Chemotherapy naive breast cancer: a correlation study between BD Cytorich™ Red cell blocks and formalin- fixed paraffin-embedded tissue blocks.

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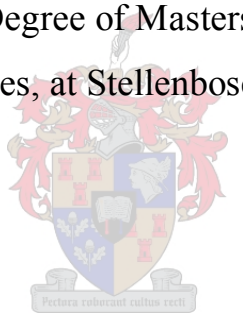
Baccalaureus Technologiae in Biomedical Technology (CPUT)

Dissertation presented for the Degree of Masters in Pathology in the Faculty of Health Sciences, at Stellenbosch University



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April 2019

Declaration

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April 2019

Abstract

Background: The ever increasing burden of breast cancer, the most common cancer among women, demands a diagnostic test that is rapid, reliable, informative and cost-effective; particularly in countries with limited financial and medical resources. FNAB cytology and cell block combination has gained worldwide utility and has been described to be accurate and reliable.

Aim: Henceforth the aim of this study was to retrospectively correlate the expression of prognostication markers, namely, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) performed on cell blocks (using the BD Cytorich™ Red method) and formalin-fixed, paraffin-embedded cell blocks (FFPET) in chemotherapy naive breast carcinomas, by immunochemistry (immunocytochemistry and immunohistochemistry respectively) and to perform fluorescence in-situ hybridization (FISH) testing for over- expression of the HER2 gene.

Methods: Between 2013 and 2016, 132 cases of primary breast carcinoma were identified that had both cytology (including Cytorich™ Red cell blocks) and histology specimens that were both chemotherapy naive. Immunostaining for ER, PR and HER2 was performed. The staining was scored according to the Allred scoring system for histology specimen and this system was slightly adapted for cytology specimens, which also took the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines into account. FISH for HER2 over-expression was performed. The grade of the carcinoma was also analysed on both the cytology specimens (using the Robertson's grading system) and the histology specimens (using the Modified Elston & Ellis system).

Results: ER and PR performed on cell blocks had good correlation with FFPET with 91% and 85% sensitivity, respectively. HER2 on cell blocks had an agreement of 88% with FFPET. 87.88% of cell blocks had more than a 100 tumour cells present on H&E sections and cytological grading had an agreement of 41.41% with histological grading.

Conclusion: The cell block technique continues to play a vital role in the diagnosis of primary, recurrent and metastatic breast carcinoma, allowing assessment of prognosis and prediction of response to therapy.

Abstrak

Agtergrond: Borskanker is die allergrootste las wat op vouens rus en is die mees algemeenste kanker onder vroue. As gevolg hiervan is diagnostiese toetse wat vinnig, bertroubaar en insiggewend is belangrik, veral in lande met beperkte mediese en finansiële hulpbronne. Fyn naald aspirasie en die selblok kombinasie het wêreldwyd nut verwerf en word beskryf as ‘n betroubare en akkurate toets.

Doelwitte: Die doelwit van hierdie studie was om retrospektief te korreleer of voorspelling merkers, naamlik estrogeen reseptor (ER), progesteroon reseptor (PR) en menslike epidermale groei faktor reseptor 2 (HER2) uitgevoer op selblokke (met gebruik van die BD Cytorich ™ Red metode) en formalien gefikseerde, paraffien inbedding weefsel blokke in chemoterapie naïve borskarsinome te korreleer deur immunochemie. Die uitvoering van FISH toetse vir die oor uitdrukking van die HER2 geen was ook gedoen.

Metodes: Tussen 2013 en 2016 was 132 gevalle geidentifiseer met ‘n primêre diagnose van borskarsinoom, wat albei sitologie (insluitend BD Cytorich™ Red selblokke) en histologie monsters het. Immunokleuring vir ER, PR en HER2 was gedoen en die kleuring was deur ‘n puntestelsel volgens die Allred stelsel vir histologie gedoen wat egter effens aangepas vir sitologie selblokke was. Die Amerikaanse vereniging van kliniese onkologie (ASCO)/ kollege van amerikaanse patoloë (CAP) riglyne was ook toegepas vir HER2 ontleding asook FISH toetse vir HER2 ekspressie. Die gradering van karsinome was ook geontleed vir beide sitologie monsters met aanwending van die Robinson’s gradering sisteem en vir histolgie monsters met aanwending van die gemodifiseerde Elston en Ellis sisteem.

Resultate: ER en PR uitgevoer op selblokke het goeie korrelasie met weefsel blokke getoon met ‘n sensitiwiteit van 91% en ‘n spesifisiteit van 62% vir ER en ‘n sensitiwiteit van 85% en spesifisiteit van 84% vir PR. HER2 op selblokke het ‘n ooreenstemming van 88% gehad met weefsel blokke en 87.88% van die selblokke het meer as 100 tumor selle teenwoordig gehad op H&E snitte. Gradering van sitologie het ‘n ooreenstemming van 41.41% met histologie gradering gehad.

Gevolgtrekkinge: Die selblok tegniek speel ‘n belangrike rol in die diagnose van primêre, terugkerende en metastase van borskarsinoom. Terselfte tyd word die berekening van prognose toegelaat asook die voorspelling van toepaslike behandeling.

Acknowledgements

I want to give praises God for granting me the strength, grace and mercy.

I would like to express gratitude to my supervisor Dr Pawel Schubert and collaborator Prof Johann Schneider for giving me the opportunity to execute this study.

Appreciation to Dr Ayanda Mfokazi, Mr Abraham Manual; Ms Greta Neethling; Miss Christine Van Wyk; Ms Ursula Paulsen and Mr Stephen Southey for all their help.

A kind thank you to Unistel laboratories for processing of FISH samples, NHLS Grant department for funding this project and Mr Carl Lombard at the Biostatistics unit at Stellenbosch University for performing statistical analysis.

Dedications

I dedicate this dissertation to my parents Pieter and Rosie Van Rooyen.

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List of Abbreviations

ASCO/ CAP:	American Society of Clinical Oncology/ College of American Pathologists
CEN17:	Centromere 17
CISH:	Chromogenic in situ hybridization
DCIS:	Ductal carcinoma in situ
DISH:	Dual in situ hybridization
ER:	Estrogen Receptor
FDA:	Food and Drug Administration
FFPET:	Formalin Fixed Paraffin Embedded Tissue block – always refers to histological specimens (biopsies or resection specimens)
FISH:	Fluorescent in situ hybridization
FNA:	Fine Needle Aspiration
FNAB:	Fine Needle Aspiration Biopsy
HER2:	Human Epidermal Growth Factor Receptor 2
ICC:	Immunocytochemistry
IDC:	Invasive ductal carcinoma / invasive carcinoma, no specific type.
IHC:	Immunohistochemistry
PR:	Progesterone Receptor
LCIS:	Lobular carcinoma in situ
MGG:	May Grunewald Giemsa

Chapter 1: Literature review

1.1 Breast cancer background

1.1.1 Incidence and mortality

In both developed and developing countries, breast cancer has now become the most common malignancy in women. In South Africa breast cancer accounts for 19.4% of all cancers observed in women compared to 10% worldwide as recorded in 2008.[1] In 1997 about 895 000 of new breast cancer cases were reported with 467 000 deaths worldwide.[2] However, in 2008 about 1 384 155 new cases were calculated from population-based cancer registries and 459 000 deaths were recorded.[2,3]

Globocan 2012 statistics indicated a startling 1.7 million women that were diagnosed with breast cancer and 522 000 related deaths.[3] The current life expectancy in South Africa is 45 years whereas life expectancy in developed countries is 82 years of age.[1] Aetiology and pathological characteristics of breast cancer are highly heterogeneous. Thus some tumours demonstrate a slow growth pattern with excellent prognosis whereas others demonstrate a highly aggressive clinical progression and poor outcome. Women in developing countries tend to have a poorer prognosis and the incidence rate is expected to increase further, mainly due to adoption of a more westernised lifestyle(s), with little or no physical activity and delayed child bearing.[3,4] However, early diagnosis and onset of treatment, improved treatment modalities and screening programs have created discrepancies between incidence and mortality.[2]

Mammography and improved therapies have allowed breast cancer to be detected earlier in developed countries.[5] South Africa has relatively sparse information regarding cancer incidence and mortality data; and having population based cancer registries can become very important in breast cancer surveillance.[6]

1.1.2 Risk Factors

Large differences exist between the geographical zones that demonstrate a high risk for breast cancer. The large differences may be due to variations in genetics, lifestyle and environmental factors.[2]

Age is however considered a major risk factor of breast cancer and cancer in general. A vast majority of breast cancer occur in women older than 50 years of age.[2] More worrying is the

2increase of breast cancer in women under 45 years of age. Age is however not the sole contributing factor. In addition to age, other risk factors include diet, reproductive and hormonal changes, genetics, exposure to chemical and hazardous substances, radiation, tobacco smoking, alcohol consumption as well as numerous unknown factors.[2]

The role of BRCA1 and BRCA2 gene expression in the aetiology of breast cancer has been studied extensively. These genes are classified as tumour suppressor genes and are mainly involved in DNA repair, transcriptional regulation, genomic stability and cell cycle control. However a disruption of these genes may increase risk for breast cancer development. The estimated life-time risk for breast carcinoma in women expressing these genes is between 50-80% compared to a risk of fewer than 10% for the general population. Therefore having knowledge of their presence in any individual allows for regular follow up for early diagnosis, quicker initiation of treatment and improved overall survival.[2,7]

Recent studies regarding hormones and its effects on breast cancer risk confirm that woman who had early menarche, remain nulliparous, have delayed childbearing, lack of breastfeeding, late onset of menopause or exposure to endogenous and exogenous estrogen hormones most frequently develop breast cancer.[2,8]

Estrogen is essential in cancer initiation and development and prolonged exposure to estrogen and progesterone contribute to the development of breast cancer. Removal of endogenous estrogen by oophorectomy decreases risk, but the primary source of estrogen in postmenopausal women is adrenal gland derived androgenic precursors, converted with the aid of aromatase enzymes to estrogen in adipose tissue. Therefore increased body fat leads to increased estrogen levels in postmenopausal women and they are more likely to develop breast cancer.[2,8]

1.1.3 Structure of the breast

The breast consists of 15 to 25 independent glandular units named breast lobes, each consisting of several lobules and compound tubule- acinar glands. These lobes are embedded in a network of adipose tissue that is subdivided by collagenous septa. The lobes are arranged radially at different depths around the nipple. The lactiferous duct is a single large duct, draining each lobe via a separate opening on the surface of the nipple. The lactiferous sinus is a dilation formed by the duct before opening onto the surface.

Each breast lobe is divided into variable numbers of breast lobules and each lobule consists of a system of ducts. Lobules are separated by moderately dense collagenous interlobular

tissue, whereas the intra- lobular supporting tissue surrounding the ducts within each lobule is less collagenous and more cellular.[9]

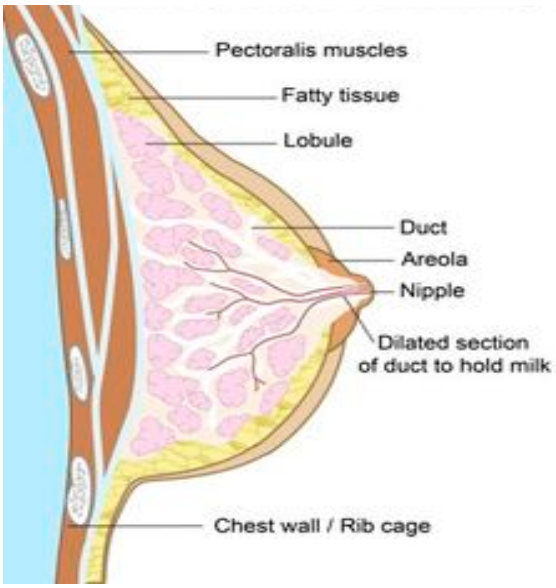


Figure 1.1 Anatomy of the female breast (https://www.kullabs.com/uploads/image00154.jpg)

1.1.4 Breast Pathology

Breast cancer is an epidemic that extensively affects women of all ages and race.[2] Breast pathology plays a vital role in the assessment of breast disease by providing initial diagnosis, prognosis and prediction of treatment (theranostics).

Material from the breast can be collected for pathological examination via either: fine needle aspiration biopsy (FNAB) for cytological diagnosis, or core needle biopsy or surgical excision for histological diagnosis. These techniques can be used to make a diagnosis of breast carcinoma, and to determine a particular tumour's susceptibility to hormonal therapy or the recently introduced Trastuzumab® for HER2 over-expression. The pathology report must be informative and accurate and should include factors important for prognosis and treatment, such as tumour size, type, grade, lymph node involvement and presence of vascular invasion.[10]

1.1.5 Types of Breast Carcinomas

1.1.5.1 Carcinoma in- situ

Carcinoma is the most common malignant neoplasm of the breast. Distinction between carcinoma in-situ and invasive carcinoma is important due to different treatment protocols.

At time of diagnosis carcinoma in-situ may be clinically asymptomatic, presents with mammary Paget's or with similar signs and symptoms as invasive carcinoma.

Ductal carcinoma in-situ (DCIS) is the proliferation of neoplastic epithelial/ductal cells within the breast ducts, which are limited by the basement membrane. DCIS may occur alone or in conjunction with invasive carcinoma. Immunohistochemistry increases the accuracy of histological diagnosis by visualization of the outer layer of myoepithelial cells. The myoepithelial cell layer is present in most benign lesions (except for micro-glandular adenosis) but is absent in invasive carcinoma. DCIS is curable by surgical excision, but mastectomy may be required in cases of extensive or multifocal disease.[10]

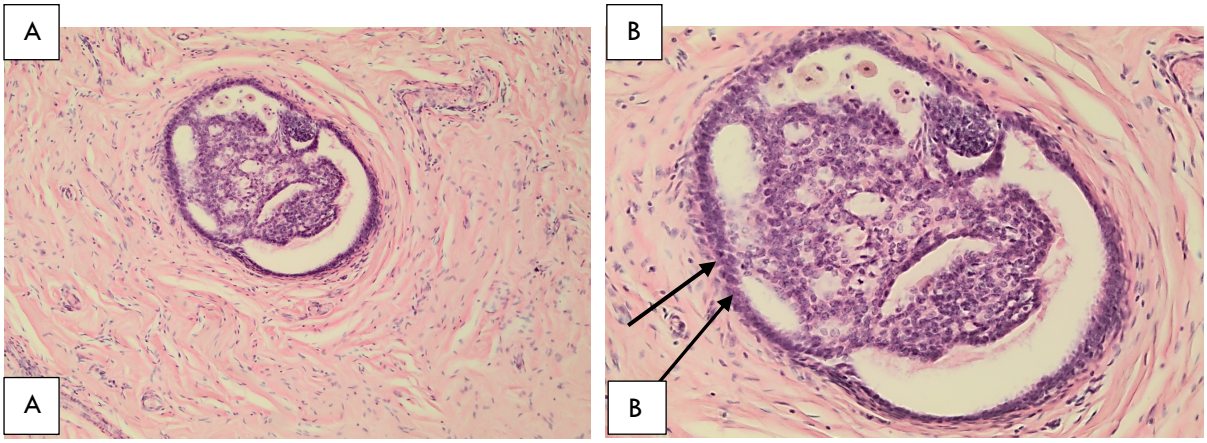


Figure 1.2 (a) Histological section indicating DCIS with malignant ductal epithelium limited to the basement membrane. (H&E stain x100). (b) Histological section indicating DCIS with the myoepithelial cell layer (arrow) that lines the basement membrane. (H&E stain x400)

Lobular neoplasia is a neoplastic change occurring within breast lobules and can range from atypical hyperplasia to lobular carcinoma in-situ (LCIS). LCIS is usually diagnosed incidentally on biopsy. LCIS is a risk factor for the subsequent development of invasive lobular cancer. Regular observation of patients is the preferred management option rather than surgery, although more aggressive subtypes such as pleomorphic LCIS may require surgical excision.[11]

1.1.5.2 Invasive carcinoma, NST (invasive ductal carcinoma, NST)

Invasive carcinoma, NST (IDC, previously known as invasive ductal carcinoma most often presents as a palpable breast mass and in some instances with skin retraction or nipple discharge. Nipple abnormalities include ulceration, retraction, and eczematous reaction as the cancerous cells grow into the epidermis of the nipple, called Paget's disease of the breast. Small asymptomatic tumours may only be identified by mammography. Invasive tumours can

vary in size and can have an irregular, stellate outline or nodular configuration. Tumour edge is usually moderately to ill-defined and lacks sharp circumscription. These tumours are hard and firm on palpation with a gritty consistency when cut with a knife. Carcinoma of no special type (NST) is the most common and comprises about 75% of all breast carcinomas. It occur more frequently in women older than 40 years. Invasive carcinoma has variable clinical presentations and pathological features. The numerous types of breast carcinoma, as defined by the current WHO classification, show a wide spectrum of morphological features and grades, which create significant challenges to the cytological diagnosis of breast cancer.[12]

Cytological aspirates are usually very cellular, composed of single cells and loosely cohesive cell groups. The single cells may have eccentric nuclei with intra-cytoplasmic lumina that may contain mucin droplets. Nuclear atypia varies depending on the grade of the tumour; from monomorphic small nuclei with vesicular chromatin to extremely pleomorphic, large nuclei with clumped, irregular chromatin chards. The background of smears varies from clean to necrotic or haemorrhagic. A limiting factor is that in-situ carcinoma cannot always be reliably distinguished from invasive carcinoma on cytology; however the absence of myoepithelial cells and abundant discohesion of tumour cells are helpful features in establishing a diagnosis of invasive carcinoma.[12]

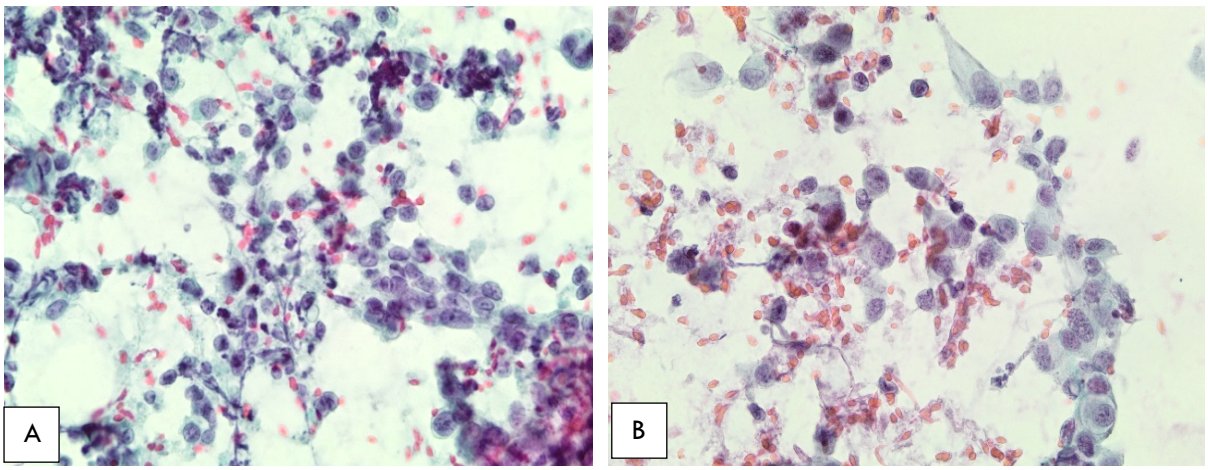


Figure 1.3 (a) Ductal carcinoma with cellular discohesion and nuclear pleomorphism. (Papanicolaou stain x400). (b) Ductal carcinoma with single cells, eccentric nuclei and large pleomorphic nuclei. (Papanicolaou stain x400).

IDC is histologically graded in Grades I, II and III by interpreting architectural arrangements (amount of tubule formation), mitotic count and nuclear pleomorphism; according the Modified Bloom and Richardson System. The presence or absence of DCIS is reported as is

the grading and extent of the DICS. Key features include prominent gland formation and infiltration of benign fibroadipose tissue.[12]

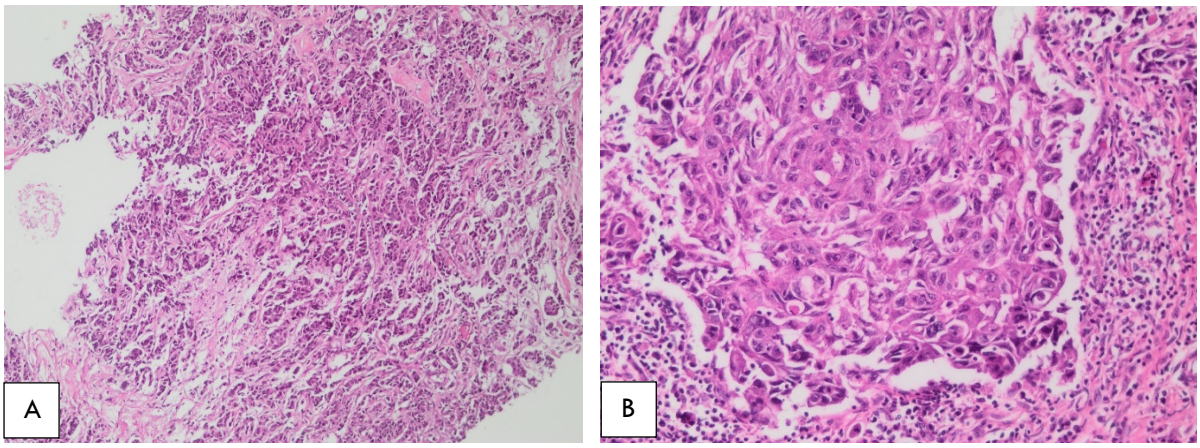


Figure 1.4 (a) Histological section showing invasive carcinoma, NST. (H&E stain x100). (b) Histological section showing invasive carcinoma, NST with pleomorphic nuclei. (H&E stain x200).

1.1.5.3 Invasive Lobular carcinoma

Lobular carcinoma comprises about 5–10% of all breast carcinomas and is the second most common breast carcinoma with a prognosis being better than the more common invasive carcinoma, NST.[13] Approximately 70-95% of lobular carcinomas are ER-positive and 60-70% are PR positive whereas HER2 expression is rare. Marked fibrosis may account for low or inadequate cellular yield on FNA. The subtle morphology of cells, low proliferative activity, and diffuse multi-focal growth pattern make it difficult to diagnose lobular carcinoma on cytology.[12,13]

Cytological aspirates usually have sparse cellularity with mostly single intact cells and few loosely cohesive groups. Cells are small to medium sized with a plasmacytoid appearance. Cytoplasm is scant to moderate and intra-cytoplasmic vacuoles may be prominent. Nuclei are small and eccentric with slight atypia and irregular membranes with small to inconspicuous nucleoli. Histological diagnosis includes observing small cells with eccentric round oval nuclei and intra-cytoplasmic lumina. Cells are usually arranged in discohesive groups, cords or singly dispersed, typically growing in a concentric fashion around native breast ducts.[12]

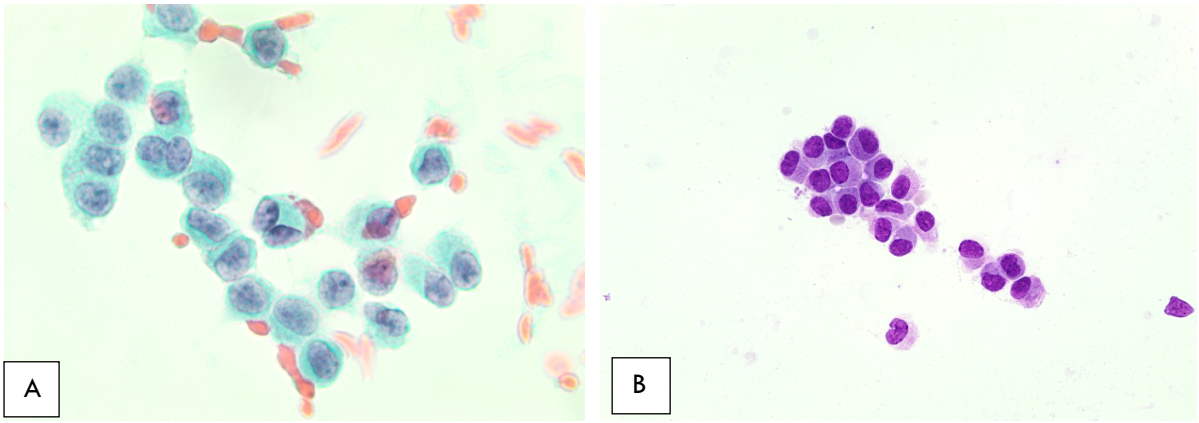


Figure 1.5 (a) Lobular carcinoma showing scant to moderate cytoplasm, eccentric nuclei and irregular nuclear membranes. (Papanicolaou stain x1000). (b) Lobular carcinoma illustrating small to medium sized cells with plasmacytoid appearance. (MGG stain x400)

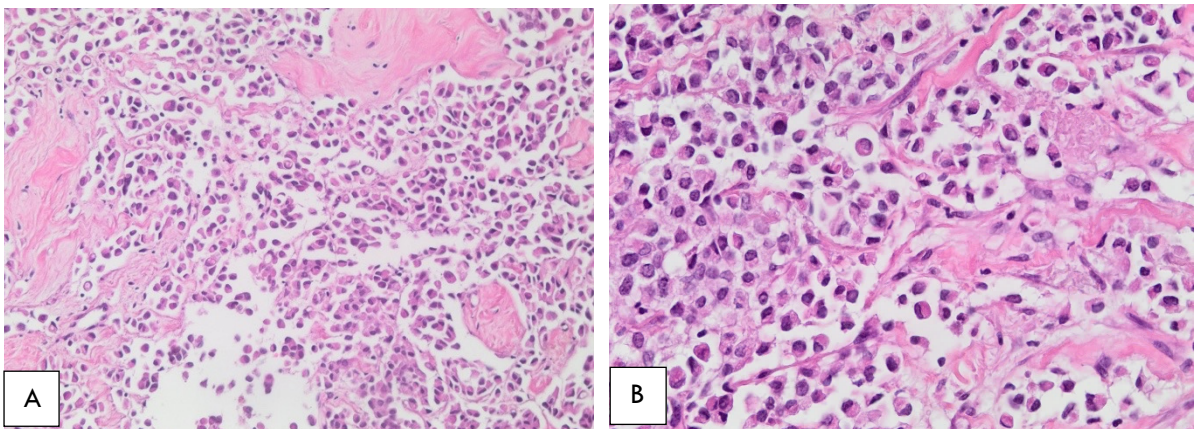


Figure 1.6 (a) Histological section illustrating invasive lobular carcinoma consisting of rather discohesive, loose lying, single intact cells. (H&E stain x200). (b) Histological section showing invasive lobular carcinoma with discohesive, plasmacytoid appearing cells with eccentric, low grade nuclei. (H&E stain x400)

1.1.5.4 Mucinous carcinoma

Mucinous (colloid) carcinoma accounts for approximately 2% of all breast carcinomas and is a mucin producing tumour with a favourable prognosis. This tumour usually occurs over a wide age range and may present with a palpable mass. They are usually well-circumscribed, soft and gelatinous. ER positivity is common and PR positivity is observed in less than 70% of mucinous carcinomas while HER2 over-expression is uncommon.[12]

Cytological characteristic of mucinous carcinoma is the presence of abundant mucinous matrix found in the background. Mucin appears metachromatic on MGG stain and green-purple on Papanicolaou stain. The malignant cells are bland with uniform nuclei and cells usually form ball like aggregates floating in the mucin pools.[12]

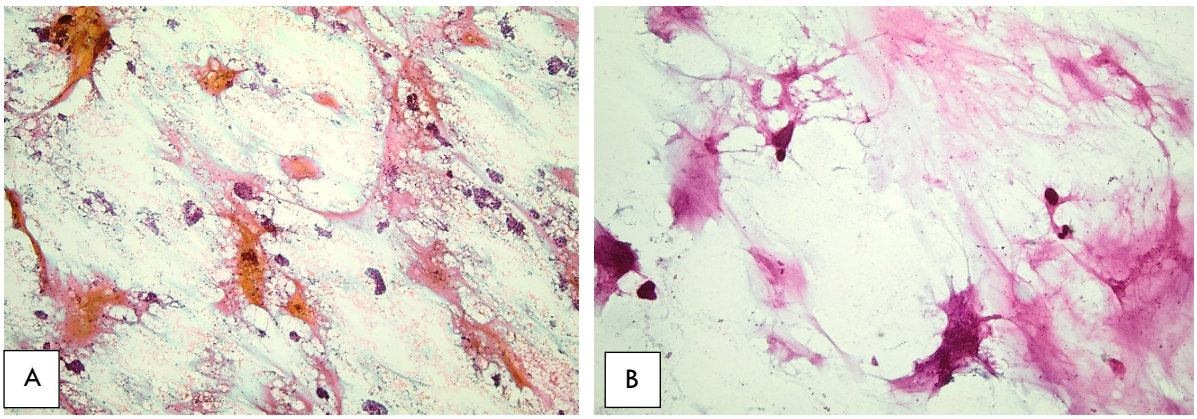


Figure 1.7 (a) Mucinous carcinoma illustrating abundant mucinous matrix in the background. (Papanicolaou stain x400). (b) Mucinous carcinoma with ball-like cellular tissue fragments floating in a sea of mucin. (Papanicolaou stain x400)

Histological features are similar to cytology with the presence of abundant extracellular mucin. The cells form tight three dimensional balls floating in mucin. Papillary, gland and trabecular arrangements can be observed. Minimal to mild nuclear atypia are seen with mitotic figures not usually observed.

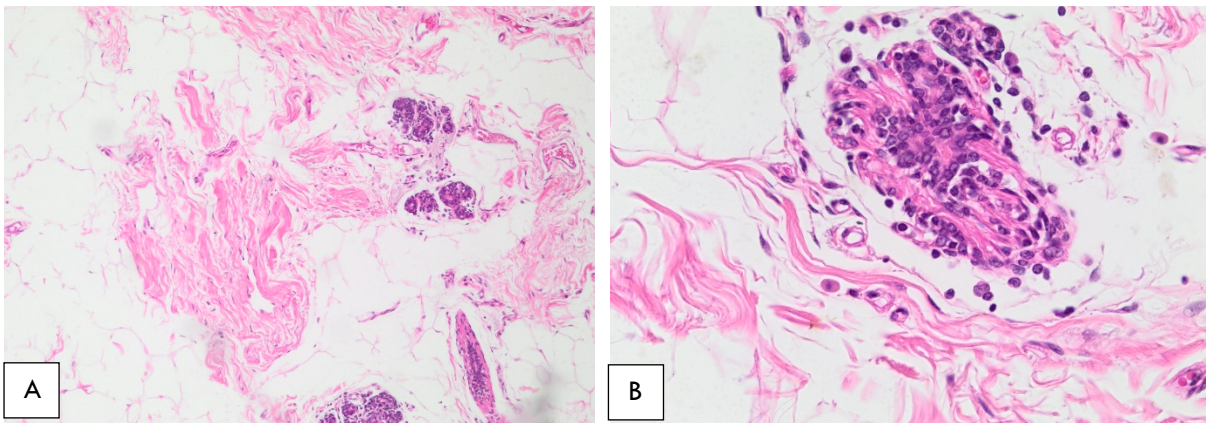


Figure 1.8 (a) Mucinous carcinoma illustrating abundant extracellular mucin in the background (H&E stain x100). (b) Mucinous carcinoma showing bland uniform nuclei. (H&E stain x400).

1.1.5.5 Carcinoma with medullary features (Medullary carcinoma)

Medullary carcinoma accounts for approximately 1-7% of all breast carcinomas and carries a better survival rate. These tumours are usually triple negative; ER, PR and HER2 negative.[12] Cytological aspirates are highly cellular and show marked atypia (high grade nuclear atypia) with bizarre nuclei which contain stripped chromatin and large nucleoli. The cytoplasm tends to be more abundant with indistinct cytoplasmic borders. Lymphoplasmacytic response in the background may raise the possibility of a medullary carcinoma but histological diagnosis is mandatory.[12] As the criteria for diagnosing medullary carcinoma are histology based, cytology may suggest this diagnosis, but final subtyping is deferred to the excision specimen. Histological features include a well circumscribed mass, syncytial growth pattern in more than 75% of the tumour and absence of tubule formation. The presence of marked lymphoplasmacytic infiltration and marked nuclear pleomorphism aids in the diagnosis.[12]

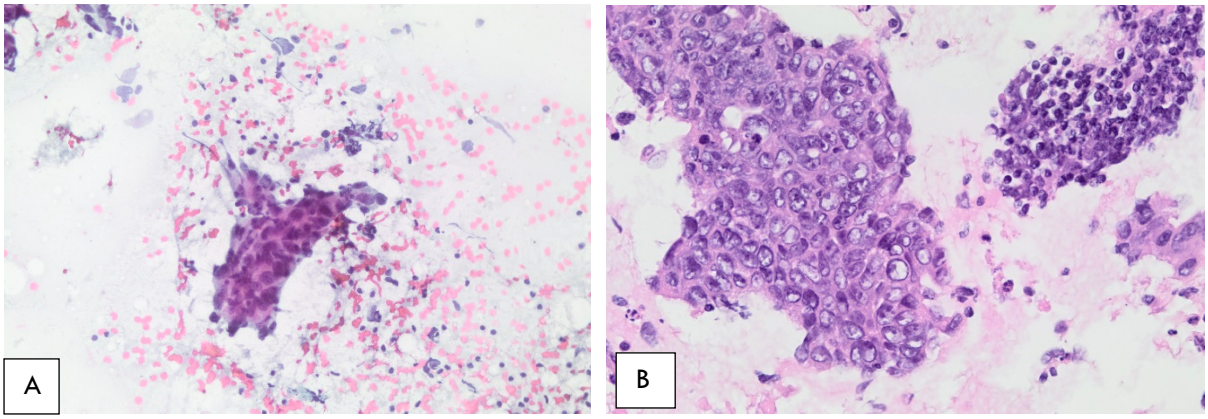


Figure 1.9 (a) Medullary carcinoma showing marked nuclear atypia. (Papanicolaou stain x200). (b) Medullary carcinoma showing pleomorphic bizarre shaped nuclei and lymphocytic infiltrate on the right. (H&E stain x400)

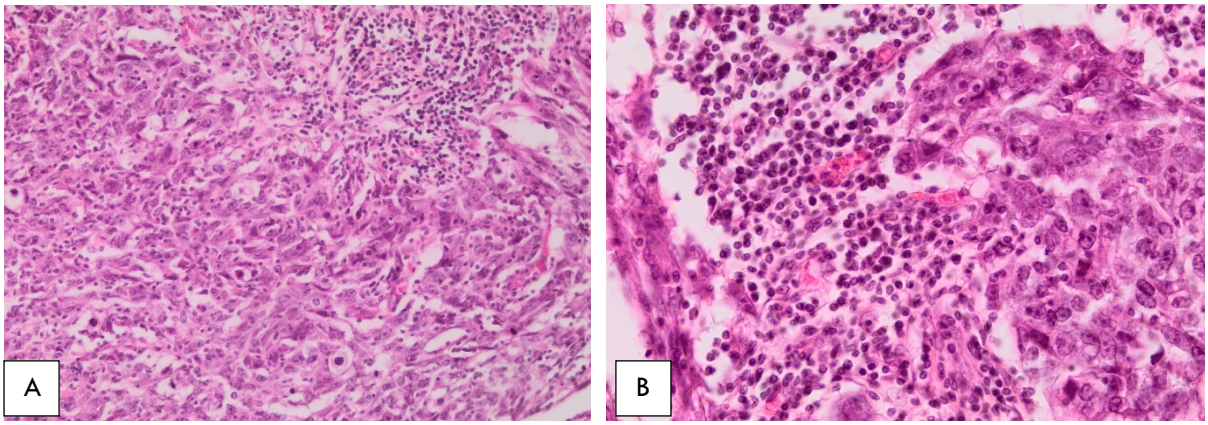


Figure 1.10 (a) Medullary carcinoma indicating a lymphoplasmacytic infiltrate top right corner. (H&E stain x200). (b) Medullary carcinoma showing lymphoplasmacytic infiltrate and marked nuclear pleomorphism. (H&E stain x400)

1.1.5.6 Papillary carcinoma

Invasive papillary carcinoma accounts for 1-2% of all breast carcinomas and has a favourable prognosis. Common clinical presentations include palpable peri-areolar mass, skin dimpling, bloody nipple discharge and nipple retraction. ER and PR positivity are usually observed and HER2 over- expression is rarely seen. Cytological diagnosis is based on very cellular aspirates showing papillary clusters with uniform tall and columnar cells surrounding true fibro vascular cores. Myoepithelial cells are usually absent. Nuclei may show varying degrees of atypia and appear elongated and bland. Occasional necrotic debris, blood in background and haemosiderin-laden macrophages may be seen.[12] Histological features include the presence of cellular islands surrounding fibro vascular cores. The evaluation of core biopsy is challenging due to the broad spectrum of papillary lesions. Apocrine differentiation and amphiphilic cytoplasm with moderately atypical nuclei may be present.[12]

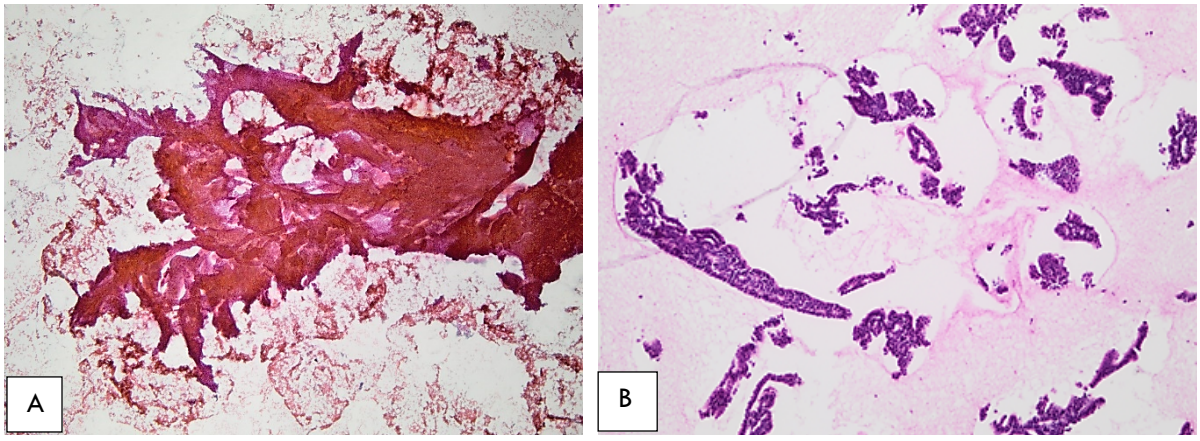


Figure 1.11 (a) Papillary carcinoma showing papillary clusters surrounding fibro vascular cores. (Papanicolaou stain x400). (b) Cell block section indicating papillary clusters. (H&E stain x100).

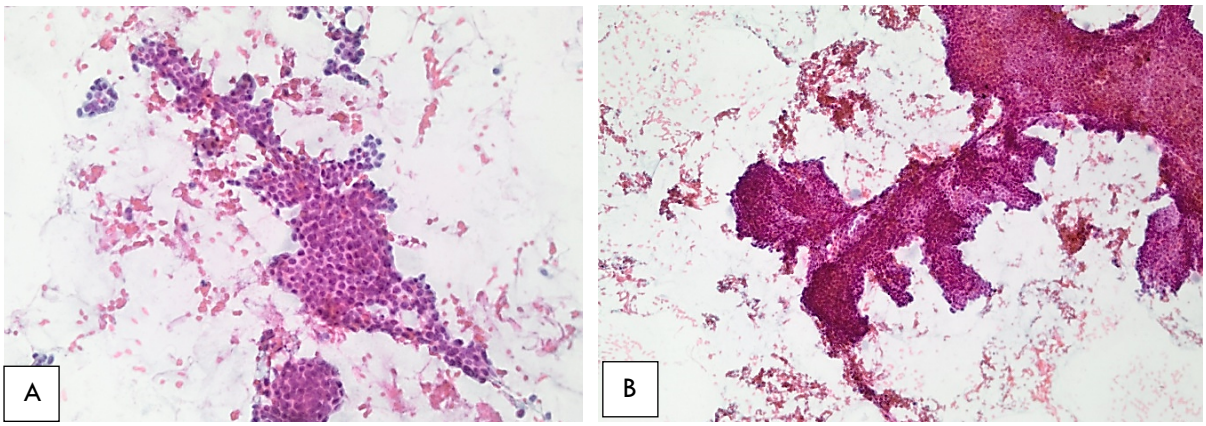


Figure 1.12 (a) Papillary carcinoma. (Papanicolaou stain x400). (b) Papillary carcinoma showing true fibrovascular cores. (Papanicolaou stain x100)

1.2 Histopathology

Histopathology is more informative and is considered the golden standard in the diagnosis of breast carcinoma. Core needle biopsy or surgical excisional biopsy for palpable breast lesions can be performed to obtain tissue for histological diagnosis. Stereotactic core needle biopsies can be performed on non-palpable lesions enabling accurate sampling of a small lesion and distinguishing between in-situ and invasive carcinoma.[14,15] Advantages of core needle biopsies include providing a diagnosis of breast abnormality as well as avoiding open surgery.[16]

Core needle biopsies, however, have numerous disadvantages such as being time-consuming, requiring anaesthesia and a highly trained operator (specialist surgeon or experienced medical doctor) to avoid pitfalls and complications and to yield representative results. Pitfalls and complications may arise if the correct sized needle is not used, with non-sampling of micro-calcifications, and if an inexperienced operator performs the procedure. Core needle biopsy is the better option when FNAB is not available or has failed to produce a diagnosis.[17]

However, core needle biopsy in the majority of cases allows for a definitive diagnosis that enables patients to receive prompt treatment. Clinical complications after core needle biopsy are low, with pneumothorax being a possible major complication.[16] Histological features of carcinomas seen in core needle biopsy correlate with the features seen in excised tumours.[15] Additionally, it allows for the determination of histological prognostic factors such as the type of tumour, grade, invasion, and the immunohistochemical assessment of ER, PR, and HER2 status. It remains unclear if the cores obtained are representative of a whole tumour because the distribution of antigens could be heterogeneous.[18]

Surgical excision of a breast lesion is considered the gold standard in the assessment of prognostic factors and is the method of choice when minimally invasive methods such as FNAB and core needle biopsy have failed. Excisional biopsy is expensive and has a greater degree of patient morbidity.[18,19] Core needle biopsy, on the other hand, is less invasive and in most instances can provide an accurate preoperative diagnosis.[20]

1.3 The Triple Assessment

The triple assessment comprises a combination of the clinical examination of the patient, and the radiological evaluation (mammography/ultrasound) and either core needle biopsy or FNAB of the breast lesion. This approach enables accurate, pre-operative diagnosis in breast pathology, and it is the most widely used test for lesions identified through screening programs or for patients presenting with symptoms.[10,21]

Clinical assessment involves a thorough history and physical examination of both breasts and regional lymph nodes. The most common clinical signs of breast cancer include a discrete lump or asymmetric thickening. Nipple retraction, skin dimpling, inflammation, swelling, oedema, bloody or serous nipple discharge, and nipple eczema are other signs indicative of breast carcinoma.[11]

Radiological assessment includes mammography and ultrasound which are the primary imaging modalities currently used. Mammography is less sensitive in younger patients (under 30 years of age), but it is the baseline method used in women over 40 years of age.[22] Ultrasound is suitable for women of all ages, and it is a useful tool for the evaluation of focal abnormalities, to determine if a lesion is solid or cystic, and in distinguishing benign from malignant (cystic) lesions.[11] Breast ultrasound has a sensitivity of approximately 93% and specificity of 97% in detecting breast carcinoma.[23] Mammography can support the diagnosis of suspicious for malignancy, record the extent of a malignant tumour and identify other non-palpable lesions.[24]

The diagnostic accuracy of breast FNAB is comparable to core biopsy when combined with physical examination and mammography, with a diagnostic error rate of less than 1% if used in the context of the triple test. If the triple test components are not in agreement or if FNAB results are equivocal or insufficient, a repeat FNAB or core needle biopsy or a larger tissue biopsy (such as surgical excision) should be performed.[10,21]

1.4 Fine Needle Aspiration Biopsy (FNAB)

Reducing discomfort for patients and using the least invasive technique to obtain a diagnosis is the ultimate goal of cytology. Cytology allows for quick, reliable diagnosis and facilitates programs for cancer diagnosis within a population.[25] FNAB is a widely accepted technique that can provide good quality material for routine diagnostic smears as well as cell block preparations that can be used for ancillary prognostic tests. [26]

Numerous studies confirmed that FNAB is a simple, rapid, cost-effective, and outpatient procedure.[27–30] FNAB is performed on clinically palpable lesions in clinical settings where a core biopsy is not available. The required level of expertise to perform FNAB is much less than for core needle biopsy and can be performed by trained nursing personnel and medical doctors who have not been trained in core needle biopsy techniques.

FNAB is also helpful to characterize breast tumours and to evaluate patients with primary, recurrent and metastatic disease.[31] Breastfeeding or lactating patients who are at higher risk of developing galactocele or milk fistula after core biopsy benefit from FNAB.

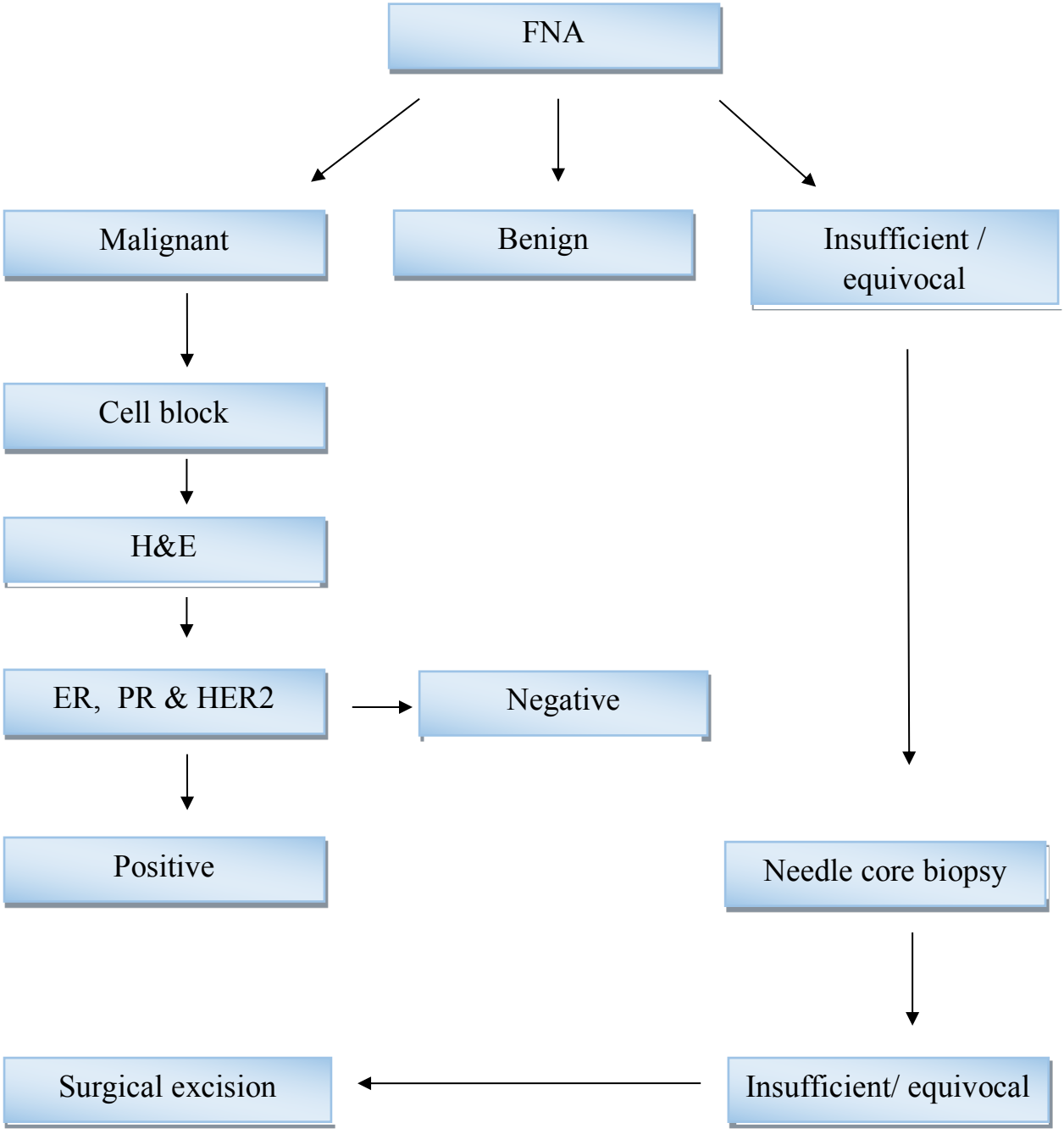
For FNAB to be successful and accurate, FNAB aspirators need to be adequately skilled, to produce aspirates of adequate quality for diagnosis, and to have access to ancillary investigation material (i.e. cell blocks). Experienced cytologists/cytopathologists should interpret FNAB derivatives, including smears and cell blocks .[32]

FNAB has few complications, mostly in the form of minor bruising or bleeding at the site of puncture.[33] Needle tract seeding is not a risk with FNAB breast specimens, which also holds for a core needle biopsy that creates an even larger tract.

The major pitfalls or disadvantages of FNAB specimens include:

- The need for high quality, cellular, and well-preserved material;
- Interpretation by experienced cytopathologists/cytotechnologists;
- Challenges to distinguish between invasive and in-situ carcinoma;
- The patient needs access to the triple test;
- Limitations to classify papillary lesions into benign or malignant with adequate sensitivity for diagnostic applications and clinical management of the patient.

Figure 1.13 Illustration of processing of a cytology breast FNAB specimen.



1.5 Cell Block Evolution

The use of cell blocks began a century ago and had become a routine service in many cytopathology laboratories. Bahrenburg first reported this technique in 1896 when he described an innovative method that was applied to ascitic fluid. Nowadays, various techniques are available, using different types of fixative and processing. The most common variants include the collodion bag technique, the thrombin method, the Millipore filtration method, the saline needle rinse method, the histogel method, and filter sedimentation and agar embedding.[30,34] Although all the different cell block methods differ with regards to the types of fixatives, materials, and techniques used, the underlying principle remains the same. The common denominator comprises of the concentration of cells by centrifugation, followed by removal of the supernatant and adding a binding agent to form a cohesive pellet, followed by processing according to standard histological procedures.

However cell block preparation methods and collection media are not yet standardized. Many of these methods are time-consuming and labour intensive, especially in the current era of strict turnaround times that apply in routine cytopathology laboratories. The different techniques also require variable technical skills that may lead to inconsistent results.[35] A wide variety of fixatives are also available for cell block preparations. Some fixatives such as B5 solution, which contains mercury, is not acceptable due to health hazards. Other fixatives such as ethanol provide optimally preserved nucleic acid and for eliminating background staining such as obscuring blood. Thus nucleic acid quality in alcohol-based fixatives may be superior compared to formalin-fixed tissue blocks.[34]

Cell blocks on the other hand can be easily stored to provide material for future research purposes; multiple sections can be cut from adequately cellular cell blocks for ancillary tests. The cell block technique has gained wide utility as a platform to perform ancillary tests and has been validated by many studies to be accurate, reliable and comparable to tissue blocks.[27,29,36] Ancillary tests include immunocytochemistry, special stains, molecular analyses, cytogenetics, polymerase chain reaction (PCR) and in-situ hybridisation.[28–30] Cell blocks often offer well-preserved cellular detail and morphological architecture, which can closely resemble histopathological features seen on tissue biopsies, thereby facilitating in diagnosis and pattern recognition.[30,37]

Sampling type, specimen size and aspiration techniques all contribute to the diagnostic accuracy and sensitivity of cell blocks, which can vary between 60% and 90%. Combining cell blocks with conventional smears improves diagnostic accuracy to 90%.[30,37]

1.6 Estrogen and Progesterone hormone Receptors

Estrogen Receptor (ER) is the most informative biomarker for breast carcinoma and the most important prognostic and predictive indicator.[5] ER is a nuclear hormone receptor that acts as a ligand-activated transcription factor. Carcinomas that are ER-positive tend to be well-differentiated, less aggressive and have an overall better prognosis (as compared to ER-negative cancers). ER-positive carcinomas account for approximately 75-80% of breast carcinomas and respond well to endocrine therapy. Greater benefit from endocrine therapy is observed in tumours that express higher levels of ER. Lobular carcinoma, mucinous (colloid) carcinoma, pure tubular carcinoma and virtually all grade 1 invasive carcinomas, NST express ER positivity.[5,38,39]

Progesterone receptor (PR) is an estrogen-regulated gene, expressed by 50% of ER-positive tumours; thus the expression of PR is highly dependent on the presence of ER. Tumours that are ER-positive and express low or absent PR expression are more proliferative, aggressive, have a poorer prognosis and develop recurrence.[5] However, tumours expressing both ER and PR have a more favourable prognosis and respond better to endocrine therapy. It is uncommon to have ER absent and detectable PR expression, which may be largely due to false negative ER or false positive PR, but not all cases account for technical errors. ER-negative and PR positive tumours are infrequently recorded, but despite limited evidence that indicates benefit from endocrine therapy, these cases are typically treated with endocrine therapy.[5,39]

PR and ER expression are associated with disease-free and improved overall survival. Thus accurate and quantitative assessment of results is critical in determining therapy. Several major factors can affect ER and PR status including tissue fixation and choice of anti-ER or anti-PR antibodies.[38] ER and PR status can be determined via IHC, which is easy, safe, specific and cheap for evaluating cytological material, fresh frozen tissue or formalin-fixed paraffin- embedded tissue specimens.[40]

1.7 Human Epidermal growth factor Receptor (HER2)

HER2 (or c-erb B2) proteins are members of subclass 1 of the receptor tyrosine kinase superfamily. The subgroup tyrosine kinase consists of four members including epidermal growth factor receptor (EGFR/ erbB1/ HER1), erbB2/ neu/ HER2, erbB3/ HER3 and erbB4/ HER4. HER1 and HER2 play a strong role in neoplastic transformation in vitro and in vivo. Normal breast epithelium express low levels of HER2 but overexpression of HER2 occurs in about 20- 30% of all breast carcinomas usually due to gene amplification, and 50% of these cases are shown to be ER-negative.[7,22,38]

HER2 is located on chromosome 17q21 and promotes tumour growth by regulating cell growth, differentiation, survival, migration and cellular responses through numerous signalling pathways.[41] The expression of HER2 usually indicates poor prognosis and aggressive behaviour but is a strong predictive biomarker to predict sensitivity to anthracycline-based chemotherapy regimens.

Trastuzumab is a monoclonal antibody attached to a chemotherapy drug that binds to the extracellular domain of HER2. Treatment with Trastuzumab® has been shown to markedly improve response rate and survival of breast carcinomas overexpressing HER2 oncogene.[38,39]

The American Society of Cancer Oncology (ASCO)/ College of American Pathologists (CAP) guidelines allow for routine evaluation of HER2 status on all primary and metastatic breast carcinomas to determine whether tumours are positive, equivocal or negative. Equivocal HER2 expression needs further assessment with fluorescence in- situ hybridization (FISH) to furthermore determine HER2 status as positive or negative.[5,39]

1.8 Fluorescent in situ hybridization

HER2 in-situ hybridization assays are aimed to visualize the HER2 gene with or without the reference target chromosome 17 centromere (CEN17) in tissue samples. The American Food and Drug Administration (FDA) has approved multiple signal detection systems for HER2 assays; namely fluorescent in-situ hybridization (FISH); chromogenic in-situ hybridization (CISH) and dual in-situ hybridization (DISH). HER2 FISH assays are performed by using manual or semi-automated protocols and a specialized fluorescent microscope to perform subsequent analysis in a dark room. HER2 ISH assay assessment comprises quantitative analysis, which includes assessment of internal control cells that are adjacent to tumour cells in the same tissue section.[42]

1.9 Breast cancer management

Surgery is the primary treatment for in-situ carcinoma and it aims to excise the entire in-situ tumour and minimise local recurrence. Local recurrences after breast-conserving therapy for DCIS display invasive carcinoma in 50% of the cases. The most effective treatment for DCIS is tumour excision or mastectomy, with or without either anti-hormonal treatment or postoperative radiotherapy. Tumour size and focality, grade, necrosis (punctate or comedo), as well as margin status and age of patient are critical elements in the reporting of DCIS.[22]

Surgery is the first line of treatment for operable primary invasive breast carcinomas, with or without involved axillary lymph nodes. Surgical options include mastectomy or breast-conserving procedures. The latter consists of the removal of a tumour with clear excision margins followed by postoperative radiotherapy.

Adjuvant treatment is given after surgical removal of a tumour and includes local treatment such as radiotherapy and systemic treatments. Adjuvant treatment aims to increase overall survival, improve disease-free and reduce the risk of local recurrence.[43]

Neoadjuvant treatment is chemotherapy given as the first line of treatment and administered before surgery for locally advanced breast cancers. The ultimate aim of neoadjuvant treatment is to reduce the size of a tumour to permit breast-conserving surgery. Assessment of the carcinoma's response to treatment involves the careful assessment and monitoring of tumour size.[10,11,43]

1.10 Breast cancer grading

Tumour grading before surgery is important to select the most suitable treatment regimen. Elston & Ellis's modification of the Scarff-Bloom-Richardson (SBR) grading system is a widely accepted tumour grading system performed on histological tissue and has been found to have a good prognostic correlation.

Cytological grading is not routinely utilised as compared to histological grading but can be a useful parameter for early breast cancer, especially when selecting neoadjuvant therapy. Various grading systems have evolved for FNA cytology based on cytological features. Grading of FNA material should be incorporated for prognostication in the era of neoadjuvant therapy. Patients with locally advanced disease, who declines surgery and older patients with accompanying chronic diseases may benefit from grading of tumours on FNA.

The Robinsons grading system is based on simplicity, specificity and reproducibility and useful for the grading of breast carcinoma on FNA material. Previous studies showed concordance rates ranging from 56.9% to 89.1% on FNA when compared to histological grading.[44]

Hence obtaining a rapid diagnosis of a breast carcinoma expedites the referral of patients to appropriate treatment centres and timely initiation of treatment, whereas a delay in the diagnosis of breast cancer reduces overall survival and increases morbidity. The increasing burden of breast carcinoma significantly increases the workload on both diagnostic and treatment facilities. In Africa, women have limited cancer education and access to screening for breast carcinoma, and 50-70% of these women that are diagnosed with breast cancer, present with advanced breast disease, which in combination with delayed access to treatment facilities result in a poor prognosis.

FNA is a quick, reliable, informative, and cost-effective diagnostic test that can significantly improve timely diagnosis of breast carcinoma and prompt referral of patients for definite treatment, especially in countries with limited resources, such as South Africa. Despite the apparent advantages of FNA and the cell block technique in the diagnostics and theranostics of breast carcinoma, there exists a significant knowledge gap regarding the accuracy and reliability of FNA and the cell block technique as an integral part of personalised patient care.

Chapter 2: Aims and Study design

2.1 Aims

- To retrospectively correlate the expression of ER and PR hormone receptors performed on BD Cytorich™ Red cell blocks and histological FFPET in chemotherapy naive breast carcinomas, by immunochemistry.

- To retrospectively compare the HER2 expression and interpretation between the BD Cytorich™ Red cell block and histological FFPET in chemotherapy naive breast carcinomas via ICC and IHC respectively. FISH testing for HER2 will be additionally performed, on cases that scored 2+ and 3+ and a random selection of negative cases.

- To grade the carcinomas on the cytology smears, using the Robinsons grading system, and compare it to histologically graded carcinoma using the Modified Richardson and Bloom grading system.

2.2 Hypothesis

- BD Cytorich™ Red cell blocks and histological FFPET are equally good in determining ER and PR hormone receptor expression by means of ICC and IHC, respectively

- BD Cytorich™ Red cell blocks and histological FFPET are equally good in determining HER2 over expression by means of ICC and IHC, respectively and have good correlation with FISH.

- Cytological carcinoma grading will be comparable to the carcinoma grading on histological samples.

2.3 Study Design and Case Selection

The archive of the Division of Anatomical Pathology, Department of Pathology, Tygerberg Hospital, Stellenbosch University and National Health Laboratory Service (NHLS) was searched for patients who had an FNAB done at the divisional FNA Clinic at Tygerberg Hospital between January 2013 and December 2015.

Patients with a primary cytological diagnosis of breast carcinoma, and who had ER, PR and HER2 ICC stains performed on BD Cytorich™ Red cell blocks, were identified. These cases were cross-checked for available histological FFPET (either core biopsy, surgical excision or mastectomy specimens), that were chemotherapy naive and on which IHC stains for ER, PR and HER2 were performed. Specimens that did not have these stains performed or that could not be retrieved from the archives, had these stains redone (were possible).

Exclusion Criteria:

- Tissue specimens collected after chemotherapy.
- Cell blocks containing insufficient material.
- Cases with a benign histological diagnosis.
- Cases with no histological follow-up.
- Cases with histological diagnosis of DCIS.

The search identified 609 patients with a primary diagnosis of breast carcinoma. 47% (n = 285) of the patients had no follow up histological specimen; 22% (n = 132) had histology done after chemotherapy administration; 7% (n = 40) of cases had no cells or too few cells on cell blocks. Additionally 3.5% (n = 20) of the cytological specimens could not be retrieved from the departmental archives.

132 Samples between 2013 and 2015 were chemotherapy naive and considered suitable for this study. All samples were reviewed by a cytotechnologist (EvR) and a pathologist (PS). The review included: confirming the original diagnosis, reviewing the grading of the carcinomas, scoring the ER, PR and HER2 immunostains on the cytology and histology specimens.

Throughout this manuscript, the use of the terms 'cell block' or 'BD Cytorich^{TM} Red' will always refer to cytology specimens. While the term 'FFPET' will always refer to histological specimens (i.e. biopsies or excision specimens that were taken and fixed directly in 10% buffered formalin for histological processing).

2.4 Breast cancer grading study population

Cytological grading was compared to corresponding FFPET grading. The same study population that were used for ER and PR analysis were utilised to perform grading. Grading was done on conventional cytology Papanicolaou stained smears. Four cases were excluded from grading as slides could not be retrieved from the archives. A total of 128 cytology cases were graded using the Robinsons grading system.

2.5 Ethical approval

Ethical approval was obtained from the Health Research Ethics Committee, Stellenbosch University. A waiver of consent was obtained due to the retrospective component of this study as the results would have no bearing on continuing patient treatment. (S15/10/236).

Chapter 3: Cytopathology Methodology

3.1 FNAB procedure

After a preliminary on-site diagnosis of breast carcinoma, a separate needle pass was performed for the preparation of cell blocks. A 22G or 24G needle and a 5-10ml syringe were used to perform the FNAB. Cellular material obtained by a separate needle pass was immediately expressed into the vial containing BD Cytorich™ Red preservative fluid. The needle was rinsed in the preservative fluid to ensure the collection of sufficient material.

BD Cytorich™ Red preservative fluid is a product manufactured to preserve cells and small tissue fragments for cytological and histological examination. The minimum fixation time is 30 minutes. The solution furthermore lyses red blood cells and solubilises proteins and is an alcohol based haemolytic fixative. The red blood cell membranes are selectively emulsified and further disrupted by a combination of alcohol and osmotic lysis.

The preservative fluid contains less than 1% formaldehyde, which cross-links with globulin proteins released from red blood cells. The proteins are cross-linked before denaturation by alcohols and prevented from forming protein super-aggregates. Cell membranes act as selective barriers that allow alcohol to pass more rapidly than formaldehyde to the cell cytoplasm. The preservative is an aqueous solution made up of ethylene glycol, formaldehyde, methanol and isopropanol. Storage of the solution should be limited to 24 months from the date of manufacture and kept at room temperature, between 15-30ºC.

The specimens were transported to the cytology specimen reception and processing area in the Division of Anatomical Pathology where the aspirated material remained in the BD Cytorich™ Red preservative fluid for a minimum of 6 hours and a maximum of 72 hours.

3.2 BD Cytorich™ Red Cell block technique

Cell blocks were processed in a biological safety cabinet to adhere to standard safety protocol, followed by centrifugation at 1500rpm for 10 minutes. The supernatant was then decanted into a sterile tube, and the pellet remained in the original cell block tube. Three to four drops of plasma were added to the pellet and mixed thoroughly with a plastic pipette. Three to four drops of Dade innovin (working solution) were added and mixed thoroughly.

Dade innovin reagent is purchased in a lypohilised form that consists of recombinant human tissue factor, synthetic phospholipids (thromboplastin, calcium ions, a heparin-neutralised compound, buffers and stabilisers (bovine serum albumin). The reagent initiates clotting via the extrinsic and common pathways. The reagent can be reconstituted with distilled or deionised water and stored at 2 to 8˚C. Contents should be mixed thoroughly to ensure complete reconstitution and should be re-mixed before use to ensure a homogenous solution. Water containing preservatives should not be used, and the reagent should not be frozen.

A clot should form within a few minutes due to the activation of the coagulation cascade by incubating plasma with thromboplastin and calcium. The clot was placed in a labelled cassette and placed in a staining dish containing 10% buffered formalin and a few drops of eosin to enhance visualisation of the clot during embedding and cutting. The cell clot remained in formalin for 6 to 12 hours before it was transferred to a tissue processor. The cell block was processed, sectioned and stained with H&E in the same manner as routine histological FFPET specimens.

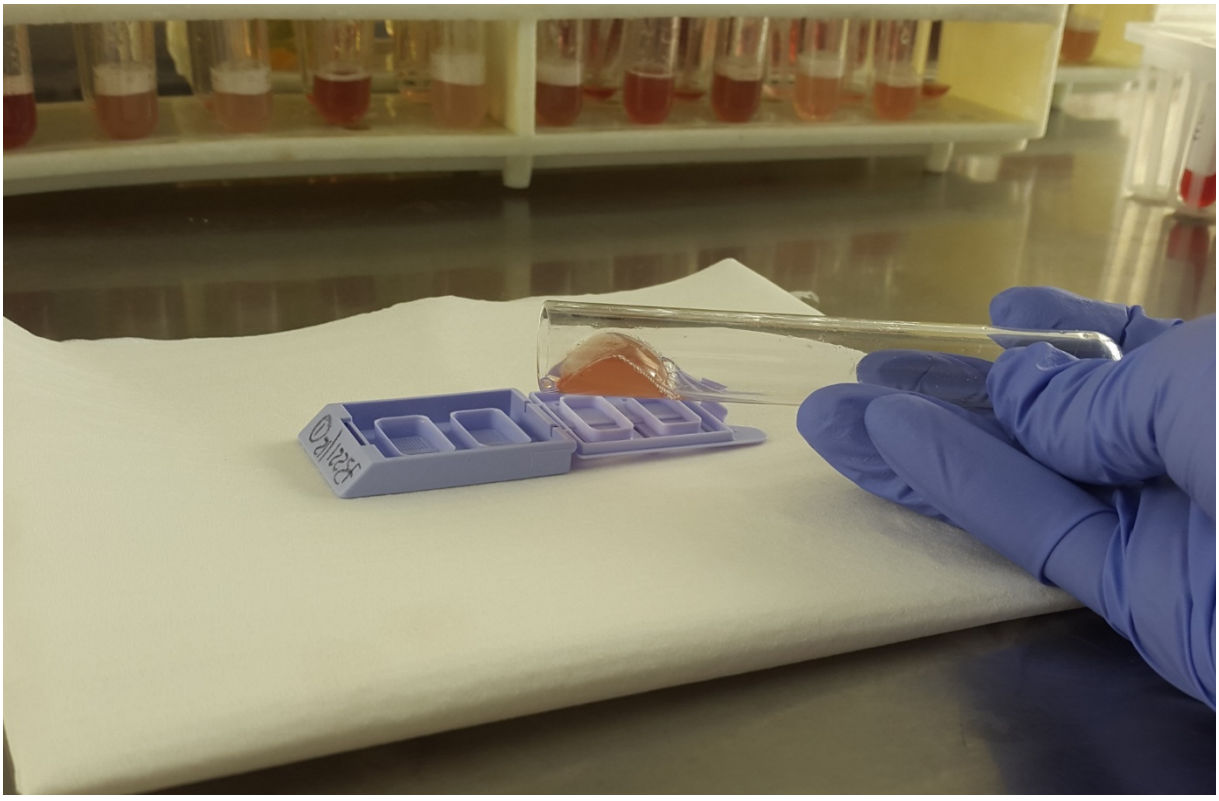


Figure 3.1: Clot formation, prior to placement in a histology cassette.

Chapter 4: Histopathology Methodology

4.1 Fixation and Tissue processing

Formalin is routinely used in histopathology laboratories as the standard preferred fixative to preserve biological specimens. Formalin is a strong disinfectant and hardens tissue. Table 4.1 illustrates the solutions used to make up 10% buffered formalin.

Table 4.1: 10% buffered Formalin preparation

Solutions	Quantity
Formaldehyde	2000ml
Tap water	18 000ml
Sodium Di- hydrogen phosphate	80g
Di- sodium hydrogen phosphate	130g

The buffering salts were dissolved separately in 1000ml tap water by stirring until dissolved. The rest of the water was added to fill up to 18 000ml. The concentrated formaldehyde was added to make up to 20 000ml.

Tissue processing (Table 4.2) allows for tissue to be embedded in a solid medium to make it firm enough, to provide adequate support and rigidity for the cut of thin tissue sections. The first step of the process involves fixation, which preserves the tissue by preventing autolysis and putrefaction resulting in tissue that is in a live-like state. The second step involves dehydration by immersing the tissue in graded alcohol to remove intracellular and extracellular water from the tissue. Alcohol is then removed from the tissue and cleared in xylene. Impregnation with paraffin wax, the last step of the tissue processing procedure, occurs at a temperature between 55ºC and 65ºC.

Table 4.2: Tissue processing program

	Solutions	Time	Temperature
1	Formalin	30 min	40ºC
2	Formalin	45 min	40ºC
3	70% Alcohol	30 min	40ºC
4	80% Alcohol	45 min	40ºC
5	96% Alcohol	45 min	40ºC
6	Absolute Alcohol	45 min	40ºC
7	Absolute Alcohol	45 min	40ºC
8	Absolute Alcohol	60 min	40ºC
9	Xylol	60 min	40ºC
10	Xylol	45 min	40ºC
11	Wax	45 min	60ºC
12	Wax	45 min	60ºC
13	Wax	45 min	60ºC
14	Wax	45 min	60ºC

4.1.1 Embedding procedure

The purpose of embedding is to support the impregnated tissue in a wax block to enable cutting of thin tissue sections. An embedding machine consists of a reservoir of molten wax which is kept at 62 to 65ºC, a hot plate, a cold plate and metal moulds of various sizes. The tissue is taken from the impregnation wax bath of the tissue processor and transferred directly to the wax reservoir in the embedding machine.

The orientation of tissue is the most important step, followed by placement in the mould. The tissue must be orientated so that it offers the least amount of resistance to the knife. A suitable size mould must be selected to allow a space of wax around the tissue, and tissue must be placed as flat as possible.

The mould is then warmed on a hot plate, and a small amount of wax is dispersed into the bottom. The tissue is removed from the cassette and orientated correctly with warm forceps in the mould. The mould is placed on a cold tray for the tissue to set in the correct position and to remove the block from the metal mould easily. The cassette is placed on top of the mould and filled with wax.

4.1.2 Microtome procedure

Rotary microtomes are used to cut sections from routine specimens, where the knife is stationary, and the block moves up and down. The microtome is used to cut a thin section of paraffin-embedded tissue and to mount the section on a slide.

After embedding and cooling of blocks on a cold plate, the block is clamped in the block holder of the microtome. The block needs to be aligned correctly and trimmed to remove excess wax covering the tissue. Trimming continues until exposure of the whole surface of the tissue. The block is released from the block holder and then placed on an ice tray to allow cooling before cutting. There is less friction between the blade and wax if the block is cooled.

The block is once again securely fitted into the microtome's block holder. The thickness setting on the microtome is 4 microns. A ribbon of sections is cut and placed in a water bath and picked up carefully onto a slide. The water bath temperature is set between 40 and 50ºC the warm water assists to remove small folds and wrinkles in the wax. Slides are then incubated for 20 minutes before staining.

4.2 H&E staining procedure

The H&E stain is the most widely used histological stain. The haematoxylin component stains the cell nuclei blue while the eosin stains the cytoplasm and most connective tissue fibres in varying shades and intensities of pink, orange and red.

Garvey's alum haematoxylin (Table 4.3) and Mayer's haematoxylin are used, and both stains use the mordant aluminium ammonium sulphate. A combination of Eosin Y and Phloxine B are used to counterstain. Two parts of eosin are mixed with one part phloxine to make up the stock solution. The working solution consists of equal quantities of stock solution and tap water is mixed and allowed to stand for 48 hours before use.

Table 4.3: Garvey's Haematoxylin ingredients

Solutions	Quantity
Haematoxylin	2.5g
Absolute alcohol	100ml
Saturated aqueous potassium alum	45g
Sodium iodate	0.3g
Citric acid	1g
Distilled water	900ml

*Solution made up to 1000ml

Method: Haematoxylin is dissolved in absolute alcohol. In a separate glass cylinder, Potassium alum is dissolved in distilled water with added heat. The two solutions are combined followed by adding sodium iodate and citric acid. The mixture is blended well and is stable for several months.

Scott's tap water is used as a blueing agent during the H&E staining procedure. Solutions used to make up Scott's tap water are tabulated in Table 4.5.

Table 4.4: Scott's tap water ingredients

Solutions	Quantity
Sodium hydrogen carbonate	3.5g
Magnesium sulphate	20g
Tap water	1000ml

Table 4.5: H&E staining program

Solutions	Time
1. De-wax sections in Xylene	5 min
2. Rinse in absolute alcohol, 96% alcohol, 70% alcohol and then water	
3. Stain in Garvey's alum haematoxylin	4- 6 min
4. Wash in water	
5. Blue in Scott's tap water	
6. Rinse in 70% alcohol	1 min
7. Stain in Eosin/Phloxine	1 min
8. Rinse in 70% alcohol	
9. Dehydrate by washing in 70% alcohol, 96% alcohol and then absolute alcohol	
10. Clear in Xylene	
11. Mount with Entellan	

4.3 Immunochemistry

Immunochemistry is the process of localising proteins in cells by antibodies binding specifically to antigens in biological tissue. Immunochemistry performed on histology specimens will be referred to as immunohistochemistry and that performed on cytological material be referred to as immunocytochemistry. The visualization of antigens via the application of a specific antibody to an antigen is performed. A secondary antibody is added to the primary antibody with a chromogenic substrate. The chromogen results in a visible reaction at the antigen site. Staining was performed by a Leica BOND III system as illustrated in Table 4.6.

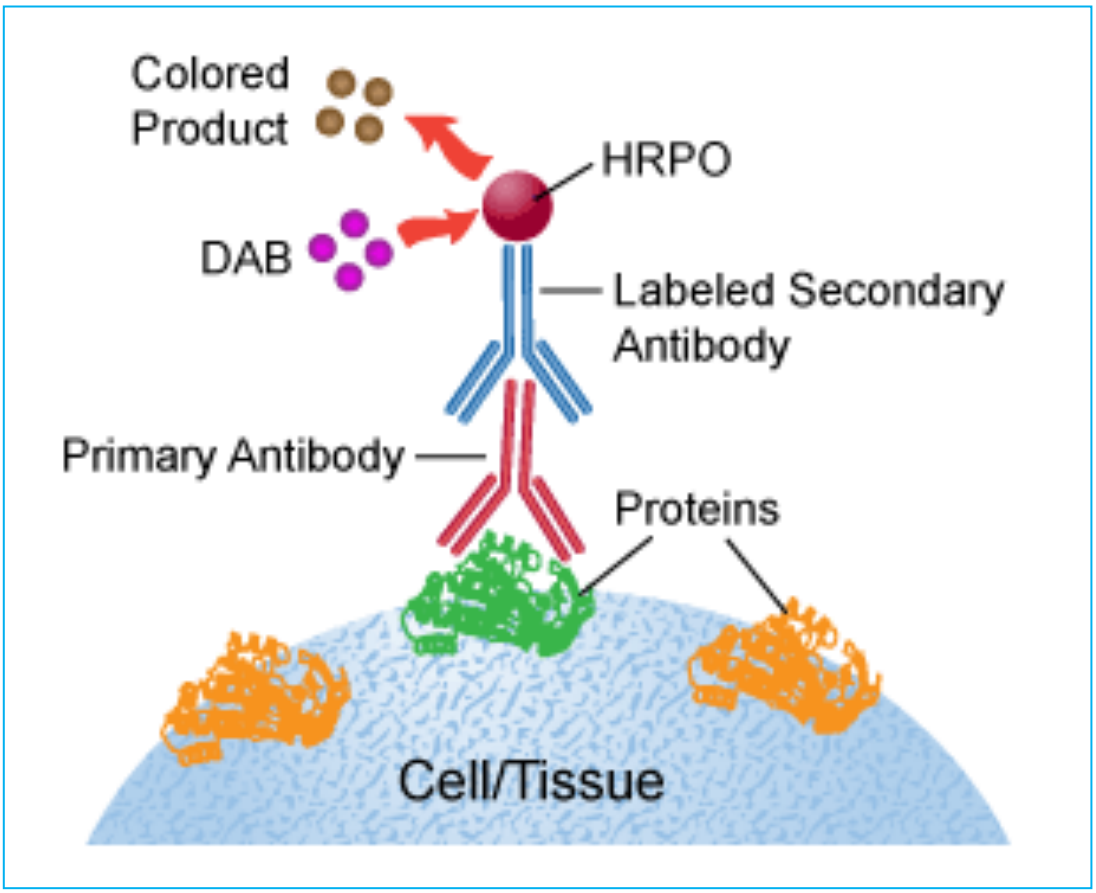


Figure 4.1 Illustration of immunohistochemistry method
(http://www.unizwa.edu.om/content_files/a69171581.jpg)

Slide preparation

Tissue sections were cut from each cell block and FFPET at a thickness of 3 micron per section and placed on charged slides, which were baked for 30 min at 70˚C. Slides were stained on the BOND machine and covertiles were placed correctly to ensure complete staining of the slides.

Bond Wash concentration

Bond wash was prepared by adding 100 ml of BOND Wash concentration to 900ml of deionised water.

Primary optimally diluted antibodies

- ER: Novocastra Estrogen Receptor diluted 1:250
- PR: Novocastra Progesterone Receptor diluted 1:500
- HER2: Novocastra HER2 oncoprotein diluted 1:250

The chromogen used was DAB (diaminobenzidine) which is the colouring substrate.

Controls

A known positive control section was included on each slide for immunochemical staining.

Table 4.6: Leica BOND III staining program

	Repeats/cycles	Time	Temperature
De-wax	X3		72˚C
100% Alcohol	X3		
Bond wash	X3		
Retrieval ER&PR	X2	20 min	100˚C
Retrieval HER2	X2	20 min	100˚C
Bond wash	X4		
Bond wash	X1	3 min	
Antibodies	X1	15 min	
Bond wash	X1	2 min	
Bond wash	X2	1 min	
Post primary	X1	8 min	
Wash	X3		
Polymer	X1	8 min	
Wash	X2	2 min	
De-ionized water	X1		
Peroxide block	X1	5 min	
Wash	X1	1 min	
Wash	X2		
Distilled water	X1		
Mixed DAB refine	X1		
Mixed DAB refine	X1	10 min	
De-ionized water	X3		
Haematoxylin	X1	5 min	
De-ionized water	X1		
Wash	X1		
De-ionized water	X1		

4.3.1 ER and PR

The Allred scoring system as illustrated in Table 4.7 was used to determine hormone receptor status. This system comprises the sum of the independent scoring of the proportion of positive staining tumour cell nuclei as well as the intensity of staining respectively.[40,45]

During assessment of ER and PR proportion, all the tumour cells on the slide were taken into account and were scored as follows (Tables 4.6 and 4.7):

Table 4.7: ER & PR proportion (Allred scoring system)

Score	Proportion
0	No staining
1	<1%
2	≥ 1% but <10%
3	≥ 11% and <33%
4	≥34% and <66%
5	≥67% and 100%

Table 4.8: Staining intensity for FFPET and Cell blocks

Score	Intensity
0	No staining
1	Weak staining
2	Moderate staining
3	Strong staining

The final interpretation of positive, negative or not interpretable was assigned as follow:

- Receptor Positive: Proportion plus Intensity score of ≥3
    - ≥1% tumour cells must stain positive.
    - Allred score ≥ 3: 1-10% weakly positive cells
- Receptor Negative : <1% of any intensity staining

4.3.2 HER 2 ASCO/ CAP guidelines[46]

The 2013 ASCO/CAP guidelines for HER2 testing state that the time from obtaining the specimen (biopsy or excision) to the initiation of tissue fixation should be as short as possible and the duration of tissue fixation should be 6 to 72 hours.

Table 4.9: HER2 scoring system

Score	Staining pattern	HER2 Status
0	No staining/ weak membrane staining in <10% of tumour cells	Negative
1+	Weak / partial membrane staining in >10% of tumour cells	Negative
2+	Moderate membrane staining in >10% of tumour cells or Strong circumferential membrane staining in <30% of tumour cells	Indeterminate
3+	Strong circumferential membrane staining in >10% of tumour cells.	Positive

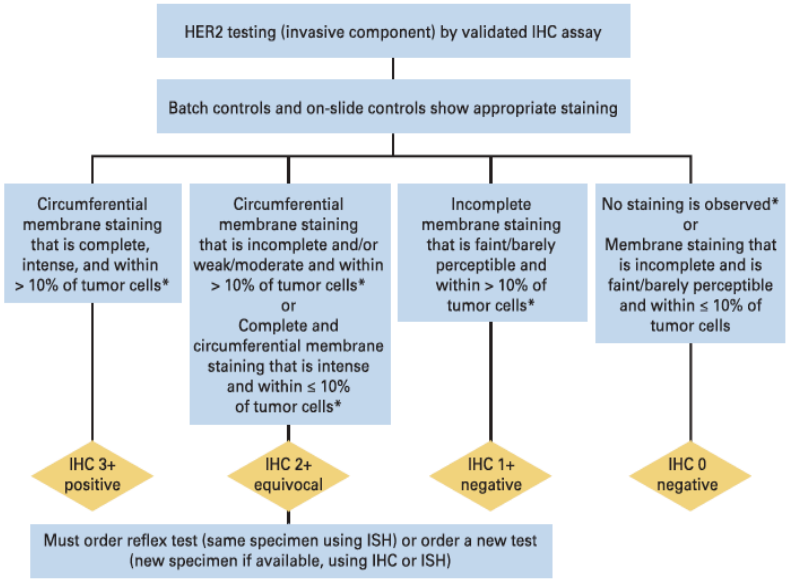


Figure 1. *Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) protein expression by immunohistochemistry (IHC) assay of the invasive component of a breast cancer specimen. Although categories of HER2 status by IHC can be created that are not covered by these definitions, in practice they are rare and if encountered should be considered IHC 2+ equivocal. ISH, in situ hybridization. NOTE: the final reported results assume that there is no apparent histopathologic discordance observed by the pathologist. (*) Readily appreciated using a low-power objective and observed within a homogeneous and contiguous invasive cell population.*

Arch Pathol Lab Med—Vol 138, February 2014 *ASCO/CAP HER2 Testing Guideline Update*—Wolff et al **249**

Figure 4.2 HER 2 assay

4.4 FISH principle and procedure

In situ hybridization is a technique that is used for localization and detection of specific DNA or RNA sequences in cells, preserved tissue sections, or an entire tissue by hybridizing the complementary strand of a nucleotide probe to a particular sequence. These hybrids can be visualised by autoradiography for probes labelled radioactively or by development of a histochemical chromogen for probes labelled non-isotopically. After a labelled probe is annealed to matching sequences in fixed cells or tissue, the hybridized probe is visualised.[47]

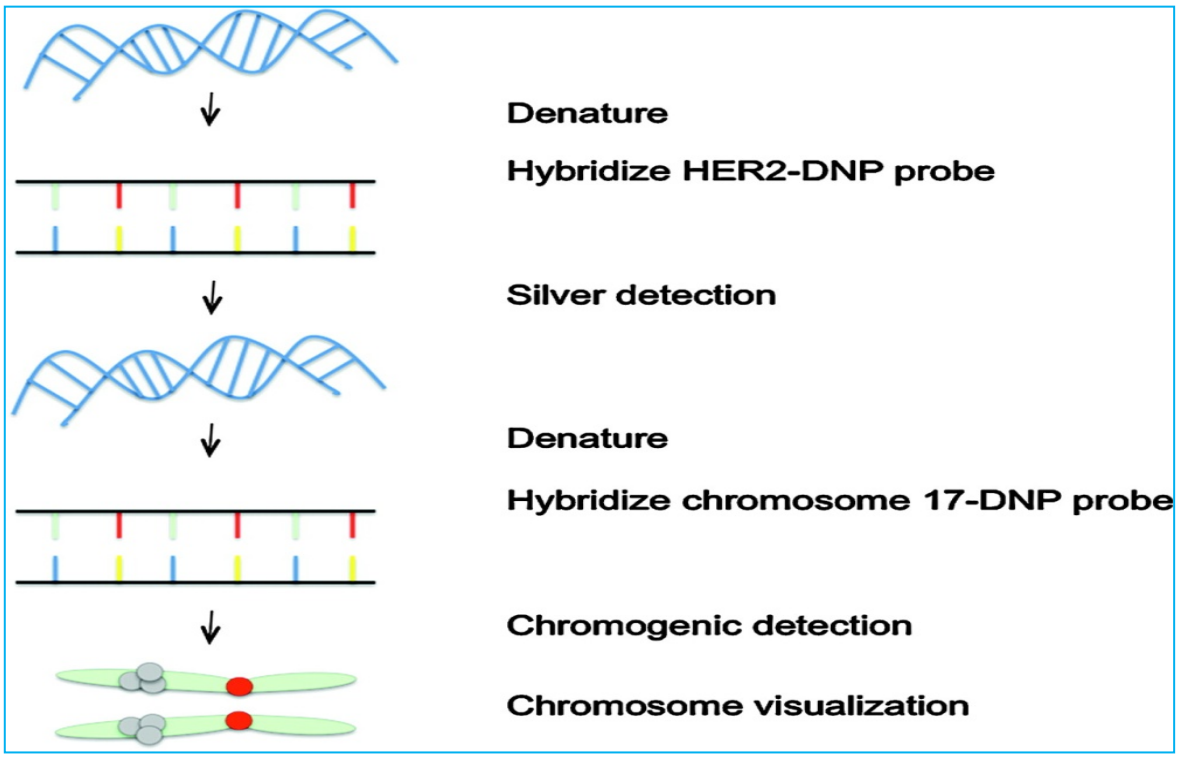


Figure 4.3 Illustration of fluorescent in situ hybridization method (http://jcp.bmj.com/content/jclinpath/63/3/210/F5.large.jpg)

FISH was performed on BD Cytorich™ Red cell blocks using the PathVysion HER 2 DNA Probe kit II and analysis was done using a Zeiss Imager Z1 fluorescence microscope. The entire section was analysed and 20 cells were selected and scored by an analyst and results were verified by a second analyst at Unistel Laboratories.

Table 4.10: FISH status ASCO/CAP guidelines.[46]

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

Figure 2. *Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) gene amplification by in situ hybridization (ISH) assay of the invasive component of a breast cancer specimen using a single-signal (HER2 gene) assay (single-probe ISH). Amplification in a single-probe ISH assay is defined by examining the average HER2 copy number. If there is a second contiguous population of cells with increased HER2 signals per cell, and this cell population consists of more than 10% of tumor cells on the slide (defined by image analysis or visual estimation of the ISH or immunohistochemistry [IHC] slide), a separate counting of at least 20 nonoverlapping cells must also be performed within this cell population and also reported. Although categories of HER2 status by ISH can be created that are not covered by these definitions, in practice they are rare and if encountered should be considered ISH equivocal (see Data Supplement 2E). NOTE: the final reported results assume that there is no apparent histopathologic discordance observed by the pathologist. (*) Observed in a homogeneous and contiguous population.*

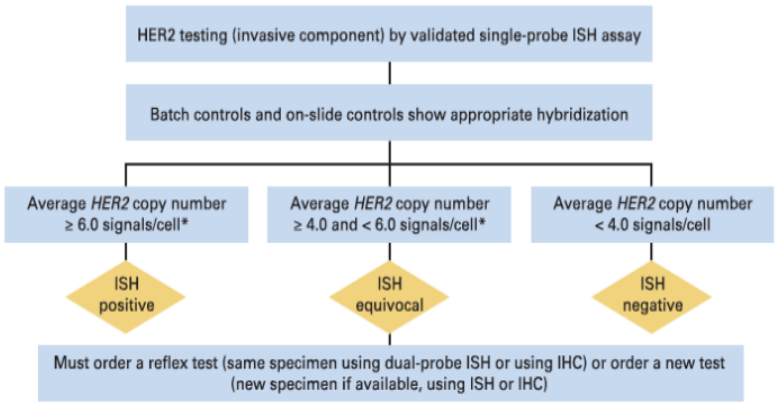


Figure 4.4 FISH assay

Chapter 5: Microscopic review methodology

5.1 BD Cytorich™ Red Cell block cellularity

The Cell block sections that contained at least 10 high power fields (x400) of tumour cells and peripheral blood cell elements with at least 10 tumour cells per field will be considered sufficient for analysis, as suggested by current published literature.[29] Table 5.1 shows the classification of cell block cellularity that was used.

Table 5.1: Cell Block cellularity scoring system

Score	Cellularity
A	Less than 20 cells
B	20 or more cells, less than 100 cells
C	100 or more cells, less than 1000 cells
D	1000 or more cells

5.2 ER and PR scoring systems

Histological FFPET cases stained with ER and PR respectively were reviewed and interpreted using the Allred scoring system for proportion and intensity, as illustrated in Table 4.7 & 4.8. The proportion and intensity of nuclear staining on cell blocks are currently not evaluated and, therefore, not reported in our laboratory. The proportion of nuclear staining on cell blocks was calculated according to an internally developed scoring system, as illustrated in Table 5.2; these results were then correlated with histological FFPET.

Table 5.2: Cell block ER and PR proportion scoring system

Score	ER and PR Proportion
0	Less than 10% tumour cells staining
1	10% or more, less than 30% tumour cells staining
2	30% or more, less than 60% tumour cells staining
3	60% or more tumour cells staining

5.3 HER2 scoring system

Scoring for reviewed cases was interpreted according to ASCO/CAP guidelines as illustrated in Table 4.9.

5.4 Grading scoring systems

5.4.1 Histology grading system

Table 5.3: Scarf- Bloom- Richardson grading system

Features	Score
Tubule formation:	
Majority of tumor >75%	1
Moderate degree 10- 75%	2
Few or little <10%	3
Nuclear pleomorphism:	
Small, uniform cells	1
Moderate increase in size, variation	2
Marked variation/ pleomorphism	3
Mitotic counts:	
0-5	1
6-10	2
>11	3

Scoring: Grade 1 (well- differentiated): 3-5. Grade 2 (moderately differentiated): 6-7. Grade 3 (poorly differentiated): 8-9.

5.4.2 Cytology grading system

Table 5.4: Robinsons grading system

	Score		
Criteria	1	2	3
Cell dissociation	Mostly clusters	Mixture of single cells and cells in clusters	Mostly single cell
Cell size	1-2 x RBC size	3- 4 x RBC size	>5 x RBC size
Cell uniformity	Monomorphic	Mildly pleomorphic	Pleomorphic
Nucleoli	Indistinct	Noticeable	Prominent or pleomorphic
Nuclear margin	Smooth	Folds	Buds/ clefts
Chromatin	Vesicular	Granular	Clumped and cleared

Grade 1: Score 6- 11. Grade2: Score 12- 14. Grade 3: Score 15- 18

RBC = red blood cell

Chapter 6: Cell block cellularity and general findings

6.1 Results

6.1.1 Cell block cellularity

Cell block cellularity as illustrated by Figure 6.1 shows that 49% (n= 65) of cell blocks were in group D, which contained 1000 cell and more, and 39% (n= 51) cell blocks were in group C, which was interpreted as more than a 100 cells but less than 1000 cells. Cell blocks that were in group B were 12% (n= 16). Forty cell blocks revealed no cells or too few cells, fitted in group A, and were excluded from the study population.

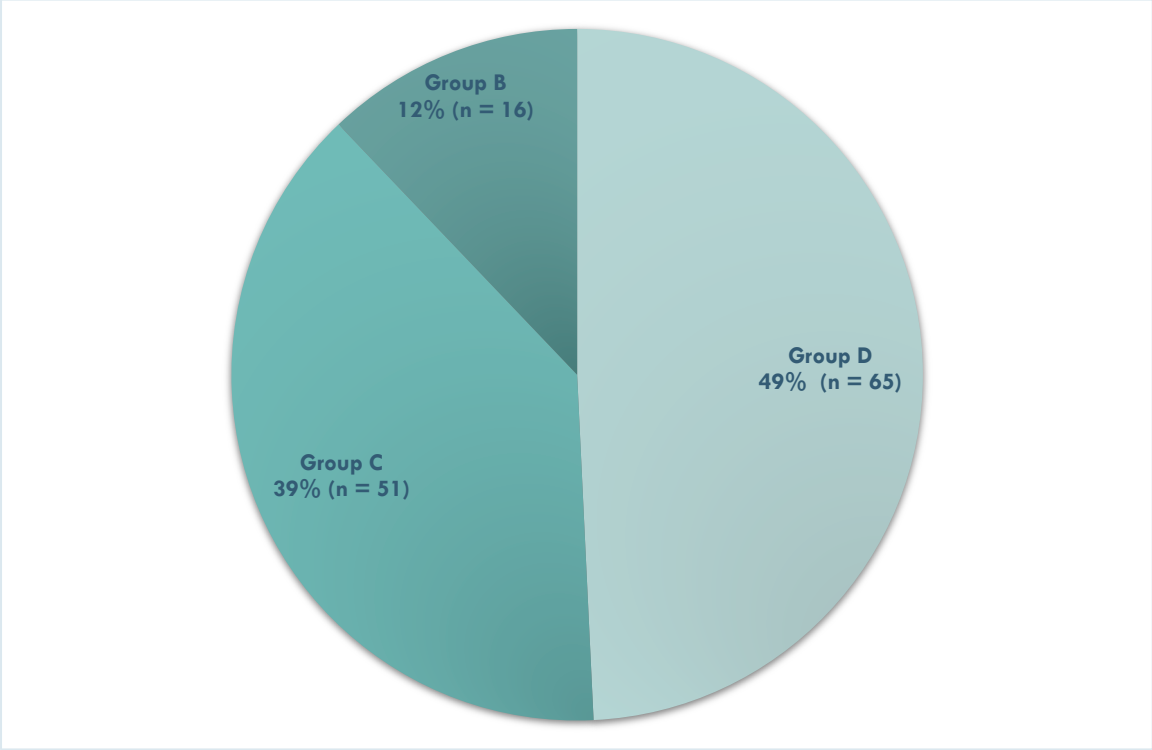


Figure 6.1: Cell block cellularity/ adequacy

6.1.2 Mean Age

Patients presenting with diagnosis of breast cancer had a mean age of 56 years. This study presented with a total of 130 females and 2 males. Females presented with a mean age of 56 and males with a mean age of 60 years. The youngest female was 28 and the oldest was 96. The youngest male was 36 and the oldest was 61.

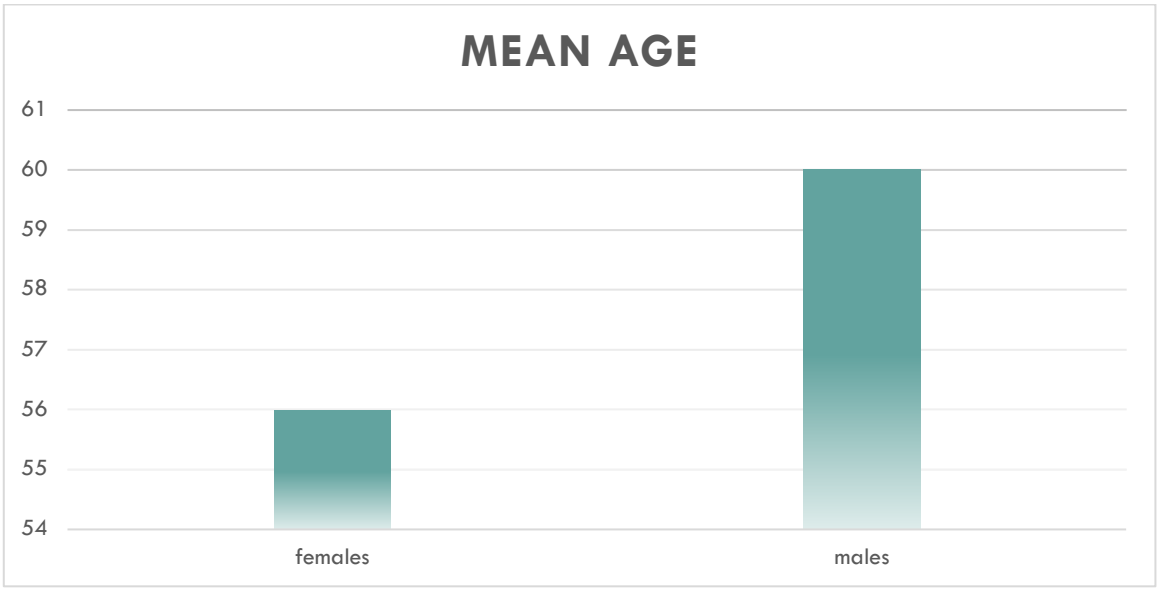


Figure 6.2 Mean age for males and females

6.1.3 Histology specimen types

Core needle biopsies were 43.18% (n = 57), tumour excisions were 30.30% (n = 40), modified radical mastectomies were 12.88% (n = 17) and simple mastectomies were 13.64% (n = 18).

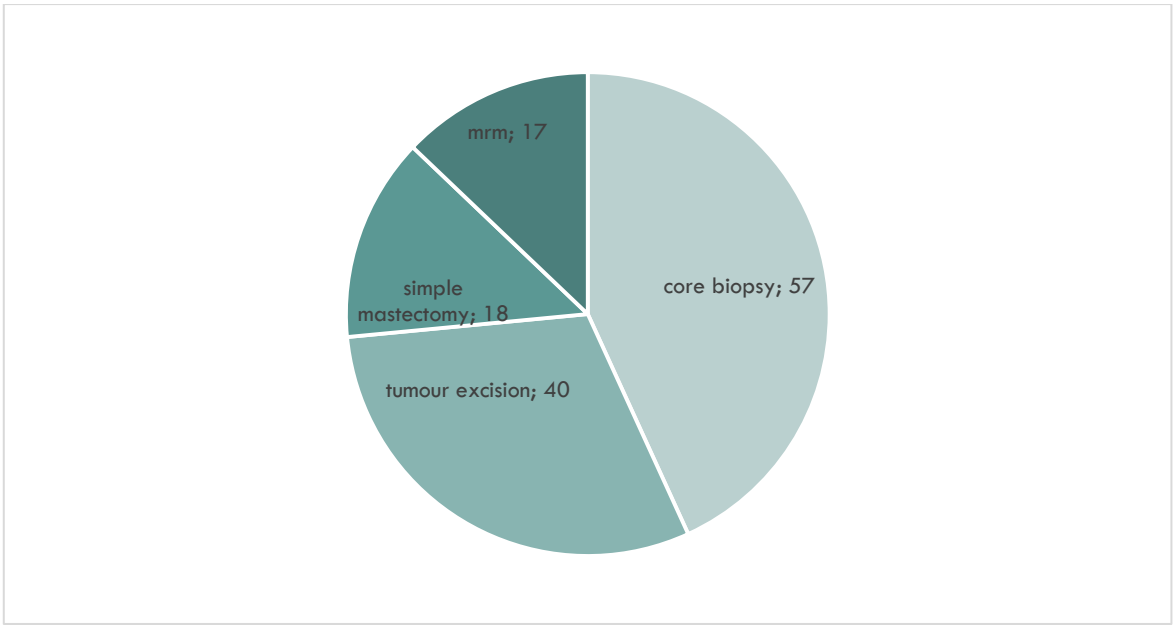
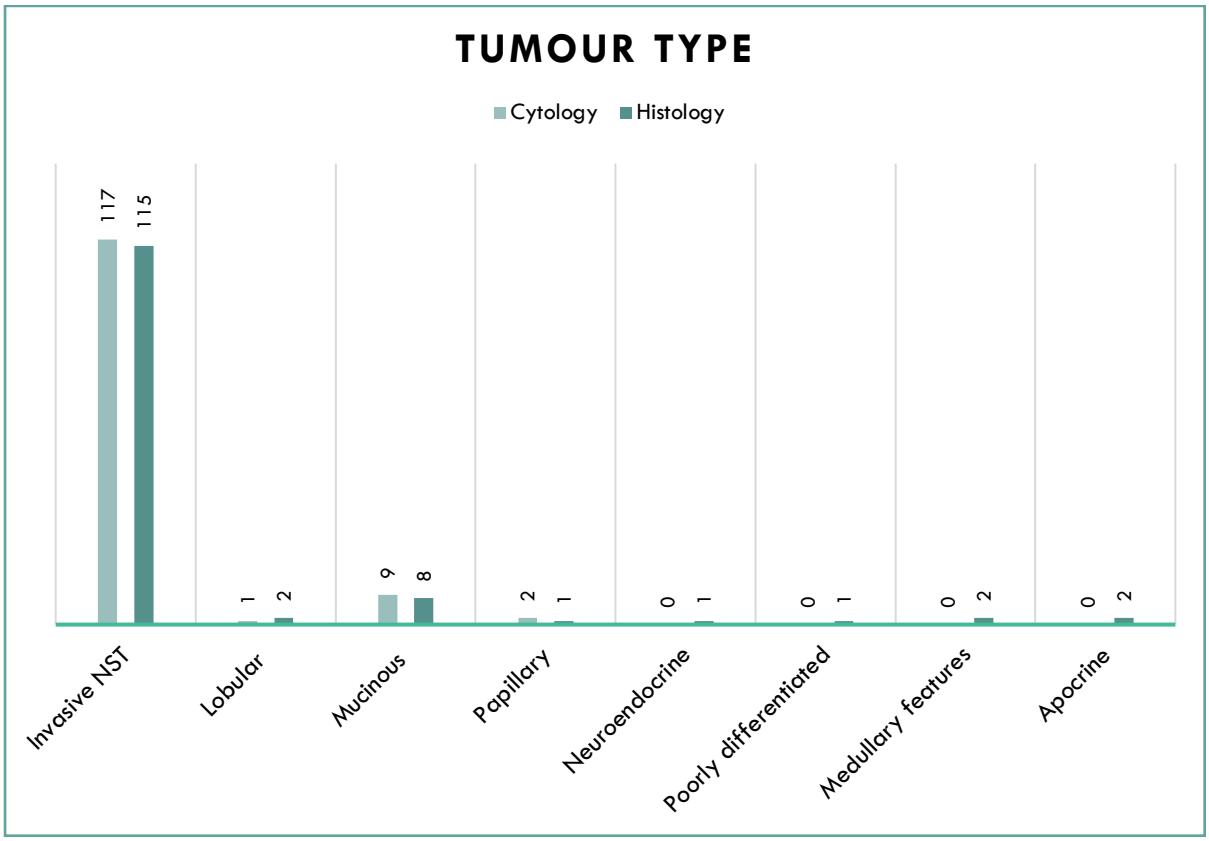


Figure 6.3 Histology specimen types. n = 132

6.1.4 Tumour types

The most common diagnosis for both cytology and histology was invasive carcinoma no specific type (NST) 89% (n = 117) and 86% (n = 114) respectively. Lobular carcinoma diagnosis was 0.76% (n = 1) on cytology and 1.52% (n = 2) on histology. Detailed results are illustrated in Figure 6.4.



*NST: No Specific Type

Figure 6.4 Cytological and Histological tumour type

6.1.5 Study population

610 cases were identified from 2013 to 2015 with a primary diagnosis of breast cancer on cytology. 285 patients had no histology follow up. 132 patients had histology done after chemotherapy. 40 patients had cell blocks with no cells or too few cells. 21 cases could not be retrieved from the archive and 132 cases were chemotherapy naive and deemed suitable for this study.

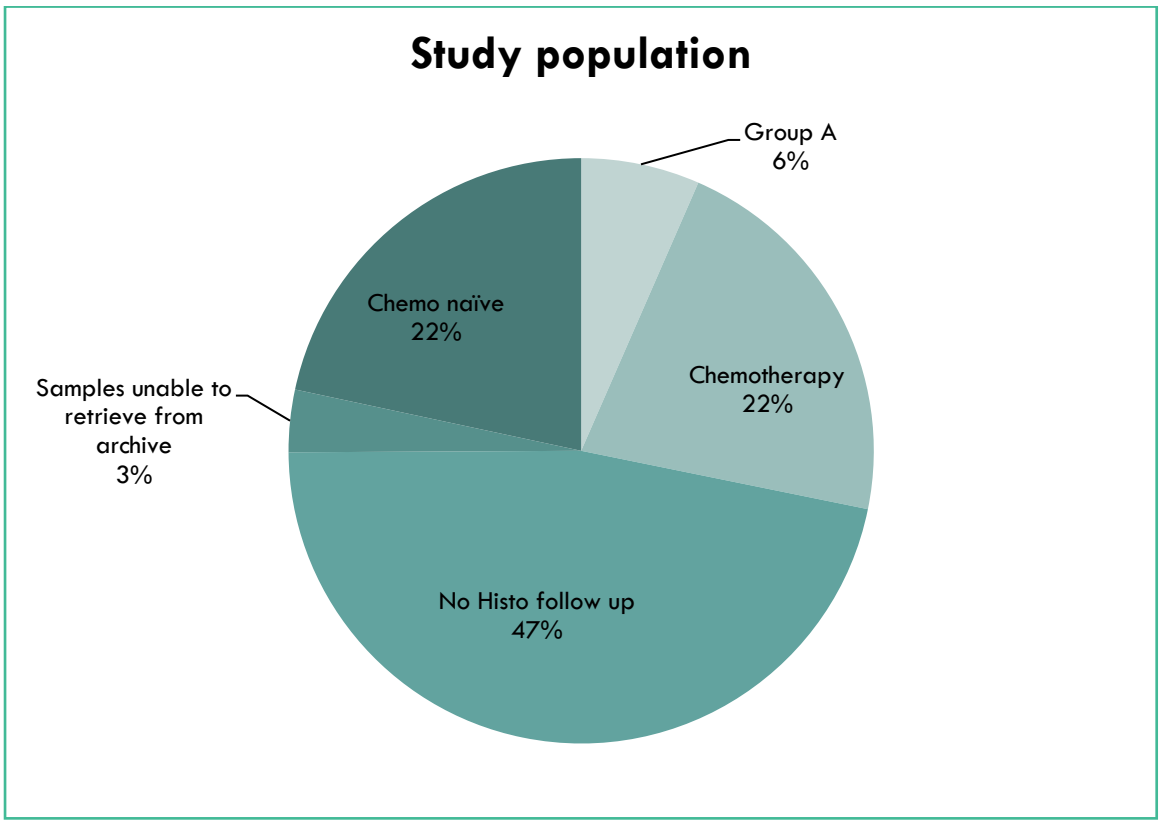


Figure 6.5 Study population

6.2 Discussion

Cell blocks of adequate cellularity are increasingly being used in adjunct with FNAB smears to improve the diagnostic accuracy of FNA cytology.[48] Cell blocks can sometimes lack adequate cellularity even if conventional smears are abundantly cellular.[49] In this study 87.88% (n=116) of our cell blocks had more than a 100 cells, which is considered sufficient for analysis according to Bueno et al. The adequacy and sensitivity of cell blocks depends on sampling type, size of specimen and the aspiration technique used.[29]

In this study the overall mean patient age was 56. Mean age for females was 56 and for males 60 years. Age is considered a major risk factor for developing breast carcinoma and the vast majority of breast carcinomas occur in women over the age of 50.[2]

In this study needle core biopsy made up 43% of all the histology specimen types followed by tumour excision (30%). Core needle biopsy is more cost-effective than tumour excision and open surgery can be avoided which lowers clinical complications. Tumour excision is however considered the method of choice because it is representative of the whole tumour but is much more expensive.[18–20]

Invasive carcinoma of no specific type comprises approximately 75% of all breast carcinomas, which correlates with the finding of this study on cytology and histology respectively. Mucinous carcinoma accounts for approximately 2% of all breast carcinomas. This study included 9 (6.82%) cases that were diagnosed on cytology and 8 (6.06%) cases diagnosed on histology.

Lobular carcinoma is seen in approximately 5-15% of all breast carcinomas. The current study had only 1 (0.76%) case diagnosed on cytology and 2 (1.52%) cases diagnosed on histology, illustrating that lobular carcinoma was uncommon in this study. These results emphasize the difficultly in correctly identifying lobular carcinoma on cytology.

There was one neuroendocrine carcinoma, two carcinomas with medullary features and two carcinomas with apocrine features diagnosed on histology, none of which were specifically diagnosed on cytology. Cytologically these were all diagnosed as carcinoma, NST. Carcinomas such as lobular, tubular, papillary or invasive carcinomas of low nuclear grade may be difficult to recognise on FNAB and should be confirmed with core needle biopsy.[22]

Chapter 7: ER and PR Analysis

7.1 Results

ER was positive in 78% (n = 103) and negative in 22% (n = 29) of the cell block cases. On histological FFPET, ER was positive in 80% (n = 105) and negative in 20% (n = 27) of the cases.

Cell block ER positivity showed a sensitivity of 91% and a specificity of 62% (with a 95% confidence interval). The positive predictive value was 90% and negative predictive value of 67%. Table 7.1 tabulates the ER-positive and ER-negative cases in cell blocks and FFPER respectively.

Table 7.1: ER staining results on cell blocks and FFPET.

		FFPET ER	
Cell block ER	**Positive**	**Negative**	**Cell block Total**
Positive	94	9	103
Negative	11	18	29
FFPET Total	105	27	132

PR was positive in 67% (n = 88) and negative in 33% (n = 44) of the cell block cases. On FFPET, PR was positive in 62% (n = 82) and negative in 38% (n = 50) of the cases.

PR positivity on cell block showed a sensitivity of 85% and a specificity of 84% (with a 95% confidence interval). The positive predictive value was 92% and negative predictive was 74%. Table 7.2 tabulates the PR-positive and PR-negative cases in cell blocks and FFPET respectively.

Table 7.2: PR staining results in cell blocks and FFPET.

		FFPET PR	
Cell block PR	**Positive**	**Negative**	**FFPET Total**
Positive	75	13	88
Negative	7	37	44
Cell block Total	82	50	132

7.2 Discussion

The correlation of immunochemical staining for ER and PR on FNAB cell blocks and on histological FFPET samples respectively has been under debate for decades. Histological FFPET remains the gold standard with proven accuracy and reliability, with a lower rate of unsatisfactory samples, and with a higher sensitivity and specificity than cell blocks; however as mentioned previously, tissue biopsies (core needle or excision biopsies) have several disadvantages such as haematoma, bleeding, increased time and cost to perform the procedure, and delays in reporting of the results.

Various studies have compared FNAB with histological samples but the latter remain superior but FNAB advantages contribute greatly as a diagnostic tool. However, FNAB offers several advantages as outlined previously, and efforts to improve its specificity and sensitivity for the detection of ER and PR remain important.[50,51]

This study confirmed the ability to prepare high quality and cellular cell blocks from FNAB material. Based on type of treatment(s) planned; ICC can be performed to determine presence and over expression of prognostic markers. Patients who would benefit from hormone therapy should be identified promptly, therefore accurate reporting is of vital importance for prognostic and predictive purposes.[31,48,52]

BD Cytorich™ Red fluid was used as a preservative fluid to conserve cellular morphology and architecture and to allow cells to appear alcohol-fixed. However cell block preparations in this study were fixed in formalin for 2 to 12 hours before histological processing. Maleki et al concluded that formalin fixation from 2 to 96 hours provides reliable IHC results. ASCO/ CAP guidelines direct that histological biopsy specimens should be fixed for a minimum of 6 hours and for not more than 48 hours for optimal biomarker assessment. This study suggests that BD Cytorich™ Red preservative fluid and 10% buffered formalin fixation has little impact on results because the BD Cytorich™ Red cell block technique has shown to have good sensitivity and specificity when compared to FFPET.

BD Cytorich™ Red cell block technique illustrates excellent performance of 91% sensitivity with a 95% confidence interval and is therefore reliable for ER assessment. Sensitivity is considered to measure the performance of a screening test, thus the sensitivity of the cell block test is the probability that someone with breast carcinoma will return a positive test

result.[53] Results obtained for specificity was 62%, specificity is also the measure of the performance of a screening test, which is the probability that someone without breast carcinoma will return a negative test result.[53] This result was calculated by the overall small quantity of ER-negative cases (n = 29) in our study population. Our results correlate well with previous studies where cell block sensitivity varies between 60% and 95%.[27,28,32]

The positive predictive value (PPV) of a test is the probability that someone who tests positive actually has breast carcinoma. BD Cytorich™ Red cell blocks have 90% PPV for ER assessment showing good performance for identifying ER-positive tumours. The negative predictive value obtained was moderate with 67%, which indicate the probability that some negative tests truly do not have ER-negative breast carcinoma. The moderate negative predictive value is therefore related to the total amount of negative cases as seen in ER specificity.

PR sensitivity of 85% indicates good performance of the cell block technique together with a specificity of 84% with 95% confidence interval. Positive predictive value of 92% and negative predictive value of 74% were obtained. PR sensitivity is lower than ER sensitivity possibly due to the conclusion made by Vohra et el. who concluded that the moderate PR concordance of 77.5% in their study was attributed to intra-tumoral heterogeneity.[27] On the other hand, PR specificity is higher than ER specificity, due to the fact that PR has a higher amount of overall negative cases (n = 44) than ER-negative cases (n = 29).

The results in this study can be considered excellent irrespective of numerous factors effecting PR positivity. Future research with a larger study population should allow identification and elimination of these factors which will ultimately increase reliability and accuracy of the BD cell block technique.

ER positivity analysis also revealed 11 false negative cases and 9 false positive cases. PR positivity showed 7 false negative cases and 13 false positive cases. It is difficult to establish the reason for these findings, but contributing factors should be identified and eliminated to avoid future error. False negative cases may be due to sampling errors, where the sections selected did not show ER or PR positivity due to tumour heterogeneity.

Generally there are many confounding factors that alter ICC results of cytological material. Various studies conclude that pre- analytical factors may have major implications on accuracy of results; such as type of fixative, length of fixation, type of antibody (sensitivity variation), inefficient antigen retrieval, staining error or analytical factors such as, interpretation of scoring systems and borderline cases.[48,54,55]

Other causes of false positive results include crushed and degenerate cells or marked necrosis, acute inflammation in the background, less specific antibody or an antibody titre that is too high. Various reports suggest that some antibodies give a false negative result with alcohol-fixation such as estrogen receptor.[31] Yet others have reported better results indicating that this variation may be attributed to variation in specific antibody manufacturing.[56]

It was suggested that discrepancies between cytology and histology samples could be ascribed mainly due to tissue heterogeneity, alteration of hormone receptors during the storage period or during execution of the tests or, methodology pitfalls.[57]

A Standardized, internationally approved scoring system for the reporting of staining of hormonal receptors on cytology cell block specimens has not been agreed on as yet. An internally developed scoring system was developed to interpret ER and PR proportion staining on cell blocks, facilitated comparison of the cell block to the histological FFPET specimens. This scoring system is listed in Table 5.2 and is a modification of the Allred scoring system, that is used on histological specimens and illustrated in Table 4.7.[58] The reporting of the staining intensity; remains unchanged between the two systems.

BD Cytorich™ Red cell block yielded excellent results and we believe that our modified scoring system provides the necessary information that is needed for determining treatment.

ER and PR biomarkers were considered positive if ≥10% of tumour cells stained positive with any intensity staining; illustrated by Figure 6.2. Thus a proportion of 0, which is staining in less than 10% of tumour cells were considered negative on the cell blocks; as illustrated in Figure 6.1. The overall positivity for this study was excellent when comparing BD Cytorich™ Red cell blocks with histological FFPET specimens considering ER and PR sensitivity results obtained.

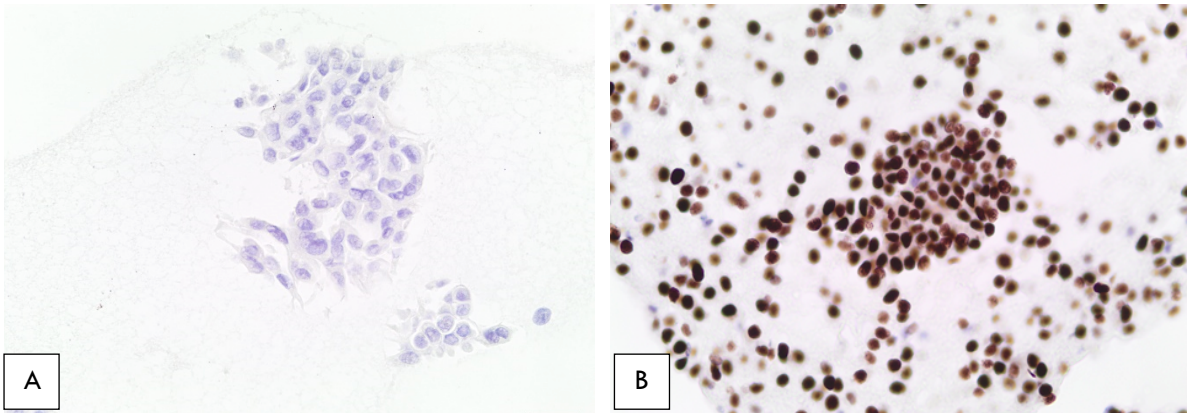


Figure 7.1 (a) Cell Block section showing ER-negative staining (x400 magnification). (b) Cell Block section showing PR positive staining (x400 magnification). BD Cytorich™ Red cell blocks.

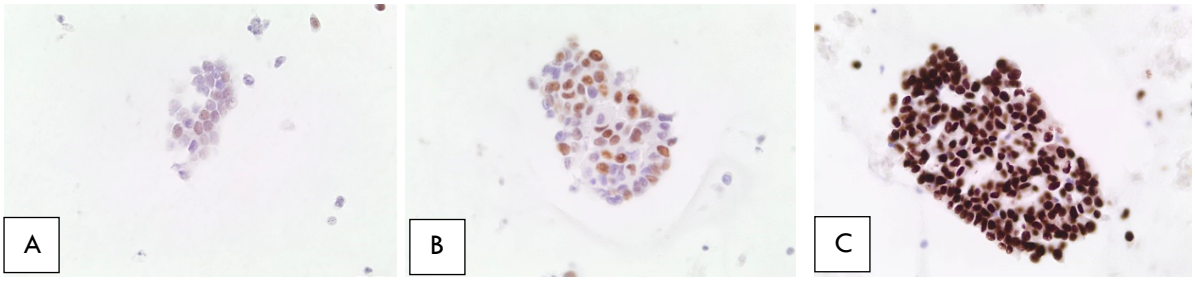


Figure 7.2 (a) Cell Block section showing ER intensity 1 staining (x400 magnification). (b) Cell Block section showing ER intensity 2 staining (x400 magnification). (c) Cell block section showing intensity 3 staining (x400 magnification). BD Cytorich™ Red cell blocks

Chapter 8: HER2 Analysis

8.1 Results

HER2 IHC staining was performed on 130 cases of the same study population. Two cases had to be excluded as the stained slides and respective tissue wax blocks could not be retrieved from the archives.

On the BD Cytorich™ Red cell blocks there were 108 negative cases; 8 cases were interpreted as equivocal (2+) and 14 cases as 3+ positive.

On the histological FFPET, HER2 was negative in 99 cases; equivocal (2+) in 19 cases and positive 3+ in 12 cases. These results are tabulated in Table 8.1.

Cell blocks had an overall agreement of 88% when compared to histological FFPET; with a Cohen kappa value of 0, 67.

Table 8.1: HER2 analysis (cytology cell blocks and histological FFPET)

	FFPET HER2			
Cell block HER2	1+	2+	3+	Total
1+	99	9	0	108
2+	0	6	2	8
3+	0	4	10	14
Total	99	19	12	130

8.2 Discussion

HER2 expression is a poor prognostic marker, which correlates with more aggressive behaviour and an overall decreased survival. HER2 testing is recommended for all breast carcinomas to determine if they are eligible for Trastuzumab® therapy.[59]

HER2 testing by immunochemistry was designed to analyse HER2 protein expression with a bright field microscope on histological FFPET sections. Immunochemistry is considered to be easier and more cost-effective to assess HER2 expression when compared to FISH. Immunochemistry is however subjective and semi- quantitative in determining expression levels of HER2 protein in breast carcinoma. Factors that influence the sensitivity and specificity of IHC include selection of antibody clones and signal detection systems.[42]

In this study cell blocks had an overall agreement of 88% when compared with histological FFPET. These findings indicate a reputable agreement between the cell block technique and histological FFPET. HER2 evaluation produced a kappa value of 0.67, indicating substantial agreement. Kappa is a quantitative measure of the magnitude of agreement between tests, therefore the cell block technique to determine HER2 status can be considered as an important modality for prediction of therapy.

Nine cases that were negative (0 or 1+) on cell block were scored 2+ (equivocal) on the FFPET. Two cases were scored 2+ (equivocal) on cell block and were scored 3+ on FFPET. Four cases that were scored 3+ on cell block; were scored 2+ (equivocal) on histological FFPET.

HER2 assessment is prone to error in both cytological and histological material having many technical and interpretive challenges. Therefore strict standardization is necessary to maintain accuracy and reliability (consistency). Patient's who may benefit from Trastuzumab® treatment, should be selected carefully due to cost and toxicity of treatment. Therefore HER2 equivocal cases need to be re- examined or submitted for FISH assays. Interpretation of HER2 status also requires intact cell membranes and cell to cell interaction.[12,27,42]

Figures 8.1 (A and B) as illustrated below were considered negative having no staining or granular cytoplasmic staining. Figures 8.2 (A and B) were deemed negative (1+) for weak or partial membrane staining in less than 10% of tumour cells.

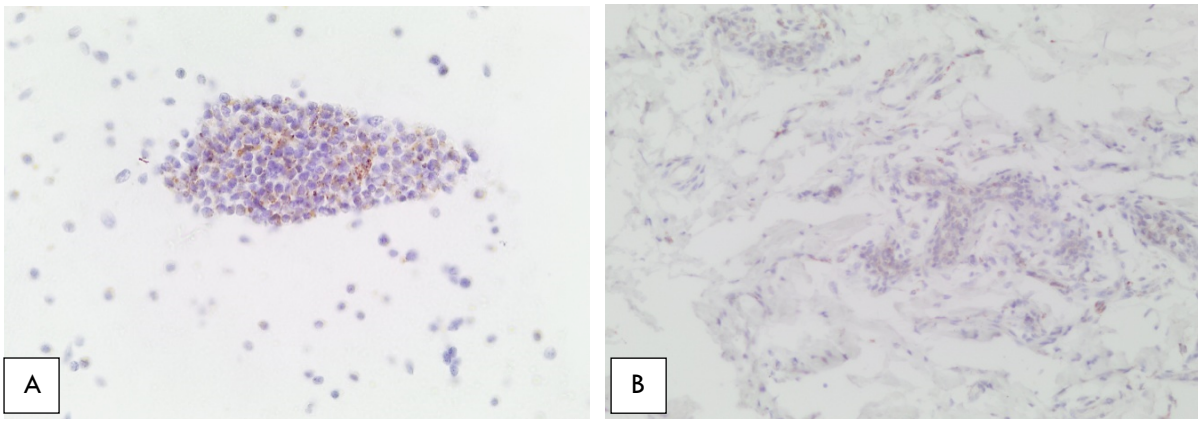


Figure 8.1 (a) Cell block section showing HER2 negative staining (x200). BD Cytorich™ Red cell block. (b) Histological section illustrating HER2 negative staining (x400).

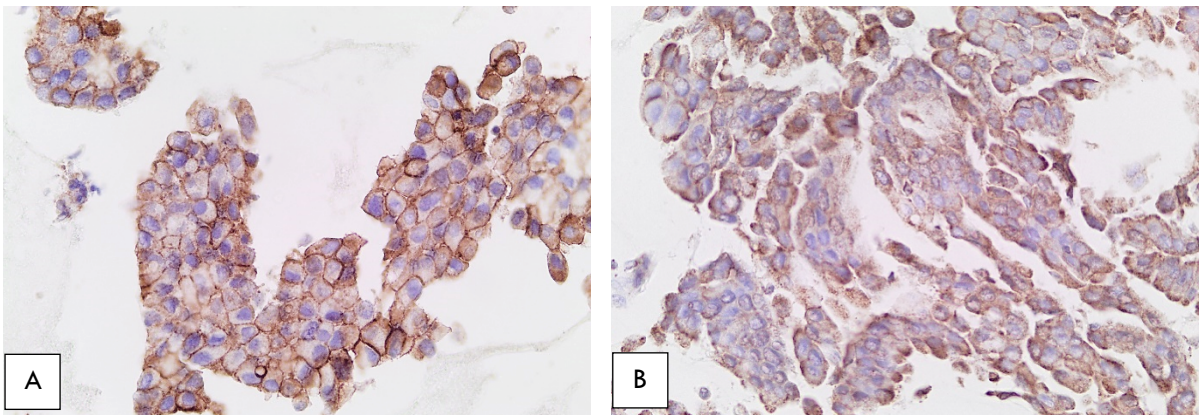


Figure 8.2 (a) Cell block showing HER2 Negative staining, scored as 1+ (x400). (b) HER2 negative staining, scored as 1+ on cell block section (x400). BD Cytorich™ Red cell blocks.

A score of 2+ is illustrated below in Figures 8.3 A and B. These cases had incomplete but strong membranous staining in more than 10% of tumour cells.

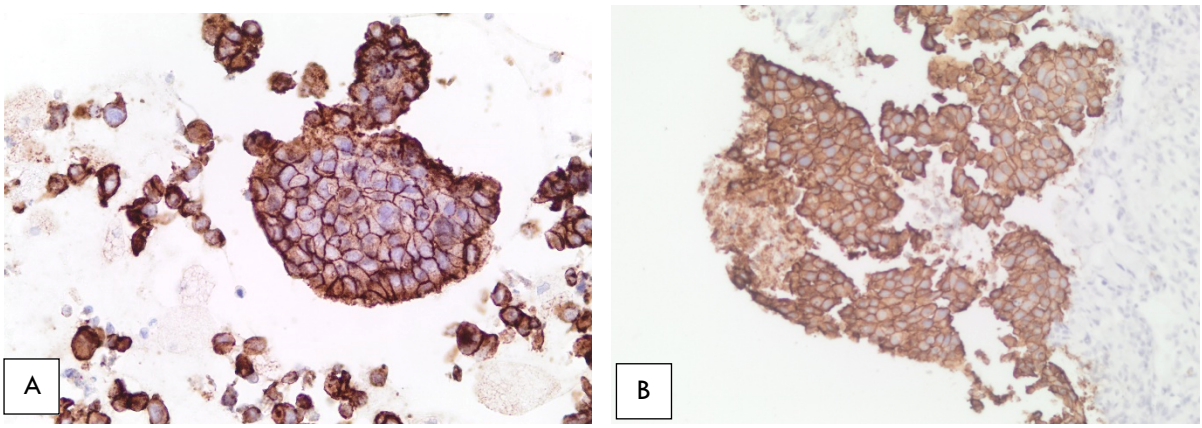


Figure 8.3 (a) Cell block showing HER2 equivocal staining, scored as 2+ (x400). BD Cytorich™ Red cell block. (b) HER2 positive staining scored as 2+ on histological section (x400).

Figures 8.4 (A and B) illustrate cases that were interpreted as 3+ on cell block sections. There were 14 cases on the cell blocks that were scored as 3+, positive staining with strong and complete, circumferential membranous staining in more than 10% of tumour cells.

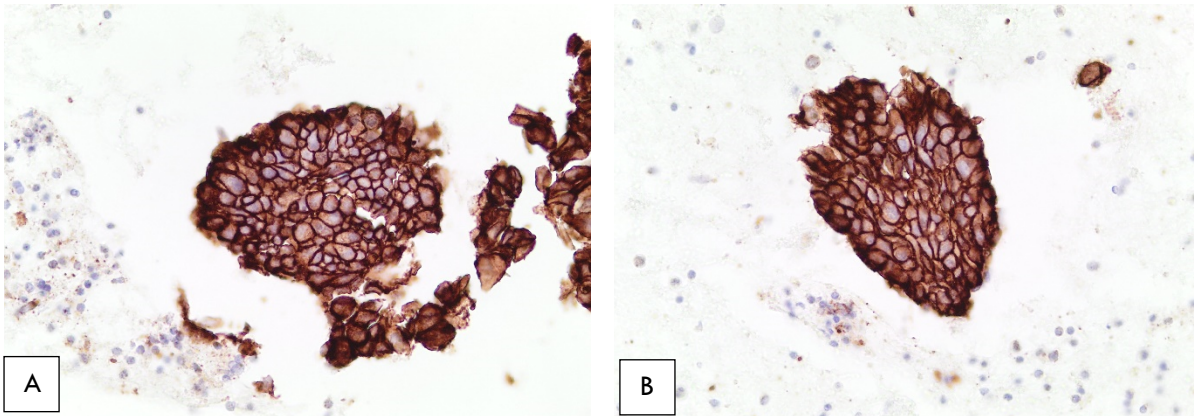


Figure 8.4 (a) Cell block showing HER2 positive staining, scored as 3+ (x400). (b) HER2 positive scored as 3+ on cell block section (x400). BD Cytorich™ Red cell block.

Chapter 9: FISH Analysis

9.1 Results

FISH analysis was performed on 25 cases that were randomly selected from the study population, and included 7 cases of positive 3+ staining, 4 cases of 2+ equivocal staining and 14 case of negative (0 or 1+) staining.

Of the seven 3+ staining cell block cases, all seven were FISH positive for amplification of HER2.

Of the four 2+ staining cell block cases, 3 were FISH positive and one FISH negative.

Of the Fourteen 1+ staining cell block cases, 3 came back as positive, 9 were negative and 2 had equivocal results, as listed in Table 9.1.

One of these fourteen 1+ cases showed a positive FISH result for HER2 amplification. This case additionally also stained as 1+ on the histological tissue.

Table 9.1: FISH analysis performed on the Cytology cell block specimens

	HER2 cell block immnostaining			
FISH Cell block	**1+**	**2+**	**3+**	**Total**
positive	3	3	7	13
negative	9	1	0	10
equivocal	2	0	0	2
Total	14	4	7	25

9.2 Discussion

All the 7 cases that were called positive on the BD Cytorich™ Red cell block immunostaining for HER2 were analysed as positive with the FISH probe.

Three of the four cases that were assessed as being 2+ (equivocal) with the immunostaining for HER2, revealed a positive FISH result. One case was FISH negative, indicating, that the 2+ score was correct and deserved FISH testing, as also required by ASCO Guidelines.

Three of the 14 cases that were called negative on HER2 immunostaining, showed a positive FISH result, with 9 being negative and 2 having an equivocal FISH results. This implies that 64% of the cases showed a correct result on immunostaining, with a further 12% being equivocal.

The three cases that had a negative score (0 or 1+) on immunostaining but a positive FISH result illustrate that more work is needed. This includes a need to improve the standardization of the reporting of the immunostaining and a need to further optimize the immunostaining technique for the HER antibody on the cell block material.

Overall, 80% of the cell block cases tested with FISH were correctly assessed on the HER2 immunostaining.

Twelve percent (3 cases) were deemed falsely negative on the HER2 immunostaining as they returned a positive FISH result. There was also 2 cases (8%) that returned an equivocal FISH result and these remain uncertain as to their true HER2 status.

These numbers are however too small to yield meaningful statistical evaluation and further studies are needed.

Chapter 10: Breast Cancer Grading

10.1 Results

10.1.1 Cytological findings

Cytology showed grade 1 in 33.59% (n= 43) of cases, grade 2 in 39.84% (n= 51) of cases, and grade 3 in 26.56% (n= 34) of cases. Detailed results are tabulated in Table 10.3.

Table 10.1: Robinsons grading parameters analysis

Parameters/variables	Cohen kappa value	Agreement associated with cytological grade
Cell dissociation	0.0831	40.30%
Cell size	0.3070	53.73%
Uniformity	0.3507	55.97%
Nucleoli	0.3339	55.97%
Margin	0.3859	59.70%
Chromatin	0.3048	54.48%

10.4.2 Histological findings

Histology scored grade 1 in 25.78% (n= 33) of cases, grade 2 in 46.09% (n= 59) of cases, and grade 3 in 28.13% (n= 36) of cases. Detailed results are tabulated in Table 10.4.

Table 10.2: Comparison of Cytological and Histological carcinoma grading

	Histological grade			
Cytological Grade	1	2	3	Total
1	15	24	4	43 (33.59%)
2	13	22	16	51 (39.84%)
3	5	13	16	34 (26.56%)
Total	33 (25.78%)	59 (46.09%)	36 (28.13%)	128 (100%)

10.4.3 Cytological and histological findings

The overall agreement was 41.41% when cytology grading was compared to histological grading. Cohen kappa value obtained was 0.1055 and z-test was 1.69. Standard error results showed a value of 0.0624 with the p-value being < 0.006 (not statistically significant).

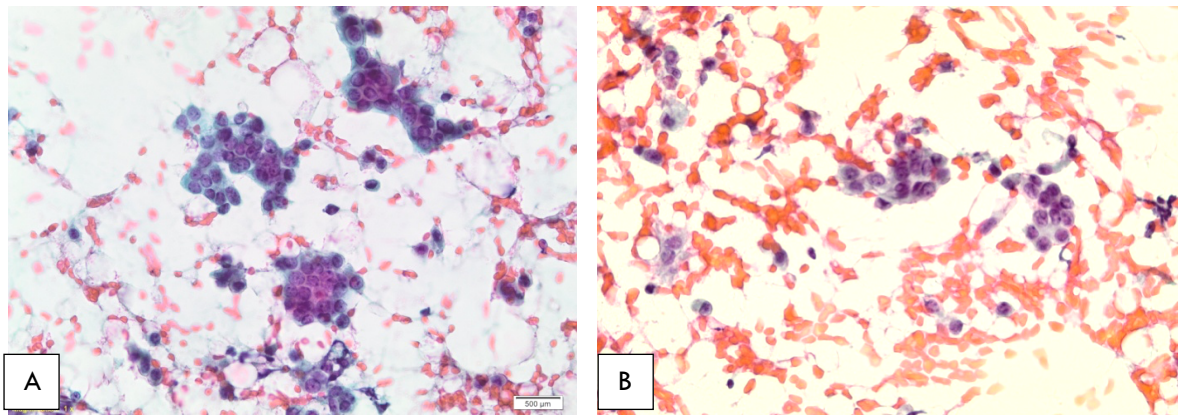


Figure 10.1 (a) Grade 1 tumour on cytology. (Papanicolaou stain x400) (b) Grade 1 tumour on cytology showing small nuclei 1- 2 times the size of a red blood cell. (Papanicolaou stain x400)

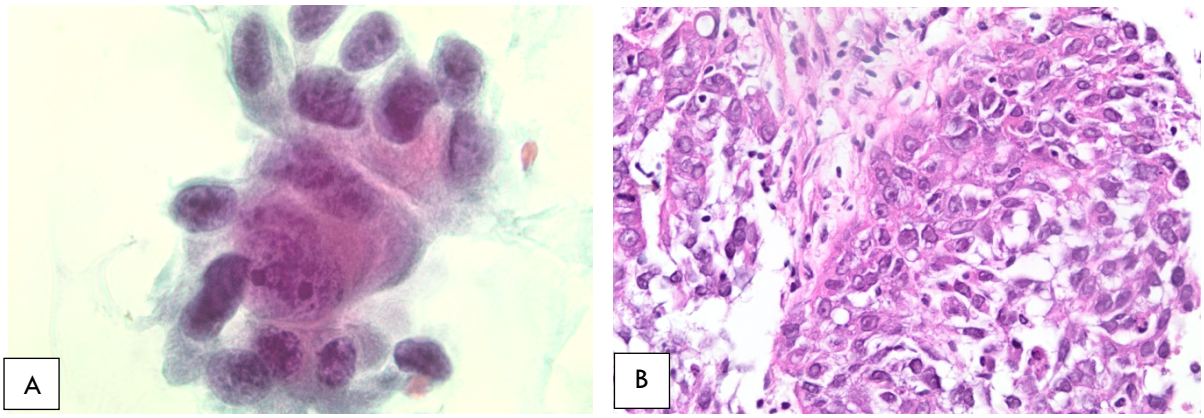


Figure 10.2 (a) Grade 3 on cytology (Papanicolaou stain x1000). (b) Highly pleomorphic cells diagnosed grade 3 on histological section (H&E stain x400)

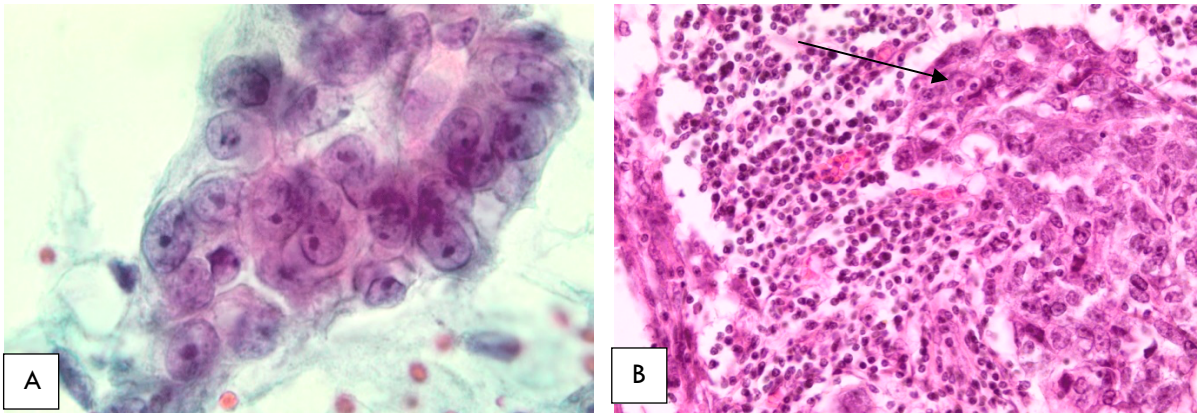


Figure 10.3 (a) Prominent nucleoli (Papanicolaou stain x1000). (b) Histological section showing pleomorphic and prominent nucleoli (H&E stain x400).

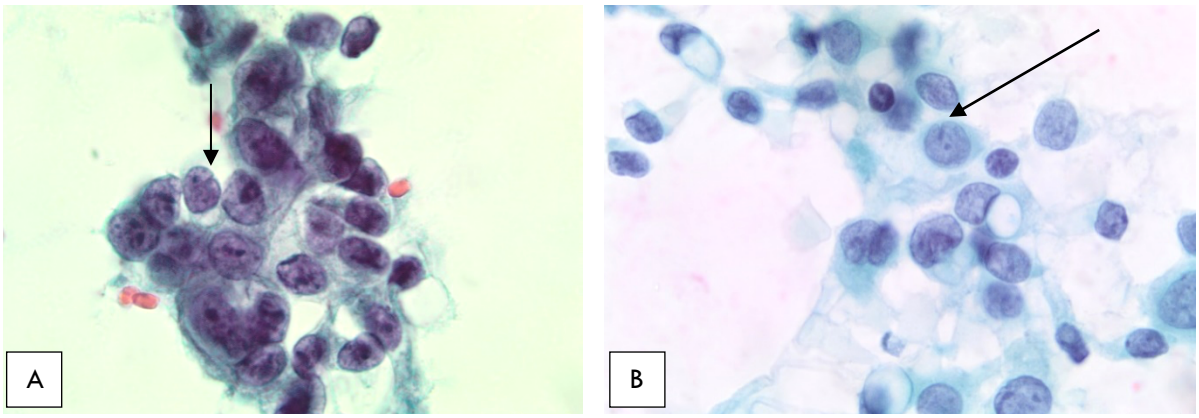


Figure 10.4 (a) Illustration of cleft formation (Papanicolaou stain x1000) (b) Illustration of grooves (Papanicolaou stain x1000)

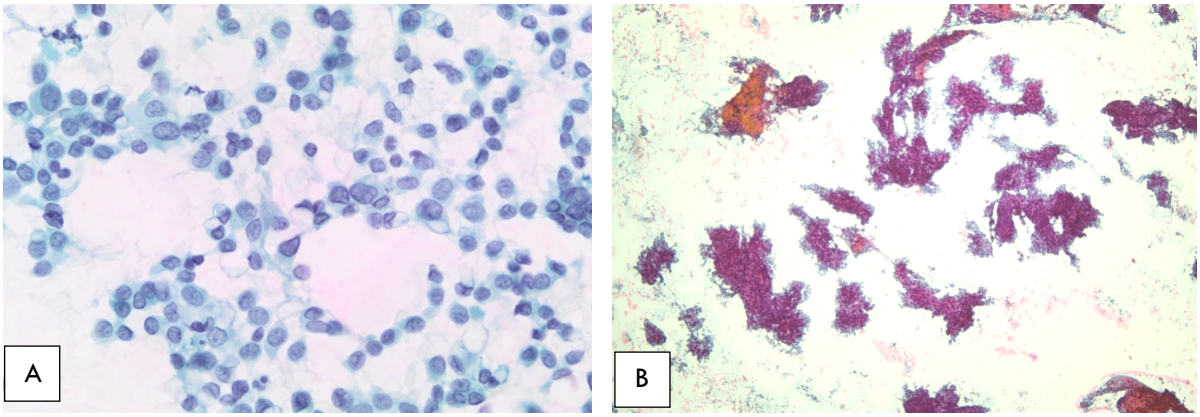


Figure 10.5 (a) Dissociation indicating mostly single cells (papanicolaou stain x400). (b) Cell dissociation illustrating mostly clusters (Papanicolaou stain x40)

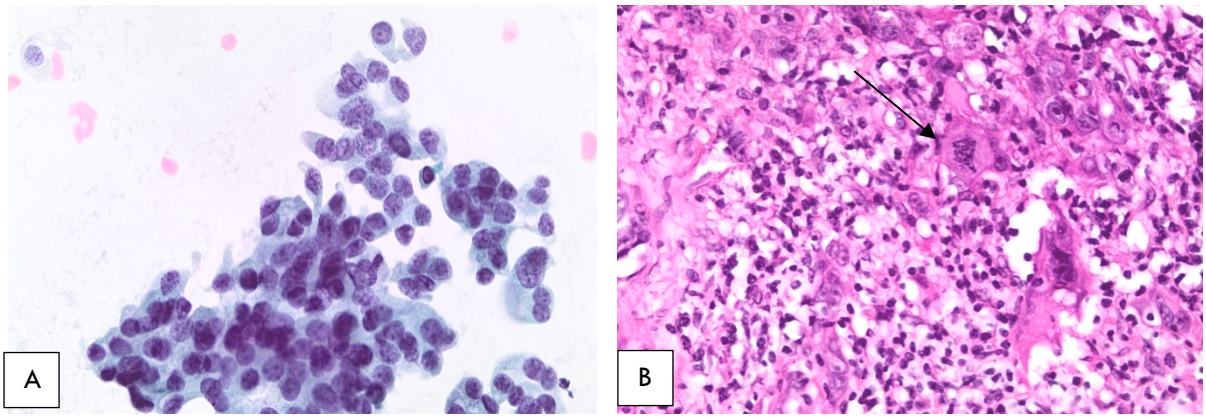


Figure 10.6 (a) Illustration of granular chromatin (Papanicolaou stain x400). (b) Illustration of mitotic figure illustrated on histological section (H&E stain x400)

10.5 Discussion

Pre-operative grading on FNA samples may be useful for determination of prognosis and neo- adjuvant therapy. Histological grading is based on the degree of tubule formation, mitosis and nuclear pleomorphism. Tubules and mitoses are difficult to assess on cytology and therefore may cause discordance between cytological and histological grading. In cytological grading nuclear size, nucleoli, nuclear membrane and chromatin pattern receive much more emphasis, which may also lead to increased disparity in grading.[44]

In this study, the overall agreement was 41.41% when cytological grading was compared to histological grading. This poor agreement implies caution when performing grading on cytology specimens.

Cohen kappa value obtained was 0.1055 and z-test was 1.69. Standard error results showed 0.0624 and p value was < 0.006. The kappa value is very low indicating a lack of agreement between cytological and histological grading.

A potential source of error may be due to inconsistency during interpretation of the measuring tools or variables which are exceptionally subjective; these variables include nucleoli, chromatin, margin, dissociation and nuclear size. Therefore these variables are subject to errors and inter- observer variation.

Detection of mitoses and tubules are notoriously difficult in FNA smears. Mitotic figures can be seen on cytological smears, but it is not as high as in histological sections. Therefore mitotic count is not measured in the Robinsons grading system.

Nuclear margin, cell uniformity and chromatin pattern are used in histology to evaluate nuclear pleomorphism. Nuclear margin was found to influence the cytological score according to Jyothi et al.[44] In this study nuclear margin is the variable that has the highest significance when compared to the final cytological carcinoma grade; and is considered to be the most important feature. Dissociation had the lowest agreement and is therefore not a significant factor/ variable to use in determining the final grade on cytological smears.

Chromatin pattern in this study revealed the lowest kappa value as tabulated in Table 10.3 and is considered not to be statistically significant. This finding might be due to subjectivity in assessment or to eye resolution limitations with the light microscope in detecting subtle grades of nuclear chromasia and granularity.[44]

Chapter 11: Conclusion

11.1 Summary of Findings

The primary aim of patients who are referred to breast clinics is to confirm or exclude presence of malignancy. FNAB can be of vital importance in the pre-operative evaluation of breast carcinoma. Ancillary techniques performed on cytological material demand standardization of pre-analytical, analytical and post-analytical methods. The amount of material obtained to conduct ancillary testing is a limiting factor. Specimen collection and processing needs to be performed in a manner to optimize both making a correct diagnosis and maximizing the amount of tissue available for ancillary studies.

In the current era of personalized medicine appropriate tissue triaging is of vital importance to avoid unnecessary sampling and/or procedures. There is huge pressure to obtain maximum amount of information from a tumour with the least amount of intervention and/or procedures. This problem is further compounded in a limited resource setting.

In many cases biopsy specimens are not available and the only source available for ancillary testing is FNA cytology material. Though cell blocks are routinely collected and prepared on malignant breast specimens, they remain plagued by collection of insufficient material.

This study has demonstrated that conventional cytology smears that were obtained via a minimally invasive, fine needle aspiration biopsy procedure (FNAB) that was performed in a dedicated FNAB clinic; could diagnose breast carcinoma correctly. The BD Cytorich^{TM} Red cell block collection method produced high cellularity cell blocks with excellent results for ER, PR and HER2 immunostaining. It also showed that BD Cytorich^{TM} Red cell blocks can be used for FISH molecular techniques. FNAB cytology offers a safe, reliable, accurate and minimally invasive alternative technique for the diagnosis and prognostication of breast carcinomas in our setting.

The grading performed on cytological material did not correlate well with histology in this study. Additional studies are needed in order to simplify grading systems that could be used on cytology smear specimens. Standardized and simple criteria that can be consistently applied are essential and need to be established on cytology FNA material to ultimately predict histological grade**.**

Bibliography

1. Vorobiof BDA, Sitas F, Vorobiof G. Breast Cancer Incidence in South Africa. 2001;19(18):125-127.
2. Sasco AJ. Epidemiology of breast cancer: an environmental disease? *APMIS*. 2001;109(5):321-332. doi:10.1111/j.1600-0463.2001.tb05823.x
3. Tao Z, Shi A, Lu C, Song T, Zhang Z, Zhao J. Breast Cancer: Epidemiology and Etiology. *Cell Biochem Biophys*. 2015;72(2):333-338. doi:10.1007/s12013-014-0459-6
4. Edge J, Buccimazza I, Cubasch H, Panieri E. The challenges of managing breast cancer in the developing world – a perspective from sub-Saharan Africa. *South African Med J*. 2014;104(5):377. doi:10.7196/SAMJ.8249
5. Patani N, Martin L-A, Dowsett M. Biomarkers for the clinical management of breast cancer: International perspective. *Int J Cancer*. 2013;133(1):1-13. doi:10.1002/ijc.27997
6. Somdyala NI, Bradshaw D, Gelderblom WC, Parkin DM. Cancer incidence in a rural population of South Africa, 1998-2002. *Int J Cancer*. 2010;127(10):2420-2429. doi:10.1002/ijc.25246
7. Keen JC, Davidson NE. The biology of breast carcinoma. *Cancer*. 2003;97(S3):825-833. doi:10.1002/cncr.11126
8. DeSantis CE, Ma J, Goding Sauer A, Newman LA, Jemal A. Breast cancer statistics, 2017, racial disparity in mortality by state. *CA Cancer J Clin*. 2017;00(00):1-10. doi:10.3322/caac.21412
9. Burkitt George, Young Barbara HJ. *Wheather's Functional Histology. A Text and Colour Atlas*. Third.; 1997.
10. Bateman AC, Shaw EC. Breast pathology. *Surg*. 2016;34(1):1-7. doi:10.1016/j.mpsur.2015.10.002
11. Sibbering M, Courtney C. Management of breast cancer: basic principles. *Surg*. 2016;34(1):25-31. doi:10.1016/j.mpsur.2015.10.005
12. Cangiarella Joan, Simsir Aylin TS. *Breast Cytohistology*. Cambridge University; 2013.
13. Egyed Z, Járay B, Kulka J, Péntek Z. Triple Test Score for the Evaluation of Invasive Ductal and Lobular Breast Cancer. *Pathol Oncol Res*. 2009;15(2):159-166. doi:10.1007/s12253-008-9083-3
14. Kocjan G, Bourgain C, Fassina A, et al. The role of breast FNAC in diagnosis and clinical management: A survey of current practice. *Cytopathology*. 2008;19(5):271-278. doi:10.1111/j.1365-2303.2008.00610.x
15. Ricci MD, Calvano Filho CMC, Oliveira Filho HR De, Filassi JR, Pinotti JA, Baracat EC. Analysis of the concordance rates between core needle biopsy and surgical excision in patients with breast cancer. *Rev Assoc Med Bras*. 2012;58(5):532-536. doi:10.1590/S0104-42302012000500008
16. Bilous M. Breast core needle biopsy: issues and controversies. *Mod Pathol*. 2010;23

Suppl 2(S2):S36-S45. doi:10.1038/modpathol.2010.34

17. Westenend PJ, Sever a R, Beekman-De Volder HJ, Liem SJ. A comparison of aspiration cytology and core needle biopsy in the evaluation of breast lesions. *Cancer Cytopathol*. 2001;93(2):146-150. doi:10.1002/cncr.9021

18. Sutela A, Vanninen R, Sudah M, et al. Surgical specimen can be replaced by core samples in assessment of ER, PR and HER-2 for invasive breast cancer. *Acta Oncol (Madr)*. 2008;47(1):38-46. doi:10.1080/02841860701441822

19. Tham TM, Iyengar KR, Taib NA, Yip CH. Fine needle aspiration biopsy, core needle biopsy or excision biopsy to diagnose breast cancer - Which is the ideal method? *Asian Pacific J Cancer Prev*. 2009;10(1):155-158.

20. Tamaki K. K, Sasano H, Ishida T, et al. Comparison of core needle biopsy (CNB) and surgical specimens for accurate preoperative evaluation of ER, PgR and HER2 status of breast cancer patients. *Cancer Sci*. 2010;101(9):2074-2079. doi:10.1111/j.1349-7006.2010.01630.x

21. Wai CJ, Al-Mubarak G, Homer MJ, et al. A Modified Triple Test for Palpable Breast Masses: The Value of Ultrasound and Core Needle Biopsy. *Ann Surg Oncol*. 2013;20(3):850-855. doi:10.1245/s10434-012-2710-y

22. Lakhani SR, Ellis IO, Schnitt SJ, Tan P-H, van de Vijver M, eds. *WHO Classification of Tumours of the Breast*. 4th ed. IARCPress; 2012.

23. Donnelly J, Iqbal M, Devaraj S, Marriott PJ, Corder AP. Role of mammography in the triple assessment of single-quadrant breast symptoms. *Br J Surg*. 2011;98(7):951-955. doi:10.1002/bjs.7490

24. Liew PL, Liu TJ, Hsieh MC, et al. Rapid staining and immediate interpretation of fine-needle aspiration cytology for palpable breast lesions: Diagnostic accuracy, mammographic, ultrasonographic and histopathologic correlations. *Acta Cytol*. 2010;55(1):30-37. doi:10.1159/000320869

25. Fischer AH, Schwartz MR, Moriarty AT, et al. Immunohistochemistry practices of cytopathology laboratories: A survey of participants in the college of American pathologists nongynecologic cytopathology education program. *Arch Pathol Lab Med*. 2014;138(9):1167-1172. doi:10.5858/arpa.2013-0259-CP

26. Karnauchow PN, Bonin RE. “Cell-block” technique for fine needle aspiration biopsy. *J Clin Pathol*. 1982;35(6):688. doi:10.1136/jcp.35.6.688

27. Vohra P, Buelow B, Chen Y, et al. Estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 expression in breast cancer FNA cell blocks and paired histologic specimens: A large retrospective study. *Cancer Cytopathol*. 2016;124(11):828-835. doi:10.1002/cncy.21745

28. Domanski AM, Monsef N, Domanski HA, Grabau D, Fernö M. Comparison of the oestrogen and progesterone receptor status in primary breast carcinomas as evaluated by immunohistochemistry and immunocytochemistry: a consecutive series of 267 patients. *Cytopathology*. 2013;24(1):21-25. doi:10.1111/j.1365-2303.2012.00997.x

29. Bueno Angela SP, Viero RM, Soares CT. Fine needle aspirate cell blocks are reliable for detection of hormone receptors and HER-2 by immunohistochemistry in breast carcinoma. *Cytopathology*. 2013;24(1):26-32. doi:10.1111/j.1365-2303.2011.00934.x

30. Jain D, Mathur SR, Iyer VK. Cell blocks in cytopathology: a review of preparative methods, utility in diagnosis and role in ancillary studies. *Cytopathology*. 2014;25(6):356-371. doi:10.1111/cyt.12174

31. Hanley KZ, Siddiqui MT, Lawson D, Cohen C, Nassar A. Evaluation of new monoclonal antibodies in detection of estrogen receptor, progesterone receptor, and Her2 protein expression in breast carcinoma cell block sections using conventional microscopy and quantitative image analysis. *Diagn Cytopathol*. 2009;37(4):251-257. doi:10.1002/dc.20989

32. Dong J, Ly A, Arpin R, Ahmed Q, Brachtel E. Breast fine needle aspiration continues to be relevant in a large academic medical center: experience from Massachusetts General Hospital. *Breast Cancer Res Treat*. 2016;158(2):297-305. doi:10.1007/s10549-016-3886-9

33. Garg S, Mohan H, Bal A, Attri AK, Kochhar S. A comparative analysis of core needle biopsy and fine-needle aspiration cytology in the evaluation of palpable and mammographically detected suspicious breast lesions. *Diagn Cytopathol*. 2007;35(11):681-689. doi:10.1002/dc.20721

34. Kalhor N, Wistuba II. Perfecting the fine-needle aspirate cell block. *Cancer Cytopathol*. 2013;121(3):109-110. doi:10.1002/cncy.21284

35. Nathan NA, Narayan E, Smith MM, Horn MJ. Cell block cytology: Improved preparation and its efficacy in diagnostic cytology. *Am J Clin Pathol*. 2000;114(4):599-606. doi:10.1309/G035-P2MM-D1TM-T5QE

36. Hanley KZ, Birdsong GG, Cohen C, Siddiqui MT. Immunohistochemical detection of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 expression in breast carcinomas: Comparison on cell block, needle-core, and tissue block preparations. *Cancer Cytopathol*. 2009;117(4):279-288. doi:10.1002/cncy.20034

37. Dahima S. iMedPub Journals Cell Block a Forgotten Tool. 2015;(January 2015):1-4.

38. Gown AM. Current issues in ER and HER2 testing by IHC in breast cancer. *Mod Pathol*. 2008;21:S8-S15. doi:10.1038/modpathol.2008.34

39. Kos Z, Dabbs DJ. Biomarker assessment and molecular testing for prognostication in breast cancer. *Histopathology*. 2016;68(1):70-85. doi:10.1111/his.12795

40. Brouckaert O, Paridaens R, Floris G, Rakha E, Osborne K, Neven P. A critical review why assessment of steroid hormone receptors in breast cancer should be quantitative. *Ann Oncol*. 2013;24(1):46-53. doi:10.1093/annonc/mds238

41. Mutlu H, Karaca H, Akca Z, Torun YA. Should Fish Test Be Performed To All Patients With Breast Cancer? *Med Sci*. 2013;2(2):539-547. doi:10.5455/medscience.2013.02.8052

42. Nitta H, Kelly BD, Allred C, et al. The assessment of HER2 status in breast cancer: the past, the present, and the future. *Pathol Int*. 2016;66(6):313-324. doi:10.1111/pin.12407

43. Flatley MJ, Dodwell DJ. Adjuvant treatment for breast cancer. *Surg*. 2016;34(1):43-46. doi:10.1016/j.mpsur.2015.10.003

44. Jyoti Prakash Phukan, Anuradha Sinha and JPD. Cytological grading of breast

carcinoma on fine needle aspirates and its relation with histological grading 1. *South Asian J Cancer*. 2014;3(2):1. doi:10.4103/2278

45. Yamashita H, Yando Y, Nishio M, et al. Immunohistochemical evaluation of hormone receptor status for predicting response to endocrine therapy in metastatic breast cancer. *Breast Cancer*. 2006;13(1):74-83. doi:10.2325/jbcs.13.74

46. Wolff AC, Hammond MEH, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *J Clin Oncol*. 2013;31(31):3997-4013. doi:10.1200/JCO.2013.50.9984

47. Press MF, Sauter G, Buyse M, et al. HER2 gene amplification testing by fluorescent in situ hybridization (FISH): Comparison of the ASCO-College of American Pathologists guidelines with FISH scores used for enrollment in Breast Cancer International Research Group clinical trials. *J Clin Oncol*. 2016;34(29):3518-3528. doi:10.1200/JCO.2016.66.6693

48. Kumar SK, Gupta N, Rajwanshi A, Joshi K, Singh G. Immunochemistry for oestrogen receptor, progesterone receptor and HER2 on cell blocks in primary breast carcinoma. *Cytopathology*. 2012;23(3):181-186. doi:10.1111/j.1365-2303.2011.00853.x

49. Ferguson J, Chamberlain P, Cramer HM, Wu HH. ER, PR, and Her2 immunocytochemistry on cell-transferred cytologic smears of primary and metastatic breast carcinomas: A Comparison Study with Formalin-Fixed Cell Blocks and Surgical Biopsies. In: *Diagnostic Cytopathology*. Vol 41. ; 2013:575-581. doi:10.1002/dc.22897

50. Ohashi R, Matsubara M, Watarai Y, et al. Diagnostic value of fine needle aspiration and core needle biopsy in special types of breast cancer. *Breast Cancer*. 2016;23(4):675-683. doi:10.1007/s12282-015-0624-9

51. Rakha EA, Reis-Filho JS, Baehner F, et al. Breast cancer prognostic classification in the molecular era: the role of histological grade. *Breast Cancer Res*. 2010;12(4):207. doi:10.1186/bcr2607

52. Srebotnik Kirbiš I, Us Krašovec M, Pogačnik A, Strojan Fležar M. Optimization and validation of immunocytochemical detection of oestrogen receptors on cytospins prepared from fine needle aspiration (FNA) samples of breast cancer. *Cytopathology*. 2015;26(2):88-98. doi:10.1111/cyt.12143

53. Webb P, Bain C. *Essential Epidemiology*. second edi. Cambridge University; 2011.

54. Jayaram G, Elsayed EM. Cytologic Evaluation of Prognostic Markers in Breast Carcinoma. *Acta Cytol*. 2005;49(6):605-610. doi:10.1159/10.1159/000326247

55. Schmitt F, Barroca H. Role of ancillary studies in fine-needle aspiration from selected tumors. *Cancer Cytopathol*. 2012;120(3):145-160. doi:10.1002/cncy.20197

56. Fowler LJ, Lachar W a. Application of Immunohistochemistry to Cytology basics when applying immunohistochemistry methods in the cytology laboratory. *Arch Pathol Lab Med—Vol Immunohistochem Appl to Cytol Lachar Arch Pathol Lab Med—Vol*. 2008;132(132):373-383. doi:10.1043/1543-2165(2008)132[373:AOITC]2.0.CO;2

57. Tafjord S, Bøhler PJ, Risberg B, Torlakovic E. Estrogen and progesterone hormone receptor status in breast carcinoma: Comparison of immunocytochemistry and

immunohistochemistry. *Diagn Cytopathol*. 2002;26(3):137-141. doi:10.1002/dc.10079

58. Marinšek ZP, Nolde N, Kardum-Skelin I, et al. Multinational study of oestrogen and progesterone receptor immunocytochemistry on breast carcinoma fine needle aspirates. *Cytopathology*. 2013;24(1):7-20. doi:10.1111/cyt.12024

59. Hanna WM, Rüschoff J, Bilous M, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. *Mod Pathol*. 2014;27(1):4-18. doi:10.1038/modpathol.2013.103