[147]

ESR1	Homo sapiens estrogen receptor 1 (ESR1)	- It's activation of ER can promote cell differentiation and prevent apoptosis[141] -Regulates the transcription of other markers such as MYC, cyclin D1, cyclin E1, and cyclin E2[141].
CCNE1	Homo sapiens cyclin E1 (CCNE1)	- Contributes to the cell cycle dysregulation[148] -It's overexpression is associated with resistance to chemotherapy -Associated with poor overall survival in triple negative breast cancer cases[148]
CENPF	Homo sapiens centromere protein F, 350/400ka (mitosin) (CENPF)	-Promotes cell growth and proliferation -Enhances progression of breast cancer[149]
CEP55	Homo sapiens centrosomal protein 55kDa (CEP55)	-Plays a role in a final stage of cell cycle. It's dysregulation and overexpression is associated with aggressive tumour biology and worse outcome[150]
FGFR4	Homo sapiens fibroblast growth factor receptor 4 (FGFR4)	-Oncogene
MMP11	Homo sapiens matrix metallopeptidase 11 (stromelysin 3) (MMP11)	-An important marker for breast cancer metastasis[141]
SFRP1	Homo sapiens secreted frizzled-related protein 1 (SFRP1)	-It has tumor suppression effect -It's expression is lost in patients with breast cancer -It's expression may serve as a useful prognostic marker [151]
TMEM45B	Homo sapiens transmembrane protein 45B (TMEM45B)	-It is expressed in some breast cancers -Plays a significant role in the development and progression of gastric cancer[152]
TYMS	Homo sapiens thymidylate synthetase (TYMS)	-Affects the stability of mRNA -The exact association in the development and progression of breast cancer is still to be determined[153]

ERBB2	Homo sapiens v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (ERBB2)	-It's overexpression is associated with poor overall outcome[10,11]
CDCA1	Homo sapiens cell division cycle associated 1 (CDCA1)	-Plays a significant role in the cell cycle through regulation of nuclear division[154]
BCL2	Homo sapiens B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein	- Inhibit programmed cell death thereby promoting development of breast cancer[141] -It's overexpression is associated with worse overall breast cancer outcome[141]
CCNB1	Homo sapiens cyclin B1 (CCNB1)	-It's overexpression is associated with genes responsible the resistance towards hormonal therapy -Prognostic marker in ER positive breast cancer in terms of overall survival, disease-free survival[141]
CDC20	Homo sapiens CDC20 cell division cycle 20 homolog (S. cerevisiae) (CDC20)	-It's overexpression is associated with progression and metastases of breast cancer[155]
NAT1	Homo sapiens N-acetyltransferase 1 (arylamine N-acetyltransferase) (NAT1)	-Associated with expression of estrogen receptors, thus luminal breast cancer subtypes -Involved in the development and progression of breast cancer[156]
ORC6L	Homo sapiens origin recognition complex, subunit 6 like (yeast) (ORC6L)	- Promotes cell growth and proliferation[141]
RRM2	Homo sapiens ribonucleotide reductase M2 polypeptide (RRM2)	-Promotes metastatic potential of a breast cancer[157]
UBE2C	Homo sapiens ubiquitin-conjugating enzyme E2C (UBE2C)	-Oncogene - It's over-expression is associated with worse outcome in breast cancer patients, esp with BRCA mutation[158]
ACTR3B	Homo sapiens ARP3 actin-related protein 3 homolog B (yeast) (ACTR3B)	- Induce cell-shape change and motility

		- May decrease the metastatic potential of tumors[159]
ANLN	Homo sapiens anillin, actin binding protein (scraps homolog, Drosophila) (ANLN)	-Plays a role in cell proliferation -Prognostic biomarker in breast cancer -Its overexpression is associated with aggressive tumour biology and poor outcome[160]
BAG1	Homo sapiens BCL2-associated athanogene (BAG1)	-Prognostic biomarker in breast cancer -Its overexpression is associated with favourable outcome[161]
BIRC5	Homo sapiens baculoviral IAP repeat-containing 5 (survivin) (BIRC5)	-Usually inhibits apoptosis of cells. Its overexpression is associated with worse overall outcome. It is also contributes towards chemo-resistance[141]
BLVRA	Homo sapiens biliverdin reductase A (BLVRA)	-Regulates growth and proliferation of cells -Over-expressed in several tumours[162]
CDC6	Homo sapiens CDC6 cell division cycle 6 homolog (S. cerevisiae) (CDC6)	-Promotes breast cancer progression -Associated with worse prognosis[163]
CDH3	Homo sapiens cadherin 3, type 1, P-cadherin (placental) (CDH3)	-Involved in the development and progression of breast cancer[164]
CXXC5	Homo sapiens CXXC finger 5 (CXXC5)	- It's overexpression is a poor prognostic factor in ER+ breast cancer[165]
EGFR	Homo sapiens epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian) (EGFR)	- Promotes cell division and, motility[166]
EXO1	Homo sapiens exonuclease 1 (EXO1)	-Oncogenic -Promotes development of breast cancer -Potential biomarker for preventive and treatment strategies[167]
FOXA1	Homo sapiens forkhead box A1 (FOXA1)	-Prognostic marker -Predominantly overexpressed in Luminal A breast cancer subtypes

		-It's overexpression is associated with favourable outcome[168]
FOXC1	Homo sapiens forkhead box C1 (FOXC1)	-Regulates various oncogenic genes -It's overexpression is associated with dysregulation of cell division, motility as well as angiogenesis[169]
GPR160	Homo sapiens G protein-coupled receptor 160 (GPR160)	-Important biomarker in triple negative breast cancer (TNBC) -Often downregulated in TNBC[170]
GRB7	Homo sapiens growth factor receptor-bound protein 7 (GRB7)	-Prognostic biomarker -It's overexpression is associated with worse outcome[171]
KNTC2	Homo sapiens kinetochore associated 2 (KNTC2)	-Promotes cell division and growth[172]
KRT5	Homo sapiens keratin 5 (epidermolysis bullosa simplex, Dowling-Meara/Kobner/Weber-Cockayne types) (KRT5)	-Prognostic biomarker commonly expressed in basal subtype breast cancer[173]
MDM2	Homo sapiens Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) (MDM2)	-Regulates the tumor suppressor gene, p53 -Rarely overexpressed in breast cancer than other cancer It's role in breast cancer development still needs to be determined[174]
MELK	Homo sapiens maternal embryonic leucine zipper kinase (MELK)	-It's dysregulation is associated with aggressive tumour biology and worse overall outcome[141]
MLPH	Homo sapiens melanophilin (MLPH)	-Overexpressed in ER + breast cancer subtypes and is associated with good overall outcome[175]
MYBL2	Homo sapiens v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2)	-Regulates cell growth and differentiation -Involved in DNA repair pathways[176]
MYC	Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC)	-Promotes cell growth and differentiation[177]

PHGDH	Homo sapiens phosphoglycerate dehydrogenase (PHGDH)	-Promotes cell growth and differentiation -Overexpressed in ER – breast cancer subtypes[178]
SLC39A6	Homo sapiens solute carrier family 39 (zinc transporter), member 6 (SLC39A6)	-Its over-expression correlates with the better outcome of breast cancer patients [179]