A dissertation on

"Correlation of Disease Severity, Estrogen, Progesterone & Epidermal Growth Factor Receptor Status with Altered Expression of Claudins 1, 3, 4 & 7, P53 & Ki67 Index in Carcinoma Breast"

Dissertation submitted
in partial fulfilment of the regulations
for the award of the degree of

M.S. DEGREE BRANCH – I
GENERAL SURGERY
Of
THE TAMIL NADU Dr.M.G.R. MEDICAL UNIVERSITY

ESIC MEDICAL COLLEGE & PGIMSR,
K.K.Nagar, Chennai- 600 078.

APRIL 2016

DECLARATION BY THE CANDIDATE

I solemnly declare that this dissertation entitled **"Correlation of Disease Severity, Estrogen, Progesterone & Epidermal Growth Factor Receptor Status with Altered Expression of Claudins 1, 3, 4 & 7, P53 & Ki67 Index in Carcinoma Breast"** is a bonafide and genuine research work carried out by me under the guidance of Prof. R.Anbazhakan, Department of General Surgery, ESIC Medical College & PGIMSR, K.K.Nagar, Chennai.

This dissertation is being submitted to Tamil Nadu Dr.M.G.R. Medical University, Chennai, towards partial fulfilment of requirements of the degree of MS (General Surgery) examination to be held in April 2016.

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I hereby declare that The Tamil Nadu Dr.M.G.R. Medical University, Chennai, shall have the rights to preserve, use and disseminate this dissertation/thesis in print/electronic format for academic/research purpose.

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CERTIFICATE OF THE GUIDE

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I would like to thank our respected Dean Dr. Srikumari Damodaram, for her auspices from the inception to the completion of this project,

I would like to thank my guide, Prof. Dr. Anbazhakan, for his steady and unwavering support and encouragement that made it possible to even believe that I could take on this project and complete it. His discretion enabled me to balance the hours that this endeavor entailed with the busy schedule of our department.

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My support system outside the hospital, my family and friends, deserve my undying gratitude for their patience and support during this process.

Lastly, I place my thesis upon the altar of science. The very essence of scientific curiosity is to question all things great and small. Without these questions, we would stagnate, and, by extension, perish. In the words of Steve Jobs, entrepreneur par excellence and a scientist of the social revolution, ‘Stay hungry. Stay foolish’.

CERTIFICATE OF APPROVAL

TO

DR. Veena Bheeman
PG in Department of General Surgery
ESIC Medical College & PGIMSR
KK Nagar, Chennai-78

Dear Dr. Veena Bheeman,

The Institutional Ethics Committee of ESI PGIMSR reviewed and discussed you application for approval of the proposal entitled "Correlation of Disease Severity, Estrogen, Progestrone and Epidermal Growth Factor Receptor Status with Altered Expression of Claudins 1,3,4,7 & TP53 and Ki67 Labelling Index in Carcinoma Breast" , No. 05/20/11/2013

The following members of the Ethics Committee were present in the meeting held on 20.11.2013 conducted at ESI PGIMSR, KK Nagar, Chennai-78.

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2.	Dr.Kamalini Sridharan, Co-ordinator/ Prof. & HOD, Dept. of Anesthesia, ESI-PGIMSR.
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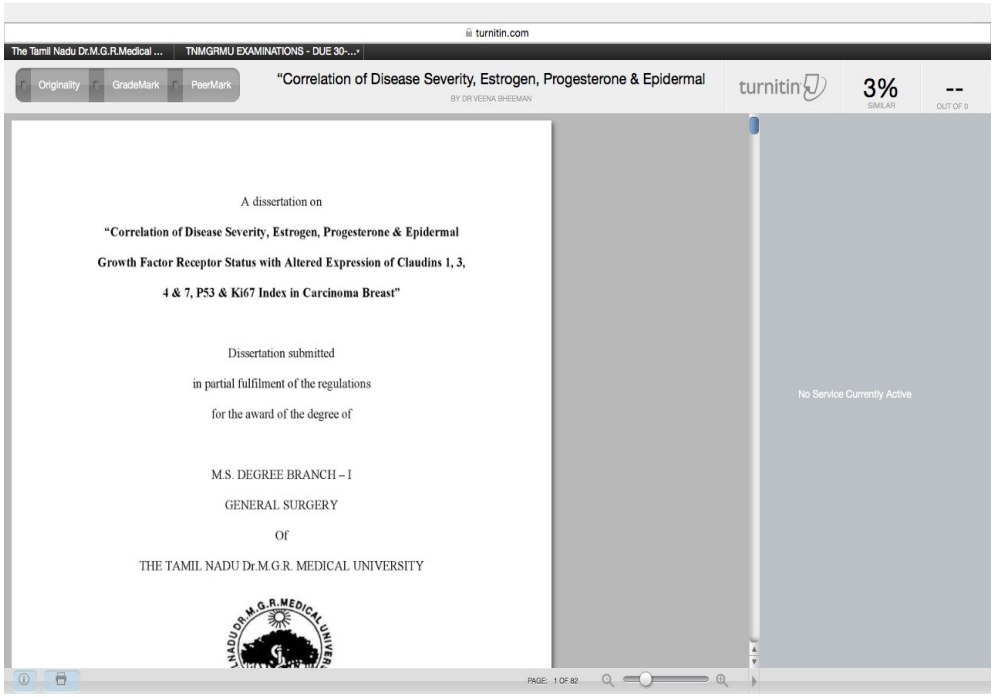
The proposal is approved to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and significant adverse effects occurring in the course of the study, any changes in the protocol and patients information / informed consent and asks to be provided a copy of the final report.

Saradha Suresh

[DR. SARADHA SURESH]
CHAIRPERSON
ETHICAL COMMITTEE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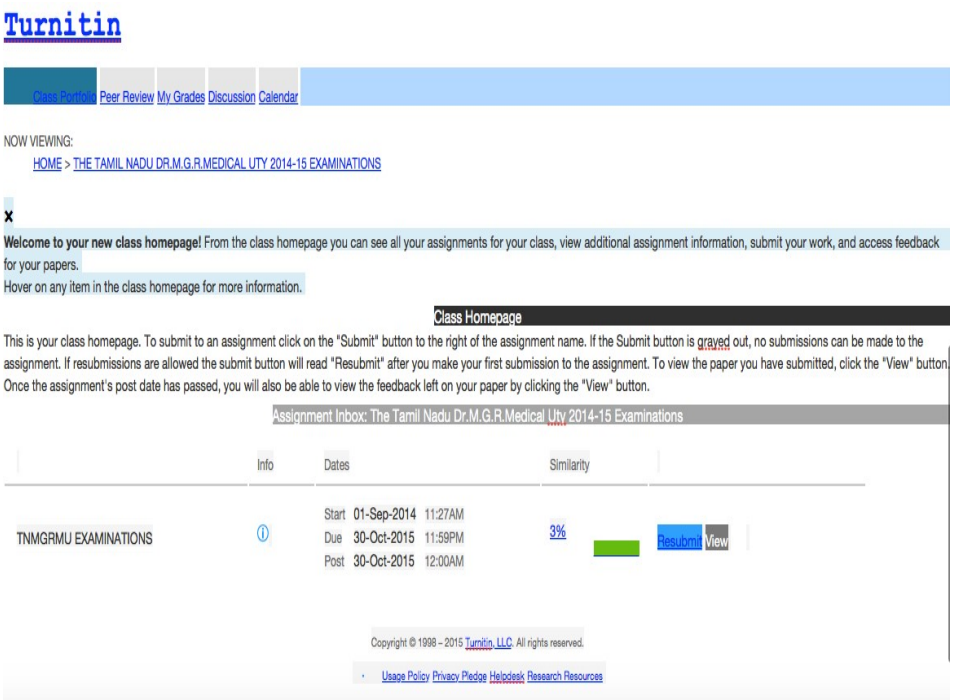
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Abstract

Introduction

Claudins [CLDN] are a new group of proteins that have of late attracted much attention as alterations in their expression is consistently associated with variations in outcomes in CA breast and many other malignancies.

Aims & Objectives

To study the association between the severity of disease, gauged by size of the primary tumour, clinical stage at presentation and the presence or absence of lymph node enlargement in breast cancer and, molecular subtype, as determined by the Estrogen and Progesterone receptor status and expression of Her2 neu, P53 Mutation, Ki 67 Index, expression of CLDNs 1, 3, 4 and 7

Materials & Methods

The study was conducted on female patients with CA breast who were managed in our department, regardless of age, stage of disease and management. Triple assessment was done and all data documented with regards to the patient, such as age, the tumour such as stage, histopathological type, hormone receptor status and HER2 neu. Immunohistochemistry was done to look for P 53 mutations and Ki 67 Index. RT PCR was done to assess the expression of Claudin genes 1, 3, 4 and 7.

Results

There was a statistically significant association between the expression of CLDNs 1, 3, 4 and 7 and the presence of clinically or radiologically suspicious lymph nodes, ER status (PR status was associated with CLDN 4 expression alone), triple negative tumours, P 53 mutation, and Ki 67 index.

There was no association, however, with the age of the patient, clinical stage at presentation, tumour size, HER 2 neu expression and that of CLDN 1, 3 and 7 with PR status.

Conclusion

The results indicate that several associations and their implications have to be kept in mind while managing breast cancer and we can no longer stop with the standard protocols. Newer methods of prognostication are required. Promising drugs that target claudins are in clinical trials and they may improve the outcome drastically in these patients. Further studies will help clarify this information further

Key Words: breast cancer, claudins, claudin low breast cancer, triple negative breast cancer, P 53, Ki 67, estrogen receptor, progesterone receptor, HER2neu, molecular subtyping of breast cancer.

INTRODUCTION

While an enormous amount of information still remains intricately hidden with the human genome, research today is slowly but steadily decoding it. Every piece of data thus garnered alters to some extent our current understanding of malignancies and opens up new avenues for their management.

Carcinoma [CA] of the breast is one of the most common malignancies encountered in the General Surgery Out Patient Department & operated on at ESIC Medical College & PGIMSR. Patients, mainly from lower socioeconomic strata, often present with either palpable lump or in a more advanced stage.

They are treated as per standard protocols with:

- Modified Radical Mastectomy followed by Adjuvant Chemotherapy, Radiation or both in early breast carcinomas,
- Neoadjuvant Chemotherapy followed by Surgery, Adjuvant Chemotherapy and Radiation in locally advanced breast cancer and
- Palliative Chemotherapy for advanced metastatic disease.

The follow up in our hospital is excellent as this is a tertiary care center for the ESI beneficiaries of this region. For a condition such as

cancer of the breast, all the patients are treated here and it is possible to follow the patient from initial presentation, through diagnosis, treatment and during adjuvant therapy as well, except in rare extenuating circumstances.

While the standard of care is being met, the number of patients who came back with poor control of the primary and recurrences was glaringly high and the need to look beyond the regular protocols to establish a new standard of care arose.

The treatment of malignancies now a day is based on the molecular biology of the cancer in general and the individual in particular. It is clear that our understanding of the molecular genetics of carcinoma of the breast is still at a very rudimentary level, as evidenced by the mortality and morbidity associated with the disease. More molecular targets need to be identified for appropriate drugs to be developed.

Claudins [CLDN] are a new group of proteins that have of late attracted much attention as alterations in their expression is consistently associated with variations in outcomes in CA breast and many other malignancies. While the overall outcomes in breast cancer have improved, those of patients with alterations in claudin expression continue to be poor. These tumours behave differently and standard chemotherapy is not as effective in their management. Claudins are a

veritable treasure trove of potential targets for treatment. These therapeutic agents are also expected to be highly specific in their site of action, thus avoiding all the unpalatable side effects of cancer chemotherapy. Promising new drugs are already in clinical trials.

In this study, the molecular subtypes according to hormone receptor status and Ki 67 index were ascertained, as were the presence P53 mutations and expression patterns of CLDNs 1, 3, 4 and 7. Associations between disease severity and these parameters and associations among these parameters were analyzed statistically.

Assessing the molecular profile of every breast cancer will soon be the norm. Only when we are able to categorically determine every genetic nuance of a malignancy will we be able to treat it with finality. Until that point there will always be lacunae.

AIMS AND OBJECTIVES

- To study the association between the severity of disease, gauged by size of the primary tumour, clinical stage at presentation and the presence or absence of lymph node enlargement in breast cancer and:
    - Molecular subtype, as determined by the Estrogen and Progesterone receptor status and expression of Her2 neu
    - P53 Mutation
    - Ki 67 Index
    - Expression of CLDNs 1, 3, 4 and 7
- To establish a baseline demographic, hormonal and genetic profile of female patients with Carcinoma breast managed in our hospital

MATERIALS & METHODS

The study was conducted on patients with Carcinoma [CA] of the breast attending the surgical Out Patient Department (OPD) of ESIC Medical College & PGIMSR, K K Nagar, Chennai

Inclusion Criteria

- All female patients with CA breast, regardless of age, stage of disease and management.

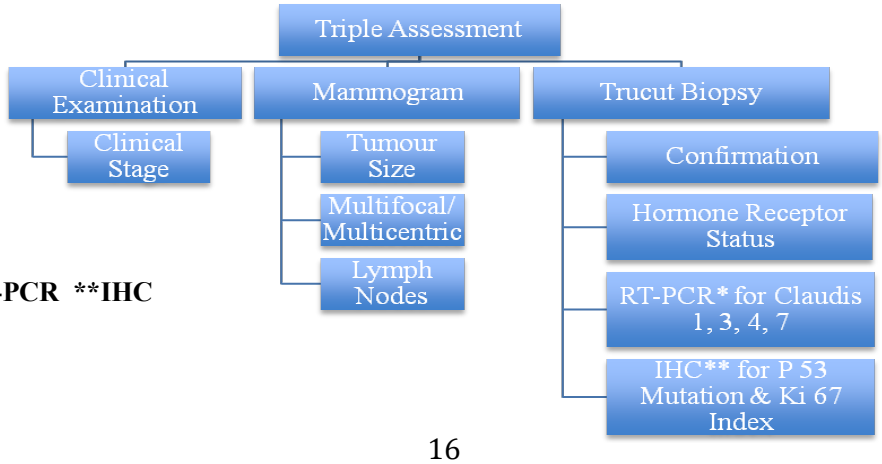
Exclusion Criteria

- Male patients with Carcinoma breast

Sample Size

In a study published in 2011, Campbell et al quoted the prevalence of claudin low subtype of breast cancer as 7 – 14%. Taking an average prevalence of 11%, the minimum number of patients needed to estimate the association with 95% confidence and a precision of 9% was found to be 46. A sample size of fifty patients was taken in this study

When a patient with a suspicious breast lump was encountered, the protocol of 'Triple Assessment was followed:



***RT-PCR** ****IHC**

This data was documented. The size of the primary tumour, clinical stage and mammographic findings were taken as markers of the severity of the disease at presentation. Trucut biopsy was used as the standard method of tissue diagnosis. Immunohistochemistry tests were run on the same samples for hormonal receptor status, P53 mutation and Ki 67 index.

Biopsy samples were placed in the preservative issued by the laboratory and frozen. RT PCR was then done for the expression of CLDN 1, 3, 4 and 7.

Patients were categorized as having Early, Locally Advanced or Metastatic Breast Cancer. They patients were subjected to Surgery, Neoadjuvant chemotherapy [NACT] followed by Surgery or Palliative Chemotherapy.

REVIEW OF LITERATURE

Embryology of the Breast[1]

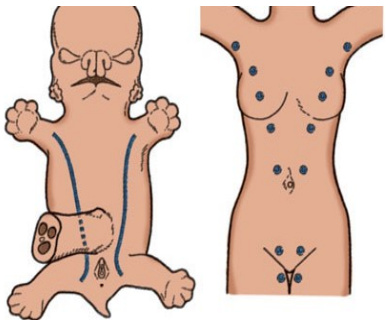


Fig 1: Milk Line

The breast is a modified sweat gland derived from the epidermis. A localized proliferation of the stratum spinosum of the epidermis of that region gives rise to the nipple. During the second month of gestation, two bands of slightly thickened ectoderm appear on the ventral body wall extending from above the axilla to below the groin. These bands are the milk lines and represent potential mammary gland tissue.

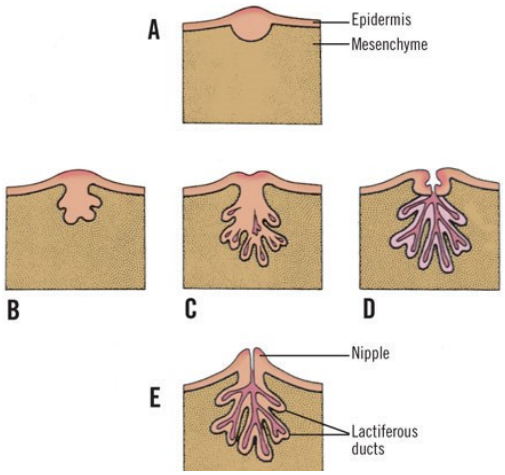


Fig 2: Development of Secretory Unit

In humans, only the pectoral portion of these bands will persist and ultimately develop into adult mammary glands. Occasionally, vestigial, or even functional, breast tissue may arise from other portions

of the milk line

The glandular tissue of the breast arises from the local thickening of the epidermis from which, 16 to 24 buds of ectodermal cells grow into the underlying mesoderm at the twelfth week of intrauterine life. These buds are initially solid, but become canalized close to term resulting in the lactiferous ducts. The terminal parts of the buds will give rise to the secretory acini at the time of lactation. The epidermal portion of the future nipple first appears as a shallow pit, which everts as the fetus approaches term. The areola is said to be visible from the fifth month onward.

Gross Anatomy [1]

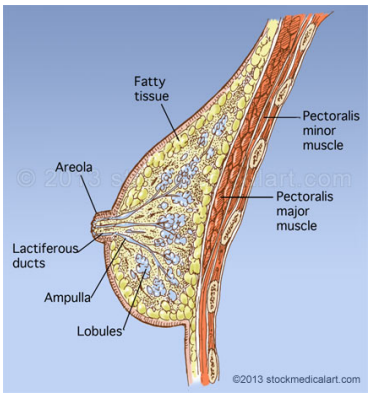


Fig 3: Anatomy of the Breast

The breast lies in the superficial fascia of the anterior thorax. The vertical extent is from the second to sixth ribs and the horizontal extent is from the sternum to the mid-axillary line. It consists of ducts and secretory lobules. The ducts converge to form 15 – 20 secretory lobules that open on to the nipple. The pigmented areola surrounds the nipple.

The connective tissue stroma that surrounds the ducts and lobules of the breast condenses to form the Suspensory ligaments of breast in some regions, which are attached to the dermis of the overlying skin and support the breast. Fat content is more abundant in non-lactating women while glandular element is more in those who are lactating.

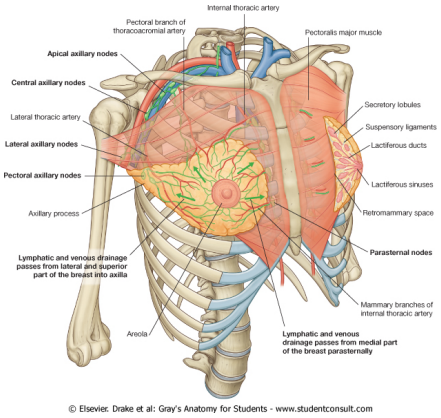


Fig 4: Relations of the Breast

The breast is related posteriorly to the deep fascia covering the Pectoralis major muscle, from which it is separated by loose connective tissue known as the retromammary space. This enables movement of the breast over the underlying structures.

The upper lateral part of the breast can extend around the lateral margin of the pectoralis major muscle and into the axilla as the axillary tail. It may extend up to the axillary apex.

Blood Supply

Arterial supply arises medially from the branches of the internal mammary and the second to fourth intercostal arteries through perforators and laterally from those of the axillary artery. Venous drainage follows the arteries and finally reach either the axillary, internal thoracic or the intercostal veins.

Nerve Supply

The breast gets its nerve supply from the anterior and lateral cutaneous branches of the second to sixth intercostal nerves. The sensory supply to the nipple is by the fourth intercostal nerve.

Lymphatic Drainage

Nearly three fourths of the lymph from the breast drains in to the axillary nodes with most of the remainder going to the internal thoracic nodes. A small quantity of lymph may drain along the lateral branches of the posterior intercostal nodes to reach the intercostal nodes.

Physiology of the Breast [2]

Several hormonal stimuli are responsible for breast development and function. They are Estrogen, Progesterone, Prolactin, Oxytocin, Thyroid hormone, Cortisol and the Growth hormone.

Estrogen is responsible for ductal development, and progesterone for differentiation of epithelium and for lobular development. Lactogenesis is stimulated by Prolactin during late pregnancy and the postpartum period. It causes upregulation of hormone receptors and aids in epithelial development. In Figure 5, the secretion of hormones that regulate the function and development of breast tissues is represented. Positive and negative feedback

mechanisms exercise stringent control of the hormones secreted from the hypothalamus and the pituitary.

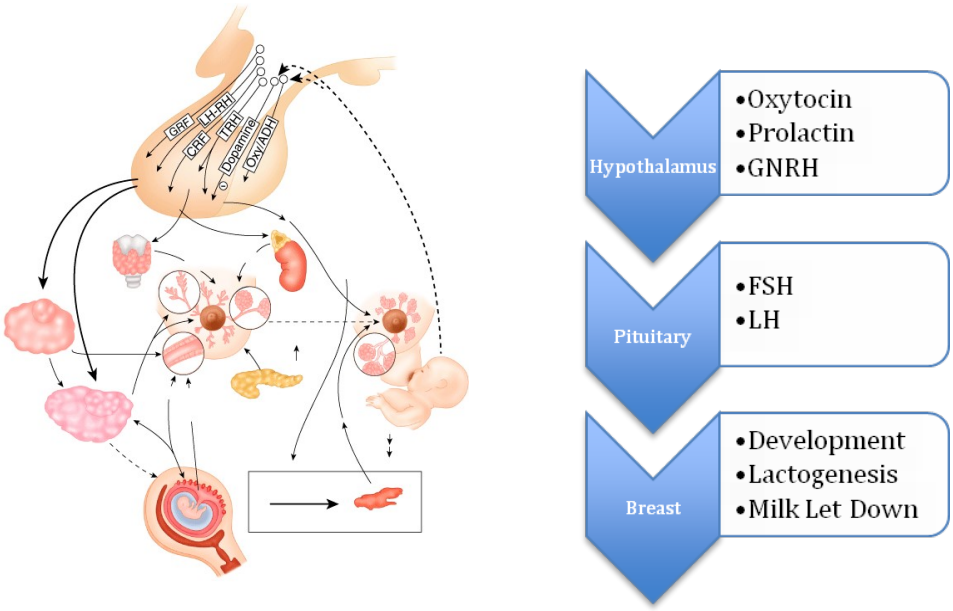


Fig 5: Regulation of Breast Development & Function

The female breast changes dramatically as it goes through the various developmental stages in adolescence, pregnancy, lactation and senescence. These changes are governed by the complex interactions among the various hormones. Imbalances in these hormones result in several conditions, both benign and malignant, involving either the glandular portion or the stroma.

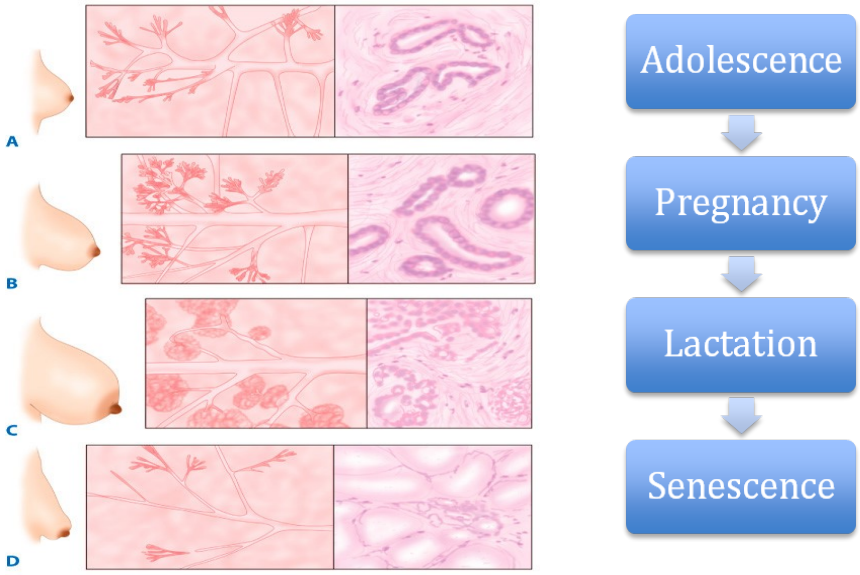


Fig 6: Physiological Stages of Breast Development

Carcinoma of the Breast

Carcinoma of the breast is epithelial malignancy arising from breast tissue and can originate from either the lobules or the ductules.

Risk Factors for Carcinoma Breast[3-9]

Hormonal Risk Factors

Breast cancer increases with the increase in exposure to estrogen. The converse is thought to be protective. The former occurs when the number of menstrual cycles is increased. This is seen with early menarche, late menopause and nulliparity. On the other hand, exercise and a longer lactation period are protective. The epithelium of the breast only attains terminal differentiation with a full-term

pregnancy and the younger the age at which this occurs, the lower the risk. Following menopause, estrogen is primarily derived from estrone, derived from androstenedione in the adipose tissue. Hence, in an obese woman the estrogen exposure is more, which translates in to a higher risk.

Nonhormonal Risk Factors

Theses include radiation exposure, especially if such exposure occurs during the time of active breast development. In the bygone era of mantle radiation therapy for Hodgkin's lymphoma, those women who received it had a substantially higher breast cancer risk than the normal population. Survivors of the nuclear fallout of World War II in Japan developed several radiation induced somatic mutations. Alcohol consumption increases the risk, as it is directly proportional to the amount of alcohol consumed. Alcohol increases estradiol levels. In addition, a high fat diet increases the breast cancer risk, also attributed to raised serum estrogen levels.

Risk Assessment Models[2]

The breast cancer risk decreases with advancing age. A fifty-year-old woman has a lifetime risk of 11% for the disease, while a seventy-year-old, 7%. There is marked interaction between the various risk factors. When several factors are present, risk evaluation is

difficult. Two risk assessment models are in popular use for such prediction. The most frequently used model is the Gail Model, which resulted from the "Breast Cancer Detection Demonstration Project", a mammographic screening programme.

Gail model	Claus model	BRCAPRO model	Tyrer–Cuzick model	BOADICEA model
• Age of the person • Age at menarche • Age at first live birth • Breast biopsies (AH) • Family history - First-degree relatives	• Age of the person • Age at menarche • Age at first live birth • Family history - First-degree relatives - Second-degree relatives	• Age of the person • Family history - First-degree relatives - Second-degree relatives - Third-degree relatives - Age at onset of breast cancer - Bilateral breast cancer - Ovarian cancer - Male breast cancer	• Age of the person • Body mass index • Age at menarche • Age at first live birth • Age at menopause • Hormone replacement therapy use • Breast biopsies (ADH, LCIS) • Family history - First-degree relatives - Second-degree relatives - Age at onset of breast cancer - Bilateral breast cancer - Ovarian cancer	• Age of the person • Family history - First-degree relatives - Second-degree relatives - Third-degree relatives - Age at onset of breast cancer - Bilateral breast cancer - Ovarian cancer - Male breast cancer

AH, atypical hyperplasia; LCIS, lobular carcinoma *in situ;* BOADICEA, breast and ovarian analysis of disease incidence and carrier estimation algorithm.

Table 1. Various Risk Assessment Models for CA Breast

The cumulative risk of breast cancer in each decade of life can be predicted using the Gail Model, where the relative risks from various categories are multiplied to arrive at an overall risk. Comparing the overall risk to that of an adjusted population then arrives at a woman's individual risk.

Data gathered in "Cancer and Steroid Hormone Study", a case-control study of breast cancer, led to the development of the Claus

Model by Claus and colleagues. It incorporates more data about family history of the disease and the breast cancer risk is estimated for the decade of life based on presence of first- and second-degree relatives with breast cancer and their age at diagnosis. Risk factors that are less consistently associated with breast cancer such as diet, use of oral contraceptives and lactation, and those that are rare in the general population like radiation exposure are included in neither model. Several other models also exist. None of these models accounts for the risk associated with mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2*.

Risk Management[10-18]

The risk of developing breast cancer will dictate several important medical decisions, such as:

- The usage of postmenopausal hormone replacement therapy
- The age at which mammography screening is to be started
- The use of Tamoxifen to prevent the disease
- The decision to perform mutilating surgery such as prophylactic mastectomy

Postmenopausal hormone replacement therapy was widely prescribed in the 1980s and 1990s because of its effectiveness in

controlling the symptoms of estrogen deficiency. However, large-scale phase III clinical trials, the results of which were released in 2002, showed conclusively that breast cancer risk is threefold to fourfold higher after >4 years of use and there is no significant reduction in cardiac or cerebrovascular events.

In women at or over the age of fifty, screening mammography, routinely used, reduces mortality from breast cancer by 33%. There are no significant risks involved and it is cost effective. Although its routine use in women less than fifty years of age is more controversial, the benefits of screening mammography between the ages of 40 and 49 years still appear to trump the risks. Reserving mammography for women known to be at a higher risk of the disease may also improve the risk – benefit ratio. For example, a mammographic abnormality in a woman with a family history of breast cancer is three times more likely to be malignant than in one without such a history. In addition, mounting data regarding mammographic breast density demonstrate an independent correlation with breast cancer risk. It is currently recommended that women undergo baseline mammography at age 35 and then have annual mammographic screening beginning at age 40.

Tamoxifen, a selective estrogen receptor modulator, has been conclusively shown to reduce the incidence of CA breast in healthy women.

Prophylactic mastectomy, where indicated, would reduce the risk of breast cancer by over 90%. However, several long-term quality of life issues remain to be addressed. The benefit varies with the risk of developing the disease. When the estimated lifetime risk is 40%, the procedure added almost 3 years of life, while with a risk of 85%, prophylactic mastectomy added more than 5 years of life.

BRCA Mutations[19-25]

Inherited germline mutations such as those of *BRCA1* and *BRCA2*account for five to ten per cent of breast cancers. They are tumor-suppressor genes, and for each gene, loss of both alleles must occur before cancer occurs. They are inherited in an autosomal dominant pattern with variable penetrance (Table 2). Hence, 50% of offspring of carriers inherit the trait. It is likely that these genes are coregulated.

Germline mutations in *BRCA1* are a predisposing genetic factor in as many as 45% of hereditary breast cancers and in at least 80% of hereditary ovarian cancers. More than 500 variations in sequence have been documented. The *BRCA2* gene is unlike any other gene identified so far, and the protein is also unique. More than 250 mutations have been found till date. In male carriers, there is an estimated breast cancer risk of 6%, which represents a hundred times higher risk than the general male population.

	BRCA1	BRCA2
Location	Chromosome 17q	Chromosome 13q
Size	100 kb	70 kb
Exons	22	26
mRNA	- 7.8 kb - Encodes a 1863 amino acid protein	- 11.2 kb - Encodes a 3418 amino acid protein
Inheritance	Autosomal Dominant	Autosomal Dominant
Functions	- Transcription - Cell cycle control - DNA damage repair pathways	- Not well defined
Distinguishing Features	- Earlier age of onset than sporadic cases - Higher incidence of bilateral breast cancer	- Earlier age of onset than sporadic cases - Higher incidence of bilateral breast cancer
Breast Cancer (Females)	90%	85%
Lifetime Risk of Ovarian Cancer (Females)	- 40% - Invasive ductal carcinoma - Poorly differentiated - Hormone receptor negative	- 20% - Invasive ductal carcinoma - Well differentiated - Hormone receptor positive
Associated Malignancies	Colon and prostate cancers.	Colon, prostate, pancreatic, gallbladder, bile duct, stomach cancers, and melanoma.

Table 2. Characteristics of BRCA1 & BRCA2 Mutations

Hereditary breast cancer is likely if:

- A family includes two or more women who developed ovarian cancer or breast cancer before age 50 years.

- Any woman diagnosed with breast cancer before age 50 years or with ovarian cancer at any age is asked about first-, second-, and third-degree relatives on either side of the family with either of these diagnoses.

- Breast and ovarian cancer in the same individual

- Male breast cancer at any age

When such a scenario arises, the most prudent strategy is first to completely analyze the sequence of both genes in the patient. When a mutation is found, relatives can be tested for that particular mutation alone. If the mutation is not identified in the relatives, complete sequencing needs to be done.

Risk management strategies for these patients are:

- Prophylactic mastectomy and reconstruction
- Prophylactic oophorectomy and hormone replacement therapy
- Diligent surveillance for breast and ovarian cancer
- Chemoprevention

Present screening recommendations in those patients who choose not to undergo prophylactic procedures are:

- Breast examination by a doctor every 6 months
- Yearly Mammography starting at the age of 25 years

The risk of breast cancer in carriers of these mutations is higher after 30 years of age. MRI is the more sensitive screening investigation in younger women with dense breasts[26].

Cancers arising in the setting of*BRCA1* mutation are usually high grade and often ER, PR and HER2 neu negative. 66% of *BRCA1*-associated DCIS lesions are estrogen receptor negative, which indicates that the hormone-independent phenotype is an early phenomenon in the evolution of the disease.

The introduction of the poly (ADP-ribose) polymerase (PARP) inhibitors has opened up new therapeutic possibilities for these patients. They have a lethal effect on affected cells by targeting an alternate DNA repair pathway in BRCA-deficient cells. A phase II trial of one PARP inhibitor was recently published, showing promising survival data in patients with triple-negative metastatic disease.

Other Hereditary Syndromes

- Cowden disease (*PTEN* mutations, in which cancers of the thyroid, GI tract, and benign skin and subcutaneous nodules are also seen)
- Li-Fraumeni syndrome (p53 mutations, also associated with sarcomas, lymphomas, and adrenocortical tumors)
- Syndromes of breast and melanoma

Presentation of CA Breast

In our country most patients present to the hospital after they detect a lump. With increase in awareness, patients are presenting earlier. However, there remains a small fraction who present with advanced disease.

When a patient presents to the hospital with a suspicious lump, the triple assessment is carried out which consists of clinical examination, radiological assessment and confirmation of the diagnosis with a tissue sample. Once the diagnosis of carcinoma breast is established, a metastatic work up is performed with the investigations warranted by the clinical scenario. We then have all the information we need to arrive at the Stage of the malignancy (Fig 7).

Then the patients can be classified into Early Breast Cancer (EBC), Locally Advanced Breast Cancer (LABC) or Metastatic disease and are treated accordingly.

Staging for Breast Cancer, With 5-Year Survival Rates By Stage

Primary Tumor (T)

TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Tis		Carcinoma in situ: intraductal carcinoma, lobular carcinoma in situ, or Paget's disease of the nipple with no tumor
T1		Tumor 2 cm or less in greatest dimension
	Tmic	0.1 cm or less in greatest dimension
	T1a	0.1 to 0.5 cm or less in greatest dimension
	T1b	More than 0.5 cm, but not more than 1 cm in greatest dimension
	T1c	More than 1 cm, but not more than 2 cm in greatest dimension
T2		Tumor more than 2 cm but not more than 5 cm in greatest dimension
T3		Tumor more than 5 cm in greatest dimension
T4		Tumor of any size with direct extension to chest wall or to skin
	T4a	Extension to chest wall
	T4b	Edema (including peau d'orange) or ulceration of the skin of the breast, or satellite skin nodules confined to the same breast
	T4c	Both T4a and T4b
	T4d	Inflammatory carcinoma

Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed (eg previously removed)
N0	No regional lymph node metastases
N1	Metastasis to movable ipsilateral axillary node(s)
N2	Metastasis to ipsilateral axillary node(s) fixed to one another or to other structures
N3	Metastasis to ipsilateral internal mammary lymph node(s)

Distant Metastasis (M)

MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastases (including metastases to ipsilateral supraclavicular lymph node(s))

Stage Grouping		Approximate 5-Year Survival Rate (%)	Stage Grouping		Approximate 5-Year Survival Rate (%)
0	Tis, N0, M0	95%–99%	IIIA	T0, N2, M0 T1, N2, M0 T2, N2, M0 T3, N1, M0 T3, N2, M0	50%–55%
I	T1, N0, M0	90%–94%			
IIA	T0, N1, M0 T1, N1*, M0 T2, N0, M0	82%–86%			
IIB	T2, N1, M0 T3, N0, M0	68%–70%	IIIB	T4, Any N, M0 Any T, N3, M0	42%–48%
			IV	Any T, Any N, M1	15%–18%

Table 3: TNM Staging of CA Breast with Approximate 5-Year Survival Rate

Evolution of Treatment Principles of CA Breast[2]

'Cancer' as described and compared to the crab is probably best exemplified by the carcinoma of the breast.

The very first documentation of the disease, the Smith Surgical Papyrus, dates back to 3000 – 2000 BC, and describes the malignancy

in, interestingly enough, a male patient with the conclusion that the condition was simply not curable. Very few other such descriptive texts have been discovered up until the first century. Celsus in the 'De Medicina' categorically states that except the 'cacoethes' or early breast cancer, this condition remains largely incurable. He observed, in fact, that attempting surgery in any of the advanced conditions only made the disease more aggressive. The Galenic system, that dictated much of medicine up until the Renaissance, shared the same principle.

Time Frame	Surgeon	Innovation
18th Century	Morgagni	Resection
	Le Dran	Theory of lymphatic spread
19th Century	Moore	Mastectomy
	Banks	Mastectomy with removal of nonpalpable lymph nodes
	Halstead & Meyer	Radical Mastectomy
	Patey	Modified Radical Mastectomy

Table 4. Milestones in Breast Cancer Management

Later on, Morgagni and others of his time started attempting surgical resections. Le Dran postulated the theory of lymphatic spread and began excising all enlarged lymph nodes during surgery.

The removal of the affected breast in toto, along with all palpable lymph nodes, was first advocated by Moore, of the Middlesex County hospital in London in the nineteenth century. Banks, supported

his methods, drew attention to the possibility of micrometastasis and emphasized that non-palpable lymph nodes should also be removed. In 1894, Halstead & Meyer presented their experience with radical mastectomy including complete excision of nodes Level I to III, long thoracic nerve and thoracodorsal neurovascular bundle with axillary contents. Then came Patey's modified radical mastectomy where the pectoralis major was preserved, while the pectoralis minor was excised to reach and remove the Level III nodes. Further modifications of this procedure preserved the pectoralis minor as well, with division or retraction being employed to access Level II lymph nodes

The Current Perspective

Carcinoma of the breast is a multifactorial and systemic disease. It is the most common malignancy in women worldwide, but for skin cancer. More than a million cases diagnosed a year.

Earlier diagnosis & better treatment modalities have improved prognosis and the focus has shifted to molecular targets for therapeutic manipulation[27]. Tailoring therapy to the individual patient has become the norm.

In the past 20 years there has been a substantial increase in our understanding of carcinogenesis and the role of genetic alterations in the diagnosis, treatment, and prevention of breast cancer. Gene expression studies have been adopted for both molecular and

phenotypic characterization and for therapeutic decision-making in breast cancer treatment.

Molecular Subtypes of Carcinoma Breast[28]

The molecular subtyping of carcinoma breast is based on the hormone receptor status and tumour grade.

Molecular Subtype	Hormonal Profile			Others
	ER	PR	HER 2	
Luminal A	+	+/-	-	Low Ki 67
Luminal B	+	+/-	+/-	High Ki 67
HER 2 Enriched				
Basal Like	-	-	-	

Table 5. Molecular Subtype of CA Breast

These breast tumour subtypes suggest arrest of development at different stages of epithelial cell differentiation.

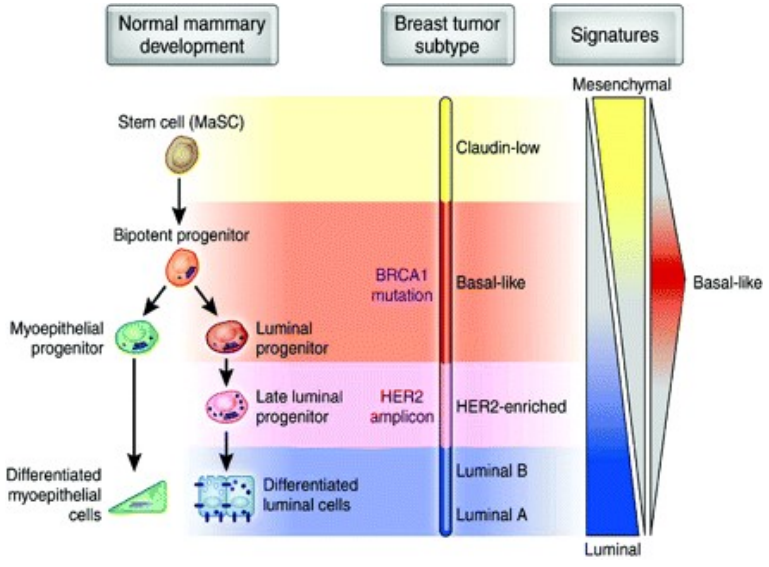


Fig 7. Mammary Cell Development and Stages Where Malignancy Develops

Basal-Like Breast Cancer

This group of tumours constitutes an average of 10%–25% of all tumors and make up about 50%–75% of the triple-negative subtype[29]. The triple-negative cancers may, less often, be of the other intrinsic subtypes. The distinguishing features are:

- Arise from progenitors early on in mammary cell development
- Express cytokeratins 5, 6, or 17, that are seen in the basal epithelial layer of the skin and airways
- Deficient in critical cell cycle regulators, p53 & RB1, thus making them highly proliferative
- Associated with BRCA1 Mutation
- High aneuploidy, several chromosomal changes, translocations, and losses.

The prognosis of basal like tumours is poor, making it imperative to identify suitable therapeutic targets in these patients[30]. The benefit of standard chemotherapy in these patients is questionable. Trials are underway to assess the responsiveness of these tumours to regimens with and without carboplatin. Potential Therapeutic Targets for these tumours include[29]:

- SRC (Non Receptor Tyrosine Kinase) Inhibition
- PARP Inhibition
- Androgen Receptor Inhibition
- Targeting Epigenetics
- EGFR Inhibition
- Phosphatidylinositol 3-kinase (PI3K)Pathway Inhibition
- Antiangiogenetic therapy (Bevacizumab)

Tight Junctions [31-33]

Tight junctions [TJ] regulate cell adhesion. They consist of transmembrane and peripheral membrane proteins.

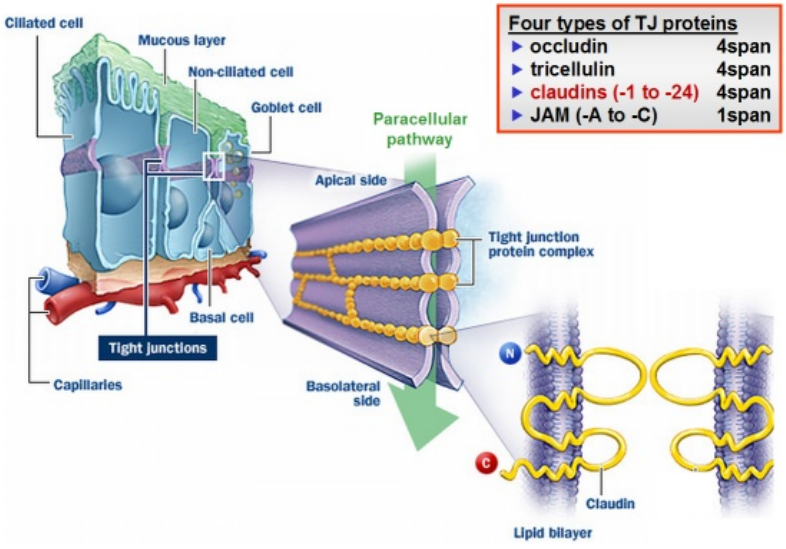


Fig 8: Components of Tight Junctions

They are involved in:

- Interactions among proteins
- Maintenance cell polarity
- Controlling paracellular ion flux.

Claudins (CLDN) are integral membrane proteins of TJs and include 24 genes in mammals[34]. In human beings, of the 23 of the 24 claudin genes have been identified, with the exception of *CLDN13*. These are small genes that have few introns. Some are located close to each other and contain strikingly similar sequences, such as *CLDN6* and *CLDN9* on chromosome 16, *CLDN22* and *CLDN24* on chromosome 4, *CLDN8* and *CLDN17* on chromosome 21, and *CLDN3* and *CLDN4* on chromosome 7. This is indicative of generation by gene duplication, and that adjacent genes may be coordinately regulated[34]. Others are diverse.

They are most commonly 20–34 kDa in size. They have four transmembrane helices with amino- and carboxyl-terminal tails extending into the cytoplasm[34, 35].

Claudins have two extracellular loops, the first extracellular loop containing charged amino acids that play a vital part in paracellular ion selectivity[36].

Gene	Chromosomal location	Transcript	Protein size (amino acids)	Molecular weight (Da)
CLDN1	3q28-q29	NM_021101.3	211	22,744
CLDN2	Xq22.3-q23	NM_020384.2	230	24,549
CLDN3	7q11.23	NM_001306.3	220	23,319
CLDN4	7q11.23	NM_001305.3	209	22,077
CLDN5	22q11.21	NM_001130861.1	218	23,147
		NM_003277.3		
CLDN6	16p13.3	NM_021195.4	220	23,292
CLDN7	17p13	NM_001307.4	211	22,390
			158	16,837
CLDN8	21q22.11	NM_199328.1	225	24,845
CLDN9	16p13.3	NM_020982.2	217	22,848
CLDN10	13q31-q34	NM_182848.2	226	24,251
		NM_006984.3	228	24,488
CLDN11	3q26.2-q26.3	NM_005602.5	207	21,993
CLDN12	7q21	NM_012129.2	244	27,110
CLDN14	21q22.3	NM_144492.1	239	25,699
		NM_012130.2		
CLDN15	7q11.22	NM_014343.1	228	24,356
CLDN16	3q28	NM_006580.2	305	33,836
CLDN17	21q22.11	NM_012131.1	224	24,603
CLDN18	3q22.3	NM_001002026.2	261	27,856
		NM_016369.3	261	27,720
CLDN19	1p34.2	NM_001123395.1	224	23,229
		NM_148960.2	211	22,077
CLDN20	6q25	NM_001001346.2	219	23,515
CLDN21	4q35.1	NA	NA	NA
CLDN22	4q35.1	NM_001111319.1	220	24,509
CLDN23	8p23.1	NM_194284.2	292	31,915
CLDN24	4q35.1	XM_001714660.1	205	22,802

Table 6: Human Claudin Genes (NA: Not Available)

The carboxy-terminal tails vary both in size and sequence among different claudin proteins that allows interaction with cytoplasmic TJ-associated proteins. This is also the site of post-translational modifications that determine localization and functions of claudins.

Claudins are expressed in different patterns in different tissues, and multiple claudins are expressed in most tissues[36].

- CLDN 2 and CLDN 15: Cation channels
- CLDN 4, 7 and 10 Anion channels/pores.

The expression of claudins is regulated by:

- Transcription factors
- Epigenetic mechanisms
- Post-transcriptional level.
- Growth factors
- Nonsteroidal anti-inflammatory drugs (NSAIDS) such as aspirin reduce claudin-7 expression in gastric epithelial cancer cells.

Several human diseases have been linked to mutations in claudin genes:

- CLDN1: Sclerosing cholangitis, ichthyosis
- CLDN16 and CLDN19: Hypomagnesemia, hypercalcinuria
- CLDN3 and CLDN4: Receptors for the Clostridium perfringens enterotoxin (CPE)
- CLDN1, 6 and 9 : Co-receptors for the hepatitis C virus
- CLDN1, 3, 4 and 7 are commonly dysregulated in cancer

These proteins have prognostic significance and are viable therapeutic targets.

Epithelial to Mesenchymal Transition

Significant heterogeneity is seen among breast cancers. There is a small population of self-renewing "cancer stem cells" or "tumor-initiating" cells (CSC/TICs), that are involved in tumour formation, chemoresistance and recurrence[37]. They can arise from fully differentiated cells by a phenomenon known as "epithelial to mesenchymal transition" (EMT)[37,38].

In the claudin low subtype of CA breast, cell–cell junction proteins, including E-cadherin, are lacking. A growing number of studies suggest that claudins are involved in the regulation of CSC/TICs and chemoresistance[39]. Features of EMT are seen in them. In addition, they are consistently associated with an intense immune cell infiltrate[40].

Claudins in Tumor Progression

Claudin expression varies in a tissue-specific manner and they have different effects on different malignancies (Table 3).

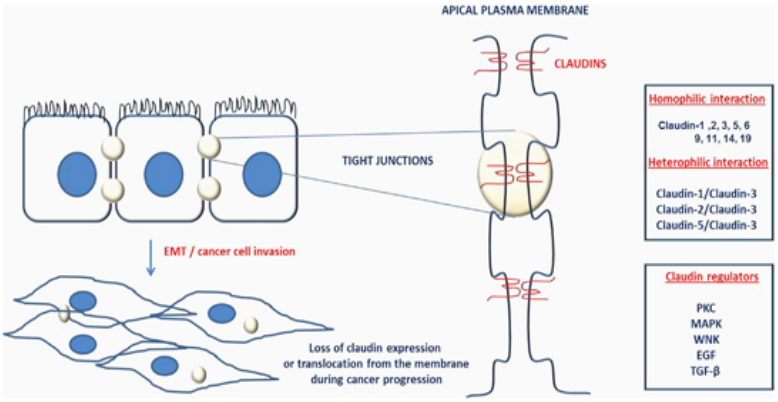


Fig 9: Role of Claudins in Cancer Cell Invasion

Claudins	Cancer	Function	*In vitro* or *in vivo*	Role
Claudin-1	Breast	Increase of cell migration	*In vitro*	Cancer promoting
	Breast	Anti-apoptotic effect	*In vitro*	Cancer promoting
	Colon	Increase of invasion and metastatic behavior	*In vitro* & *in vivo*	Cancer promoting
	Liver	Increase of invasion	*In vitro*	Cancer promoting
	Liver	Induction of EMT	*In vitro*	Cancer promoting
	Melanoma	Increase of cell motility and invasion	*In vitro*	Cancer promoting
	Oral	Increase of invasion	*In vitro*	Cancer promoting
	Gastric	Inhibition of tumorigenicity	*In vivo*	Tumor suppressive
	Lung	Inhibition of cell migration and invasion, *in vivo* metastasis	*In vitro* & *in vivo*	Tumor suppressive
Claudin-3	Ovarian	Increase of invasion	*In vitro*	Cancer promoting
	Ovarian	Promoting *in vivo* tumor growth and metastasis	*In vivo*	Cancer promoting
	Ovarian	Inhibition of *in vivo* tumor growth and metastasis	*In vitro* & *in vivo*	Tumor suppressive
	Ovarian	Suppression of EMT	*In vitro* & *in vivo*	Tumor suppressive
Claudin-4	Ovarian	Increase of invasion	*In vitro*	Cancer promoting
	Ovarian	Stimulation of angiogenesis	*In vitro* & *in vivo*	Cancer promoting
	Gastric	Inhibition of migration and invasion	*In vitro*	Tumor suppressive
	Ovarian	Suppression of EMT	*In vitro* & *in vivo*	Tumor suppressive
	Pancreatic	Suppression of cell invasion and metastasis	*In vitro* & *in vivo*	Tumor suppressive
Claudin-6	Gastric	Increase of proliferation, migration and invasion	*In vitro*	Cancer promoting
	Breast	Inhibition of anchorageindependent growth	*In vitro*	Tumor suppressive
	Breast	Inhibition of anchorageindependent growth, migration and invasion	*In vitro*	Tumor suppressive
Claudin-7	Colorectal	Increase of cell proliferation and tumorigenicity	*In vitro* & *in vivo*	Cancer promoting
	Ovarian	Increase of invasion	*In vitro*	Cancer promoting
	Esophageal	Decrease of cell growth and invasion	*In vitro*	Tumor suppressive
	Lung	Inhibition of migration and invasion, *in vivo* tumor growth	*In vitro* & *in vivo*	Tumor suppressive
Claudin-11	Bladder	Inhibition of cell invasion	*In vitro*	Tumor suppressive
	Gastric	Inhibition of cell invasion	*In vitro*	Tumor suppressive

Table 3: Role of Claudins in Human Cancers

Epithelial integrity is lost during malignant transformation due to loss of epithelial integrity that results from deranged TJ function that causes cells to lose polarity[41]. So it was assumed that loss of claudin expression contributed to tumor progression. However, growing evidence suggests that increased expression of claudins maybe involved in invasion and metastasis (Table 3). Mislocalization of claudins may have in addition, a role in tumorigenesis and increases paracellular permeability.

A number of signaling pathways participate in claudins mediated malignant change. For example, increased expression of claudin 2 is associated with increased incidence of hepatic metastasis.

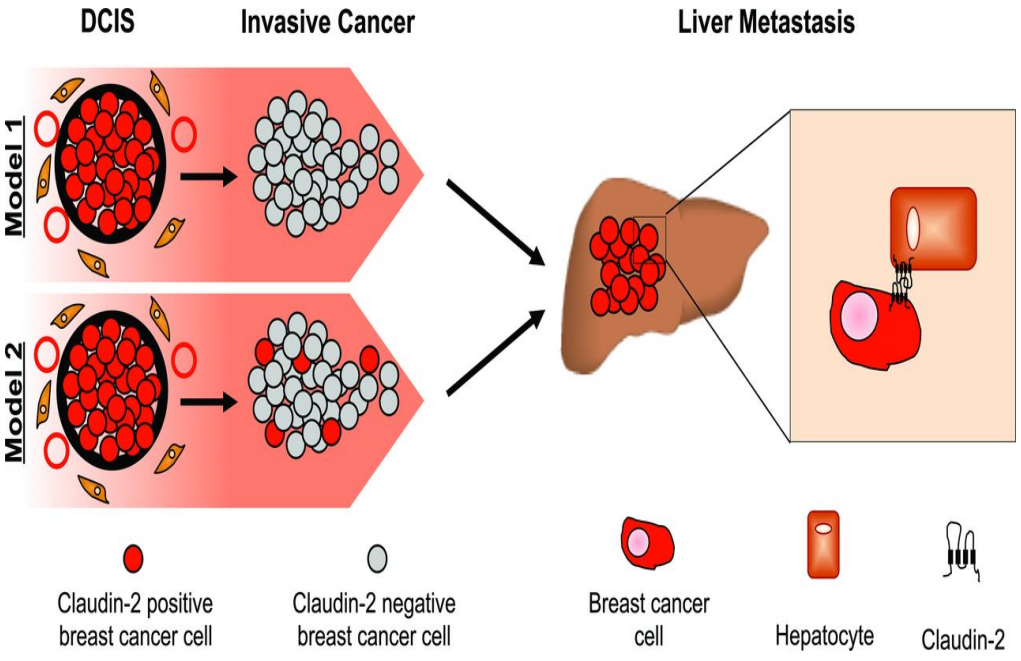


Fig 10: Claudin 2 & Hepatic metastasis in CA Breast

Claudin 1

Claudin 1 is deranged in several human cancers. It is probably the most commonly affected claudin. It has different roles in different cancers, causing in tumour suppression in some and progression in others.

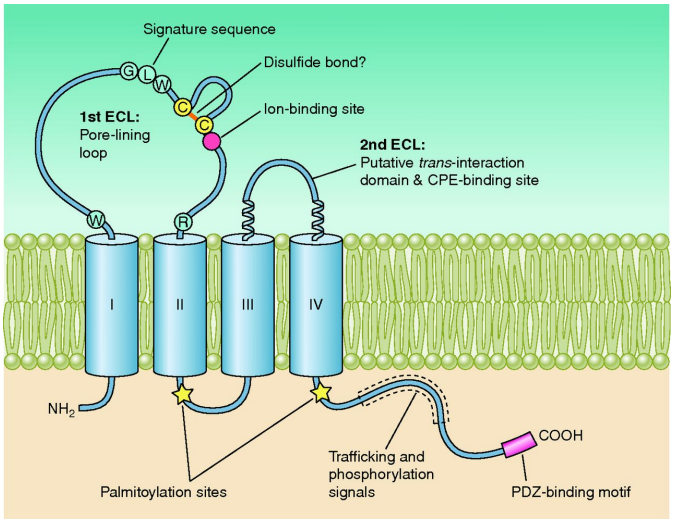


Fig 11: Claudin 1: Molecular Structure

Invasion and metastasis are probably the most commonly mediated phenomena in cells with altered claudin expression. Loss of CLDN1 in basal-like breast cancer cells decreases cell migration[42].

However, it has an anti-apoptotic effect in tamoxifen-treated human breast cancer cells and a tumor suppressive role in gastric cancer and lung cancer.

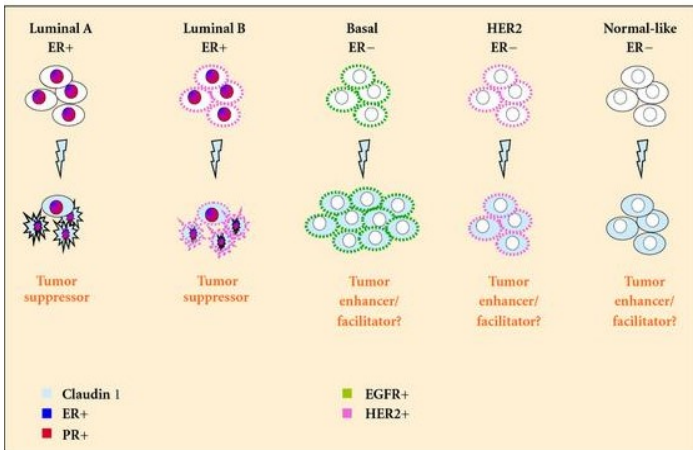


Fig 12: A hypothetical model of claudin 1 expression in different breast cancer subtypes.

Claudin 3 and Claudin 4

These genes are frequently dysregulated in ovarian cancer. Claudin 4 increases the production of angiogenetic factors. Metastasis of breast or ovarian cancers may be enabled by CLDN4 by interactions with extracellular matrix proteins[43].

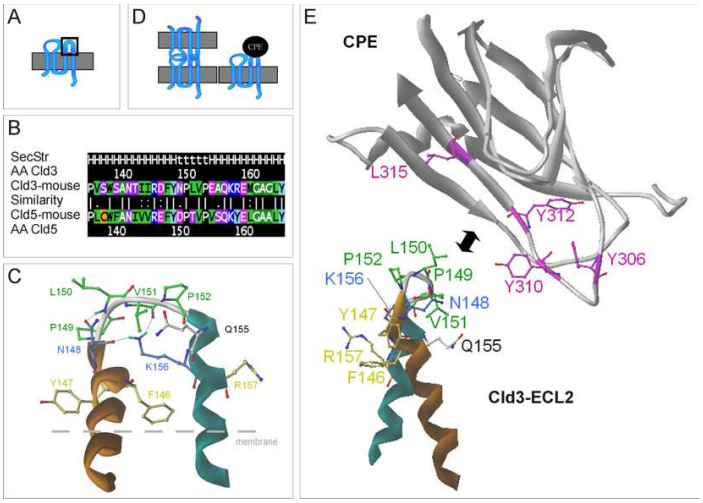


Fig 13: Claudin3: Molecular Structure

The loss of expression of CLDN 3 and 4 during tumorigenesis, maybe responsible for the low expression of E-cadherin, CLDN1 and 7 in the claudin-low breast cancer subtype[44,45].

A tumor suppressive role has also been associated with these genes in many cancers. Metastatic potential has been found to be reduced in pancreatic cancer. Several reports indicate that decreased expression of CLDN4 is linked to bad prognosis in breast, esophageal, colon and pancreatic cancers. It is thus postulated that claudin-4 probably plays a tumor suppressive role in these malignancies.

Claudin-7

Loss of Claudin-7 expression has been shown to be associated with rapid cell growth and invasion, probably as it causes loss of E-cadherin. Re-expression of normal levels of CLDN7 resulted in restoration of E cadherin function[46].

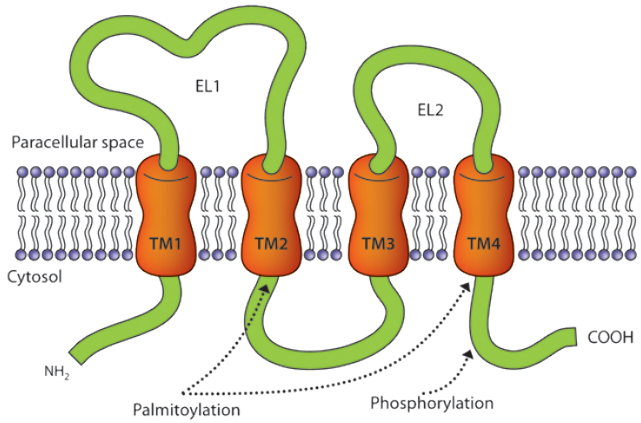


Fig 14: Claudin 7: Molecular Structure Molecular Structure

Evidence suggests that loss of CLDN 7 expression correlates with the grade of ductal carcinoma in situ and infiltrating ductal carcinoma. Alterations are also seen in lobular carcinoma in situ. It may thus promote tumor progression. CLDN7 overexpression in colorectal cancer cells disrupts cell polarization and enhances β-catenin activity and cell proliferation. This promotes tumor formation *in vivo* in xenograft mice injected with CLDN7 overexpressing colorectal cancer cells. Similar effects are seen in ovarian cancer cells.

Claudin Low Molecular Subtype of Breast Cancer

This newer molecular subtype of CA breast has lowered expression of CLDN1, CLDN3, CLDN4, CLDN7 and CDH1due to deranged tight junctions. These tumors are often triple-negative breast cancers, which lack the expression of estrogen, progesterone and epidermal growth factor receptor 2 (HER2). Stromal contamination or tumour-associated fibroblasts may be responsible for the mesenchymal features of these tumours. Several aspects of these tumours are not well understood and further evaluation is needed to understand them better[45]. They are characterized by an intense inflammatory infiltrate.

Several recent studies have shown that in the claudin low tumours, there in an increased generation of breast CSC/TICs. They are capable of better self-renewal *in vitro*, enhanced *in vivo* tumorigenicity and increased resistance to conventional chemotherapy.

There is sufficient evidence to suggest that they are generated by EMT[44].

Whether the loss of claudin expression is a cause or effect of this process remains to be conclusively determined in the claudin-low subtype of breast cancer. But there is little doubt that the role of claudins in CSC/TICs is becoming more apparent, and they may be promising new targets in the treatment of cancer.

Claudins in Chemoresistance

Failure of therapy usually occurs due to non-responsiveness of a particular tumour to chemotherapy[47]. Similar to antibiotic resistance, deciphering the causes for chemoresistance is imperative.

In a recent study it was seen that claudin 7 expression in lung cancer indicates sensitivity to cisplatin by the activation of caspases.

Low claudin expression has been linked to chemoresistance. A recent study showed that the malignant cells, which remain following conventional neoadjuvant chemotherapy in the low claudin group of breast cancer, are those with high CSC/TIC potential. This is likely to be due to CSC/TICs surviving chemotherapy[48].

CLDN11 is involved in DNA methylation mediated development of resistance to cisplatin[49]. Lowered CLDN 1 expression

is also associated with chemoresistance

The mechanisms involved in claudin induced drug resistance are thought to involve mechanisms of drug transport, apoptosis and CSC/TICs.

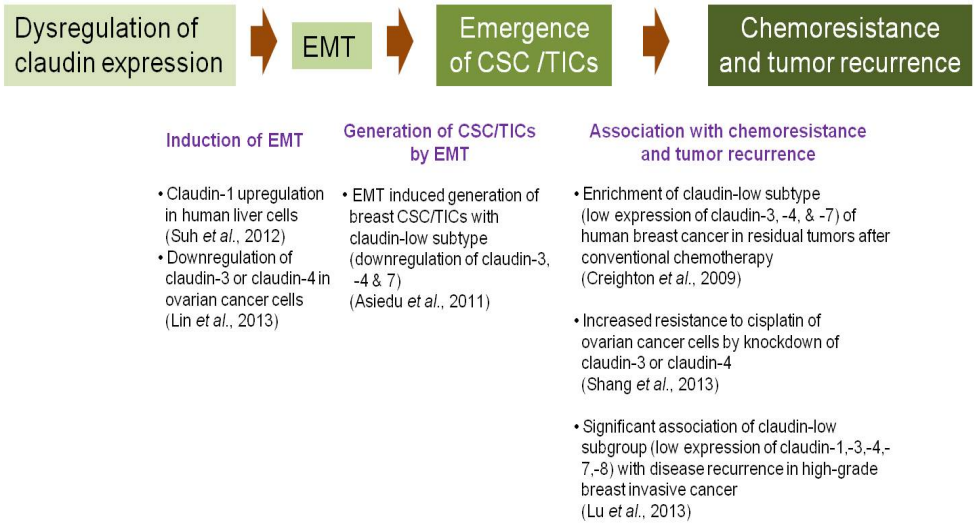


Fig 15: The Emerging role of claudins in EMT, CSC/TICs, and chemoresistance or recurrence.

The relationship between claudins and chemoresistance according to cancer type also remain to be determined, underlining the need for more studies in this area.

Claudins as Biomarkers and Therapeutic Targets

Claudin expression patterns in specific cancers are extremely consistent. This may translate into a role in detection and diagnosis. Recently CLDN 4 was detected in peripheral blood in circulating ovarian cancer cells.

The prognostic significance of variations of claudin expression is also being studied to establish clinical utility.

Decreased expression of claudin-1 indicates poor outcome in many malignancies. In addition, it is a reliable predictor of tumor recurrence. When claudin-1 expression is low in hepatocellular carcinomas, the degree of dedifferentiation and portal invasion are seen to be more. In breast cancer claudin-1 expression is positively correlated with a short disease-free interval. Variations in claudin-1 expression in different molecular subtypes of breast cancer, can mean that they can be used in the identification of certain subtypes. Loss of claudin-2 expression is linked with increased incidence of metastasis in breast cancer.

Increased CLDN4 expression is seen to markedly shorten overall survival and recurrence-free survival, indicating a poor prognosis[50]. In addition, studies indicate that this prognostic significance of CLDN3 or CLDN4 varies with the breast cancer subtype. For instance, a Japanese group showed that "the combination of claudin-4 and E-cadherin expression called CURIO accurately predicts relapse-free survival in breast cancer. CURIO was shown to predict prognosis, especially in luminal A and triple-negative subtypes of breast cancer: high expression of CURIO is related to worse prognosis and the converse is also true. The distinctive prognostic significance of claudin-3 and

claudin-4 in triple-negative and luminal types of breast cancer was analyzed in a recent report in which positive expression of claudin-3 was associated with poor prognostic factors, whereas claudin-4 expression was related to better prognostic factors in TNBCs."

Decreased CLDN7 expression has been linked to a poor prognosis several cancers[51]. In breast cancer, loss of CLDN7 expression is associated with:

- A higher histological grade
- A shorter time recurrence free interval
- Increased rate of metastasis

Different combinations of CLDN expression can predict disease recurrence, as shown in a recent study by Lu *et al.*[52], in which "the relationship between the expression of claudins 1, 3, 4, 7 and -8 and patient survival was analyzed in high-grade invasive breast cancer including several molecular subtypes. Low expression of all five claudins was mostly detected in basal-like cancers (77%), and patients with claudin-low tumors had significantly shorter recurrence-free survival, suggesting that low levels of claudin expression predict disease recurrence."

Claudins as Drug Targets in Cancer

The four transmembrane domains and two extracellular loops that CLDNs possess, are potential targets for antibodies that can be

developed. However, they have a low immunogenicity and this is a roadblock in the development of appropriate antibodies.

Recently, antibodies against the extracellular loops have been produced for CLDN 3 and 4. The antibody against CLDN4 has shown promising antitumour activity both *in vitro* and *in vivo*. Another development has been the preparation of a monoclonal antibody that acts against both CLDN 3 and 4, which was effective *in vitro* and *in vivo*. This antibody induced antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) *in vitro* and inhibited tumor formation *in vivo*[53].

Claudin-3 and claudin-4 are receptors for CPE. The exploitation of this property in anticancer therapy has been considered. Small interfering RNA (siRNA) that target CLDNs are seen to suppress tumour growth and metastasis[54].

With the limited studies that are available indicate the potential value of CLDNs as targets for drug therapy. However the transition of such research into clinical application will require more extensive research and conclusive evidence.

TUMOUR PROTEIN 53 GENE

The TP53 gene is situated on the short arm of chromosome 17 and codes Tumour Protein p53. This protein is critical in assessing if

damaged DNA can be salvaged by repair, or if the damage is too extensive, in which case, further cell division will be prevented and the cell will undergo apoptosis. It is the most commonly mutated gene in human cancer

Hereditary breast cancer can be seen as part of Li-Fraumeni syndrome. Somatic mutations occur in 20 – 40% of all cases of CA Breast.

In any malignancy, the presence of this mutation indicates a poor response to treatment, and shortened survival[55]. There are in fact, different types of mutations, each of which affects the prognosis differently. Specifically, those affecting the DNA-binding motifs have been linked with a guarded prognosis.

Several studies have assessed the prognostic importance and predictive value of TP53 mutation in breast cancer. Most of them indicate a poor prognosis for tumours that possess this mutation, especially more so for those involving the DNA binding motifs[56]. In fact, the*TP53* mutation has now emerged as an independent predictor of poor prognosis. Missense mutations as well as non-missense mutations of residues that bind DNA resulted in a poor prognosis, the latter being worse. Non missense mutations cause loss of protein expression, and thereby, loss of function[57].

Although TP53 mutation independently is seen to predict poor prognosis, it has not been compared to a great extent with other markers of poor prognosis so far. For instance, in a study of women with node-negative breast cancer, a higher risk of recurrence and death was seen when there was *P53* mutation along with expression of HER 2 neu. Similar results have also been reached in expression profiling using micro-arrays, where it was seen that TP53 mutation was seen in association with profiles that indicated a poor outcome. When these expression profiles were excluded, the presence of a TP53 mutation status and size of the primary tumour were the strongest predictors of breast cancer survival. However, gene expression-based classification and tumor size gained precedence as independent predictors of survival when the former was included. The TP53 mutation is also strongly associated with the basal like molecular subtype of breast cancer[58]. An expression signature for *TP53* mutation status has been identified in human breast cancer that is a good predictor of patient survival.

The prognostic significance of TP53 mutations is substantial with the risk of death from breast cancer being in between 2 and 5.

Ki-67 PROLIFERATIVE INDEX

The **Ki-67** protein is exclusively seen in proliferating cells. It is a non-histone protein. It has a critical role in polymerase I-dependent ribosomal RNA synthesis. As expected, it is absent in resting cells (G_0). In the G1 and S phases of the cell cycle, Ki-67 levels are low and it seen to be at its highest level during mitosis. During interphase, the Ki-67 antigen is seen within the cell nucleus alone. It migrates to the surface of the chromosomes during mitosis.

Since it is only seen during the active phases of the cell cycle, it is an excellent marker for the determination of the fraction of dividing cells in a given cell population[59].While exact cut off levels to categorize tumours based of fraction of proliferating cells have not been arrived at as yet, arbitrarily, levels above 10%–14% are taken to be high risk lesions in terms of prognosis.

Its levels in a malignant tumour are determined by immunohistochemistry. A number of studies have verified its credibility as a proliferative index[60].

Rather than using the qualitative values directly to all tumours, it may be more prudent to consider its value in conjunction with other markers that have a prognostic significance, such as the IHC4 panel, which consists of the ER, PR and HER2 neu along with Ki67 index.

In the determination of residual risk in patients being treated prophylactically with Letrozole or Tamoxifen for the reduction of early breast cancer risk, Ki67 index is included in the algorithm. Further studies are required to accurately gauge its importance in these regards

Predictive Role of Ki67.

Various studies have of late shed light on the predictive role of expression of the response to specific chemotherapeutic agents. Several centres have tried to establish cutoff levels of Ki67 that can differentiate between Luminal A and Luminal B molecular subtypes of breast cancer[61].

Studies have reported that those malignancies with high Ki67 index responded better to the addition of docetaxel to fluorouracil and epirubicin chemotherapy in adjuvant treatment of ER-positive tumors. But there is also evidence that suggests that Ki67 index has no predictive value when cyclophosphamide, methotrexate, and fluorouracil were added to endocrine therapy in hormone-responsive node-negative disease. Thus, the determination of areas of utility remains unsatisfactory.

ER negative tumours are generally believed to be more responsive to adjuvant chemotherapy than their ER positive counterparts[62]. This may be explained by the fact that these tumours

overall are highly proliferative. Hence, the measurement of the Ki 67 index may help predict response to neoadjuvant chemotherapy.

Neoadjuvant Therapy and Pharmacodynamic Role of Ki67

In LABC, after the completion of NACT, the response of the tumour to such therapy is assessed and when there is a pathological complete response, it is a reliable predictor of disease free and overall survival of the patient.

The results of the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) study[63]and P024 study[64] of neoadjuvant letrozole vs tamoxifen demonstrated clearly that "the difference in the degree of Ki67 suppression between the study arms was the same as the difference in recurrence in equivalent large adjuvant trials, Arimidex, Tamoxifen Alone or Combined (ATAC) trial[65] and Breast International Group (BIG) 1–98 trial, respectively."The neoadjuvant study American College of Surgeons Oncology Group (ACOSOG) Z1031[66]also produced similar results, with no difference in Ki67 suppression between exemestane and anastrozole. This concurs with the results of the MA.27 trial where similar rates of disease-free survival were observed in patients when these drugs were used in adjuvant therapy.

Hence, Ki 67 index is an excellent primary endpoint in determining the effectiveness of neoadjuvant therapy, mainly but not limited to endocrine therapy[67]. Additionally, in one therapeutic neoadjuvant trial that tested the activity of gefitinib when added to anastrozole, Ki67 was chosen to be the primary endpoint, instead of tumor shrinkage. When this trial showed no benefit from gefitinib by either the Ki67 index or by clinical response, phase III clinical trials were abandoned in patients with early breast cancer.

In the P024 study, at the end of 4 months of neoadjuvant endocrine therapy with letrozole or tamoxifen, Ki67 index, pathological tumor size, node status, and ER status were seen to be independently associated with recurrence-free and overall survival[64]. A Preoperative Endocrine Prognostic Index (PEPI) derived from a combination of these factors was validated as predictive of long-term outcome in an independent dataset from the IMPACT trial.

Ki67 as a Pharmacodynamic Intermediate Endpoint.

If the Ki67 index remains unchanged early on in treatment, it might be due to therapeutic failure. In the IMPACT trial, the Ki 67 index following two weeks of endocrine therapy had a stronger association with time to recurrence compared with pretreatment Ki67 level. In addition, pretreatment Ki67 level was not significantly associated with time to recurrence in a multivariable model that

including the pretreatment and 2-week Ki67 values[68]. The pretreatment value is known to have prognostic importance and the change in the index in 2 weeks has a predictive importance. Hence, at the end of two weeks, it is the residual risk after endocrine therapy that is arrived at. The possible advantage of measuring 2-week Ki67 instead of pretreatment Ki67 is under evaluation in the 4000-patient "Perioperative Endocrine Therapy for Individualizing Care (POETIC)" window-of-opportunity study.

Using tumor samples from a phase II neoadjuvant trial with letrozole, Ellis et al. identified a group of patients in whom the Ki67 index was 10% or greater after 4 weeks of treatment. Thus the early evaluation of the Ki 67 index can be used to segregate ER-positive patients and subject them to NACT rather than neoadjuvant endocrine therapy[69]. Trials are now underway to further clarify this.

Ki67 as an Eligibility Criterion for Neoadjuvant Trials.

When a neoadjuvant trial is undertaken, it is usually to determine is a new therapeutic agent shows any activity against the malignancy being studied. Those patients with a low Ki67 index at the time of detection of the disease are unlikely to show any significant improvement. In this scenario, the pretreatment Ki67 index may be used to exclude such patients from trials[70].

Neoadjuvant Chemotherapy.

As of now, the value of Ki67 is greater with neoadjuvant endocrine therapy than chemotherapy. Studies have shown that the Ki67 index decreases in most patients undergoing neoadjuvant treatment and it is possible that a greater degree of reduction is associated with a better response[71]. Ki 67 index determined in residual tumours has a strong prognostic significance. This may help identify patients who would benefit from adjuvant therapy, as they would be the ones to benefit most from it.

RESULTS

In this study, the average age of women included was 49.96 years. They were arbitrarily classified in to age groups of less than or equal to 50 years and more than fifty years.

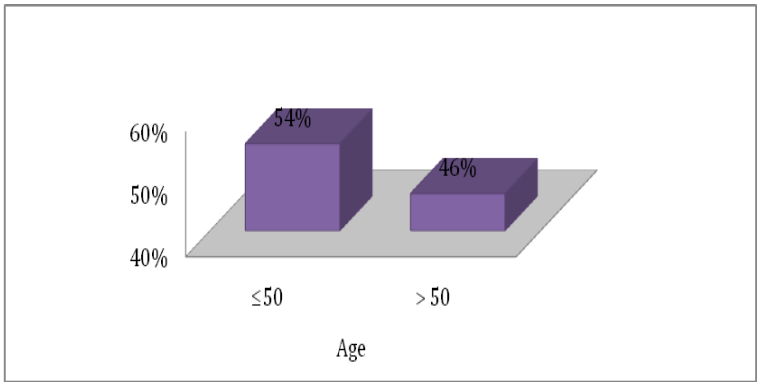


Chart 1: Age

There were 23 (46%) women of age more than fifty and 27 (54%) younger than or of fifty years of age. The cancer was present in the right breast in 29 (58%) and in left breast in 21 (42%) of these women.

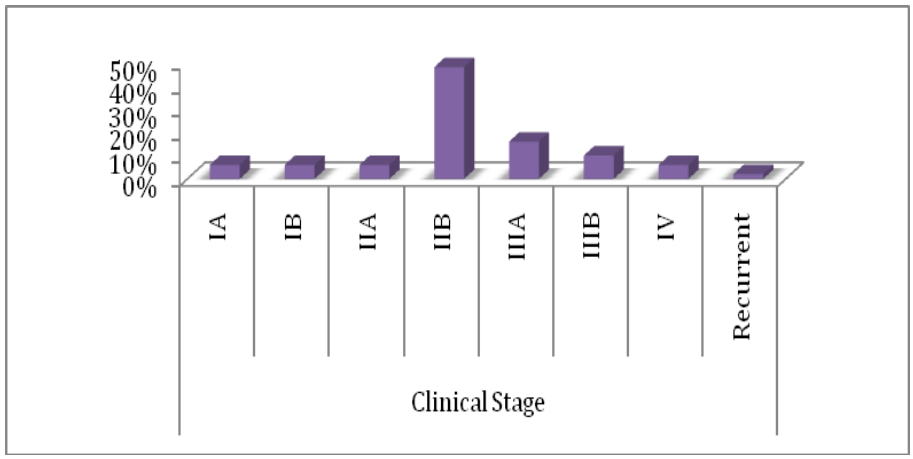


Chart 2: Clinical Stage at Presentation

When the clinical staging was initially assessed, it was seen that 3 (6%) women had Stage IA disease, 3 (6%) IIB, 3 (6%) IIA, 24 (48%) IIB, 8 (16%) IIIA, 5 (10%) IIIB and 3 (6%) metastatic Stage IV disease. 1 patient (2%) was a case of recurrent carcinoma. Hence Stage IIB disease was by far the commonest stage of presentation. The sizes of the primary tumours varied widely. Lesions as small as 2x2 cm and as large as 12x10 cm were seen.

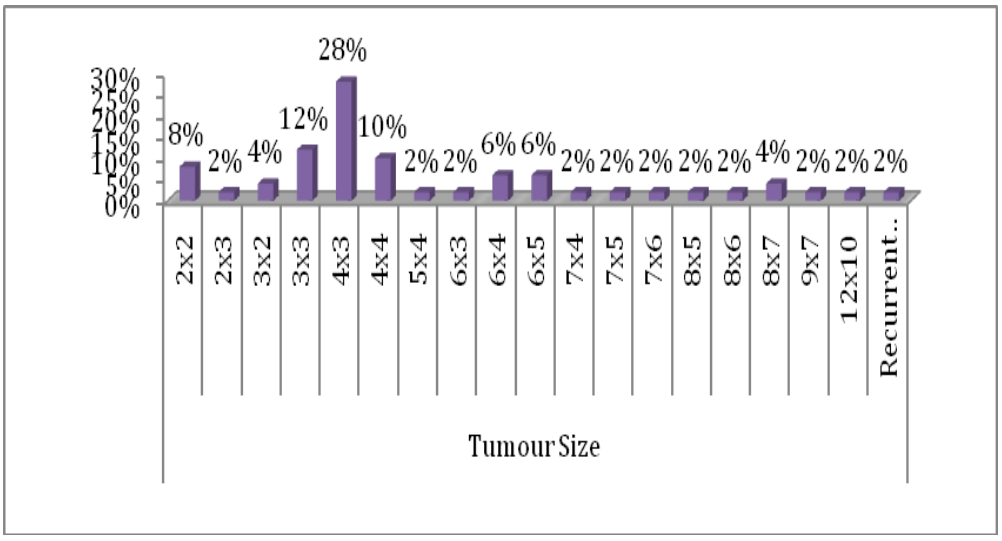


Chart 3: Tumour Size

In the patient with recurrence, it presented as a 2x1 cm nodule. They were all single lesions clinically. Mammogram confirmed the absence of multicentricity and multifocality in all patients.

Clinical or radiological presence of suspicious lymph nodes was seen in 26 (52%) of patients.

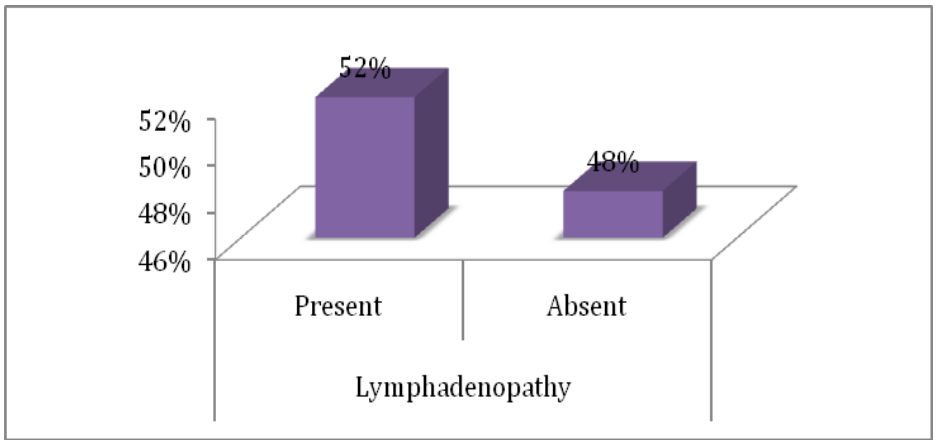


Chart 4: Clinical/Radiological Lymphadenopathy

Trucut biopsy taken for the confirmation of diagnosis showed that they were all uniformly infiltrating ductal carcinomas. Based on clinical stage, 33 (66%) were categorized as early breast cancer, 13 (26%) as locally advanced breast cancer and 4 (8%) were metastatic.

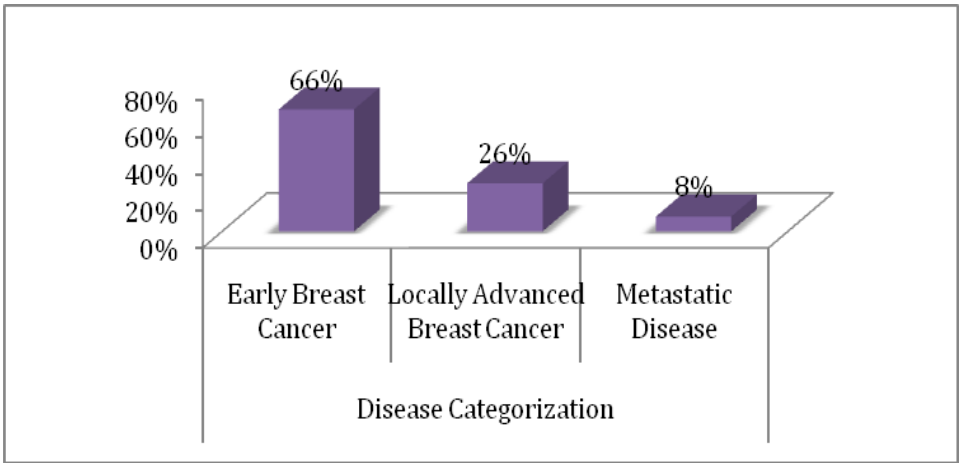


Chart 5: Disease Categorization

These patients were subjected to primary surgery, neoadjuvant chemotherapy followed by surgery and palliative care, respectively.

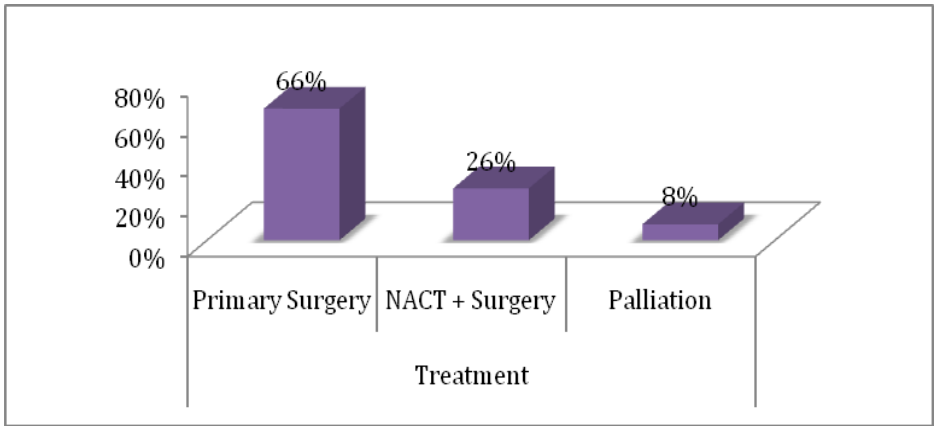


Chart 6: Treatment

Immunohistochemistry for hormonal receptor status showed that 30 (60%) of tumours were Estrogen Receptor positive, 25 (50%) Progesterone Receptor positive and 17 (34%) expressed the Human Epidermal Growth Factor Receptor, HER 2 neu.

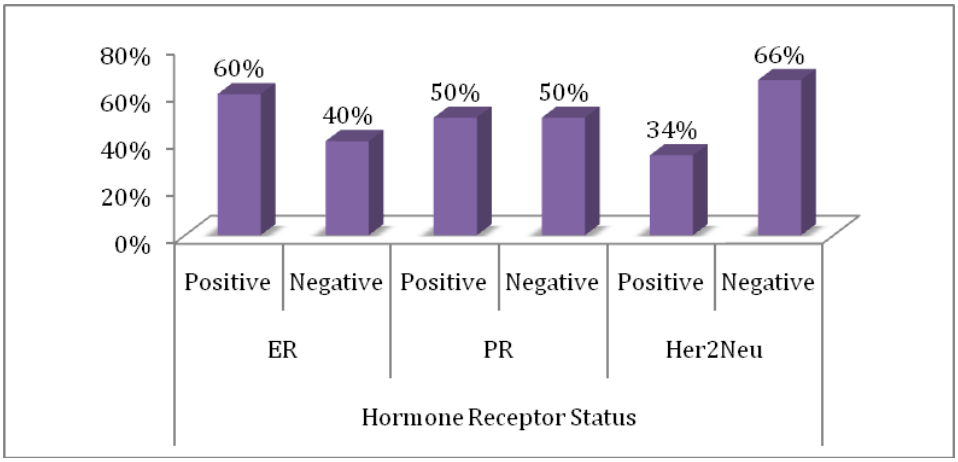


Chart 7: Hormone Receptor Status

Taken together, Luminal A molecular subtype was the commonest, and seen in 22 (44%) of tumours, while Luminal B tumours constituted 8 (16%), Her 2 enriched 9 (18%) and the Basal Like or Triple negative tumours accounted for 11 (22%).

Mutations of the Tumour Protein 53 and Ki 67 index were also assessed by Immunohistochemistry. P 53 mutations were seen in 12 (24%) of tumours.

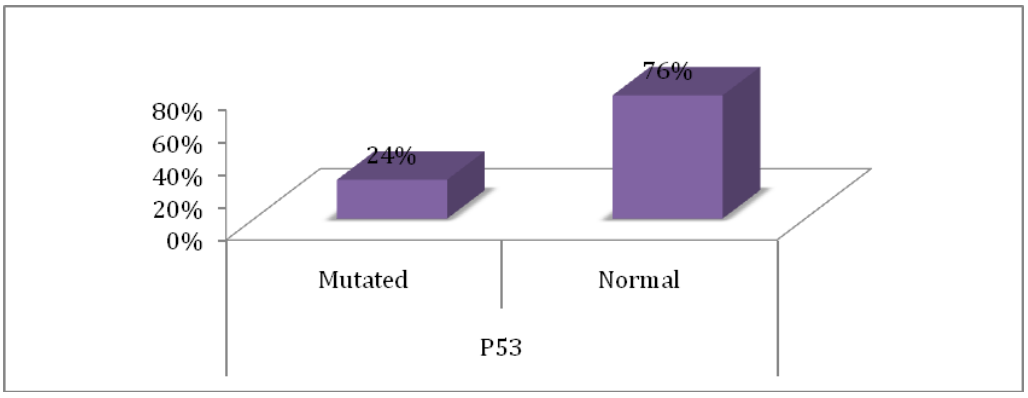


Chart 8: P53 Mutation

Ki 67 Index was quantified and the patients were divided into those with a high index (>15%) and a low index (≤15).

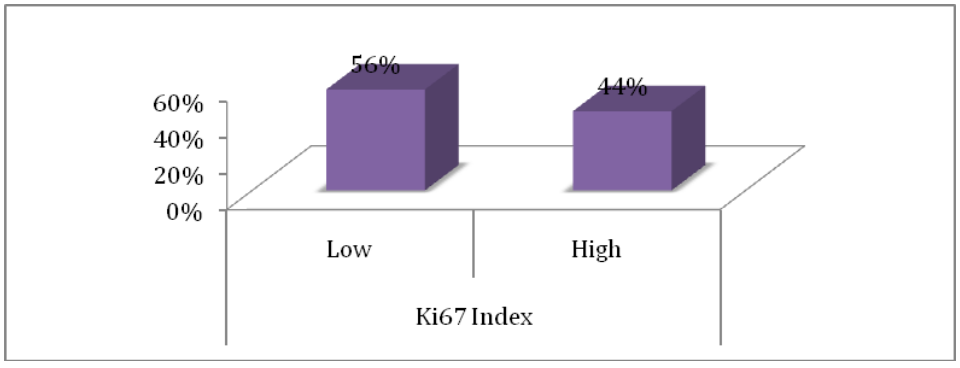


Chart 9: Ki 67 Index

There were 28 (56%) patients who had a low Ki67 Index and 22 (44%) with a high Index.

RT-PCR was done to assess the expression of Claudins 1, 3, 4 and 7. While CLDN 1, -3 and -4 showed low expression in 7(14%) patients, CLDN 4 showed low expression in 6(12%) of the patients who had low expression of the three other CLDNs and high expression in the seventh

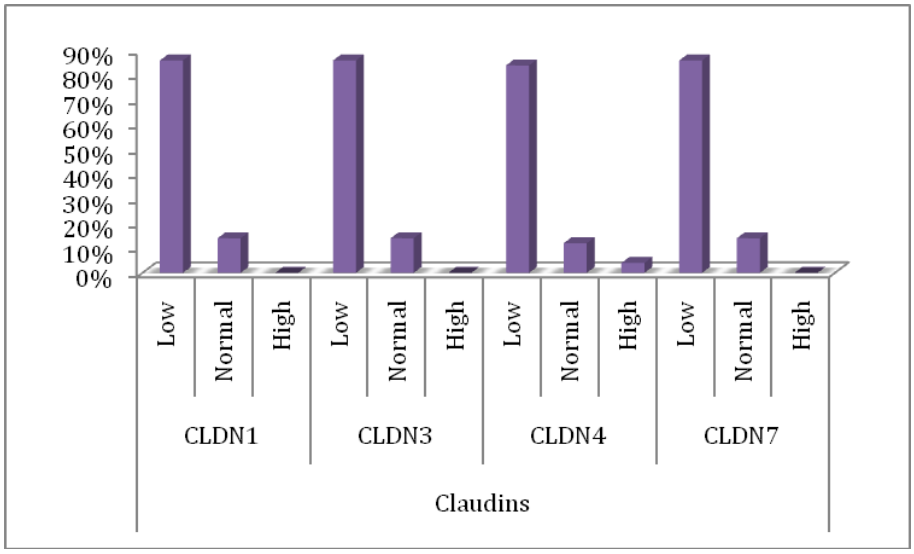


Chart 10: Expression of Claudins 1, 3, 4 & 7

patient (Patient No. 11), as well as in one patient (Patient 19) who had normal levels of expression of the other three. Thus, 2 patients (4%) had increased expression of Claudin 4. Normal levels were seen in the others.

These results were analysed using Statistical Package for the Social Sciences (SPSS). It was seen that the association between the expression of CLDN1, -3 and -7 and the age of the patients was not significant, with p = 0.524. Even with the application of a continuity correction of 0.053, the p value remained at 0.819

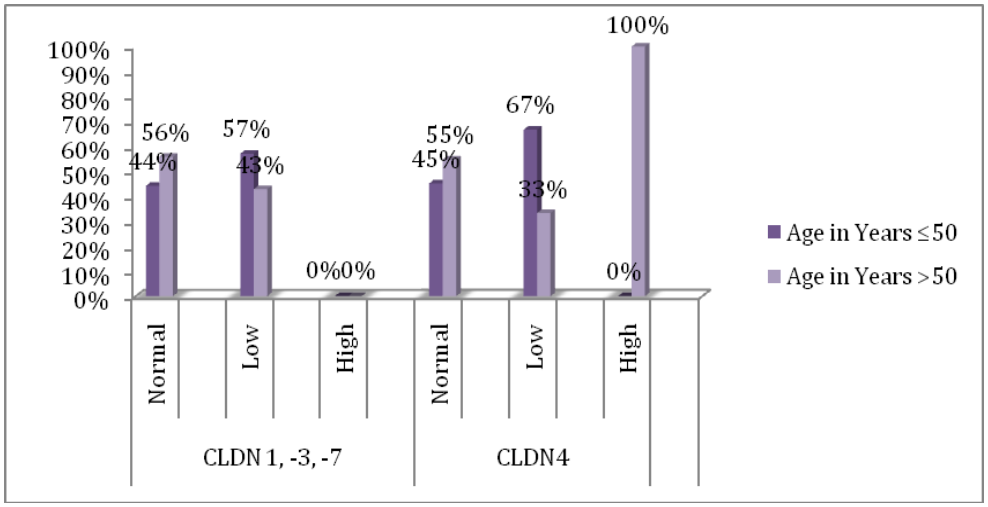


Chart 11: Expression of CLDN 1, 3, 4, and 7 vs. Age

Similarly, the association of CLDN 4 with age had a p value of 0.253 and hence, was also, not significant.

CLDN		Age in Years			Chi-Square Test		
		<=50	>50	Total	Value	df	p Value
CLDN1	Low	19 (44%)	24 (56%)	43 (100%)			
	Normal	4 (57%)	3 (43%)	7 (100%)			
	High	0(0%)	0(0%)	0(0%)			
CLDN3	Low	19 (44%)	24 (56%)	43 (100%)			
	Normal	4 (57%)	3 (43%)	7 (100%)	0.407	1	0.524
	High	0(0%)	0(0%)	0(0%)			
CLDN7	Low	19 (44%)	24 (56%)	43 (100%)			
	Normal	4 (57%)	3 (43%)	7 (100%)			
	High	0(0%)	0(0%)	0(0%)			
CLDN4	Low	19(42%)	23(55%)	42(100%)			
	Normal	4(67%)	2(33%)	6(100%)	2.745	2	0.253
	High	0(0%)	2(100%)	2(100%)			

Table 4: Association of Expression of CLDN 1, 3, 4 & 7 with Age

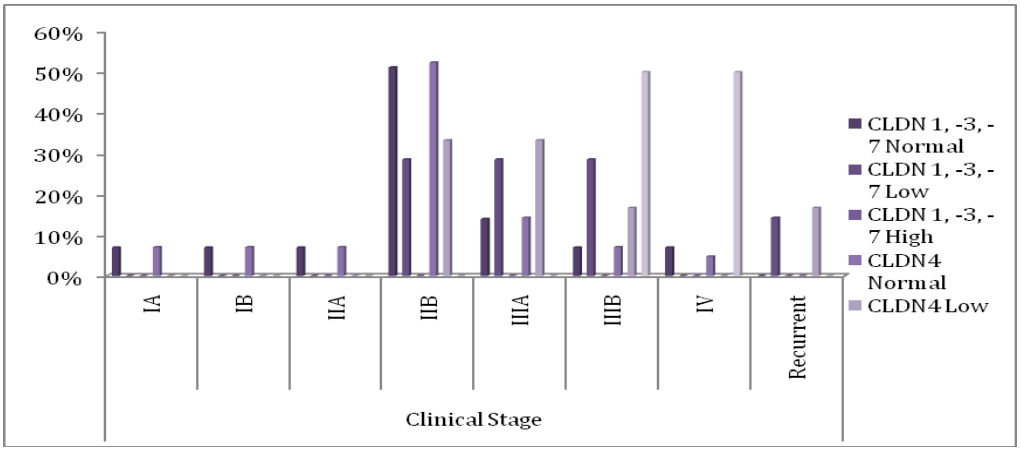


Chart 12: Clinical Stage vs. Expression of Claudins 1, 3, 4 & 7

When the association of clinical stage of breast cancer with levels of CLDN 1, -3, -4 and -7 was analysed, it was seen that there was no significant association between the clinical stage of disease and the expression of CLDNs with $p = 0.090$.

The same was true for the size of the primary tumour, and the association was deemed not significant at a $p = 0.065$.

CLDN		Clinical Stage									Chi-Square Test		
		IA	IB	IIA	IIB	IIIA	IIIB	IV	Recurrent	Total	Value	df	p Value
CLDN1	Normal	3 (7%)	3 (7%)	3 (7%)	22 (51)	6 (14%)	3 (7%)	3 (7%)	0 (0%)	43 (100%)	12.348	7	0.090
	Low	0 (0%)	0(0%)	0 (0%)	2 (29%)	2 (29%)	2 (29%)	0 (0%)	1 (14%)	7 (100%)			
	High	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)			
CLDN3	Normal	3 (7%)	3 (7%)	3 (7%)	22 (51)	6 (14%)	3 (7%)	3 (7%)	0 (0%)	43 (100%)			
	Low	0 (0%)	0(0%)	0 (0%)	2 (29%)	2 (29%)	2 (29%)	0 (0%)	1 (14%)	7 (100%)			
	High	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)			
CLDN7	Normal	3 (7%)	3 (7%)	3 (7%)	22 (51)	6 (14%)	3 (7%)	3 (7%)	0 (0%)	43 (100%)			
	Low	0 (0%)	0(0%)	0 (0%)	2 (29%)	2 (29%)	2 (29%)	0 (0%)	1 (14%)	7 (100%)			
	High	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)			
CLDN4	Normal	3(7%)	3(7%)	3(7%)	22 (52%)	6 (14%)	3(7%)	2(5%)	0(0%)	42 (100%)	22.698	14	0.065
	Low	0(0%)	0(0%)	0(0%)	2 (33%)	2(33%)	1(17%)	0(0%)	1(17%)	100.00%			
	High	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(50%)	1(50%)	0(0%)	6			

Table 5: Association of Expression Claudins 1, 3, 4 & 7 with Clinical Stage

When the association of presence of clinically and radiologically suspicious lymph nodes with expression of CLDN 1, -3, -7 was tested, it was seen to be significant with p = 0.006. (With a continuity correction of 5.44, p value was 0.02). The association of CLDN4 with clinical or radiological lymphadenopathy was significant at 0.012. The difference was thus, not due to chance.

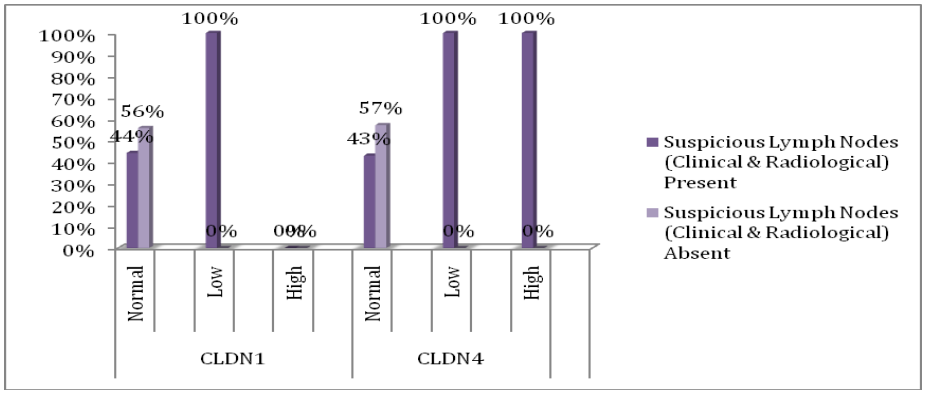


Chart 13: Clinical/Radiological Lymphadenopathy vs. Expression of Claudins 1, 3, 4 & 7

CLDNs		Suspicious Lymph Nodes (Clinical & Radiological)			Chi-Square Test		
		Present	Absent	Total	Value	df	p Value
	Normal	19 (44%)	24 (56%)	43(100%)			
CLDN1	Low	7 (100%)	0 (0%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
	Normal	19 (44%)	24 (56%)	43(100%)			
CLDN3	Low	7 (100%)	0 (0%)	7(100%)	7.513	1	0.006
	High	0 (0%)	0(0%)	0 (0%)			
	Normal	19 (44%)	24 (56%)	43(100%)			
CLDN7	Low	7 (100%)	0 (0%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
	Normal	18(43%)	24(57%)	42(100%)			
CLDN4	Low	6(100%)	0(0%)	6(100%)	8.791	2	0.012
	High	2(100%)	0(0%)	2(100%)			

Table 6: Association of Expression Claudins 1, 3, 4 & 7 with Clinical/Radiological Lymphadenopathy

On analysing the association of expression of CLDNs 1, 3, and -4 with Estrogen receptor (ER) positivity, a significant p value of 0.008 was arrived at. (With a continuity correction of 5.046, the p value was 0.025.) ER positivity was also found to be significantly associated with CLDN 4 expression (p = 0.004).

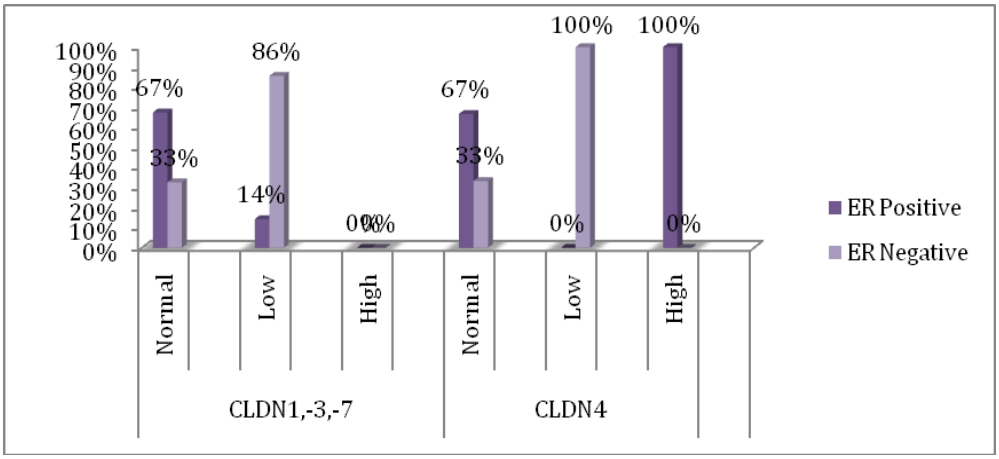


Chart 14: Estrogen Receptor vs. Expression of Claudins 1, 3, 4 & 7

CLDNs		ER			Chi-Square Test		
		Positive	Negative	Total	Value	df	p Value
CLDN1	Normal	29(67%)	14(33%)	43(100%)	7.087	1	0.008
	Low	1(14%)	6(86%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN3	Normal	29(67%)	14(33%)	43(100%)			
	Low	1(14%)	6(86%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN7	Normal	29(67%)	14(33%)	43(100%)			
	Low	1(14%)	6(86%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN4	Normal	28(67%)	14(33%)	42(100%)	11.111	2	0.004
	Low	0(0%)	6(100%)	6(100%)			
	High	2(100%)	0(0%)	2(100%)			

Table 7: Association of Expression of Claudins 1, 3, 4 & 7 with Estrogen Receptor

The association of the expression of CLDNs 1, 3, and 7 with the Progesterone receptor (PR) was not significant as, although the Pearson Chi-Square test showed a p value of 0.042, after applying a continuity correction of 2.658, the p value was 0.103. That of CLDN 4, however, was significant with p = 0.015.

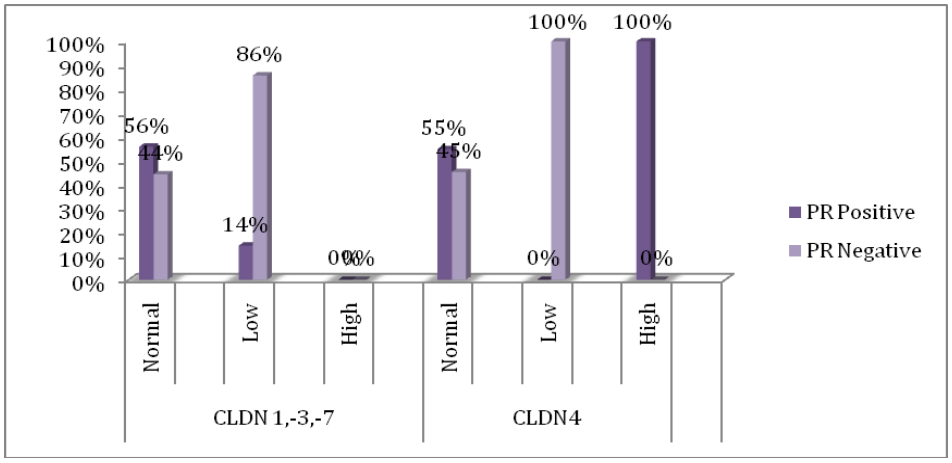


Chart 15: Progesterone Receptor vs. Expression of Claudins 1, 3, 4 & 7

CLDNs		PR		Total	Chi-Square Test		
		Positive	Negative		Value	df	p value
CLDN1	Normal	24(56%)	19(44%)	43(100%)	4.153	1	0.042
	Low	1(14%)	6(86%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN3	Normal	24(56%)	19(44%)	43(100%)			
	Low	1(14%)	6(86%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN7	Normal	24(56%)	19(44%)	43(100%)			
	Low	1(14%)	6(86%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN4	Normal	44(55%)	19(45%)	42(100%)	8.381	2	0.015
	Low	0(0%)	6(100%)	6(100%)			
	High	2(100%)	0(0%)	2(100%)			

Table 8: Association of Expression of Claudins 1, 3, 4 & 7 with Progesterone Receptor

When association of HER2 neu expression with that of CLDNS 1, 3 and & 7 and CLDN4 was, however not significant with p=0.744 and p =0.581, respectively.

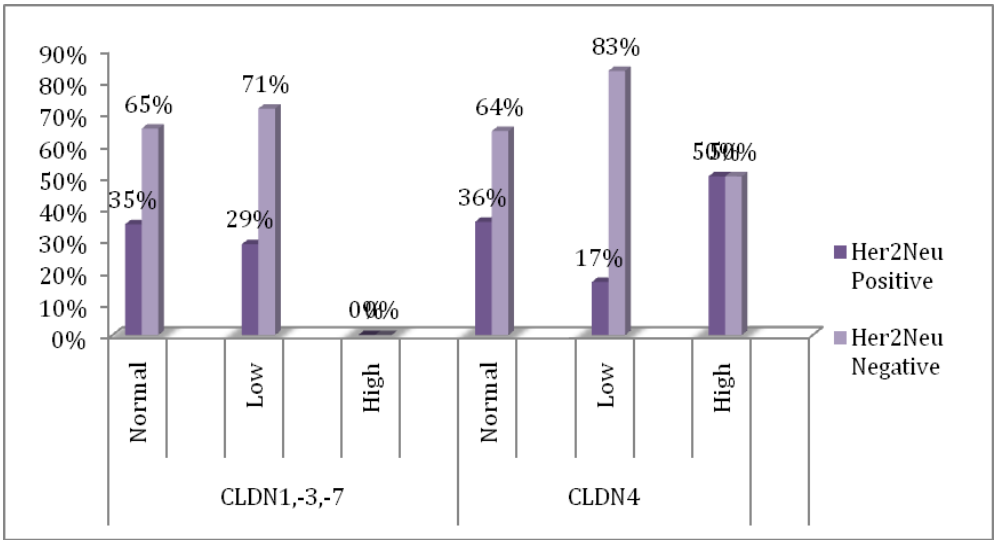


Chart 16: HER 2 neu vs. Expression of Claudins 1, 3, 4 & 7

CLDNs		HER 2 neu		Total	Chi-Square Test		
		Positive	Negative		Value	df	p value
CLDN1	Normal	15(35%)	28(65%)	43(100%)	0.107	1	0.744
	Low	2(29%)	5(71%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN3	Normal	15(35%)	28(65%)	43(100%)			
	Low	2(29%)	5(71%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN7	Normal	15(35%)	28(65%)	43(100%)			
	Low	2(29%)	5(71%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN4	Normal	15(36%)	27(64%)	42(100%)	1.086	2	0.581
	Low	1(17%)	5(83%)	6(100%)			
	High	1(50%)	1(50%)	2(100%)			

Table 9: Association of Expression of Claudins 1, 3, 4 & 7 with HER 2 neu Expression

By determining ER, PR and HER 2 neu, the tumours were categorised into their molecular subtypes. As the area of focus of this study is the triple negative tumours, their association with the expression of CLDNs 1, 3, 4 and 7 was determined and it was found to be highly significant.

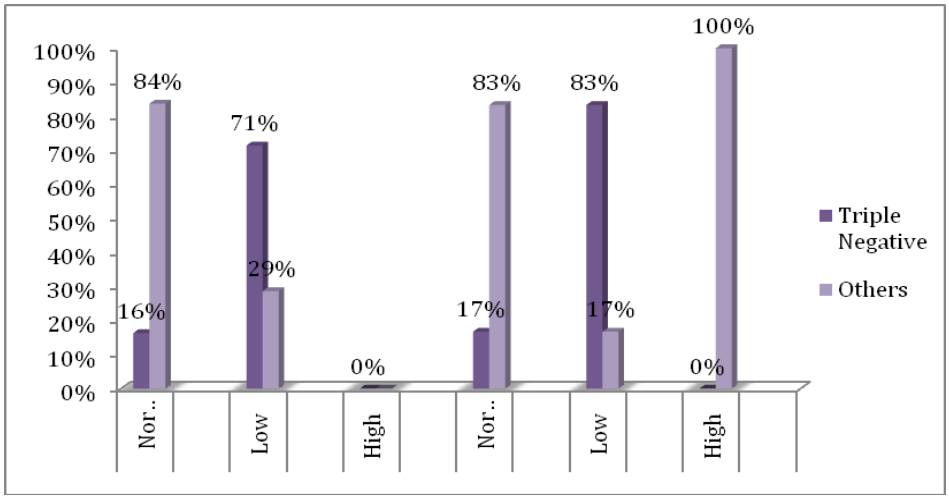


Chart 17: Triple Negative Tumours vs. Expression of Claudins 1, 3, 4 & 7

CLDN		Triple Negative Tumours			Chi sqare test		
		Yes	No	Total	Value	df	p Value
CLDN1	Normal	7 (16%)	36 (84%)	43 (100%)	10.038	1	0.002
	Low	5 (71%)	2 (29%)	7 (100%)			
	High	0(0%)	0(0%)	0(0%)			
CLDN3	Normal	7 (16%)	36 (84%)	43 (100%)			
	Low	5 (71%)	2 (29%)	7 (100%)			
	High	0(0%)	0(0%)	0(0%)			
CLDN7	Normal	7 (16%)	36 (84%)	43 (100%)			
	Low	5 (71%)	2 (29%)	7 (100%)			
	High	0(0%)	0(0%)	0(0%)			
CLDN4	Normal	7(17%)	35(83%)	42 (100%)	13.450	2	0.001
	Low	5(83%)	1(17%)	6 (100%)			
	High	0(0%)	2(100%)	2 (100%)			

Table 10: Association of Expression of Claudins 1, 3, 4 & 7 with Triple Negative Tumours

The loss of TP53 was analysed with respect to the expression of CLDN 1, 3, 4 and 7. A strong association was found with a highly significant p value (p = 0.000).

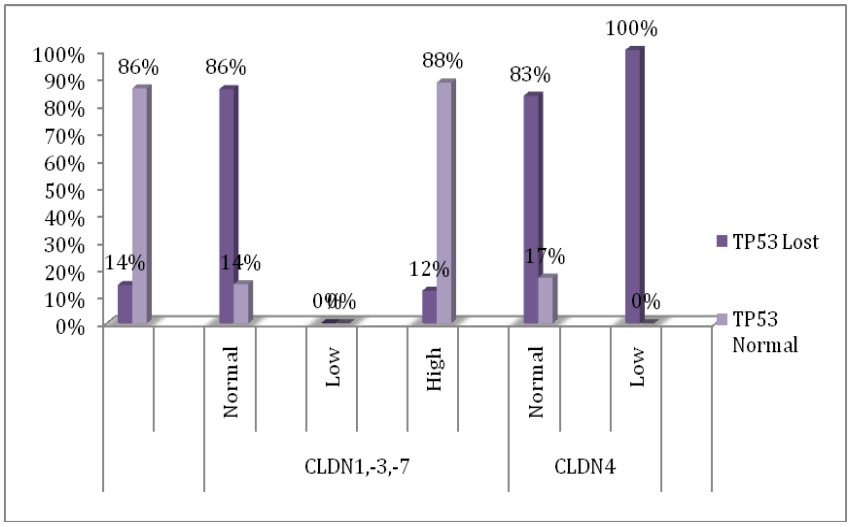


Chart 18: Loss of P53 vs. Expression of Claudins 1, 3, 4 & 7

CLDNs		TP53		Total	Chi-Square Test		
		Lost	Normal		Value	df	p value
CLDN1	Normal	6(14%)	37(86%)	43(100%)	16.996	1	0.000
	Low	6(86%)	1(14%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN3	Normal	6(14%)	37(86%)	43(100%)			
	Low	6(86%)	1(14%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN7	Normal	6(14%)	37(86%)	43(100%)			
	Low	6(86%)	1(14%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN4	Normal	5(12%)	37(88%)	42(100%)	21.282	2	0.000
	Low	5(83%)	1(17%)	6(100%)			
	High	2(100%)	0(0%)	2(100%)			

Table 11: Association of Expression of Claudins 1, 3, 4 & 7 with Loss of TP53

Finally, the Ki 67 index of these patients was found to be significantly association with the expression of CLDN 1, 3, 4 (p = 0.001) and CLDN 7 (p = 0.005)

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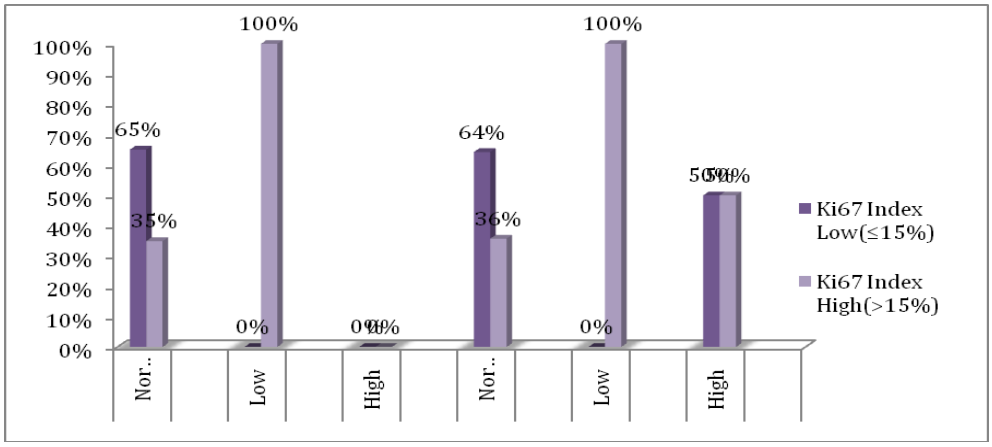

Chart 19: Ki 67 Index vs. Expression of Claudins 1, 3, 4 & 7

CLDNs		Ki 67 Index		Total	Chi-Square Test		
		Low(≤15%)	High(>15%)		Value	df	p value
CLDN1	Normal	28(65%)	15(35%)	43(100%)	10.359	1	0.001
	Low	0(0%)	7(100%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN3	Normal	28(65%)	15(35%)	43(100%)			
	Low	0(0%)	7(100%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN7	Normal	28(65%)	15(35%)	43(100%)			
	Low	0(0%)	7(100%)	7(100%)			
	High	0 (0%)	0(0%)	0 (0%)			
CLDN4	Normal	27(64%)	15(36%)	42(100%)	8.836	2	0.005
	Low	0 (0%)	6(100%)	6(100%)			
	High	1(50%)	1(50%)	2(100%)			

Table 12: Association of Expression of Claudins 1, 3, 4 & 7 with Ki 67 Index

DISCUSSION

Among the patients included in this study, 58% had right-sided breast cancer, whereas cancer of the left breast is more common in literature. The average age of the women included in this study was 49.96 years, which concurs with data from several epidemiological studies done in India by Bhadoria et al[72]and Kokiwar et al[73], among others. It is slightly less than the median age in the Western population, which is 53 years[74]. The most common clinical stages at initial assessment were Stages IIB and IIIA. 48% presented in Stage IIB and 16% in IIIA. This data also coincides with that in the above studies.

All tumours were single lesions and they were all uniformly infiltrating ductal carcinomas. This could be attributed to the limited number of subjects in this study. A larger number would have yielded variations.

52% of patients presented suspicious lymph node enlargement. This data also coincides with the available demographic data for the Indian Subcontinent[75]. Metastasis to axillary nodes is generally regarded as the single most important variable in the prognostication of CA breast. Szasz et al, in a study published in 2011, describe regional lymph node metastasis in association with CLDN 1, 3, 4, 5 and 7. It was seen that CLDN 1 expression was lesser in primary tumours that

presented with lymph node metastasis, than in the lymph nodes themselves. CLDNs 3, 4 and 7 were expressed at a lower level in the lymph nodes, than in the primary tumour. CLDN 5 expression, however was seen to be higher in the metastases, than in the primary lesion.

60% of tumours showed Estrogen receptor positivity, 50 % Progesterone receptor positivity and 34% expressed the HER2 Receptor. This is much higher than the levels seen in studies in the Indian population. Desai et al[76] found ER positivity of 32.6% and PR positivity of 46.1% in a study conducted in 2000. More recent studies, such as that by Kaul et al[77] in 2011 showed ER positive tumours to constitute 34.5 and PR positive tumours, of 36.4 in their study. The expression of Her 2 neu was slightly higher than the general consensus of 25 – 30%[78,79].

22% of tumours were triple negative. This is higher than the rate found in studies in the West (11.2%) such as Dent et al[74]. There is a marked variation in the rates found in the Indian population in studies by Ram Prabhu et al (24.5%)[80] and Suresh et al (12.5%)[81]. Ko et al, in a paper presented at the 2013 ASCO Annual Meeting, declared that the percentage of low claudin expression in their study the included 342 cases of triple negative breast disease was 20.5%. 5 out of the 12 triple negative cases (42%) showed low expression of CLDNs 1, 3, 4 and 7.

TP53 mutations were found in 12 patients (24%) patients. This is marginally lower when compared with literature with most studies quoting a range of 25 – 40% in sporadic breast cancer[82,83,84]. In an interesting study published in June, 2014 by O'leary et al, the authors showed that expression of the mammotrophic hormone, prolactin or its receptor decreased the latency of tumours when there were preexisting p53 mutations. Most tumours thus detected were also triple negative. The role of prolactin in breast tumourigenesis is gaining prominence as several epidemiological studies have demonstrated an association.

The Ki 67 index was seen to be low in 28(56%) and high in 22(44%) of tumours, where low Ki67 was taken as ≤15% and high was >15%. This concurs with the available literature[85,86] Several studies indicate that while simple assessment of ER, PR and HER 2 neu status of tumours maybe insufficient to accurately classify breast cancer into its intrinsic subtypes, this stratification of Ki 67 index in to high and low can help differentiate Luminal A from Luminal B tumours.

The expression of CLDNs 1, 3, 4 and 7 determined by RT PCR yielded low expression of CLDN 1, 3 and 7 in 7 (14%) patients. Low expression of CLDN 4 was seen in 6 (12%) and increased expression in 2 (4%) of patients. Tokés et al reported similar results when studying Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions in 2005[87]. In literature, several variations in claudin expression that affect the tumour phenotype in different

ways have been described[88].

Claudin gene expression showed significant association with:

- The presence of clinical or radiologically suspicious lymph nodes
- ER status
- PR status was only significantly associated with the expression of CLDN 4, and not with that of CLDN 1, 3 and 7.
- Triple negative tumours
- P 53 mutation, and
- Ki 67 index.

Every one of these associations indicates an aggressive tumour phenotype. Regardless of the stage of presentation, these patients must be expected to have a shorter disease free survival and overall survival[89]. These tumours are often only found to have partial response to NACT[48].

In a study consisting of 446 cases or primary sporadic breast cancer, Ricardo et al[88] showed that in the low claudin subtype of breast cancer was significantly associated while CLDN 1 was not

significantly associated with tumour size, the association with CLDNs 3, 4 and 7 were significant. The presence of lymph node enlargement, while associated with expression of CLDNs 3 and 7, was not associated with the expression of CLDN 1 and 4. Histological grade was significantly associated with CLDNs 1, 3 and 4 but not with CLDN 7. ER and PR positivity was significantly associated with CLDN 1 and 4, but not with 3 and 7.

In this study, significant association was not seen with age, clinical stage and size of primary tumour at initial assessment and HER 2 neu expression. In addition only CLDN 4 had a significant association with PR status. In a study by Blanchard et al, published in 2013, high expression of CLDN 1 was significantly associated with triple negative tumours in women of or above the age of 55, while CLDN 4 was not. In the same study, there was no association between the expression of CLDN 1 and tumour size. Lanigan et al in a study published in 2009 also found no significant association between CLDN 4 expression and age, tumour size, histological type or lymphadenopathy.

In the study by Ricardo et al quoted above, HER 2 neu expression only showed significant association wit CLDN1, but not with the rest. Ki 67 index showed no statistical significance in its association with any of the claudins studied. Other studies have shown

that the patterns of survival in patients expressing HER 2 neu varied with the expression of ER and PR.

While most of the available data state the lack of an association between HER 2 neu and claudin expression, several studies quote that there is a significant association with the presence of p53 mutations. But this association was not analyzed in this study, and could be ascertained from the available data as an extension.

In a study with 226 consecutive patients conducted between 1996 and 2009, Lu et al showed that HER2 expression was not significantly associated with either increased or decreased expression of CLDNs 1, 3, 4, 7 and 8. This is in concurrence with our study.

The rate of growth of the primary and progression of the disease may be better parameters than a single measurement of tumour size and the stage of the disease at initial assessment. This maybe attributed to the difference in threshold of these women to visit the hospital. Some may rush to the doctor at the slightest suggestion of a lump, while others, albeit rare in today's scenario, may only come with the onset of pain or ulceration.

SUMMARY

The present study was conducted to find the association between disease severity and hormone receptor status, P 53 mutations, KI 67 index and expression of CLDNs 1, 3, 4 and 7. The study was conducted in the General Surgery Department of ESIC Medical College & PGIMSR over a period of 18 months. Fifty women with CA breast were included in the study. The mean age of women in this study was 49.96 years. Right-sided lesions were more common. The size of the primary tumour varied from 2x2 cm to 12x10 cm. The commonest stages of presentation were Stage IIB (48%) and Stage IIIA (16%). All tumours were single lesions and were histopathologically infiltrating ductal carcinomas. 52% had suspicious lymph node enlargement. 60% shower ER positivity, 50% had PR positivity and 24% showed expression of HER 2 neu. P 53 mutations were found in 24% of patients. Ki 67 index was low in 56% of patients and high in 44%. Low expression of CLDN1, 3 and 7 was found in 14%, while low expression of CLDN 4 was seen in 12% of patients. Increased expression of CLDN 4 was found in 4%.

There was a statistically significant association between the expression of CLDNs 1, 3, 4 and 7 and:

- The presence of clinically or radiologically suspicious

lymph nodes

- ER status
- PR status was associated with CLDN 4 expression alone
- Triple negative tumours
- P 53 mutation, and
- Ki 67 index.

There was no association, however, with the age of the patient, clinical stage at presentation, tumour size, HER 2 neu expression and that of CLDN 1, 3 and 7 with PR status.

CONCLUSION

The present study concludes that there is a significant association between the alterations in expression of CLDNs 1, 3, 4 and 7 and:

- The presence of clinical or radiological lymphadenopathy
- ER status
- PR status was associated only with CLDN 4 expression
- Triple negative tumours
- Loss of TP53, and
- Ki67 Index

There was no significant association between the expression of these CLDNs and the age of the patient, clinical stage of presentation, HER2 neu expression and that of CLDN 1, 3 and 7 with PR status.

While this is purely an observational study, it has helped establish a baseline demographic profile of patients with breast cancer in the present set up. This information can be carried forward and used to formulate larger studies that can verify the above findings and their statistical authenticity. It can also help design studies where parameters such as response to chemotherapy by pathological cure rate, recurrence free survival and overall survival can be ascertained.

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ANNEXURE I

S. No.

PROFORMA

Name:

Age/Sex:

Side and Size of Tumour:

Clinical Stage:

Multifocality/Multicentricity by Mammogram:

Lymph Nodes (Clinical/Radiological) (Yes/No):

- **Trucut Biopsy:**
    - **Diagnosis:**
    - **IHC:**
    - **ER:**
    - **PR:**
    - **HER 2 neu:**
    - **P 53:**
    - **Ki67 Index:**

RT-PCR for Expression of:

- **CLDN1:**
- **CLDN3:**
- **CLDN4:**
- **CLDN7:**

ANNEXURE II

INFORMED CONSENT

Informed consent for patients who are operated on in ESI-PGIMSR hospital, and whom we are inviting to participate in the research titled **"Correlation of Disease Severity, Estrogen, Progesterone & Epidermal Growth Factor Receptor Status with Altered Expression of Claudins 1, 3, 4 & 7, TP53 & Ki67 antibody in Carcinoma Breast" AT ESIC Medical College & PGIMSR, KK Nagar, Chennai, 2013-14**. Dr. Veena Bheeman, M.S. (General surgery) post graduate is the principal investigator of this research under ESIC Medical College & PGIMSR, Chennai.

Part I: INFORMATION SHEET

Introduction

We, **Dr. Veena Bheeman**, 1st Year General Surgery PG, guided by Dr**.R.Anbazhakan**, Professor & HOD of Surgery, are going to give you information and invite you to be a part of this research. Before you decide, you can talk to anyone of us you feel comfortable with about the research. This consent form may contain words that you do not understand. Please ask us to stop as we go through the information and we will take time to explain. If you have questions later, you can ask us.

Purpose of the research

We will be studying whether there is correlation between the disease severity, estrogen, progesterone & epidermal growth factor receptor status with altered expression of Claudins 1, 3, 4 & 7, TP53 & Ki67 antibody in carcinoma breast.

Type of Research

This research will involve your participation in a non-experimental manner, with assured privacy and confidentiality.

Right to Refuse or Withdraw

Your participation is strictly voluntary. Refusal to participate will not affect subsequent services to you

Procedures

If you give consent to participate in this study, we will ask you questions regarding your disease. A history will be taken recording symptoms, duration of disease and a family history of similar illness. As per the research protocol, we will perform the diagnostic, staging, preanaethetic and general investigations required to assess your health in general and your illness in particular. After assessment of your illness, the available treatment options will be discussed with you. If surgery is feasible and you decide to undergo the same, the tissue that is operated on and removed inclusive of the tumour and glands of the armpit will be sent for histopathological examination as is routinely done with all operated specimens. Pathological grading of your the will be performed along with gene

expression studies for Claudins 1, 3, 4 and 7, p53 and ki67 antibody and immunohistochemistry for estrogen, progesterone & EGFR status.

The severity of your disease as assessed in the preoperative, intraoperative and postoperative periods will be correlated with the expression of Claudins 1, 3, 4 & 7, TP53 and Ki67 antibody and positivity or negativity of estrogen, progesterone and EGFR status.

Confidentiality

All information you provide will be kept confidential. Your name will not be used in any way.

Whom to Contact

If you have any questions, you can ask them now or later. If you wish to ask questions later, you may contact:

Dr. Veena Bheeman , General Surgery PG

Ph no: 9952372160

Guide: Dr R,Anbazhakan, Professor & HOD Of Surgery & Dr. Uday Shamrao Kumbhar, Associate Professor Of Surgery, Department of General Surgery, ESIC Medical College & PGMSR , KK NAGAR, Chennai-78

This proposal has been reviewed and approved by Institute Ethics Committee, which is a committee whose task is to make sure that research participants are protected from any harm.

If you have any questions regarding any part of the study, please feel free to ask.

Part II: CERTIFICATE OF CONSENT

I have read the information in the consent form (or it has been read to me.) I was free to ask any questions and they have been answered. I understand what is being requested of me as a participant in this study. I have been given satisfactory answers to my questions. I certify that I am more than 18 years of age. I freely consent to participate in the study called "**Correlation of Disease Severity, Estrogen, Progesterone & Epidermal Growth Factor Receptor Status with Altered Expression of Claudins 1, 3, 4 & 7, TP53 & Ki67 antibody in Carcinoma Breast"** at ESIC Medical College & PGIMSR,KK Nagar,Chennai, 2012-13.

I have read and understood this consent form and the information provided to me.

I have been explained about the nature of the study.

My rights and responsibilities have been explained by the investigator

I agree to cooperate with the investigator.

Currently I am not participating in any research study.

I hereby give permission to the investigators to release the information obtained from me as a result of participation in the study to the regulatory authorities, government agency, ethics committee. I understand that they may inspect my original records.

My records will be kept confidential

I have decided to participate in the study.

As I was not able to read, the consent form has been read out to me by the investigator and all my questions have been answered and I give my consent with my free will.

_____

Name of Participant

_____ _____

Sign of Participant Signature of Investigator

Date

ANNEXURE III

Informed Consent in Tamil

மருத்துவர் வீணா பீமன் தனது முதுநிலை அறுவைச்சிகிச்சை பட்டப்படிப்பு முழுமைபெறுவதற்காக மேற்கொள்ளும் மார்பக புற்று நோய் ஆய்வினைப்பற்றி எனக்கு என் தாய்மொழியில் விளக்கப்பட்டது. இந்த ஆய்வில் Claudins 1, 3, 4 & 7, TP53 Ki-67 ஆகியவற்றின் அளவு மார்பக புற்று நோயின் தன்மையயும் அதன் Estrogen, Progesterone & Her2 receptor நிலையயும் எவ்வாறு பாதிக்கிறது என்பதை ஆறாயிந்து கண்டறிய உள்ளார் என புறிந்துகொண்டேன்.

இந்த ஆய்வின் அனைத்து அம்சங்களும் விளக்கப்பட்டது. இதில் என் முழு விருப்பத்துடன் கலந்து கொள்கிறேன். எனினும் எந்த நேரத்திலும், எந்த காரணமுமின்றி இந்த ஆய்விலிரிந்து விலகிக்கொள்ள எனக்கு முழு உரிமையுள்ளது. இதனால் எனது நலனில் எந்த பாதிப்பும் ஏற்படாது என்று உறுதியளிக்கப்படுகிறது. இந்த ஆய்வின் அறிக்கையில் என்னுடய தனிப்பட்ட விவரங்கள் அனைத்தும் ரகசியமாய் பாதுகாக்கப்படும் என்று எனக்கு உறுதியளிக்கப்பட்டிருகிறது. மேற்கூறிய உறுதிமொழிகள் யாவும் பின்பற்றப்படும் பட்சத்தில் நான் இந்த ஆய்வுக்கட்டுரைக்கு என்னுடய விவரங்களை அளிக்க முன்வருகிறேன். இதற்கு முழுமனதுடன் என்னுடய ஒப்புதலை தந்து இந்த ஆய்வறிக்கையில் பங்கேற்கிறேன்.

இப்படிக்கு,

(கையொப்பம்)