# "DIGITAL Vs MANUAL ASSESSMENT OF Ki67 IN BREAST CARCINOMA PATIENTS A HOSPITAL BASED STUDY"


**By**

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# LIST OF ABBREVIATIONS USED

| | | |
|---|---|---|
| AI | = | Artificial intelligence |
| AJCC | = | American Joint Committee on Cancer's system |
| ANOVA | = | Analysis of variance |
| ASCO | = | American Society of Clinical Oncology |
| BLBC | = | Basal like breast cancer |
| BRCA | = | Breast cancer gene |
| CK5/6 | = | Cytokeratin 5/6 |
| C.C | = | Correlation Coefficient |
| CDK1 | = | Cyclin-dependent kinase 1 |
| C.I | = | Confidence interval |
| DALY | = | Disability-adjusted life years |
| DCIS | = | Ductal carcinoma in situ |
| DIA | = | Digital image analysis |
| EGFR | = | Epidermal growth factor receptor |
| ER | = | Estrogen receptor |
| FFPE | = | Formalin fixed paraffin embedded |
| FHA | = | Forkhead-associated domain |
| GLOBOCAN | = | Global cancer observatory |
| HER2 | = | Human epidermal growth factor receptor |
| H&E | = | Haematoxylin and Eosin |
| HR | = | Hazard ratio |
| IARC | = | International Agency for Research on Cancer |
| IHC | = | Immunohistochemistry |

| | | |
|---|---|---|
| IKWG | = | International Ki67 in Breast Cancer Working Group |
| ICC | = | Intraclass correlation coefficient |
| IDC | = | Invasive ductal carcinoma |
| ILC | = | Invasive lobular carcinoma |
| KI67 LI | = | Ki67 Labelling index |
| LCIS | = | Lobular carcinoma in situ |
| LR domain | = | Leucine and arginine rich domain |
| LVI | = | Lymphovascular invasion |
| MC | = | Mucinous adenocarcinoma |
| MIB-1 | = | Mindbomb E3 Ubiquitin Protein Ligase-1 |
| NCBC | = | Non classifiable Breast Cancer |
| PCL | = | Peri-chromosomal layer |
| PDL1 | = | Programmed death L1 |
| PHA | = | Phytohemagglutinin |
| PI | = | Proliferative index |
| PI3KCA | = | Phosphoinositide 3-kinase |
| PNI | = | Perineural invasion. |
| PP1 | = | Protein phosphatase |
| PR | = | Progesterone receptor |
| RUNX1 | = | Runt-related transcription factor 1 |
| RV | = | Reference value |
| SEER | = | Surveillance, Epidemiology, and End Results |
| TC | = | Tubular carcinoma |
| VA | = | Visual assessment |
| WHO | = | World Health Organization |

# ABSTRACT

## DIGITAL Vs MANUAL ASSESSMENT OF Ki67 IN BREAST CARCINOMA PATIENTS A HOSPITAL BASED STUDY

**BACKGROUND:**

The Ki67 antigen is widely used as a proliferation marker in breast carcinoma. Expression of this nuclear protein (pKi67) is associated with the proliferative activity of intrinsic cell populations in tumours, allowing it to be used as a marker of tumour aggressiveness. The Ki67 protein is substantially used as a prognostic and predictive marker for cancer diagnosis and treatment.

Ki67's value for treatment methods is restricted by its uncertain analytical validity. Ki67 might be a useful prognostic marker, but the available information is still sparse and inconsistent. The clinical use of Ki67 IHC in breast cancer treatment is now restricted to determining prognosis in cases of stage I or stage II breast cancer.

Digital pathology with advancements in automated scoring could help to overcome the current restrictions in standardization of Ki67, thereby reducing inter- and intra-pathologist variability and accrediting automation to amplify routine practise and thus improving reliability and reproducibility of the results.

**OBJECTIVES:**

1.To compare Digital Vs Manual Assessment of Ki67 in histologically diagnosed cases of breast carcinoma

2.To correlate Ki67 expression with clinic-pathological parameters.

**METHODOLOGY:**

Fifty cases of histologically diagnosed breast carcinoma cases were taken. H& E and Ki67 IHC staining was done using the Ki67 SP6(vitro) antibody.

1. Manual Ki67 assessment was done by 3 different observers using printed images on 500 tumour cells from the hotspot areas in 50 breast cancer cases.
2. Digital image analysis of the Ki67 assessment was done using Qupath open software, and a positive Ki67 tumour cell count was measured.
3. Comparison between manual and digital methods was done.

**RESULTS:**

Manual assessment of Ki67 showed excellent agreement between inter-observer's (r: 0.956, p value <0.0001). Digital image analysis of Ki67 measurements showed excellent correlation with manual Ki67 measurements (r = 0.957, r = 0.937, and r = 0.932, p= 0.0001). A comparison between clinicopathological parameters and Ki67 (DIA) showed no significant correlation between them except for fibrosis.

**Conclusion:** Digital Ki67 assessments exhibit the same variability and reproducibility as manual Ki67 assessments. Given the non-inferiority and substantial time savings, the present study supports more widespread adoption of automated digital image analysis of Ki67 in routine clinical practice.

**Key words:** Breast cancer, Ki67, Manual, Digital, Qupath

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# INTRODUCTION

In every country in the world, cancer is the main cause of death and a significant hurdle to raising life expectancy.[1] Most breast cancer patients have lost more disability-adjusted life years (DALYs) globally than any other type of cancer. Breast cancer occurs at any age after puberty, but with increasing rates in later life .[2]

According to GLOBOCAN, the number of breast cancer deaths is also expected to rise by more than 50%, from 685,000 in 2020 to 1 million in 2040 .[3]

In spite of significant advances in research, breast cancer remains a crucial health problem and constitutes a top biomedical research priority .[4]

In comparison to earlier staging that was solely based on morphological aspects of the disease, the most recent update of breast cancer staging that included biologic markers improved outcome prediction, Validation studies involving the reassessment of the Surveillance, Epidemiology, and End Results (SEER) database, according

The AJCC manual's 8th edition proved to have more accurate prognostic information, which included Ki67 as one of the prognostic biomarkers. [5]

The Ki67 antigen is widely used as a proliferation marker. Expression of this nuclear protein (pKi67) is associated with the proliferative activity of intrinsic cell populations in tumours, allowing it to be used as a marker of tumour aggressiveness. The Ki67 protein is substantially used as a prognostic and predictive marker for cancer diagnosis and treatment .[6]

The following are three clinical applications for Ki67 IHC: 1) To estimate whether extra adjuvant chemotherapy is required in early-stage disease, 2) To ascertain whether chemotherapy may or may not be active, and 3) To follow-up patients during or after neoadjuvant endocrine or chemotherapy to assess whether the chosen regimen is effective or if an alternative strategy should be used .[7]

Ki67's value for treatment methods is restricted by its uncertain analytical validity. Ki67 might be a useful prognostic marker, but the available information is still sparse and inconsistent.[5]

The World Health Organization (WHO) released a consensus document in 2019 entitled "Recommendations on digital interventions for health system strengthening," recognising that artificial intelligence (AI) and digital technologies can offer boundless possibilities to advance health management and accomplishment. AI-based systems assist pathologists in analysing digital images to perform quantification, diagnosis, and prognostication.

Digital pathology, which includes the digitising of glass slides to construct whole slide images, enables the application of AI in pathology and provides reliable, objective, and reproducible results, thereby reducing inter- and intra-pathologist variability and accrediting automation to amplify routine practise.[8]

The clinical use of Ki67 IHC in breast cancer treatment is now restricted to determining prognosis in cases of stage I or stage II breast cancer. Additional advancements in automated scoring could help to overcome some current restrictions.[7]

# OBJECTIVES

**Primary objective:**

To compare Digital vs. Manual assessment of Ki-67 in histologically diagnosed cases of breast carcinoma

**Secondary objective**:

Quantitative analysis of Ki67 expression and its correlation with clinicopathological parameters in breast carcinomas

# REVIEW OF LITERATURE

## DEVELOPMENT OF BREAST

Breast development is the first significant somatic change in females, which commences at puberty and is established between 4 and 6 weeks of gestation.

Ectodermal cells on the anterior body wall thicken to form a ridge known as the "milk line" or "milk ridge." Except in the pectoral region, where the mammary gland grows in relation to the second to sixth rib, the milk ridge runs the length of the axilla to the groin, the thickening regressing later.

Generally, a newborn's breast can be felt at birth, with varied quantities of tissue and no obvious gender differences. From the first two years of life until puberty, the typical gland is dormant. Due to the effect of sex hormones, particularly oestrogen, sexually dimorphic breast development begins during puberty. [9]

## ANATOMY OF BREAST

The breast is a modified sebaceous gland, although it is present in both sexes, but in males it is rudimentary and is located within the pectoral region's superficial fascia.[10]

The mammary primordium is the origin of the breast tissue, which forms lactiferous ducts and mammary glands (parenchyma). The mesenchyme that surrounds this becomes the fibrous and fatty portions of the breast (stroma). The nipple appears at 8 months' gestation. During thelarche, oestrogen promotes adipose

and ductal tissue growth, while progesterone encourages lobular growth and alveolar budding.

The terminal end bud, or more precisely, the location of the mammary stem cells in the terminal end bud's cap cell layer, is where ductal elongation and complicated branching first appear. Segmental and subsegmental ducts are formed from the primary ducts. The terminal duct is formed by the subsegmental ducts and differentiates into multiple terminal ductules, or acini.[9]

The tubulo-alveolar gland secretes milk; each gland has 15-20 lobes, and the lactiferous duct drains each lobe, which is made up of a collection of alveoli. The lactiferous ducts converge and open on the nipple. Each duct has a lactiferous sinus, which is a dilatation near its terminus.[10]

The stroma serves as the gland's supporting structure. There are both fatty and fibrous parts to it. The Cooper suspensory ligaments are septa made of the fibrous stroma that hold the skin and glands to the pectoral fascia. The main mass of the gland is made up of fatty stroma.[10]

**VASCULAR SUPPLY:**

Arterial supply to the breast is from branches of the axillary, internal thoracic, and intercostal arteries.[11] Following the arteries are the veins; the internal thoracic vein receives blood from the superficial veins, and the axillary and posterior intercostal veins get blood from the deep veins.[10]

**NERVE SUPPLY:**

The anterior and lateral cutaneous branches of the fourth to sixth intercostal nerves supply the breast.[10]

**LYMPHATIC SUPPLY:**

Except for the nipple and areola, the skin over the breast is drained by the superficial lymphatics. The parenchyma is drained by deep lymphatics, which also drain the nipple and areola.[10]

Because lymphatic pathways are primarily involved in the spread of metastatic disease, the breast's lymphatic system has significant clinical significance.[11]

**PHYSIOLOGY OF BREAST:**

The breast is a physiologically distinctive organ for lactation, which involves the synthesis, secretion, and ejection of milk. The secretory units' ability to produce milk is controlled by a complex network of hormones and growth factors.

Oestrogen is the primary female hormone responsible for breast growth and maintenance. It causes the ductal system to grow and the nipples to mature and develop, which in turn causes the ductal epithelium, myoepithelial cells, and surrounding stroma to proliferate.

Progesterone is responsible for the development of the terminal duct and lobuloalveolar units. Both oestrogen and progesterone are induced to activate in the presence of hormones like insulin-like growth factors and growth hormones

In the development of ducts and lobulo-alveoli, prolactin works in conjunction with oestrogen and progesterone. Prolactin aids in the differentiation of alveolar cells into milk-secreting cells along with cortisol and insulin.

Release of oxytocin is stimulated by sucking reflux. Myoepithelial cells constrict due to oxytocin, which forces milk out of the lobules and into the lactiferous ducts.[12]

**HISTOLOGY OF BREAST:**

Age, stage of the menstrual cycle, pregnancy, and lactation all affect the histology of breast tissue. It is made up primarily of the ductal-lobular system, the stroma, and the nipple areolar complex.[11]

Ducts and acini are lined by two layers of cells: the inner luminal layer is lined by epithelial cells, and the outer basal layer is lined by myoepithelial cells, which are flattened cells.[13] There is a single layer of columnar or cuboidal cells in the smaller ducts, but there are two layers in the larger ones[11]; Numerous discontinuous stellate myoepithelial cells of ectodermal origin are in close contact with the bases of these cells.[13]

Each lobe of the breast is drained by lactiferous ducts, and each lactiferous duct is joined to a network of ducts and lobules that are all encircled by connective tissue stroma to form a breast lobule. The glandular components of the lobule have a secretory function. In accordance with their hormonal state, their structure changes.

Each lobule of the adult resting breast is made up of a collection of blind-ended, branched ductules, but these lack the mature terminal alveoli (acini) that the

lactating breast needs for milk secretion. Collagenous interlobular tissue encircles a glandular lobule.[11]

**EPIDEMOLOGY OF BREAST CANCER:**

Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer , with 2.3 million women diagnosed in 2020, resulting in 6,85,000 deaths worldwide. By the end of 2020, there will be 7.8 million women alive who have been diagnosed with breast cancer in the past 5 years, making it the world's most prevalent cancer.[1]

Based on the GLOBOCAN predictions of cancer incidence and death compiled by the International Agency for Research on Cancer (IARC), the burden of newly diagnosed cases of invasive breast cancer globally is expected to increase by over 40% by 2040, reaching nearly 3 million cases annually.[3]

**PROGNOSTIC FACTORS IN BREAST CANCER:**

Traditionally, breast cancer was contemplated as a homogenous entity[14], but now breast cancers are considered heterogenous, with variable morphologic and biological features, and thus show different clinical behaviour, response to molecular features, and therapeutic treatment.[15]

Breast cancer prognostic factors are those that describe the course or consequences of the disease, either with or without regular treatment. These are connected to the likelihood of benefiting from a particular treatment. The parameters that aid in predicting and determining prognosis in breast cancer should be included in every breast cancer pathology report. Clinical teams can make more customized

therapy choices for each patient with breast cancer using these predictive prognostic factors.[16]

The biomarkers ER and HER2 continue to be the principal predictors of prognosis in early-stage breast cancer. Germline BRCA testing, PDL1 immunoassays, and PI3KCA mutation testing for specific subgroups of stage 4 patients are predictive of drug effectiveness.

The size of the tumour, histological type, grading, and hormone receptors all have clinical implications.[16]

Breast cancer classification aims to provide an accurate diagnosis and prediction of tumour behaviour to facilitate oncologic decision-making.

Breast cancer assessment mainly evaluates:

- How bad the tumour is (typing and grading) based on the histologic subtypes
- How extensive is the tumour (staging)? WHO tumour classification (TNM staging)
- ER, PR, and HER2 expression[17]

Traditional histologic classification of breast cancers is based on tumour cell type, extracellular secretion, architectural features, and an immunohistochemical profile.[17] Depending on its relationship to the basement membrane, breast cancer can be either invasive or non-invasive. There are two main forms of noninvasive breast tumours: lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS). Invasive breast cancer is broadly divided into lobular and ductal types based on

histologic type; the most predominant type, invasive ductal cancer, also known as infiltrating ductal carcinoma, makes up 50% to 70% of all invasive breast cancers.[18]

There are 20 different histologic types of invasive breast cancer. The most common is IDC/NST (70–80%) of all invasive cancers, and invasive lobular carcinomas (ILC) make up around 10% of all invasive cancers.[17]

The WHO's classification of invasive breast cancers is the one that is most commonly used; it has updated its classification of breast cancer in its 5$^{th}$ edition, and the current update mainly focuses on morphologic classification. Changes were made in the mitotic count expression, which now uses a defined area expressed as $mm^2$ in the denominator; the molecular classification; and the expression profile are also included in the current WHO classification ([19]). IDC-NST, also known as invasive ductal carcinoma, is by far the most prevalent histologic form of invasive breast cancer (IDC).

Based on the current WHO (5$^{th}$ edition), invasive breast carcinoma is classified into the following histologic subtypes: infiltrating duct carcinoma (NOS), oncocytic carcinoma, lipid-rich carcinoma, glycogen-rich carcinoma, sebaceous carcinoma, lobular carcinoma NOS, tubular carcinoma (TC), cribriform carcinoma NOS, mucinous adenocarcinoma (MC), mucinous cystadenocarcinoma NOS, Invasive micropapillary carcinoma of breast, Metaplastic carcinoma NOS.[19]

TC, MC, cribriform carcinoma, and adenoid cystic carcinoma are some examples of pure, specific histologic subtypes that are recognised clinically as "favourable histologic subtypes."[16]

All breast cancers can be classified into the following biomarker-defined subgroups for therapeutic purposes based on the morphologic classification of breast cancers, which has been supported by a substantial amount of additional molecular and immunohistochemical evidence. Incorporating age, menopausal status, ER, HER2, Ki-67, tumour size, grade, and lymph node status into the National Health Service's calculation of the overall differences in 5-, 10-, and 15-year survival with and without additional therapies.[16]

Before treatment, a physical examination and imaging investigations are used to stage breast cancer clinically; after definitive surgical treatment, a pathologic evaluation of the primary tumour and regional lymph nodes is used to stage breast cancer pathologically. Staging is done to classify individuals into risk groups that define the prognosis and direct treatment options for patients with a similar prognosis.

The TNM classification system for breast cancer divides patients into four stage groups depending on the size of the main tumour (T), the status of the regional lymph nodes (N), and the presence of any distant metastases (M). The American Joint Committee on Cancer's system(AJCC) is the one that is most commonly used.[18]

**TNM STAGING**

TNM staging is based on the AJCC 8$^{th}$ edition staging system.

T-Tumour size: based on the size of the largest, most invasive component in the largest dimension.

T1- T1mi -( microinvasive, tumour <1mm), T1a-(tumour >1mm and <5mm), T1b-(tumour >5mm and <10mm), T1c-( >10mm and <20mm).

T2- Tumour size >20mm to <50mm

T3- Tumour size >50mm.

T4- T4a (any size involving chest wall), T4b- (macroscopic skin changes-ulcer, oedema, satellite nodules, not meeting inflammatory breast cancer criteria), T4c- (T4a+T4b), T4d- (diffuse oedema and erythema - Peu d'orange, inflammatory breast cancer at least involving 1/3$^{rd}$ of breast).

N- Node

cNx- nodes removed but which cannot be examined by imaging or physical examination.

cN0- Evaluation of lymph nodes is possible, but no regional lymph nodes have been examined by physical or imaging examination.

cN1- Level 1 and 2 lymph nodes, ipsilateral

cN2- Metastasis to fixed or matted ipsilateral level 1 and 2 lymph nodes or metastasis to ipsilateral internal mammary lymph nodes with no axillary lymph node involvement

cN3- Ipsilateral level 3 lymph node with or without level 1 and 2 involvement, supraclavicular lymph node ipsilateral, or internal mammary lymph node ipsilateral with level 1 and 2 lymph node involvement

M- Metastasis

cM0- No distant metastasis (clinically/ imaging)

cM1- Distant metastasis seen (clinically/ imaging)

pM1- Distant metastasis based on pathological findings.[20]

Early breast cancer has a remarkably good prognosis. Both stage 0 and stage I have a 5-year survival rate of 100%. Stage II and stage III breast cancer have 5-year survival rates of approximately 93% and 72%, respectively. The prognosis of the illness drastically deteriorates when it spreads throughout the body. The 5-year survival rate for stage IV breast cancer patients is only 22%.[18]

**Grading**

Grading of breast cancer based on tubule formation, nuclear pleomorphism, and proliferation as indicated by the mitotic index.

The Nottingham modification of the Scarff Bloom Richardson grading is used to evaluate each parameter with a numerical scoring system of 1 to 3 to produce a summation score for grade assignment.[17] Because neoadjuvant therapies can alter a cancer's presentation, grading may be more important before treatment than after post-neoadjuvant therapy.[16]

**Table 1.Nottingham modification of the Scarff Bloom Richardson grading**

| FEATURES | SCORE |
|---|---|
| 1.Tubule formation (extent within tumor) | |
| >75% | 1 |
| 10-75% | 2 |
| <10% | 3 |
| 2.Nuclear pleomorphism | |
| Small, regular, uniform | 1 |
| Moderate variation in shape and size | 2 |
| Marked variation in shape and size | 3 |
| Mitotic count/10 hpf (dependent on microscopic field area) | |
| 3.Field diameter 0.59-mm diameter/0.274-mm2 area | |
| 0-9 | 1 |
| 10-19 | 2 |
| >20 | 3 |
| 4.Field diameter 0.44-mm diameter/0.152-mm2 area | |
| 0-5 | 1 |
| 6-11 | 2 |
| >12 | 3 |

Total score: 3-5, grade 1, as well differentiated,

6-7 grade 2 as moderately differentiated,

Grades 8-9 are poorly differentiated (hpf, high power field).[16]

**HORMONAL RECEPTORS:**

The estrogen receptor (ER) and the progesterone receptor (PR) are the two most commonly used hormonal receptors in the prognosis of breast cancer.

Histology, prognosis, and treatment response are all highly correlated with hormone receptor status. Hormone marker expression is related to less aggressive histologic features and positive prognostic characteristics.

Estrogen and progesterone receptor expression is a very potent and helpful predictor. Evaluation of the receptor expression profile enables prediction of breast cancer response to hormone therapy since the presence of estrogen and progesterone receptors is associated with the response rate to hormonal therapy in breast cancer.[21]

ESTROGEN RECEPTOR:

Estrogen receptor has two subtypes-ERα and Erβ.

Breast cancer diagnosis, prognosis, and treatment selection, the ERα subtype is considered. ERβ was identified as the second estrogen receptor after Erα, which is expressed in myoepithelium, breast epithelium, and 20–30% of breast cancers.[22]

Women's breast tumours are considered to exhibit ER in between 70 and 80 percent of cases. With the right treatment plan, these tumours have a better prognosis and slower growth, which is correlated with the duration of survival following surgical removal.[21] Overall and disease-specific survival are independently predicted by ER expression. It is a nuclear stain.[22]

Interpretation of ER: Allred scoring method

Combining the proportion (0–5) and the intensity (0–3) of ER positive tumor cells to create a score (0–8 out of 8)

Proportion:

0: 0%, 1: < 1%, 2: 1 - 10%, 3: 11 - 33%, 4: 34 - 66%, 5: > 66%.

Intensity:

0: negative, 1: weak, 2: moderate, 3: strong.

Interpretation of scores 0 and 2 as negative; interpretation of scores 3 to 8 as positive.

Other scoring methods for ER interpretation: ASCO/CAP guidelines and H scoring system.[22]

**PROGESTERONE RECEPTOR:**

PR is induced by ER and modifies ER's behavior. PR depends on estrogen for its expression. Tumor differentiation and resistance to chemotherapy are both influenced by PR activity. It is a nuclear marker, scored by the Allred scoring system (similar to ER) and ASCO/CAP guidelines.

Regardless of ER, PR expression is linked to better clinical outcomes.[22]

Although the usefulness of PR to further stratify ER-positive cases into prognostic groups can be attributed to the fact that PR expression is typically more heterogeneous than ER expression, the validity of specific PR thresholds for this purpose is not well established.[16]

**HUMAN EPIDERMAL GROWTH FACTOR 2 (HER2):**

HER2, also known as ERBB2 (Erb-B2 receptor tyrosine kinase 2), is a prognostic and predictive factor in breast cancers. HER2 belongs to a group of growth factor receptors that influence the survival, development, and proliferation of normal cells. Approximately 15–20% of breast tumours have HER2 expression, which is associated with aggressive invasive cancers and comedocarcinoma. Chemotherapy combined with anti-HER2 therapy reduces death, metastasis, and recurrence in patients with HER2-amplified breast cancer.

Interpretation: ASCO/CAP guidelines

Positive- >10% of tumor cells exhibit full, strong, and circumferential membrane staining

Equivocal- More than 10% of tumor cells exhibit weak-to-moderate full membrane staining.

Negative- Incomplete membrane staining, faint in >10% of tumor cells (1 +) or no staining, faint in <10% of tumor cells (0).[16]

**MOLECULAR SUBTYPING OF BREAST CANCER**

The evolutionary biomolecular era began in 1953, with the introduction of the structure of deoxyribose nucleic acid by Crick, Franklin, and Watson, leading to a better understanding of the molecular basis of disease and the utility of biomarkers in clinical practice. A biomarker is defined as a normal biologic, pathologic, or pharmacological response to therapeutic intervention.[23]

Biomarkers are of several types: diagnostic, predictive, prognostic, and therapeutic, which may influence management aiming for personalised disease treatment. A prognostic biomarker mainly influences the likelihood of a clinical response but has limitations like interindividual variability and intraindividual variability (like Ki67 measurement), sensitivity, and specificity inferences.[24]

In breast cancer clinical practice, at least 4 heterogeneous molecular subtypes are acknowledged based on the expression of ER, PR, HER2, and Ki67 according to ASCO/CAP guidelines.[15,25]

The molecular classification of breast cancer has been used as the gold standard in recent years due to its characterization of breast cancer and its use in gene profiling models for prognostic outcome.[26]

Invasive ductal carcinoma is divided in to 7 molecular subtypes based on ER, PR, HER2, Ki67, CK5/6, EGFR IHC status.

ER and PR are reported using the Allred scoring system, HER2 using the ASCO/CAP protocol, Ki67 reporting is based on Ki LI (Ki67 cut-offs are 14%, 14–19%, and >14%), CK5/6 cytoplasmic positivity >20% positive tumour cells are taken as positive, and EGFR >10% positive tumour cells are taken as positive for molecular classification.[33]

The seven molecular subtypes are as follows:

1. Luminal A
2. Luminal B
3. Luminal HER2 PR+
4. Luminal HER2 PR-
5. HER2 Enriched
6. Basal like (BLBC)
7. Non classifiable (NCBC) ([33])

**Table 2.Molecular Subtyping Of Breast Cancer [33]**

| | **Molecular subtypes** | **ER** | **PR** | **HER2** | **Ki67** | **CK5** | **EGFR** |
|---|---|---|---|---|---|---|---|
| **LUMINAL** | LUMINAL A | + | + | - | <14 | - | - |
| | LUMINAL B Ki67 >14% | + | >20 | - | >14 | - | - |
| | Luminal B PR<20% | + | <20 | - | +/- | - | - |
| **HER 2+ve** | Luminal Her 2 PR+ | + | + | + | +/- | - | - |
| | Luminal Her 2 PR -ve | + | - | + | +/- | - | - |
| | Her 2 Enriched | - | - | + | +/- | - | - |
| **TRIPLE NEGATIVE** | BASAL LIKE (BLBC) | - | - | - | +/- | + | + |
| | NONCLASSIFIABLE (NCBC) | - | - | - | +/- | - | - |

Molecular classification based on IHC was recommended in St. Gallen guidelines in 2013: the cut-point between "high" and "low" values for Ki-67 varies between laboratories.[15]

In 2015 St Gallen guidelines proposed lab specific median Ki67 LI in hormone receptor positive and Her 2 negative cancers to distinguish Luminal A like from Luminal B like breast cancer.[27]

The St. Gallen 2019 consensus centred on primary optimal treatment in breast cancer. In grade 3 breast cancer, Ki 67 would be the parameter to decide the inclusion of an aromatase inhibitor as adjuvant endocrine chemotherapy.[28]

There have been several studies conducted on the validity of Ki-67 as an important biomarker in invasive breast cancers. According to the study done by Enrico et al. in 2018 in 506 breast cancer patients (stages I–III), an optimal cut-off of 23.4% Ki67 expression was suggested as a clinically significant independent prognostic marker for recurrence and survival in HR+ breast cancer cases.[29]

In 2019, Wu et al. described a Ki67 cut-off > 40% that has predicted disease-free survival with a hazard ratio of 2.30, a confidence interval of 1.54–3.44, and overall survival with a HR of 2.95 and a CI of 1.67–5.19 in 7716 triple-negative breast cancer patients in their meta-analysis study.[30]

Zhu et al. in 2020 conducted a study on 1800 early-stage triple-negative breast cancer patients using a 30% Ki67 cut-off, which has significant prognostic and predictive value for overall survival with HR: 1.947, CI: 95% (1.108–3.421).[31]

Furthermore, Tian et al. outlined that the expression of Ki67 15% is correlated with the 70 gene MammaPrint assay, and 81.4% were genetically low-risk following 70 gene signature stratification, suggesting inclusion of genomic testing in therapeutic decision-making in ER+ and HER2-negative breast cancer patients[32]

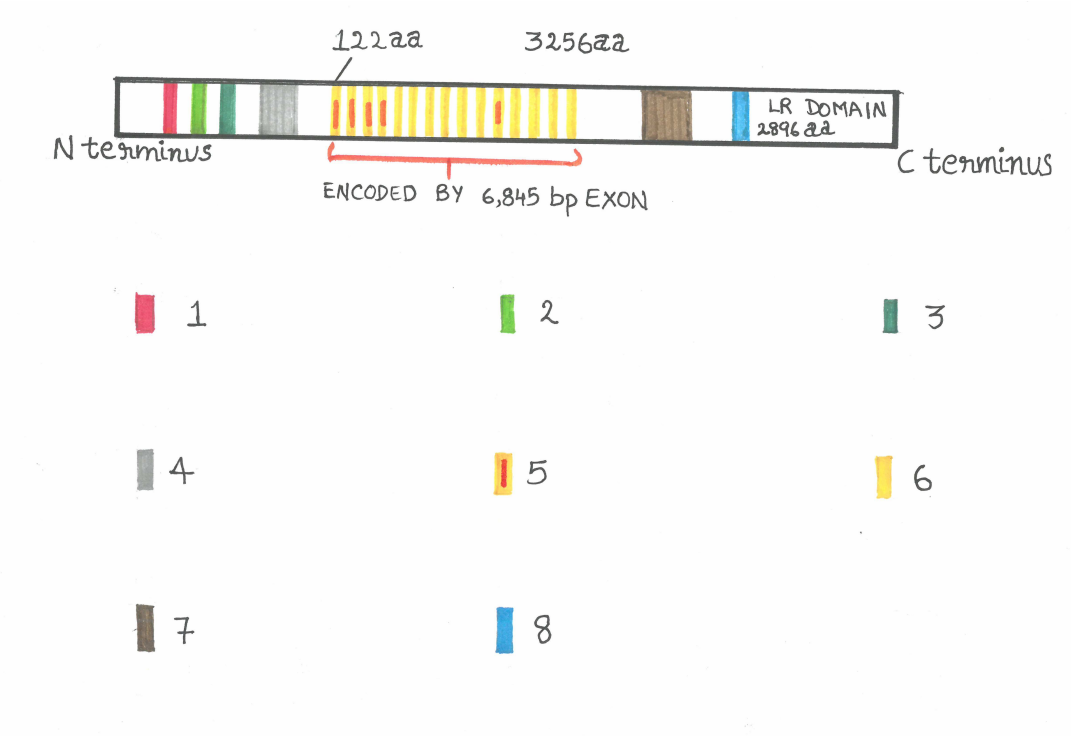
According to the current guidelines of the International Ki67 in Breast Cancer Working Group (IKWG 2021), the recent updated recommendation in the assessment of Ki67 in the prognosis of breast cancer has included Ki67 values of 5% or less (low) and 30% or more (high). The clinical utility of Ki67 is included only for prognostic estimation in luminal cases to help therapeutic decision-making in the use of adjuvant chemotherapy.[7]

**Ki67**

Ki67 was initially isolated from the Hodgkin lymphoma cell line L428 and named after the antibody that identified it. The Ki word is derived from Kiel (Germany), and 67 is being taken from the 96-well plate number.[34]

Ki67 is a proliferation marker seen throughout all active phases of the cell cycle; its expression is staunchly associated with tumour cell proliferation and growth.[35] These characteristics made Ki67 a useful biomarker for evaluating proliferation in clinical specimens from breast cancer patients.[36] Ki67 is a nuclear protein that is encoded by the MK167 gene in humans and encodes two protein isoforms (small, 320 kD, and large, 359 kD).[35] Ki67 has five variants with similar central tandem repeats and C-terminal regions but differing at the N-terminal region (the FHA domain), a PP1 binding domain (protein phosphatase), and the central region of tandem repeats.[37]

**Photomicrograph 1. Domain Structure of Ki67 In Humans [38]:**




| | | | |
|---|---|---|---|
| 1.Forkhead-associated domain | 2.PP1binding domain | 3.Conserved domain | 4.Nucleolar targeting region |
| 5.Concatenated repeats with PEST sites | 6.Concatenated direct repeats (Ki67 repeats) | 7.Kon 21 domain | 8.ATP/GTP binding domain. |

Ki67 has a chain of 16 direct repeats of 122 amino acids with few PEST sites. The PEST sites are vulnerable to proteolytic degradation, which is responsible for antigen detection by the MIB1 antibody. The N terminus has the FHA domain (transcription factor binding site), the PP1 domain (phosphoregulation of nucleophosmin/ B23 by casein kinase II), the conserved domain (31 amino acids), and a few nucleolar targeting regions.

The C terminus mainly has the Kon 21 domain for heterochromatin maintenance, the ATP/GTP binding domain, and the LR domain, which has 2896 amino acids and helps in DNA binding during chromosome targeting of Ki67. The LR domain can change the order of chromatin structure.[38]

The N-terminus has a forkhead-associated domain (FHA), and cell proliferation is increased with overexpression of N-terminal fragment The FHA domain recognises two important phosphoproteins during mitosis. 1. Hklp2 or Kif15 (a kinesin-like motor protein) plays a role in spindle bipolarity maintenance. 2. NIFK (nucleolar protein): used in cell proliferation and cancer metastasis (for example, lung cancer with RUNX1 transcription factor destabilisation and promotion of the WNT/-catenin signalling pathway).

Protein phosphatase 1 interaction domain:

Ki67 binds specifically to PP1 and PP1 isoforms in vivo; during anaphase, Ki67 targets PP1 isoforms, which is a critical step for mitotic exit for histone dephosphorylation.

In anaphase, PP1-mediated dephosphorylation of H3S10P is useful for rebinding HP1 to chromatin, which leads to re-establishing heterochromatin in post-mitotic cells.

Ki67 central tandem repeats:

During mitosis, CDK1 phosphorylates the tandem repeats, which help in Ki67 mitotic localization and DNA binding affinity. Ki67 acts as highly positive electrostatic charges, preventing hyperaggregation of chromosome arms.

C-terminal domain: rich in leucine and arginine (LR domain), it is useful for binding of Ki67 to all three isoforms of heterochromatin protein 1 (HP1), which in turn helps in the formation of heterochromatin rich in centromeres and telomeres in all cell types.[37]

Expression of Ki67 during the cell cycle:

Ki67 expression is controversial in the G1 phase (initial phases negative) and expressed in the later G1 phase.[14] According to phytohemagglutinin (PHA) studies on peripheral mononuclear blood leukocytes (PBL), Ki67 mRNA levels increase in the G1 phase. Variable degrees of quiescence lead to variations in Ki67 levels in the G1 phase after cell cycle re-entry.[37]

Through the S phase, increased staining for Ki67 was seen, and in the G2 phase, more increased Ki67 staining intensity was observed.[35]

Ki67 protein cellular distribution changes dramatically during the cell cycle and is not constant. G0 (the resting cell) has no expression of Ki67. Ki67 is found in the nucleoplasm in the early G1 phase, the nucleoli reassembly in the mid-G1 phase, and the heterochromatin region in the late G1 phase. Staining of Ki67 during the S phase is strictly seen in the nucleoli. The G2 phase of cell cycle expression of Ki67 is controversial; some authors found it to be present throughout the nucleus.

During mitosis, Ki67 protein is redistributed; Ki67 is absolutely detected in interphase; in prophase, fine meshwork with condensed chromatin is seen; in metaphase, bright staining covers the surface of each chromosome; and in anaphase and telophase, granular staining is detected.[35]

Ki67 helps in heterochromatin maintenance and peri-chromosomal assembly in mitotic chromosomes.[37]

Ki67 on chromosomes:

Chromosomes are covered by a proteinaceous sheath termed the peri-chromosomal layer (PCL), which has Ki67 as an important foundational component. Ki67 acts as a surfactant on the mitotic chromosomal surface due to its large size and high density with positively charged amino acids on molecular dissection.[37]

There are different commercially available Ki67 antibody clones in the form of concentrate (conc) or ready-to-use (RTU). 1) MIB-1; conc (Dako); 2) MM1; conc (Leica); 3) SP6; conc (Thermofisher Scientific); 4) 8D5; conc (Cell Signaling Technology); 5) 1297A; conc (Novus Biologicals Inc.); and 6) 30-9; RTU (Ventana).

Over the past decades, numerous efforts have been undertaken to assess the predictive power of the Ki67 proliferative index[25], Despite these efforts, this biomarker has not yet been fully adopted as a routine component of clinical decision-making or pathological reporting[39], mainly due to its standardization issues.[7,1] Ki67 independently predicts outcome in breast cancer cases, and its expression is an essential prognostic determinant in breast cancer.[40]

There are different proliferative markers that are helpful for prognostic and predictive purposes in breast cancer; the most commonly used nowadays are ER, PR, HER2, and Ki67. Other important prognostic biomarkers in breast cancer are Mib-1, E-cadherin, circulating circular RNA, P53, microRNA, tumour-associated macrophages, inflammation-based models[5], nuclear antigens like Repp86 (restricted expressed proliferation associated protein), thymidine kinase, cyclin D and E, S phase

fraction/flow cytometry, mini chromosome maintenance (MCM), and proliferating cell nuclear antigen (PCNA).[42]

Proliferation markers like Ki67, STK15, Survivin, Cyclin B1, and $MYBL_2$ are assessed by polymerase chain reaction in the multigene panel Oncotype DX 21-gene recurrence score signature, generating a recurrence score that indicates the likelihood of recurrence of the disease. In this expression, the Ki67 score is correlated with the recurrence risk.[24]

Among the different proliferative markers, Ki67 is most commonly used in breast cancers; although this use is still under consideration, the IKWG does acknowledge the value of employing Ki67 IHC in clinical trial settings for both monitoring and prediction.[7]

## Ki67 PROLIFERATIVE INDEX

The Ki67 proliferative index (PI) is defined as the percentage of positively stained cells within the total number of malignant cells. Ki67 PI is a predictive and prognostic biomarker and has shown a positive correlation with a pathological complete response to chemotherapy.[36]

According to the current IKWG, Ki67 has three important clinical uses: (1) determining prognosis in early-stage brest cancer to determine the need for adjuvant chemotherapy; (2) estimating chemotherapy whether active or not; and (3) monitoring breast cancer patients during or after neoadjuvant chemotherapy or endocrine therapy to determine whether the regimen chosen is useful or an alternate should be used. The clinical value of IHC Ki67 in breast cancers is now restricted to prognostic assessment in early stage I and II breast cancers, based on the IKWG.[7]

Ki67 cut-offs for clinical use are 5 (low) and >30% (high).

Ki67 staining is heterogeneous within the tumor; during the International Ki67 in Breast Cancer Working Group, Ki67 values between 5% and 30% showed considerable inter- and intra-observer variability; because of this, ASCO has recommended the inclusion of multi-parameter gene assays for prognosis to decide whether chemotherapy is needed or not (33). The IKWG has recommended that no chemotherapy be used if the Ki index is less than 5% and that chemotherapy be used if the Ki index is greater than 30%.[7]

The Ki67 is used to differentiate between Luminal A (low Ki67) and Luminal B (high Ki67) breast cancers. A high Ki67 value indicates a poor prognosis but good results with chemotherapy.[43]

The 14th St. Gallen International Breast Cancer Conference in 2015 suggested that the in-house median value at each laboratory be used to determine cut-off values because of interlaboratory differences.[27]

In 2017, the St. Gallen International Breast Cancer Conference did not include cutoffs and stated that low and high Ki67 categories and the specific laboratories could have mid-range Ki67 values accordingly.[28]

The drawbacks of Ki67 use in clinical utility are due to a lack of analytical validity and standardization. The present IKWG suggests that Ki67-IHC analytical validity can be reached by careful observation of preanalytical issues and calibrated, standardized visual scoring. For preanalytical issues, close attention is needed to minimize prefixation delays; fixation of surgical specimens (5–10 mm thick) in neutral buffered formalin for 6–72 hours according to ASCO and CAP guidelines.[7]

The validation of the Ki67 labelling index is affected by different parameters, like the type of specimen (biopsy or resection) and the availability of the number of cells counted in different regions.[7]

WHO's recommended evaluation of Ki67 PI is based on hotspot areas (with highest Ki67 nuclear positive) and a count of $\geq$ 500 cells in these areas, which has better implications for the pathologists.[7]

Ki67 PI (proliferation index), stain intensity of tumour cells which are positive, and stain intensity of tumour cells that are negative are the three parameters that are used to estimate the staining quality of Ki67 slides.[8]

The evaluation of Ki67: According to Zsuzsanna Varga et al., there are two techniques. Ki67 cut-off values are taken at 10%, 14%, 20%, and 30%.

1. Exact counting: 2000 tumor-positive cells were counted in the periphery region of a randomly selected high Ki67 positive hotspot area.
2. Counting 500 cells by eyeballing five randomly chosen tumour regions.

According to their study, the exact counting method showed the predictive value of Ki-67 with a 10% cut-off.[41]

In gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs), grading is based on Ki67 PI in the hotspot area or mitotic activity.[45]

According to Dordi Lea et al., in their study, 500 to 2000 tumour cells were assessed for Ki67 with the Viziopharm program. Excellent agreement is seen between the DIA and manual Ki67 methods. The reliability and reproducibility of grading the

tumour have improved with DIA. The study also states that the size of the hotspot might influence the grade.[44]

A study on 60 breast cancer patients at the University of Toronto in Canada and King Abdulaziz University in Saudi Arabia discovered that digital image analysis of Ki67-expressed breast cancers provides accurate and reproducible quantitative data faster than manual counts. The automated algorithm provided results within a few seconds, compared to up to 55 minutes per analysis when performed manually. This study inferred that multiple analyses of a single sample using variable numbers of cells and variable ROI regions of interest could alter the result in clinically significant ways.[45]

A study of Ki67 labelling index estimation by automated digital image analysis in 164 breast cancer patients was done at the National Center of Pathology, an affiliate of Vilnius University Hospital, Vilnius, Lithuania, where they found that proper quantitative validation methodologies have to be employed to establish and ensure accuracy of IHC Ki67 estimation by digital image analysis. Reference value (RV) (n = 30) obtained by 3 independent observers The three Ki67 counts did not significantly differ according to a one-way ANOVA, but there was a high degree of pairwise correlation between the results. The agreement within the same confidence interval among all three measurements was 69%, whereas the pairwise agreement varied from 83 to 86%.[46]

A comparative study between manual assessment on optical microscopy and digital quantitative assessment of Ki67 in 100 patients diagnosed with breast cancer was done at Cairo University, Cairo, Egypt. Their study concluded Automated assessment of Ki67 value appears comparable to visual Ki67 assessment on optical

microscopy, but the overall agreement between manual and automated assessment of Ki67 indices using 14% as well as 20% as cut-off points was only substantial (kappa estimated at 0.623, p value=0.01). Manual methods of Ki67 assessment using optical microscopy lack perfect accuracy, especially in cases with a Ki67 index ranging between 10 and 35%, leading to an improper distinction between luminal A and B subtypes of breast cancer. A high level of inter-observer agreement was found when using optical microscopy, with the correlation coefficient (CC) estimated at 0.878 (p value 0.01).[47]

A comparison of visual assessment (VA) and automated digital image analysis of Ki67 in 155 cases of breast cancer was done at Fudan University, Shanghai, China, and concluded excellent agreement between visual assessment and digital image analysis of Ki67 in breast cancer in a mixed cohort study. The heterogeneity of the tumour may slightly affect the concordance between visual and digital assessment of Ki67. A perfect agreement was demonstrated between visual assessment and digital image analysis of Ki67 by intra-class correlation coefficient analysis (P 0.0001) in the whole cohort. All cases are classified into three groups based on VA values (10%, 11–30%, and >30%).[48]

A study of digital image analysis and manual assessment in 279 breast cancer-diagnosed patients was done at the Department of Oncology and Pathology, Karolinska Institute, Stockholm, Sweden. The study shows the difference in Ki67 scores in luminal A and B subtypes was significant. 67 out of 279 cases in cohorts 1 and 2 combined (24%) had the "hot spot" area of the highest Ki67 intensity within 1 mm of the tumor’s invasive margin. In this study, all tested DIA (digital image analysis) methods of scoring Ki67 outperformed manual scores in terms of sensitivity

and specificity for the luminal B subtype. Applying the >20% cutoff, interobserver concordance for pathologist 1 and 2 scores of Ki67 clone 30-9 was 80% (k = 0.57). Independent samples in manual and digital image analysis (DIA) methods found a significant difference in Ki67 scores in Luminal A and B PAM50 (P 0.002).[49]

Factors affecting Ki67 IHC: According to the 2021 IKWG

1. 1.Preanalytical: specimen type (core biopsy preferred), fixation—delays in prefixation affect morphologic nuclear integrity—method of storage—prolonged storage affects Ki67.

2. 2.Antigen retrieval specific antibodies are required, as is high temperature antigen retrieval (MIB 1 is widely validated); colorometric detection; Optiview+Amp (Ventana) is powerful; counterstain completeness and intensity of stain (all negative nuclei are counterstained); and finally, quality assurance and quality control are required.

Interpretation and scoring: count all positive cells within the region, and scoring requires a percentage of positive cells to the total number of invasive tumour cells. The read area of the slide-controversial global vs. hotspot (non-statistically significant differences), digital imaging—automated scoring is validated, data formatted, and cut off—categorical or continuous.[7]

The inconsistency in Ki67 IHC scoring shows that automated scoring may help this biomarker become a valuable diagnostic in a clinical setting. There are numerous open-source or commercial platform methods for picture analysis and quantification ([7]). Ki67 LI can be evaluated using DIA platforms, and numerous studies have been done to evaluate manual scoring with DIA platforms. Nevertheless,

several platforms have distinctive algorithms to classify and categorise cellular and tissue compartments.[50]

IKWG conducted research to determine the viability of introducing automation to Ki67 scoring using a variety of platforms and software. A pilot study was conducted using Qupath. However, after being correctly implemented, this programme once again produced ICCs of about 0.9. Plans for cross-platform standardisation were not included in any of the research on automated analysis.[7]

## MANUAL METHODS FOR Ki67 ASSESMENT

Ki67 must be assessed in at least 500 cells from the regions with the highest labelling (hotspot areas) by manual counting on a printed image, according to current WHO classification.[8]

The Ki67 labelling index is counted by different methods:

Global Method: average score among all the available tissues for assessment of Ki-67.

The percentage of tumour positive cells with a high, medium, low, or negligible Ki67 score is used to assess first staining heterogeneity.

A total of up to 4 fields are counted; in each field, count 100 invasive tumour nuclei using a typewriter pattern.

a) Unweighted global method: the average Ki67 value is calculated by counting different fields with different intensities

b) Weighted global method: total positive tumour area is calculated as high, medium, low, or negligible Ki67 staining, and the weighted Ki67 score is calculated as a percentage of total positive tumour area.[51]

METHOD OF HOTSPOT: Positive tumour cells were manually counted in one photomicrograph in a high staining hot spot area, and 500 cells were counted in a typewriter pattern.[51]

AVERAGE METHOD: Manual counting of positive tumour cells in three photomicrographs and calculating the average percentage of positive tumour cells.[52]

Other Manual Method: Using a light microscope at 40X, 500 neoplastic cells are counted by eyeballing (center to periphery for 500 cells).[52]

**DIGITAL IMAGE ANALYSIS FOR Ki67 ASSESMENT:**

In recent years, with the emergence of digital analysis platforms, the automation of biomarker evaluation has greatly improved. The assessment of Ki67LI using digital image analysis (DIA) gives fast, accurate values when compared with manual assessment.[45,47,53,46,48,54]

There are different digital analysis platforms like Halo, QuantCenter, Qupath, Image J, Cog M software, Visiopharm (custom-designed algorithm), Virtuoso software (Ventana), Nuclear algorithm (Leica Biosystem), Definiens tissue studio scanner (3DHISTECH), etc,

HALO, QauntCenter, and Qupath softwares use colour deconvolution and cell segmentation algorithms for identifying tumour cells, stromal cells, immune cells, and others. These machine learning methods were used for the evaluation of Ki67LI.[50]

HALO (IndicaLab, Corrales, NM, USA) uses a random forest supervised classifier, while QuantCenter (3DHistechh, Budapest, Hungary) uses wavelet-based multilevel feature extraction for pattern recognition. Cell segmentation and optimal colour deconvolution settings are used in both DIA platforms.[50]

Machine learning classification training is to identify tissue pattern scoring (tumour cells).[50]

The last step is cell segmentation in Halo and QuantCenter. blue, non-tumour cells; yellow, 1+; orange, 2+; and red, 3+, positive tumour cells.

In Qupath, cell segmentation is followed by machine learning classification to identify subpopulations of cells to be classified

Green: stromal cells; Purple: immune cells; Blue: non-tumor cells; Yellow: 1+; Orange: 2+; Red: 3+; positive tumour cells.[50]

Cognition Master Professional Suite (CogM) Ki67 Quantifier software: Interpretation: red, positive tumour cells; green, negative tumour cells; black, non-tumor cells.[55]

The Visiopharm Program is a customised analysis protocol package for Ki67 quantification and detection based on both size and morphology of nuclei. Tumor regions of interest are marked by outlined masks of tumour areas. Ki67-positive cells were distinguished by their brown DAB staining. Ki67-negative cells identified by blue H&E stain.[56]

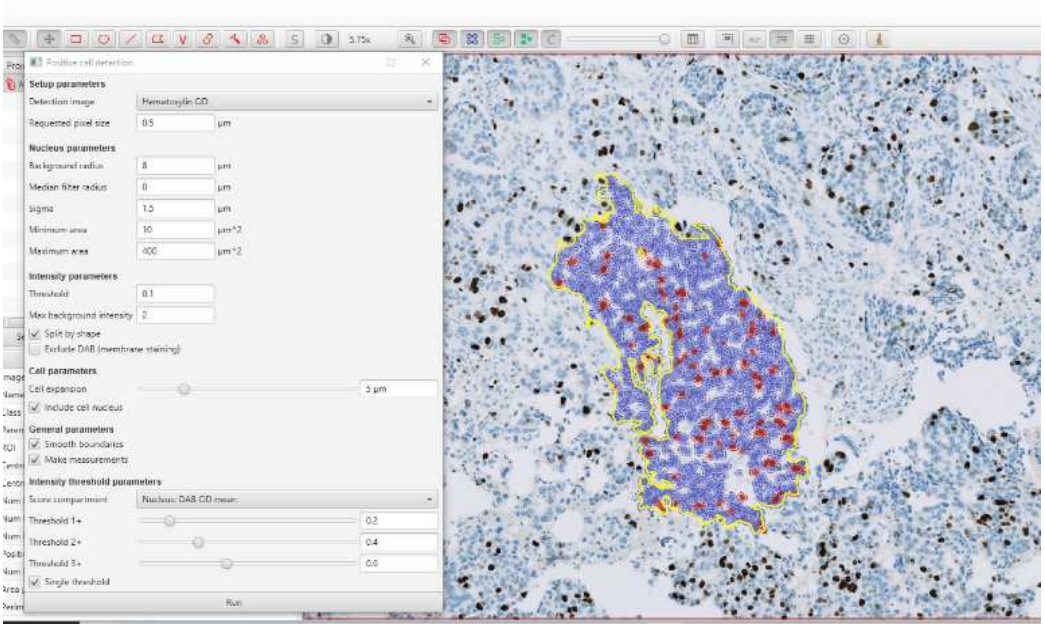
QUPATH is an open-source software for digital pathology and whole-slide image scanning that provides annotations and visualisation tools. The distinguishing feature that makes it different from other image analysis software's is the incorporation of an "object-based data model" in it.

The main objective is to exclude non-tumor cells and quantify the proportion of Ki67-positive (brown) tumour cells. This is also known as the "Ki67 labelling index." [57]

The object denotes a structure or region within the tumour area; these regions can be created using the interactive drawing tool, and these can also be manipulated (e.g., annotation of areas of interest) according to the user. The object is of different types, e.g., detection and annotations. Annotations are manually drawn around tumour cells to identify them, and QuPath can then rapidly find the cells and determine the percentage of positive cells inside each annotation. To detect nuclei and cells of interest, Qupath also has automated segmentation commands that are user-friendly. Create an annotation around a region containing tumour cells that have to be recognised and counted using some of the tools provided in Annotating Pictures. Qupath has built-in commands or scripting that helps in the assignment of classifications and measurements and the linking of parent and child-object in a way that can be queried immediately and manipulated accordingly.

The Qupath's built-in cell algorithms detect millions of cells as objects in a single whole slide image, identify cell morphology, and note the expression of biomarkers. Start the positive cell detection command under "Analyze > Cell Analysis." When a dialogue box appears, the majority of the options are related to how the cells are detected. Options referring to how identified cells will be classified

as positive or negative can be found at the dialog's bottom. When you click the Run button in the dialogue, the outcomes should start showing up.[57]




Interpretation- Counts no of positive tumour cells per $mm^2$ .

Tumour cells- Red, Stroma- Green, Immune cells- Purple, Necrosis- Black, Other- Yellow, White space- white.[57]

# MATERIAL AND METHODS

The study has a sample of 50 breast cancer cases. Patients clinically and histologically diagnosed with breast carcinoma operated in the year 2021, from January 1st to December 31st, at Dr. Prabhakar Kore Hospital were included in the study. The breast carcinoma cases with modified radical mastectomy and breast conservative surgery specimens were taken in the present study.

**Study design:** Hospital-based prospective descriptive study

**Study period:** 1st January to 31st December 2021

**Study population:** Patients clinically and histologically diagnosed with breast carcinoma were included in the year 2021 in Dr. Prabhakar kore Hospital.

**Inclusion criteria:** Histologically proven cases of breast carcinoma, Modified radical mastectomy (MRM) specimens and Breast conservative surgery (BCS) specimens.

**Exclusion criteria**: 1. Inadequate biopsies /core biopsies

2. Improperly fixed specimens

**Sample size:** Fifty breast carcinomas in total. All the cases fulfilling the inclusion criteria were taken in the study (Universal sampling).

**Ethical clearance:** The present study was approved by Jawaharlal Nehru medical college's Institutional Ethics committee on Human Subjects research. (Ref.: MDC/DOME/124.

**Data collection:** The breast carcinoma cases with modified radical mastectomy and breast conservative surgery specimens that are operated on at Dr. Prabhakar Kore Hospital were taken for this study, these specimens were grossed according to the standard procedure, and bits were given. All the formalin-fixed paraffin-embedded tissue blocks were screened and stained with H&E, and slides were screened. Tissues with the highest tumour areas were selected for Ki67 IHC staining with the SP6 (vitro) antibody.

Information regarding clinical data were obtained from Medical Records Department (MRD) records.

**Statistical analysis:** Descriptive statistics such as mean, percentages, and for comparison between observers (manual), comparison between manual and DIA Ki67 the Pearson correlation coefficient was used. Comparison between DIA Ki67 and clinico-pathological parameters Pearson's chi-square was used.

**IHC MANUAL STAINING PROTOCOL:**

Tissue sections of 3 microns is cut with microtome and then collected on a coated slide. Steps for IHC staining for Ki67 is as follows:

1. Bake the slide for overnight at 37degree centigrade.
2. Before test bake it at 60degree centigrade for 1 hour.

**DEPARAFFINIZE STEP**

3. Xylene I- 10minutes
4. Xylene II-10minutes
5. Absolute alcohol I-10minutes
6. Absolute alcohol II-10minutes

7. Rinse in water for-5minutes
8. Rinse in distilled water for-1minute
9. **Antigen retrieval**- (Tris buffer +EDTA)- Buffer solution
10. Required amount of buffer is prepared and then cook the slides in pressure cooker for 3 whistles
11. Cool to room temperature-15minutes
12. Wash with wash buffer 2times with gap of 30seconds each
13. 3% Hydrogen peroxide-8 to 10 minutes
14. Wash with wash buffer 3times with gap of 30seconds each
15. **Primary antibody** incubated for 45-60 minutes at room temperature in a closed room
16. Wash with wash buffer 3times with gap of 30seconds each
17. Apply polymer HRP for 25-30 minutes at room temperature in a closed chamber
18. Wash with wash buffer 3times with gap of 30seconds each
19. Apply **DAB substrate** for 10minutes
20. Was in water-2minute
21. Wash with distilled water-1minute
22. Haematoxylin counter stain-3minutes
23. Blueing in warm water-1minute
24. Clear in xylene and mount with DPX.

MANUAL Ki67 ASSESSMENT: Manual counting of Ki67-positive, nuclear-stained cells from the captured image in 40X OLYMPUS U-TVIX-2 (BX41) was done using GRYPHAX-JENOPTIK.

Hotspot areas were marked and Ki67 assessed in 500 cells from the regions of highest labelling (Hotspot areas, as proposed by the IKBCWG) by manual counting on a printed image by three observers. Blinding was done to prevent bias between the observers.

Ki67 positive tumour cells were counted (brown in colour), and other positive non-tumour cells (inflammatory cells, stromal cells, and endothelial cell nuclei) were excluded by morphologic assessment.

DIGITAL Ki67 ASSESMENT:

Griffax Olympus Pentahead was used to take images of the slides, and Qupath open-source software was used for digital image analysis.

In this study, the parent Ki67 image was taken from the publicly available OpenSlide test data (OS-2. ndpi), images were uploaded in Qupath software, automated "dearraying" was used to get tumour positive areas, and then this dearrayed grid was annotated by tracing manually around the tumour cells. QuPath is able to swiftly find the cells and determine the positive percentage inside each annotation.

Watershed cell detection was used to segment the cells according to the following settings:

Annotations, detection image, optical density sum, requested pixel size-0.5µm, background radius-8µm, median filter radius-0µm, sigma-1.5µm. minimum cell area: 10 $m^2$, maximum cell area: 400 $m^2$, threshold: 0.1 to 0.5, maximum background intensity: 0.5 to 2.

INTERPRETATION-

- Counted no of positive tumour cells per $mm^2$
- Tumour cells- Red
- Stroma- Green
- Immune cells- Purple
- Necrosis- Black
- Other- Yellow
- White space- white

## RESULTS

Fifty histologically proven cases of breast cancer were taken into the study, among them 49 cases were of invasive ductal carcinoma and only one case was of mucinous carcinoma.

Majority of the cases i.e,74% of cases were grade 2 and 26% of cases were of grade 3.

INTER-OBSERVER VARIABILITY ON MANUAL ANALYSIS USING VISUAL IMAGES (Table 3 and Fig. 1, 2, 3)

Three different observers estimated the Ki67 proliferation index independently and were blinded to other observer's results, Ki67 assessment for each of the 50 cases was done by using visual images from the highest labelling areas and counting of 500 tumour nuclei. Inter-observer agreement was calculated by using continuous scores.

The statistical comparison between three different observer groups was done by measuring Significance of difference using Correlation coefficient.

Interpretation of Correlation coefficient: 0.00-0.20 as slight correlation; 0.21-0.40 as fair correlation; 0.41-0.60 as moderate correlation; 0.61-0.80 as substantial correlation; and >0.80 as almost perfect correlation.[47]

A p value of <0.05 was considered statistically significant, p value <0.01 was considered highly significant and p value >0.05 statistically insignificant.

Inter-observer agreement between each pair of observers was done using Pearson correlation coefficient and then mean of Correlation coefficient was calculated.

The three observers' assessments of Ki 67 manually were compared, which revealed almost perfect agreement and was highly significant (mean r: 0.956, p value <0.0001).

**Table 3: Inter-observer variability on manual analysis by three different observers using Pearson correlation coefficient (r) and p value.**

| Manual (VIA) | Observer 1 | | Observer 2 | | Observer 3 | |
|---|---|---|---|---|---|---|
| | r | Significance | r | Significance | R | Significance |
| Observer 1 | 1 | - | 0.963 | <0.0001 | 0.960 | <0.0001 |
| Observer 2 | 0.963 | <0.0001 | 1 | - | | |
| Observer 3 | 0.960 | <0.0001 | 0.946 | <0.0001 | 1 | - |
| Mean r | 0.956 | | | | | |
| N=50 cases | | | | | | |

CORRELATION OF MANUAL COUNTING BETWEEN OBSERVERS:(Fig.1,2,3)

Bland-Altman plots of manual counting compared between three different Observer's, skewness was negligible within the given data range. Correlation for inter-observer agreement between observer 1 and 2, observer 1 and 3 and observer 2 and 3 was high.

Analysis among the observers shows excellent inter-observer correlation.

Correlation between observer 1 with observer 2 and 3 ($r_=0.963$ and $r_=0.960$, $p<0.0001$) respectively shows very good correlation.

Correlation between observer 2 with 1 and 3 respectively ($r_=0.963$ and $r_=0.946$, $p<0.0001$) shows excellent correlation.

Correlation between observer 3 with observer 1 and 2 respectively also shows very good correlation ($r_=0.960$ and $r_=0.946$).

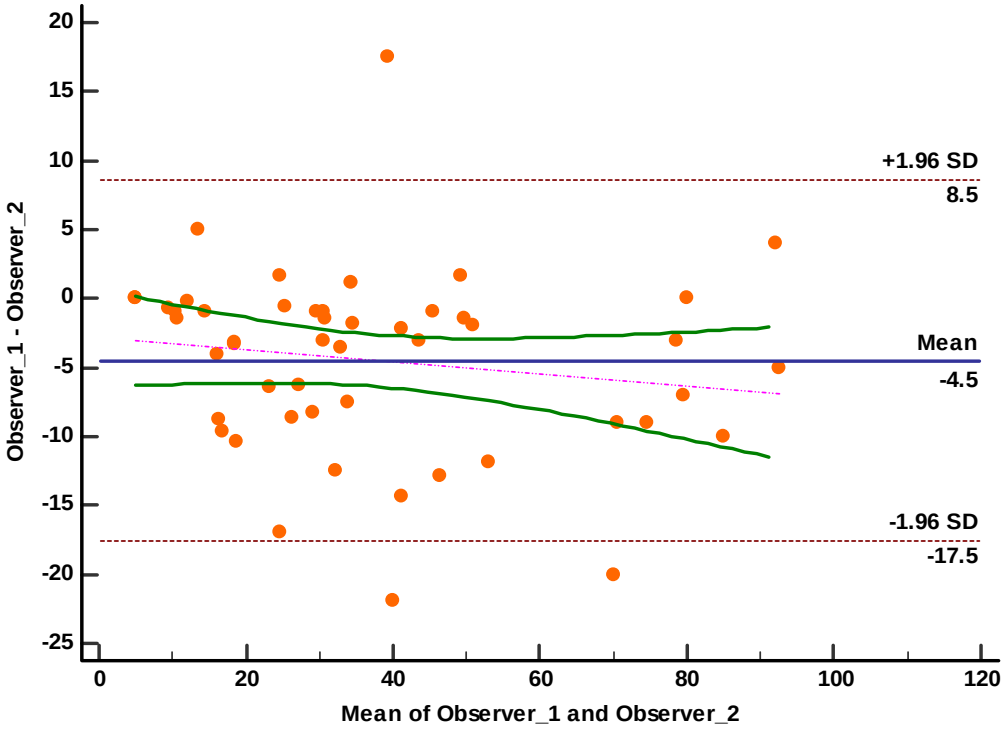



**Fig.1 Bland-Altman plot illustrating agreement between observer 1 and 2 (manual) VIA methods. Difference between observer 1 and 2 compared with mean of observer 1 and 2.**

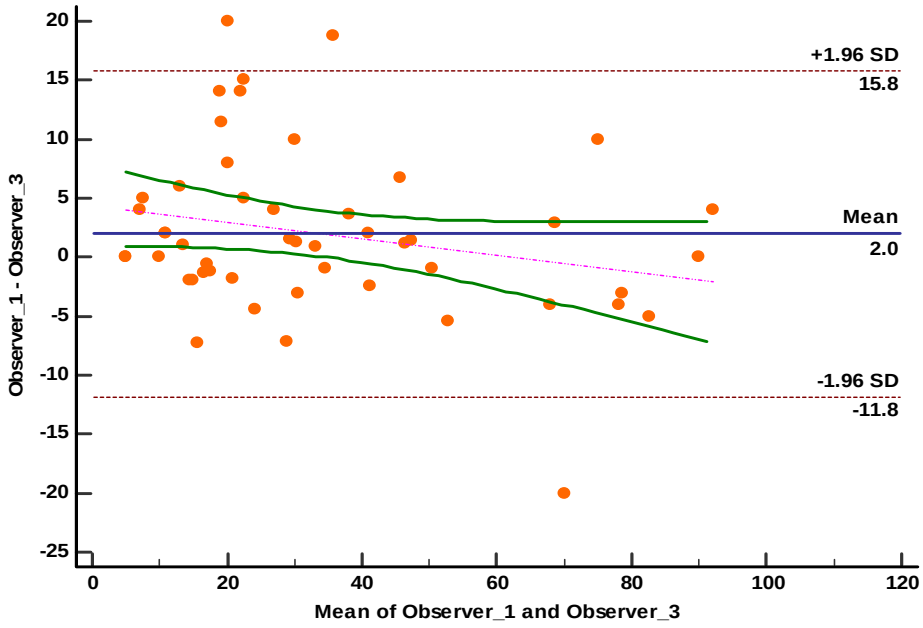



**Fig 2. Bland-Altman plot illustrating agreement between observer 1 and 3 (manual) VIA methods. Difference between observer 1 and 2 compared with mean of observer 1 and 3.**

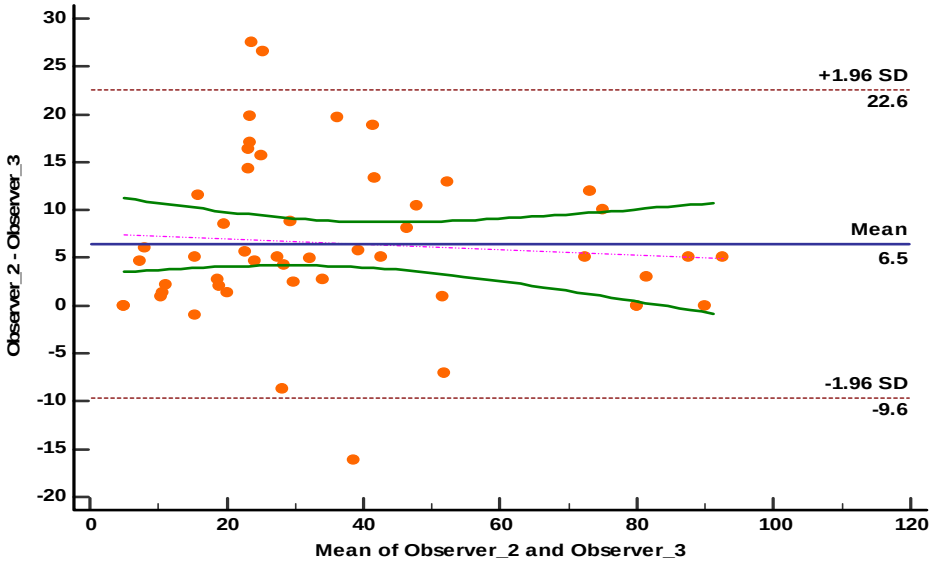



**Fig.3 Bland-Altman plot illustrating agreement between observer 2 and 3 (manual) VIA methods. Difference between observer 1 and 2 compared with mean of observer 2 and 3.**

COMPARISION BETWEEN MANUAL AND DIGITAL Ki67 MESUREMENTS: (Table 4, Fig.4)

Pearson correlation coefficient (r) is used to evaluate correlation between manual and digital Ki67 counts.

A correlation coefficient >0.80 denotes almost perfect correlation between observers and methods and $n_=$ no. of total cases.

Evaluation of correlation between the two methods Manual (observer 1, 2 and 3) and DIA Ki67 measurements shows excellent correlation with Pearson correlation coefficient ($r_=$0.957, $r_=$0.937 and $r_=$0.932) respectively and p<0.0001 which is highly significant.

Bland-Altman plots of manual counting by three different observers compared with DIA, skewness was negligible within the given data range in the present study. Correlation for agreement between observer 1, 2, 3 (manual) with DIA was high.

**Table 4: Comparison between Manual and DIA Ki67 assessment**

| | | Manual | | |
|---|---|---|---|---|
| | | Observer 1 | Observer 2 | Observer 3 |
| Digital Image Analysis (Qupath) | Correlation coefficient(r) | 0.957 | 0.937 | 0.932 |
| | Significance Level P | <0.0001 | <0.0001 | <0.0001 |

**Correlation between Manual counting (VIA) between three observers and DIA.**

**Table 5. Systematic difference:** No significant difference seen between observers and DIA method(n=50).

| Reference method: DIA | | |
|---|---|---|
| Systematic differences | | |
| Variable | Differences(variable-reference) (95% CI) | |
| | Mean | SD |
| Observer 1 | -8.2720 | 7.3751 |
| Observer 2 | -3.7720 | 8.8554 |
| Observer 3 | -10.2540 | 9.3415 |

**Table 6. Limits of agreement:**

| Reference method: DIA | | |
|---|---|---|
| Limits of agreement | | |
| Variable | Limits of agreement (95%CI) | |
| | Lower limit | Upper limit |
| Observer 1 | -22.7272 | 6.1832 |
| Observer 2 | -21.1286 | 13.5846 |
| Observer 3 | -28.5633 | 8.0553 |

Since there is 95% of the observations lie with in Upper limit and Lower limit, good relation and agreement is seen between observer 1 and DIA, observer 2 and DIA, observer 3 and DIA. (n=50).

Above two methods of comparison showed excellent agreement between manual (observers 1,2,3) and DIA.

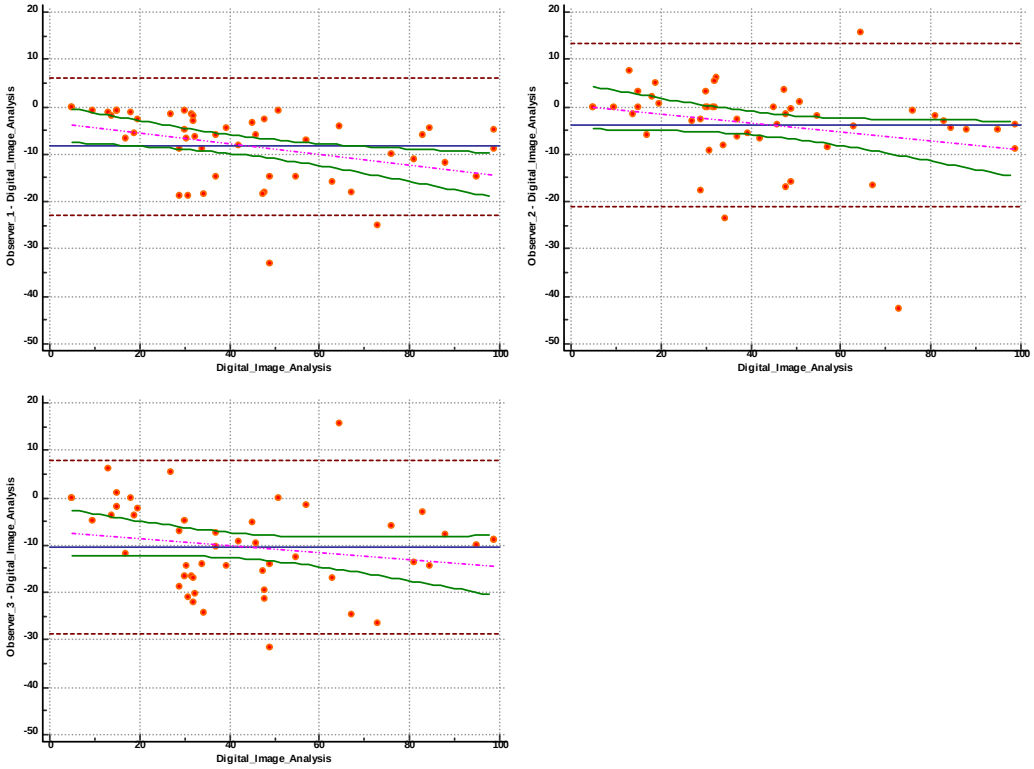



**Figure 4: Bland Altman plot is used for evaluation of agreement between two methods.**

In each panel DIA plotted on x axis and manual counts are plotted on y axis.

Strong correlation seen between observer 1, 2, 3 with DIA.

Concordance of DIA (Qupath) and observers(manual) methods: (Fig;5)

Intraclass correlation is used to measure degree of absolute agreement among measurements (observers1, 2, 3 and DIA). This is a two-way model and n=50.

(ICC: 0.9167, 95%CI;0.821-0.9576) of single measure, gives reliability of single rating which is highly reliable and average measure (ICC:0.9778, 95%CI;0.9484-0.9891) which is showing excellent reliability among average ratings.

Concordance was very high among the observers and DIA.

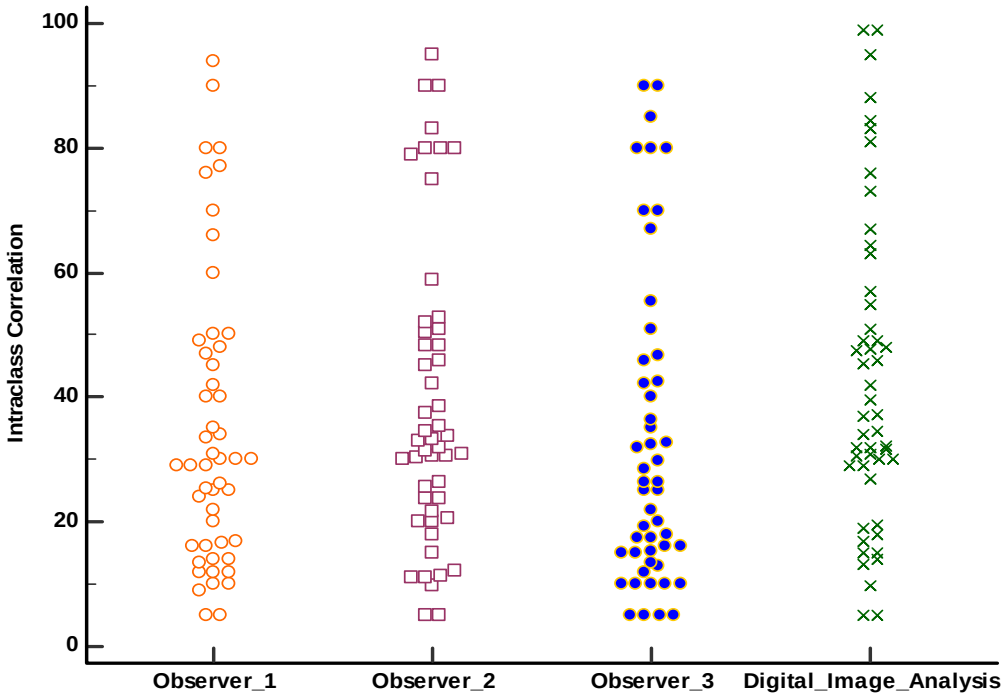



**Figure 5: Intraclass correlation of DIA (Qupath) and observers(manual)**

**Flow of Ki67(%) values are plotted by different methods: Fig 6**

For example- in 1st case, according to observer 1 Ki67 expression was 29%, for observer 2 and DIA there was an overlap (30%) and Ki67 was found to be 25% for observer 3, which shows close match was found between all the methods.

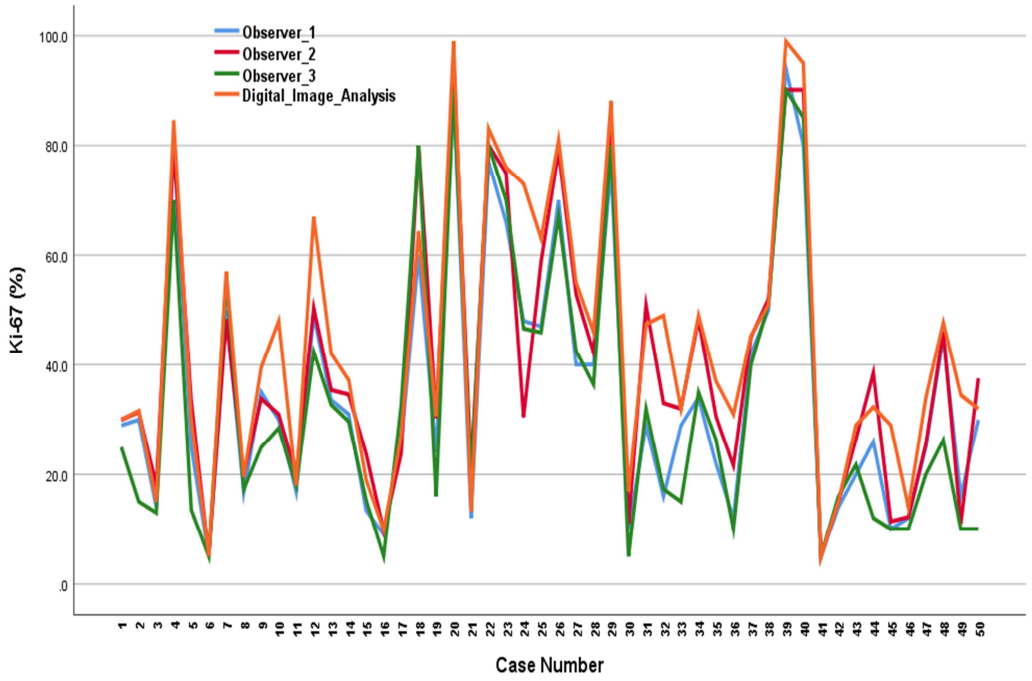



**Figure 6: Flow of Ki67 PI (%) values by all the methods.**

On x axis total number of cases (n=50) and y axis -mean Ki67 values (%) were taken.

Coloured lines indicate each method of Ki67 measurement

Better close match seen between observers and DIA.

Also it was noted that the average time taken for manual assessment for each image was 12minutes (ranging from 10-15minutes) as compared to Digital method where the time taken was around 1minute.

The mean age of the study was 53 years, (SD: 11.829) and the mean size of the tumor was 4.414, (SD: 2.6699).

**Table 7. Age and gross tumour size**

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| Age | 50 | 24 | 75 | 53.00 | 11.829 |
| Gross Tumor Size | 50 | 1.0 | 14.5 | 4.414 | 2.6699 |

Comparison between nipple and areola involvement with Ki67 (DIA) showed no significant association between them ($p=0.205$, Pearson chi-square =3.165).

**Table 8. Comparison between nipple and areola involvement with Ki67**

| | | DIA Ki67 | | | |
|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL |
| Nipple and Areola Involvement | No | 29 | 13 | 1 | 43 |
| | | 67.4% | 30.2% | 2.3% | 100.0% |
| | Yes | 7 | 0 | 0 | 7 |
| | | 100.0% | 0.0% | 0.0% | 100.0% |

Comparison analysis between skin involvement and Ki67 (DIA) showed no significant association between them ($p=0.429$, Pearson chi-square=1.691).

**Table 9. Comparison between skin involvement and Ki67**

| | | DIA Ki67 | | | |
|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL |
| Skin Involvement | No | 32 | 13 | 1 | 46 |
| | | 69.6% | 28.3% | 2.2% | 100.0% |
| | Yes | 4 | 0 | 0 | 4 |
| | | 100.0% | 0.0% | 0.0% | 100.0% |

Comparison between Infiltrative margins with Ki67 (DIA), has no significant association between them ($p=0.724$, Pearson chi-square=0.647)

**Table 10. Comparison between infiltrating margins and Ki67 (DIA) values.**

| | | DIA Ki67 | | | |
|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL |
| Infiltrating margins | No | 13 | 4 | 0 | 17 |
| | | 76.5% | 23.5% | 0.0% | 100.0% |
| | Yes | 23 | 9 | 1 | 33 |
| | | 69.7% | 27.3% | 3.0% | 100.0% |

Comparison between Axillary lymph node involvement and Ki67 (DIA), there is no significant association between them ($p=0.697$, Pearson chi-square= 0.721).

**Table 11. Comparison between Axillary lymph node involvement and Ki67 (DIA).**

| | | DIA Ki67 | | | |
|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL |
| Axillary Lymph Node Involvement | No | 15 | 5 | 0 | 20 |
| | | 75.0% | 25.0% | 0.0% | 100.0% |
| | Yes | 21 | 8 | 1 | 30 |
| | | 70.0% | 26.7% | 3.3% | 100.0% |

7(14%) cases showed necrosis, 43(86%) cases had no necrosis. Comparison of necrosis with Ki67(DIA), showed no significant association ($p=0.669$, Pearson chi-square 0.805).

**Table 12. Comparison between necrosis and Ki67 (DIA).**

| | | DIA Ki67 | | | |
|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL |
| Necrosis | No | 30 | 12 | 1 | 43 |
| | | 69.8% | 27.9% | 2.3% | 100.0% |
| | Yes | 6 | 1 | 0 | 7 |
| | | 85.7% | 14.3% | 0.0% | 100.0% |

Fibrosis is seen in 6(12%) cases, significant association is seen between fibrosis with Ki67 values ($p_{=}0.19$, Pearson chi-square=7.933).

**Table 13. Comparison between fibrosis and Ki67 (DIA).**

| | | DIA Ki67 | | | |
|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL |
| Fibrosis | No | 33 | 11 | 0 | 44 |
| | | 75.0% | 25.0% | 0.0% | 100.0% |
| | Yes | 3 | 2 | 1 | 6 |
| | | 50.0% | 33.3% | 16.7% | 100.0% |

In total 49 cases of IDC, 36 cases are of Grade 2 and 13 are of Grade 3. Most of the cases show High Ki67, which is about 36 (73.5%) cases. No significant association was found between grades and Ki67 (DIA) count ($p=0.708$, Pearson chi-square=0.689).

**Table 14. Comparison between Grade and Ki67 (DIA).**

| | | | DIA_Group | | | Total |
|---|---|---|---|---|---|---|
| | | | High | Intermediate | Low | |
| Grade | 2 | Count | 27 | 8 | 1 | 36 |
| | | % within Grade | 75.0% | 22.2% | 2.8% | 100.0% |
| | 3 | Count | 9 | 4 | 0 | 13 |
| | | % within Grade | 69.2% | 30.8% | 0.0% | 100.0% |
| Total | | Count | 36 | 12 | 1 | 49 |
| | | % | 73.5% | 24.5% | 2.0% | 100.0% |

33(66%) cases showed lymphocyte response and in 17(34%) cases not seen. No significant association seen between lymphocyte response with Ki67(DIA) ($p=0.458$, Pearson chi-square=1.561).

**Table 15. Comparison between lymphocyte response and Ki67 (DIA).**

| | | DIA Ki67 | | | |
|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL |
| Lymphocyte response | No | 11 | 6 | 0 | 17 |
| | | 64.7% | 35.3% | 0.0% | 100.0% |
| | Yes | 25 | 7 | 1 | 33 |
| | | 75.8% | 21.2% | 3.0% | 100.0% |

Among 50 cases, 49(98%) cases noted LVI and PNI, 1(2%) case with no LVI and PNI involvement. When LVI & PNI are compared with Ki67(DIA), there is no significant association (($p_{=}$0.820, Pearson chi-square=0.397).

**Table 16. Comparison between LVI and PNI with Ki67 (DIA).**

| | | DIA Ki67 | | | | Pearson chi-square | P value |
|---|---|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL | | |
| LVI | No | 1 | 0 | 0 | 1 | 0.397 | 0.820 |
| | | 100.0% | 0.0% | 0.0% | 100.0% | | |
| | Yes | 35 | 13 | 1 | 49 | | |
| | | 71.4% | 26.5% | 2.0% | 100.0% | | |
| PNI | NO | 1 | 0 | 0 | 1 | 0.397 | 0.820 |
| | | 100.0% | 0.0% | 0.0% | 100.0% | | |
| | YES | 35 | 13 | 1 | 49 | | |
| | | 71.4% | 26.5% | 2.0% | 100.0% | | |

Insitu component is seen in 14(28%) of cases and not seen in 36(72%) of cases, no significant association is seen between Insitu component and Ki67(DIA) ($p=0.382$, Pearson chi-square=1.923).

**Table 17. Comparison between Insitu component with Ki67 (DIA).**

| | | DIA Ki67 | | | |
|---|---|---|---|---|---|
| | | HIGH | INTERMEDIATE | LOW | TOTAL |
| Insitu component | No | 24 | 11 | 1 | 36 |
| | | 66.7% | 30.6% | 2.8% | 100.0% |
| | Yes | 12 | 2 | 0 | 14 |
| | | 85.7% | 14.3% | 0.0% | 100.0% |

COMPARISON BETWEEN Ki67 EXPRESSION AND HORMONAL RECEPTORS (ER, PR) and HER2:

Comparison between Ki67 values by DIA method and ER, PR and HER2 status was done by using Pearson chi-square test for categorical variables and significance p values.

Among the 50 cases, for 38 cases ER, PR and HER2 profile was done.

Ki67 expression <5% taken as Low, 5-30% as Intermediate and >30% as High ([7]).

In our study among the 38 cases most of them showed High Ki67 values which is about 26(68.4%) cases, 11 (28.9%) are intermediate Ki67 and 1(2.6%) case of low Ki67 value by DIA assessment.

In 50 cases, 14(36.8%) cases were positive for ER, 24 (63.2%) cases were negative for ER. Association between ER with Ki679 (DIA) values showed results which are not statistically significant with P value 0.736 (>0.05) and Pearson chi-square test of 0.614.

**Table 18. Comparison between ER and DIA Ki67**

| | | | DIA_Group | | | Total |
|---|---|---|---|---|---|---|
| | | | High | Intermediate | Low | |
| ER | Negative | Count | 16 | 7 | 1 | 24 |
| | | % within ER | 66.7% | 29.2% | 4.2% | 100.0% |
| | Positive | Count | 10 | 4 | 0 | 14 |
| | | % within ER | 71.4% | 28.6% | 0.0% | 100.0% |
| Total | | Count | 26 | 11 | 1 | 38 |
| | | % | 68.4% | 28.9% | 2.6% | 100.0% |

10 (26.3%) cases showed PR positivity, 28(73.7%) of cases showed PR negativity, and there was no association found between Ki67(DIA) with PR with a P value of 0.832(>0.05) and Pearson chi-square value of 0.367 which is statistically insignificant.

**Table 19. Comparison between PR and DIA Ki67**

| | | | DIA_Group | | | Total |
|---|---|---|---|---|---|---|
| | | | High | Intermediate | Low | |
| PR | Negative | Count | 19 | 8 | 1 | 28 |
| | | % within PR | 67.9% | 28.6% | 3.6% | 100.0% |
| | Positive | Count | 7 | 3 | 0 | 10 |
| | | % within PR | 70.0% | 30.0% | 0.0% | 100.0% |
| Total | | Count | 26 | 11 | 1 | 38 |
| | | % | 68.4% | 28.9% | 2.6% | 100.0% |

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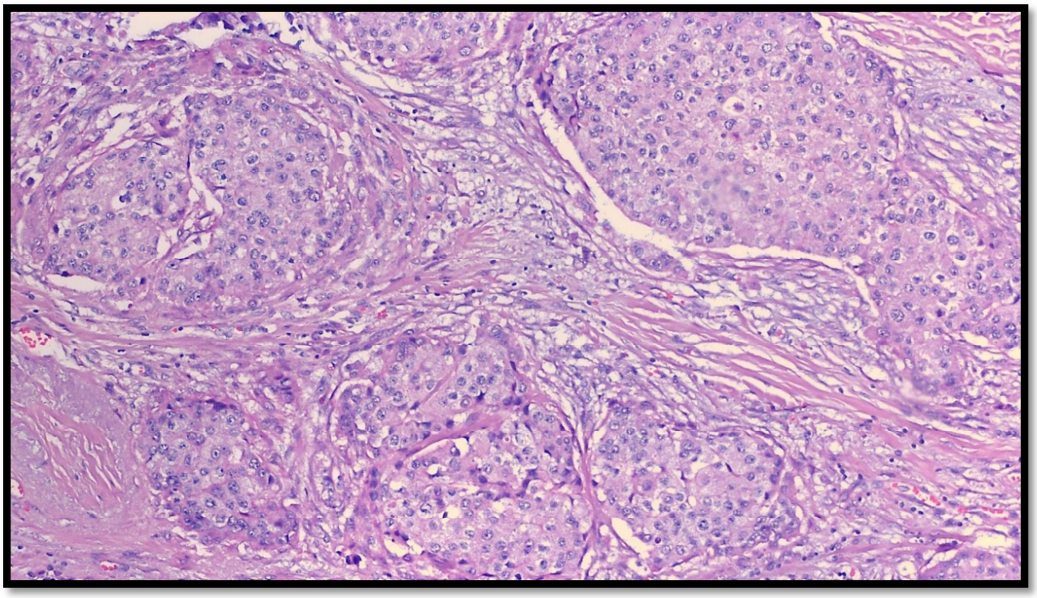
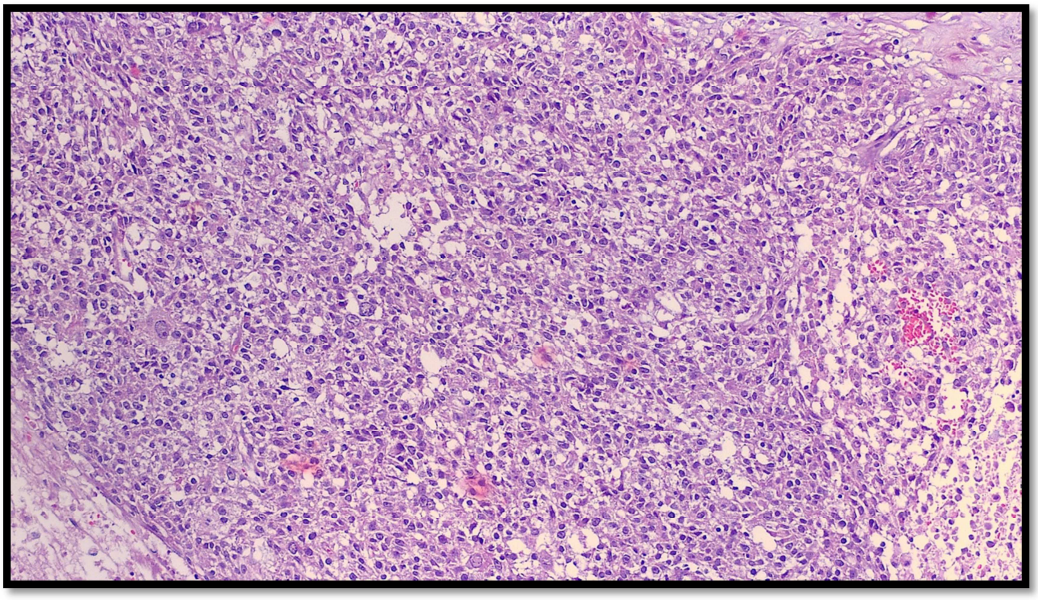
Expression of HER2 is seen in 15(39.5%) of cases, negative in 23(60.5%) of cases, no association was found between HER2 and Ki67(DIA) categorical values P=0.128 and Pearson chi-square =4.106 which is not statistically significant

**Table 20. Comparison between HER2 and DIA Ki67**

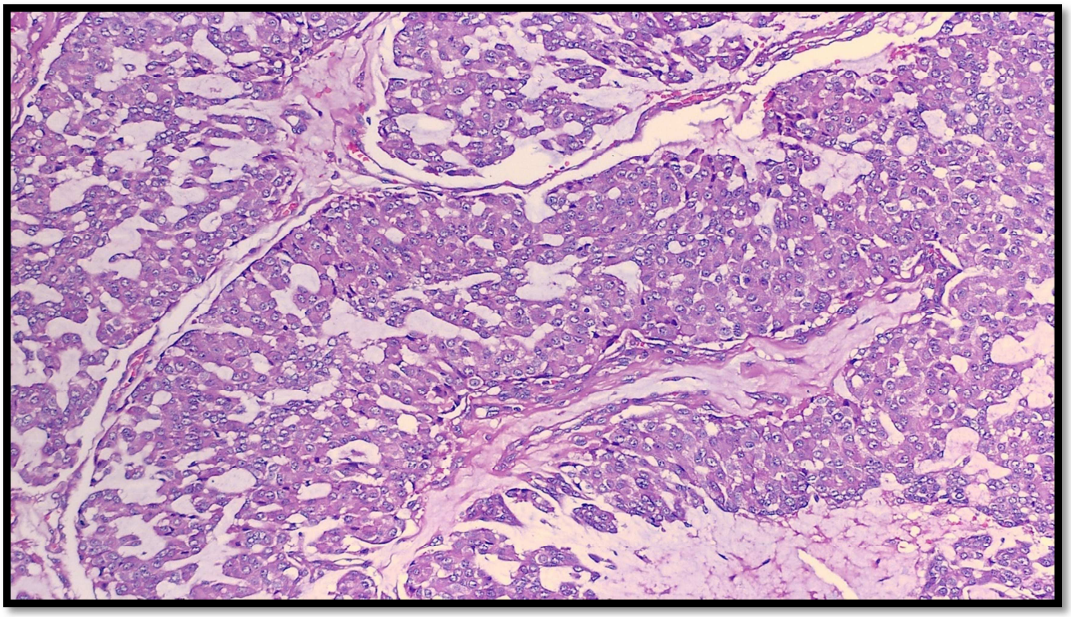
| | | | DIA_Group | | | Total |
|---|---|---|---|---|---|---|
| | | | High | Intermediate | Low | |
| HER_2 | Negative | Count | 14 | 9 | 0 | 23 |
| | | % within HER_2 | 60.9% | 39.1% | 0.0% | 100.0% |
| | Positive | Count | 12 | 2 | 1 | 15 |
| | | % within HER_2 | 80.0% | 13.3% | 6.7% | 100.0% |
| Total | | Count | 26 | 11 | 1 | 38 |
| | | % | 68.4% | 28.9% | 2.6% | 100.0% |



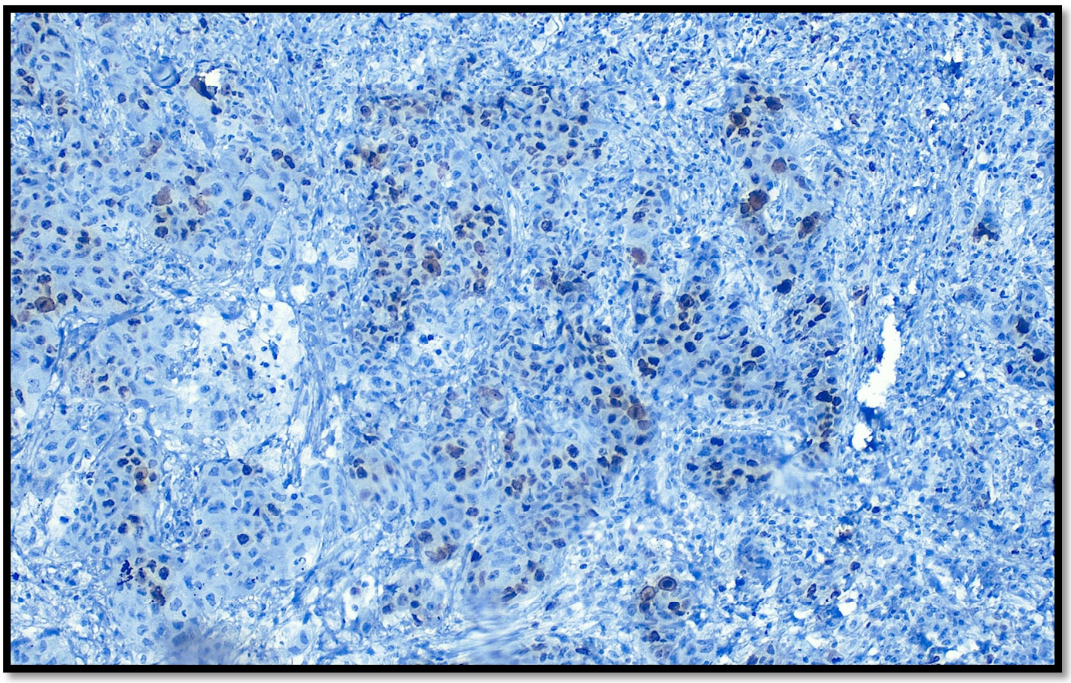
**Photomicrograph 2 : IDC Grade 2 (H&E, 10x)**



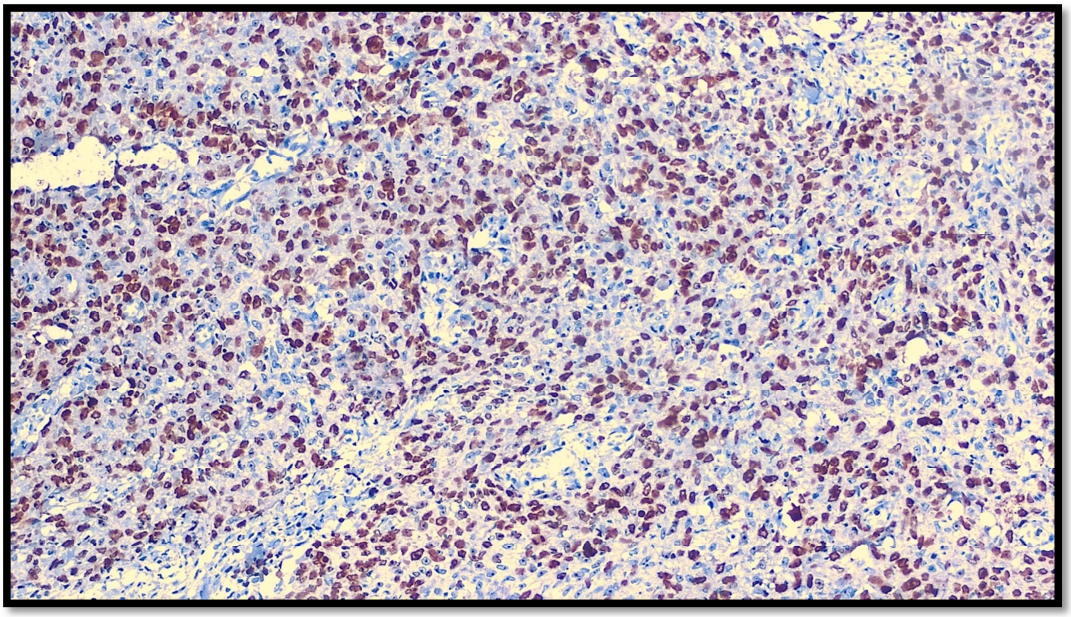
**Photomicrograph 3 : IDC Grade 3 (H&E, 10x)**



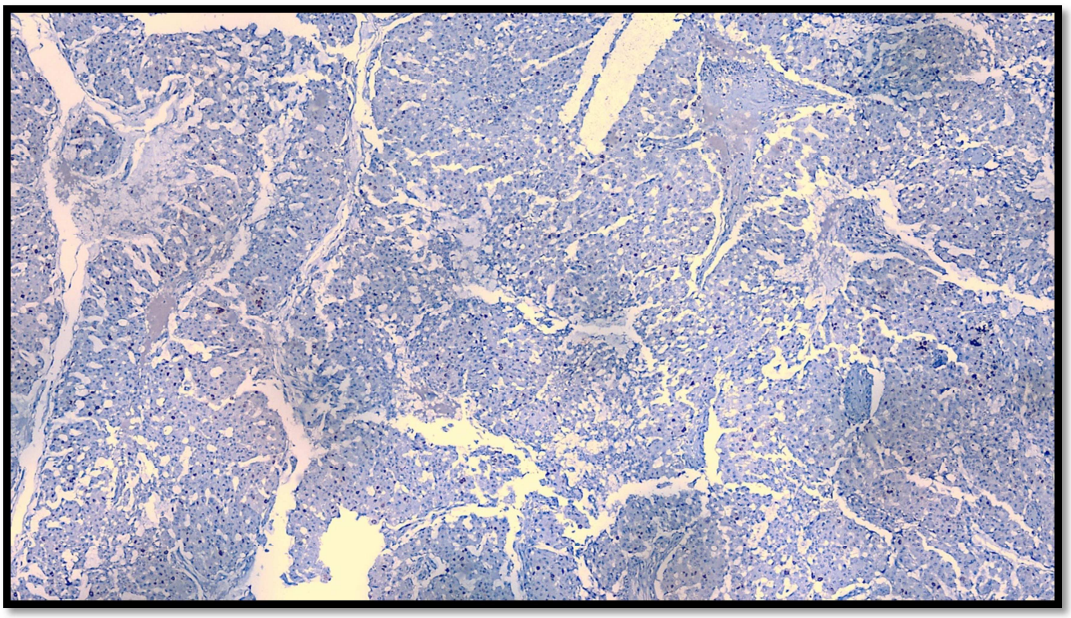
**Photomicrograph 4 : Mucinous carcinoma (H&E, 10x)**



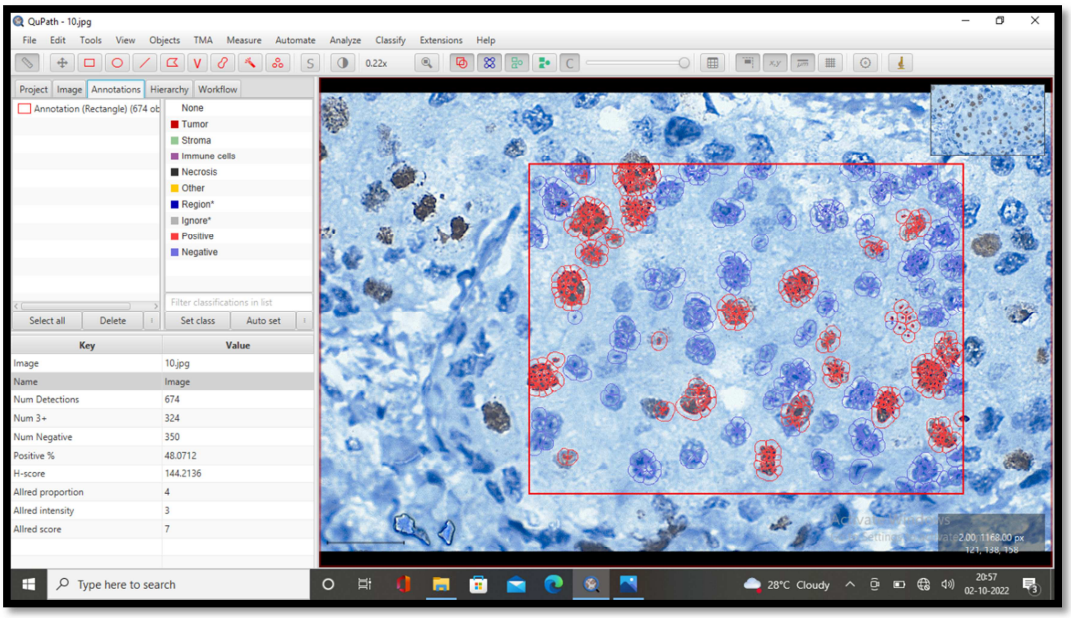
**Photomicrograph 5 : Shows Ki67 expression in IDC Grade 2 (10x)**



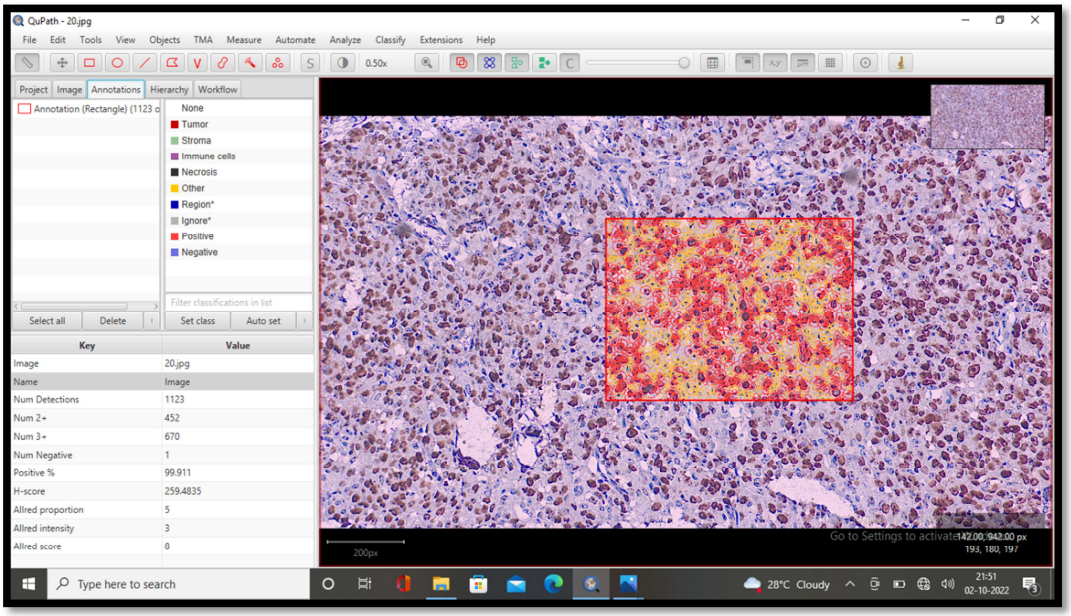
**Photomicrograph 6 : Shows Ki67 expression in IDC Grade 3 (10x)**



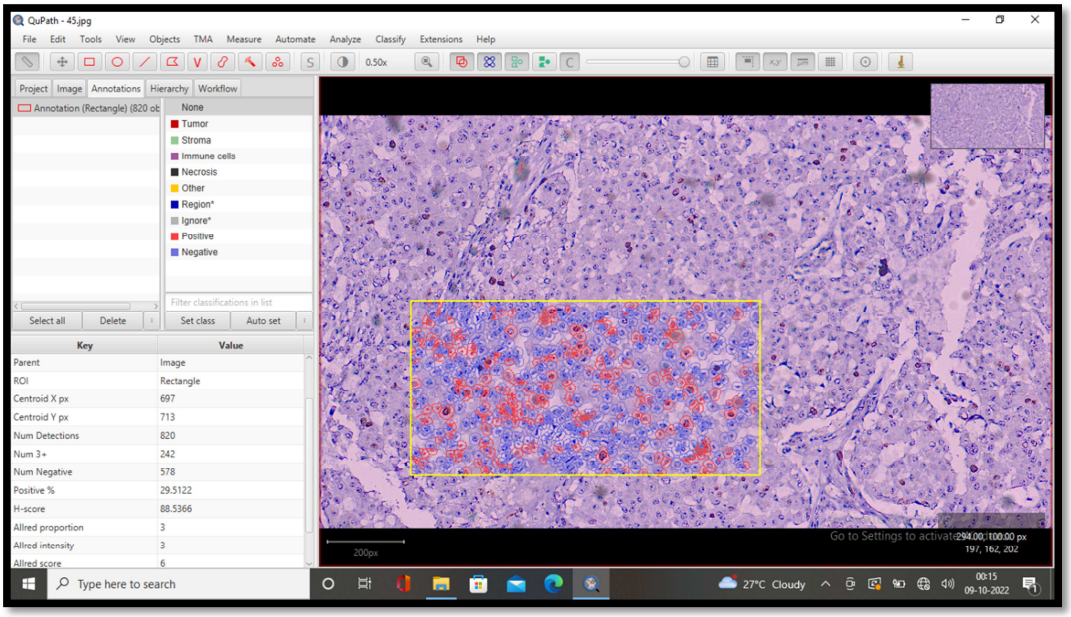
**Photomicrograph 7 : Shows Ki67 expression in Mucinous carcinoma (4x)**




**Photomicrograph 8 : Shows Ki67 cell count (48%) in QUPATH software (IDC Grade 2)**




**Photomicrograph 9 : Shows Ki67 cell count (99%) in QUPATH software (IDC Grade 3)**




**Photomicrograph 10 : Shows Ki67 cell count (29.5%) in QUPATH software**

**(Mucinous carcinoma)**

# DISCUSSION

According to St Gallen guidelines, clinical decision making for breast cancer has recommended IHC based molecular classification. Based on ER, PR, HER2, Ki67, CK5/6, EGFR Invasive breast cancer is divided in to 7 molecular subtypes: 1) Luminal A, 2) Luminal B, 3) Luminal HER2 PR+ 4) Luminal HER2 PR- 5) HER2 Enriched, 6) Basal like 7) Non classifiable.[15,33]

Ki67 is widely used as a proliferation marker, expression of which is strongly associated with tumour aggressiveness.[35] Ki67 is expressed in all active phases of cell cycle ($G_1$, S, $G_2$, M) except $G_0$ and exclusively seen during interphase of the cell cycle ([24]), IHC Ki67 is quantitatively expressed as percentage of immunoreactive neoplastic cell nuclei as Ki67 Labelling Index (Ki67LI).[6]

The purpose of this study was to study Ki67 expression in Breast carcinoma cases, and assessment of IHC Ki67 manually by visual image analysis and digitally by automated Digital image analysis method using Qupath open software. Comparison between manual and digital method was done. Expression of Ki67 was also compared with clinicopathological parameters.

In the present study, 50 histologically proven breast cancer cases were taken, among these 49 cases are invasive ductal carcinoma and 1 is mucinous breast carcinoma.

Ki6 was assessed and characterized by the level of agreement between manual and automated digital based Ki67 proliferation index (%) by several metrics. Ki 67 measurement by DIA showed excellent agreement with manual counting values with

high correlation coefficients suggesting a high degree of agreement in the present study.

Digital image analysis of Ki67 measurement using Qupath software shows an excellent correlation with manual (VIA) Ki67 counting; Which was performed on 500 tumor cells each, by three different observers. Pearson correlation coefficient (r) was used for correlation between digital and manual method of Ki67 assessment. Correlation coefficient between DIA and Manual observer 1 is $r_{=}0.957(p_{=}<0.0001)$, DIA with observer 2 is $r_{=}0.937$ ($p_{=}<0.0001$) and DIA with observer 3 is $r_{=}0.932$ ($p_{=}<0.0001$). Intraclass correlation coefficient (ICC: 0.977, 95%CI:0.948-0.989) of average measures (DIA, Observer -1,2,3) using Bland-Altman plots which shows highly reliable and reproducible correlation values suggesting that DIA can be standardized to independent automation of Ki67LI measurement platform.

Fangfang Zhong et al reported that in their study involving 155 cases of primary invasive breast cancer, IHC Ki67 was performed and comparison between Visual assessment (VA) and DIA was done. Visual assessment of Ki67 by 5 different pathologists using eyeballing Ki67 scoring by two different methods 1) hotspot and 2) average score method on 1000 tumor cells. DIA performed by VENTANA Virtuoso Digital pathology Image Analysis software version 5.3 using same scoring method as VA. Perfect agreement is seen between VA and DIA Ki67 LI by the two score methods. Comparison was done using intraclass correlation coefficient using 95% confidence interval. Hotspot score method ICC = 0.957, 95%CI 0.941, 0.968, P<0.0001 and Average score method ICC = 0.974, 95%CI 0.964, 0.981, P<0.0001. Which shows concordance with our study.[48]

Study done by SaragA. boukhar et.al on 20 GEP-NET cases, assessed level of agreement between manual and semiautomated DIA method of Ki67 measurement. Manual method using Visual image analysis on 1000 tumour cells by three different observers and DIA by two software types HALO and QUANTCENTER. Pearson correlation coefficient is used to compare by using 95% CI (r,0.91; 95% CI, 0.84-0.96, P < 0.0001), their study showed excellent agreement between VIA and DIA as in our study.[54]

A comparison study on visual and digital Ki67 assessment in 248 invasive breast carcinoma cases by Anette H. Skjervold et al, in their study for visual assessment 100 and 500 tumour cells were counted in hotspot areas by 2 observers and for DIA Qupath software is used with same no. of cells taken as VA. Comparison done by using Bland Altman plots with 95%CI which shows agreement between VA and DIA which is similar to our study.[43]

Ki67 expression assessment for breast cancer subtype classification was done by Meagan A Healey et al on 2653 invasive breast cancer cases, DIA Ki67 assessed by Definiens Tissue Studio software and Manual Ki67 is scored by one observer using continuous Ki67 score. Strong agreement seen between DIA and Manual with a Spearman correlation coefficient of $\rho$=0.86 which is concordance with our study, suggesting inclusion DIA to assess Ki67 for validation and standardization of Ki67 assessment for clinical use.[53]

Timco Koopman et al conducted a study on 154 breast cancer cases for clinical validation on DIA Ki67 assessment. Manual count done by 2 observers on 600 tumor cells whole section scoring protocol, at least 3 regions of interest were selected and DIA with two different platforms. Bland Altman plots and scatterplots

were used for correlation and agreement. Manual with platform A(DIA) r=0.94, P<0.001 and Manual with platform B(DIA) r=0.93, P<0.001 in similarity to our study, suggesting DIA as an alternative and accurate method for Ki67 assessment to manual scoring method.[58]

Garazi serna et al in their study included 329 breast cancer cases, manual scoring of Ki67 by 3 observers taking average Ki67LI and DIA by Kiquant software, ICC=0.905, 95% CI:0.878-0.926 demonstrating an excellent agreement between manual scoring and DIA Ki67 scoring, suggesting inclusion of DIA scoring method (KiQuant) in clinical settings, which is similar to our study, but the software used for DIA in our study is Qupath.[59]

A study on 140 breast cancer cases, manual Ki67 counting on 200 tumor cells and DIA based on virtual double staining method produced precise correlation between them (r >0.97, ICC:0.78-0.92) in the study done by Rasmus Roge et al is in concordance with our study, recommending introduction of DIA methods for Ki67 proliferative index measurement in breast carcinomas due to its reproducibility and accuracy which is similar to the gold standard manual scoring methods.[60]

Arvydas Laurinavicius et al provided methodology based on calibration, validation, error correction using stereology grid count for achieving accurate DIA Ki67 LI, study sample of 164 breast cancer cases, 5 observers performed visual estimate and DIA with the help of Aperio genie/nuclear algorithm (ANOVA

($P < 0.05$), Regression analyses-R-square = 0.90). DIA shows accurate agreement with manual count which is in concordance with our study.[46]

Correlation of Ki67 expression with Axillary lymph node status, PR, HER2 shows no statistical association with p value 0. 0.697, 0.832 and 0.128 which is in concordance with the study done by Mowafak Masoud.B et al. with P value 0.971, 0.892, 0.418 respectively, but it is not in concordance with Ki67 correlation with grade of the tumor, necrosis and ER which showed significance in there study($p=$0.001,0.047, and 0.002 respectively), in our study comparison of Ki67 with Grade($p=$0.708, Pearson chi-square=0.689), necrosis($p=$0.669, Pearson chi-square=0.805) and ER($p=$0.736, Pearson chi-square=0.614) showed no association.[64]

Research by Militza Petric et al. shows that no correlation between High grade with Ki67 expression in 95 breast cancer cases treated with preoperative chemotherapy (Pearson r=0.076, $p=$0.237). In our study correlation between grade 2 and 3 with Ki67 expression (Low, intermediate and high) was done, no agreement was found between them which shows similar findings as the above study ([5]). Similar results were observed by Renuka Patil et.al, who also found no significant association between tumor grade and Ki67 expression($p=$0.720).[61]

Mohamed A et al. found no statistical association between Ki67 expression with lymphovascular invasion (LVI), ER positivity, PR positivity (P=0.689, 0.377, 0.149) which is similar to our study (LVI:P=0.820; Pearson chi-square=0.397, ER:P=0.736; Pearson chi-square=0.614 and PR:P=0.832; Pearson chi-square=0.367). In the same study Ki67 is compared with other parameters like Grade, lymph node metastasis, HER2 showed statistical significance but not in our study, due to different Ki67 cut-offs and sample size.[62]

Statistically significance is noted with Ki67 expression correlation with fibrosis (p value 0.019, Pearson chi-square=7.933), according to Maria J et al. women who have fibrosis had a lower risk of developing breast cancer, as in our study low Ki67 expression is statistically significant with presence of fibrosis.[63]

In our study Ki67 expression was compared with involvement of skin, nipple and areola, infiltrating margins, in-situ component, PNI, and lymphocyte response which showed no significant association between them, not many studies have compared these parameters with Ki67 expression.

# CONCLUSION

Our study evaluated the expression of Ki67 manually and compared it with Digital image analysis of Ki67 expression by using Qupath software in 50 cases of breast carcinoma. DIA Ki67 values were taken as standards and compared with various clinicopathological parameters.

Manual counting of Ki67 was done by 3 different observers, and the correlation between the observers showed excellent inter-observer agreement.

A comparison between the manual and DIA Ki67 assessments showed perfect agreement between them.

DIA Ki67 values were taken as standards and compared with various clinicopathological parameters, which showed no significant association between them except for fibrosis.

In conclusion, Digital Ki67 assessments exhibit the same variability and reproducibility as manual Ki67 assessments. Given the non-inferiority and substantial time savings, the present study supports more widespread adoption of automated digital image analysis of Ki67 in routine clinical practice.

# SUMMARY

1. A total of 50 histologically diagnosed cases of breast carcinoma were included in the study for manual and digital measurement of Ki67 expression and their comparison. DIA ki67 measurements were compared with clinicopathological parameters.

2. Manual assessment of Ki67 by different observers using the same hotspot visual image revealed almost perfect agreement between interobservers and was highly significant (mean r: 0.956, p value 0.0001).

3. Digital image analysis Ki67 measurement with Qupath software shows excellent correlation (r = 0.957, r = 0.937, and r = 0.932) with manual (VIA by 3 observers) Ki67 measurement and p= 0.0001, which is highly significant.

4. When DIA Ki67 measurements were used as standards and compared to different clinicopathological parameters, there was no significant correlation between them.

5. When DIA Ki67 measurements were used as standards and compared to different clinicopathological parameters, there was no significant correlation between them except with fibrosis ($p_{=}0.19$, r=7.933).

6. The current study supports wider usage of automated digital image Ki67 assessment in everyday clinical practise due to the non-inferiority and significant time savings.

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# ANNEXURE I

## WHO CLASSIFICATION OF BREAST TUMOURS (2019)

1. Epithelial tumours
2. Invasive breast carcinoma-Infiltrating duct carcinoma (NOS), Oncocytic carcinoma, Lipid rich carcinoma, Glycogen rich carcinoma, Sebaceous carcinoma, Lobular carcinoma NOS, Tubular carcinoma, Cribriform carcinoma NOS, Mucinous adenocarcinoma, Mucinous cystadenocarcinoma NOS, Invasive micropapillary carcinoma of breast, Metaplastic carcinoma NOS.
3. Rare and salivary gland type tumors- Secretory carcinoma, Acinar cell carcinoma, Mucoepidermoid carcinoma, Polymorphous adenocarcinoma, Adenoid cystic carcinoma: Classic adenoid cystic carcinoma, Solid basaloid adenoid cystic carcinoma, Adenoid cystic carcinoma with high grade transformation and Tall cell carcinoma with reversed polarity
4. Neuroendocrine neoplasms- Neuroendocrine tumor, NOS, Neuroendocrine tumor, grade 1, Neuroendocrine tumor, grade 2, Neuroendocrine carcinoma NOS, Neuroendocrine carcinoma, small cell, Neuroendocrine carcinoma, large cell.
5. Epithelial - myoepithelial tumors- Pleomorphic adenoma, Adenomyoepithelioma NOS, Adenomyoepithelioma with carcinoma, Epithelial-myoepithelial carcinoma, Non invasive lobular neoplasia: Atypical lobular hyperplasia, Lobular carcinoma in situ NOS; Classic lobular carcinoma in situ and Florid lobular carcinoma in situ, Lobular carcinoma in situ, pleomorphic, Ductal carcinoma in situ (DCIS); Ductal carcinoma, non infiltrating, NOS-DCIS of low nuclear grade, DCIS of intermediate nuclear grade, DCIS of high nuclear grade

6. Benign epithelial proliferations and precursors- Usual ductal hyperplasia, Columnar cell lesions including flat epithelial atypia, Atypical ductal hyperplasia, Adenosis and benign sclerosing lesions, Sclerosing adenosis, Apocrine adenoma, Microglandular adenosis, Radial scar / complex sclerosing lesion
7. Papillary neoplasms- Intraductal papilloma, Ductal carcinoma in situ, papillary, Encapsulated papillary carcinoma, Encapsulated papillary carcinoma with invasion, Solid papillary carcinoma in situ, Solid papillary carcinoma with invasion, Intraductal papillary adenocarcinoma with invasion, Adenomas, Tubular adenoma, Lactating adenoma, Duct adenoma NOS.
8. Mesenchymal tumours- Vascular tumours, Angiomatosis,

   Hemangioma NOS: Perilobular hemangioma, Venous hemangioma, Cavernous hemangioma, Capillary hemangioma. Atypical vascular lesion: Lymphatic atypical vascular lesion resembling lymphangioma, Vascular atypical vascular lesion resembling hemangioma. Postradiation angiosarcoma: Epithelioid angiosarcoma. Angiosarcoma: Epithelioid angiosarcoma, Fibroblastic and myofibroblastic tumours: Nodular fasciitis, Myofibroblastoma, Desmoid type fibromatosis, Inflammatory myofibroblastic tumour.

   Peripheral nerve sheath tumours: Schwannoma NOS, Neurofibroma NOS, Granular cell tumour NOS, Granular cell tumour, malignant, Smooth muscle tumours.

   Leiomyoma NOS: Cutaneous leiomyoma, Leiomyoma of the nipple and areola.

   Adipocytic tumours: Lipoma NOS, Angiolipoma NOS, Liposarcoma NOS.

   Other mesenchymal tumours and tumour-like conditions: Pseudoangiomatous stromal hyperplasia

9. Fibroepithelial tumours- Fibroadenoma NOS, Phyllodes tumour NOS: Periductal stromal tumour.
10. Tumours of the nipple- Nipple adenoma, Syringoma NOS, Paget disease of the nipple
11. Malignant lymphoma- Diffuse large B cell lymphoma NOS, Burkitt lymphoma NOS/Acute leukemia, Burkitt type: Endemic Burkitt lymphoma, Sporadic Burkitt lymphoma, Immunodeficiency associated Burkitt lymphoma.

    Breast implant associated anaplastic large cell lymphoma, Mucosa associated lymphoid tissue lymphoma and Follicular lymphoma NOS
12. Metastatic tumours
13. Tumours of the male breast: Gynecomastia, Carcinoma-Invasive carcinoma and In situ carcinoma.

## ANNEXURE II – CONSENT FORM

## COMPARISON OF DIGITAL Vs MANUAL ASSESSMENT OF Ki67 IN BREAST CARCINOMA.

**Purpose of the study**: The purpose of this study is to determine the use of Quantitative digital analysis of Ki67 in breast carcinoma.

**Procedure:** During this study, you will be asked questions regarding history and background and you are supposed to answer to the best of your knowledge .If you agree to enroll yourself in this study, you will be interviewed regarding your present, past and family history and your clinical manifestations.

**Risks and benefits:** There are no risks involved in taking part in this study and benefit is we will be able to save time for pathologists for diagnosis.

**Alternatives:** Taking part in this study is voluntary. You may choose not to take part in this study or if you decide to take part now, you can later change your mind and withdraw from the study. The study doctor may terminate your participation in this study anytime.

**Privacy and confidentiality:** All information collected about you during the course of this study will be kept confidential to the extent permitted by law. The code numbers will identify you in this research record. Information from this study will be published but your identity will be confidential in any publication. No information about you or information provided by you during research will be disclosed to other without your written permission except:

1. In emergency to protect your rights and welfare.

2. If required by law.

**Financial incentives for participation:** You will not be paid / offered any gift /incentives for participating in this study.

**Authorization to publish results:** The results of this study would be forwarded to the JNMC, KAHER, Belagavi as a part of requirement towards the completion of MD degree, review and publishing.

In case of the queries during the study or in future you may contact following persons:

1. If you have any queries about your rights as a study subject, you may call Dr. Harsha Hegde, Chairman of J.N. Medical College, IEC & scientist D, ICMR, National Institute of traditional medicine, phone number 9480422500, Belagavi.

**CONSENT STATEMENT**

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any legal rights by signing this form. My signature below indicates that I have read, or it has been read to me, this entire consent form and have had all my questions answered.

Name of the participant: (Signature/Thumbprint)

Name of the witness: (Signature)

Name of the investigator: (Signature)

Date:

# ANNEXURES III

## PROFORMA FOR BREAST CARCINOMA

PATIENT HISTORY

Name:

Age:

EXAMINATION:

BREAST

Skin changes

Axillary lymph node involvement

Nipple, areola

INVESTIGATIONS:

Gross findings

Nipple and areola Skin involvement H and E findings

Histological type Grade

Necrosis Fibrosis

Insitu-component

Infiltrating margins

Pagets

Lymphovascular involvement

Perineural involvement

Lymphocyte response

Lymphnode metastasis

IHC:

Ki-67

MANUAL ASSESSMENT: Observer 1,2,3.

DIGITAL IMAGE ANALYSIS: Qupath

Hormonal receptors:

ER

PR

**HER2**

# ANNEXURE IV-MASTER CHART

| SL NO | AGE | SKIN CHANGES | AXILLARY LYMPH NODE INVOLVEMENT | NIPPLE AND AREOLA INVOLVEMENT | INFILTRATING MARGINS | SKIN INVOLVEMENT | HISTOLOGICAL TYPE | GRADE | NECROSIS | FIBROSIS | INSITU COMPONENT | PAGETS DISEASE | LYMPHOVASCULAR INVASION | PERINEURAL INVASION | LYMPHOCYTE RESPONSE | LYMPH NODE METASTASIS | OBSERVER - 1; IHC - % OF KI-67 IN MANUAL ASSESSMENT | OBSERVER - 2; IHC - % OF KI-67 IN MANUAL ASSESMENT | OBSERVER - 3; IHC - % OF KI-67 IN MANUAL ASSESSMENT | DIGITAL IMAGE ANALYSIS IHC- % OF KI 67 | ER | PR | HER - 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 55 | NO | YES | NO | NO | NO | IDC | 3 | NO | NO | NO | NO | YES | YES | NO | YES | 30 | 62.3 | 25 | 30 | | | |
| 2 | 69 | NO | YES | NO | NO | NO | IDC | 2 | NO | YES | NO | NO | YES | YES | NO | YES | 32.3 | 39.4 | 15 | 15.6 | POSITIVE | POSITIVE | POSITIVE |
| 3 | 44 | NO | YES | NO | PRESENT | NO | IDC | 3 | NO | NO | YES | NO | YES | YES | MILD | YES | 15 | 18 | 13 | 4.6 | | | |
| 4 | 44 | NO | YES | NO | PRESENT | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | MILD | YES | 69.4 | >80 | 70 | 72 | | | |
| 5 | 67 | NO | NO | NO | NO | NO | IDC | 2 | NO | NO | YES | NO | NO | NO | NO | NO | 15 | 33.3 | 13.5 | | POSITIVE | POSITIVE | NEGATIVE |
| 6 | 51 | NO | YES | NO | PRESENT | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | YES | 1 | <5 | 5 | | | | |
| 7 | 53 | NO | NO | NO | NO | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | MILD | NO | 50 | 48.3 | 55.4 | 66 | NEGATIVE | NEGATIVE | POSITIVE |
| 8 | 56 | NO | NO | NO | YES | NO | IDC | 2 | NO | YES | NO | NO | YES | YES | MILD | NO | 15.7 | 20.9 | 17.3 | 19.5 | POSITIVE | POSITIVE | NEGATIVE |
| 9 | 56 | NO | NO | NO | PRESENT | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | NO | 35 | 33.8 | 25 | 39.5 | NEGATIVE | NEGATIVE | NEGATIVE |
| 10 | 63 | NO | YES | NO | NO | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | YES | YES | 30 | 31 | 28.5 | 48 | NEGATIVE | NEGATIVE | POSITIVE |
| 11 | 55 | NO | YES | NO | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | YES | NO | 16.8 | 20 | 18.6 | 17 | | | |
| 12 | 63 | NO | NO | NO | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | NO | 45 | 50.4 | 42.3 | 67 | POSITIVE | POSITIVE | NEGATIVE |
| 13 | 45 | NO | YES | NO | YES | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | NO | YES | 33.6 | 35.4 | 32.7 | 42 | | | |
| 14 | 46 | NO | NO | NO | NO | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | NO | NO | 31 | 44.6 | 29.7 | 37 | positive | NEGATIVE | NEGATIVE |
| 15 | 54 | NO | YES | NO | NO | NO | IDC | 3 | NO | NO | NO | NO | YES | YES | YES | YES | 13.4 | 23.8 | 15.3 | 19 | NEGATIVE | NEGATIVE | positive |
| 16 | 57 | NO | NO | NO | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | NO | 9 | 10 | <5 | 9.8 | NEGATIVE | NEGATIVE | NEGATIVE |
| 17 | 49 | NO | NO | NO | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | NO | NO | 25.4 | 23.8 | 32.5 | 27 | positive | positive | NEGATIVE |
| 18 | 59 | NO | NO | NO | PRESENT | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | NO | NO | 72 | 80 | 80 | 64.2 | NEGATIVE | NEGATIVE | NEGATIVE |
| 19 | 53 | YES,ULCERATION | YES | NO | YES | YES | IDC | 3 | NO | NO | NO | NO | YES | YES | NO | YES | 14 | 41.3 | 16 | 14.8 | | | |
| 20 | 35 | NO | YES | NO | YES | NO | IDC | 3 | YES | NO | NO | NO | YES | YES | MILD | YES | 8.2 | >9.5 | 9 | | | | |
| 21 | 26 | NO | YES | NO | NO | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | NO | YES | 15 | 20.7 | 19.3 | 13.3 | NEGATIVE | NEGATIVE | NEGATIVE |
| 22 | 50 | NO | YES | YES,INVERTED NIPPLE | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | YES | 75 | 80 | 80 | 56.8 | NEGATIVE | NEGATIVE | NEGATIVE |
| 23 | 58 | NO | YES | NO | NO | NO | IDC | 2 | YES | NO | NO | NO | YES | YES | MILD | YES | 66 | 80 | 70 | 40.3 | NEGATIVE | NEGATIVE | positive |
| 24 | 62 | NO | YES | NO | NO | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | MILD | YES | 40 | 30.5 | 46.6 | 39 | NEGATIVE | NEGATIVE | positive |
| 25 | 68 | NO | YES | NO | YES | NO | IDC | 3 | NO | NO | NO | NO | YES | YES | MILD | YES | 42 | 58.8 | 45.9 | 47 | positive | positive | NEGATIVE |
| 26 | 54 | NO | NO | NO | NO | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | MILD | NO | 65 | 79 | 67.1 | 69.7 | NEGATIVE | NEGATIVE | positive |
| 27 | 40 | NO | NO | NO | NO | NO | IDC | 3 | NO | YES | NO | NO | YES | YES | MILD | NO | 40 | 52.9 | 42.5 | 39.2 | | | |
| 28 | 72 | NO | NO | YES,RETRACTED NIPPLE | YES | NO | IDC | 2 | YES,COMEDO NECROSIS | YES | YES | YES | YES | YES | MILD | NO | 40 | 42.2 | 36.4 | 46 | NEGATIVE | NEGATIVE | positive |
| 29 | 24 | YES,BLACKISH DISCOLORATION | NO | YES, CYSTIC&NECROTIC | NO | YES | INFILTRATING DUCTAL CARCINOMA | | YES | NO | NO | NO | YES | YES | MILD | NO | 76 | 90 | 80 | 78.5 | NEGATIVE | NEGATIVE | NEGATIVE |
| 30 | 35 | NO | YES | NO | YES | NO | IDC | 3 | YES | YES | NO | NO | YES | YES | MILD | YES | 10 | 11 | 5 | 16.9 | NEGATIVE | NEGATIVE | NEGATIVE |
| 31 | 65 | NO | NO | NO | YES | NO | IDC | 3 | NO | NO | NO | NO | YES | YES | MILD | NO | 29 | 50.9 | 32 | 18.5 | NEGATIVE | NEGATIVE | positive |
| 32 | 48 | NO | YES | NO | YES | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | MILD | YES | 16 | 33 | 17.3 | 28 | positive | positive | NEGATIVE |
| 33 | 62 | NO | YES | NO | YES | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | MILD | YES | 29 | 34 | 15 | 32 | NEGATIVE | NEGATIVE | positive |
| 34 | 45 | NO | NO | YES,PURULENT DISCHARGE FROM NIPPLE | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | MILD | 29 | 48.3 | 35 | 15.3 | positive | positive | NEGATIVE |
| 35 | 63 | YES,ULCERATION | YES | YES,MASS SEEN | YES | ULCERATED | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | YES | 22 | 30.6 | 26.4 | 15.5 | positive | NEGATIVE | NEGATIVE |
| 36 | 45 | NO | YES | NO | YES | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | MILD | YES | 12 | 21.6 | 10 | 16.3 | positive | NEGATIVE | NEGATIVE |
| 37 | 64 | NO | YES | NO | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | YES | 49 | 71.5 | 40 | 28.5 | | | |
| 38 | 64 | NO | YES | NO | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | YES | 55 | 78 | 57.7 | 43 | NEGATIVE | NEGATIVE | positive |
| 39 | 46 | NO | NO | NO | YES | NO | IDC | 3 | NO | NO | NO | NO | YES | YES | NO | NO | 94 | 90 | 90 | 79.5 | | | |
| 40 | 52 | NO | NO | NO | YES | NO | IDC | 3 | NO | NO | NO | NO | YES | YES | MILD | NO | 80 | 90 | 85 | 40.5 | NEGATIVE | NEGATIVE | positive |
| 41 | 51 | NO | YES | NO | YES | NO | IDC | 2 | NO | YES | NO | NO | YES | YES | MILD | YES | 1 | <5 | <5 | 0.06 | NEGATIVE | NEGATIVE | positive |
| 42 | 62 | NO | YES | NO | YES | NO | IDC | 3 | NO | NO | NO | NO | YES | YES | NO | YES | 14 | 26 | 16.8 | 15 | NEGATIVE | NEGATIVE | NEGATIVE |
| 43 | 48 | NO | YES | NO | NO | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | NO | YES | 20 | 26.4 | 21.8 | 14.7 | NEGATIVE | NEGATIVE | positive |
| 44 | 38 | NO | YES | YES,MASS SEEN | NO | NO | IDC | 2 | NO | NO | YES | NO | YES | YES | MILD | YES | 26 | 38.5 | 12 | 29.5 | NEGATIVE | NEGATIVE | positive |
| 45 | 75 | NO | NO | NO | NO | NO | MUCINOUS CARCINOMA | | NO | NO | NO | NO | YES | YES | NO | NO | 10 | 11.4 | 10 | 29 | Positive | NEGATIVE | NEGATIVE |
| 46 | 70 | NO | NO | NO | YES | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | MILD | NO | 12 | 12.2 | 10 | 14 | positive | positive | NEGATIVE |
| 47 | 67 | YES, ULCEROPROLIFERATIVE | YES | YES | YES | YES | IDC | 2 | YES | NO | NO | YES | YES | YES | MILD | YES | 25 | 25.6 | 20 | 34 | positive | positive | NEGATIVE |
| 48 | 38 | NO | YES | NO | YES | NO | IDC | 3 | NO | NO | NO | NO | YES | YES | NO | YES | 45 | 75.6 | 26.3 | 42.6 | | | |
| 49 | 32 | YES,ULCERATION/ABCESS | NO | NO | YES | NO | IDC | 2 | YES | NO | NO | NO | YES | YES | NO | NO | 16 | 11 | 10 | 34.5 | NEGATIVE | NEGATIVE | NEGATIVE |
| 50 | 52 | NO | YES | NO | NO | NO | IDC | 2 | NO | NO | NO | NO | YES | YES | NO | YES | 35 | 37.5 | 10 | 30 | | | |