"A STUDY TO EVALUATE THE PRESENCE OF DISSEMINATED TUMOUR CELLS IN THE BONEMARROW AS AN INDEPENDENT PROGNOSTIC FACTOR IN BREAST CARCINOMA"

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CERTIFICATE

This is to certify that the Dissertation **"A STUDY TO EVALUATE THE PRESENCE OF DISSEMINATED TUMOUR CELLS IN THE BONEMARROW AS AN INDEPENDENT PROGNOSTIC FACTOR IN BREAST CARCINOMA"** presented herein by **Dr. P.GAYATHRI** is an original work done in the Department of Pathology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D. (Branch III) Pathology under my guidance and supervision during the academic period of 2008 - 2011.

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CERTIFICATE

I hereby certify that this work embodied in the dissertation entitled **"A STUDY TO EVALUATE THE PRESENCE OF DISSEMINATED TUMOUR CELLS IN THE BONEMARROW AS AN INDEPENDENT PROGNOSTIC FACTOR IN BREAST CARCINOMA"** is a record of work done by Dr.P.Gayathri, in the Department of Pathology, Tirunelveli Medical College, Tirunelveli, during her postgraduate degree course in the period 2008-2011. This work has not formed the basis for any previous award of any degree.

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This is to Certify that the Institutional Ethical Committee of Tirunelveli Medical College & Hospital, Tirunelveli -11 in its meeting held on 9th October 2009 has unanimously approved the dissertation titled A Study To Evaluate The Presence Of Disseminated Tumour Cells In The Bone Marrow As An Independent Prognostic Factor In Breast Carcinoma the work proposed by Dr. P. Gayathri, Postgraduate Student (MD) in Pathology of the Department of Pathology , at Tirunelveli Medical College.

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DECLARATION

I solemnly declare that the dissertation titled **" A Study To Evaluate The Presence of Disseminated Tumour Cells In The Bonemarrow As An Independent Prognostic Factor In Breast Carcinoma"** is done by me at Tirunelveli Medical College hospital, Tirunelveli.

The dissertation is submitted to The Tamilnadu Dr. M.G.R.Medical University towards the partial fulfilment of requirements for the award of M.D. Degree (Branch III) in Pathology.

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CONTENTS

INTRODUCTION

Breast cancers are one of the most common Non dermatological cancers occurring in women and are one of the leading causes of cancer related deaths in women. It is reported that more than 1000000 cases occur world wide annually (Parkins DM et al 2001)[1]

It is quintessential in clinical practice to understand the growth, spread, and outcome of these tumours to give the patients a significant and comfortable long term survival. This needs a strict pattern of diagnostic, prognostic and treatment methods that shall take into account the behaviour of the tumour, characteristics of the treatment and milieu of the patient.

The important prognostic information in breast cancers is provided by the clinical staging of the tumour and pathological grading. The various grading and staging methods use parameters like nuclear grade, tumour size, presence or absence of lymphatic and vascular invasion, axillary lymphnode involvement, sanctity of the resection margins and steroid receptor status.

Nonetheless, 20-30% of these patients who had been accorded a favourable prognosis during or immediately after completion of treatment, do suffer relapses / recurrences within 5 years, while paradoxically some patients who were assigned a poorer prognosis initially, do survive for more than 10 years (Vincent Salomon A et al 2007)[2]. Hence this throws up a very interesting question of what makes this difference.

This paradox indicates the existence of certain factors that point to some specific biological nature of the tumour that subsumes all such existing and

accepted parameters of prognostication this disease. The haematogenous dissemination of breast cancers is not significantly addressed in the present prognostic criteria, though they can occur independent of the spread by lymphogenous routes (Akiyama H et al 1994)[3]. Hence it is primarily imperative that all patients need to be searched for evidence for such haematogenous dissemination. The presence of bone marrow micro-metastases have been shown to be associated with such early treatment failures and hence to be considered as a poor prognostic factor in the treatment and management of patients with cancers of breast (Braun S et al 2005)[4.] The presence of bone marrow disseminated tumour cells (DTC) is associated with a poor outcome for patients in stage I to III disease in Breast cancer. (Braun S et al loc. cit 2005). The dissemination is represented as either isolated or microaggregates of tumour cells, which have the potential to establish overt metastases at a later time, and are not detectable by serological tests such as tumour markers or by radiological imaging (Liotta LA et al 1991)[5]. These micro-metastases have been detected in approximately 90% of patients who have had curative therapy according existing protocols. This explains the frequent early tumour recurrences that occur in patients after such curative treatments (O'Brien et al 1995)[6]. Early spread of tumour cells usually remain undetected even by very high resolution imaging technologies. The CT scan detects peritoneal or hepatic metastases with a sensitivity of 21 % and 47% respectively (Posner MR et al 1994)[7].

Sensitivity and specificity of serum tumour antigen is limited. These techniques analyse the primary tumours for its DNA content, Oncogenes, Tumour

suppressor genes and proliferative markers. They do not provide a direct measure of tumour burden of metastatic spread (Pandha HS et al 1995) [8].

In contrast, the clearance of such metastatic cells from the bone marrow either spontaneously or as a response to targeted antibody therapy is seen to be associated with an improved patient survival . (Akiyama H et al loc.cit 1994).

In this context, there is a real need for newer and more accurate methods for the identification of these disseminated tumour cells in the bone marrow. The current challenge for the pathologist is to improve and standardise the methods of early detection of DTC and hence this study is taken.

AIMS AND OBJECTIVES

1. To evaluate the Bone Marrow of patients with Breast Carcinoma for Disseminated Tumour Cells.
2. To evaluate the relationship of the presence of Disseminated Tumour Cells in the Bone Marrow in patients with Breast Carcinoma with their Clinical stage, and Pathological parameters of the Primary Tumour.
3. To compare and correlate the significance of the presence of Disseminated Tumour Cells in the Bone Marrow in relation to other prognostic factors.

REVIEW OF LITERATURE

The incidence of breast cancer has slowly increased over the past 20 years but now appears to have levelled off. Death rates have decreased during the last 25 years because of earlier detection and better therapy. Currently, one in eight American women may be expected to develop breast cancer, one quarter of whom will die of the disease. In Western industrialized countries with high rates of breast cancer, the incidence of this tumour continues to increase throughout life.

Epidemiology of Breast Cancers

Breast Cancers are more common in women in socio economically developed countries like in North America, Western Europe and Scandinavia (Harris JR et al 1992)[9]. The disease is uncommon before the age of 35 years. (Holford TR et al1991)[10]. Breast cancer is four to five times more frequent in Western industrialized countries than in developing countries. It has been suggested that diet, in particular dietary fat, may in part explain differences in the geographical distribution of breast cancer, but this concept remains controversial. Breast cancers accounts for nearly 32% of cancers in women (Jemal A et al 2003)[11]

Classification of Breast Cancers

Breast Cancers are classified as Epithelial Tumours, Myoepithelial Tumours, Mesenchymal Tumours, Fibroepithelial Tumours, Tumours of the Nipple, Malignant Lymphomas and Metastatic Tumours (Tavossoli F A et al 2001)[12]. Of these Epithelial Tumours / Carcinomas of the Breast are more common than the others. Epithelial Tumours of the Breast (Breast Carcinomas) are further

classified into Ductal and Lobular Carcinoma (Susan C et al 2004)[13]. Of these, the Infiltrating Ductal Carcinoma of the Breast are more common constituting 90%. The most common histological subtype is NOS type of ductal carcinoma, that forms almost 70% of the cases (Dixon JM et al 1985)[14.] The other variants of ductal carcinoma constitute 15-20%. Lobular Carcinomas, though unique accounts for 10-15% of the cases of breast carcinomas. The distribution of the different types of breast carcinomas (Table No 1) shows a similar worldwide variation.

Table.1.Histologic types of invasive breast cancer in four large series before the widespread use of mammographic screening

	Histologic Type								
Study	**No. of Cases**	**Ductal[a] (%)**	**Lobular (%)**	**Medullary (%)**	**Mucinous (%)**	**Tubular (%)**	**Tubular Mixed (%)**	**Mixed (%)**	**Other (%)**
Fisher and colleagues[15]	1,000	53	5	6	2	1	-	32	-
Rosen [16]	857	75	10	9	2	2	-	-	-
Ellis and colleagues[17]	1,547	49	16	3	1	2	14	14	2
Page and Anderson [18]	Not stated	70	10	5	2	3	2		8

Pathobiology of breast carcinoma

The pathogenesis of breast cancer is poorly understood but epidemiologic, molecular and genetic studies outline complex risk factors. Breast cancers also exhibit diversity in histopathology, molecular features and overall patient outcomes. Hence, the disease can be viewed as a multifaceted and complex epithelial malignancy. The strongest association with an increased risk for breast

cancer is a positive family history, specifically breast cancer in first-degree relatives (mother, sister & daughter). The risk is greater when the relative is afflicted at a young age or with bilateral breast cancer. Some of the major risk factors include the following.

Table 2. Risk Factors associated with breast cancer.

Factor	Relative Risk
Well-Established Influences	
Geographic factors	Varies in different areas
Age	Increases after age 30yrs
Family history	
First-degree relative with breast cancer	1.2-3.0
Premenopausal	3.1
Premenopausal and bilateral	8.5-9.0
Postmenopausal	1.5
Postmenopausal and bilateral	4.0-5.4
Menstrual history	
Age at menarche <12yrs	1.3
Age at menopause >55yrs	1.5-2.0
Pregnancy	
First live birth from age 25 to 29yrs	1.5
First live birth after age 30yrs	1.9
First live birth after age 35yrs	2.0-3.0
Nulliparous	3.0
Benign breast disease	
Proliferative disease without atypia	1.6
Proliferative disease with atypical hyperplasia	>2.0
Lobular carcinoma in situ	6.9-12.0

Less Well-Established Influences	
Exogenous estrogens	
Oral contraceptives	
Obesity	
High-fat diet	
Alcohol consumption	
Cigarette smoking	

Bilimoria MM,et al.(1995) [19].

The factors also include: **1. Hormonal Status:** A link between breast cancer and the hormonal status of women is strongly suggested by the association of (1) early menarche, (2) late menopause, and (3) older age at first pregnancy with an increased risk of disease. Nulliparous women, or those who become pregnant for the first time after age 35 have a two to three fold higher risk of breast cancer than women whose first pregnancy occurred before age 18 (Wang DY et al 1985)[20]. **2. Radiation:** The female breast is susceptible to radiation induced neoplasia. The risk of breast cancer was increased in atomic bomb survivors and in women irradiated for postpartum mastitis Shore RE. et al.(1977)[21] and Hodgkin disease; the highest risk occurred when exposure took place in childhood and adolescence (Hildreth NG et al 1983)[22]. Modern mammographic techniques use extremely low doses of radiation that are unlikely to pose a hazard (Goss PE et al 1998)[23] **3. Previous Cancer of the Breast:** Women who have previously had breast cancer have at least a 10-fold increased risk of developing a second primary breast cancer in the same or in the contra lateral breast. **4. Fibrocystic Change:** Women with proliferative fibrocystic change and particularly those demonstrating

atypical hyperplasia are at increased risk for cancer (Kern WH et al 1969)[24] , (Steinhoff NG et al 1970)[25].

Genetic Factors in Breast Cancers: The major factors include: a. The BRCA1 gene (breast cancer 1), a tumour suppressor gene located on chromosome 17q21 has been implicated in the pathogenesis of hereditary breast and ovarian cancers and possibly prostate and colon cancer. Mutations in this tumour-suppressor gene are thought to be carried by 1 in 200 to 400 people in the United States. Germ line point mutations and deletions in BRCA1 confer a 60% to 85% lifetime risk for breast cancer, with more than half of the tumours developing before 50 years of age. It is currently suspected that mutated BRCA1 is responsible for 20% of all cases of inherited breast cancer and is responsible for about 3% of all breast cancers. Somatic mutations in BRCA1 are infrequently detected in sporadic breast cancers (Futreal PA et al 1994)[26]. **b**. The BRCA2 gene, located on chromosome 13q12, has been incriminated in approximately 20% of hereditary breast cancers. Women with one copy of a mutated BRCA2 gene have a 30% to 40% lifetime chance of developing breast cancer. Like patients with BRCA1, these women have an increased risk of ovarian cancer. BRCA2 mutations also put male carriers at increased risk of breast cancer. Mutations of BRCA2 are particularly common among Ashkenazi Jewish women (Struewing JP 1997)[27].

c. The p53 gene is mutated in the Li-Fraumeni syndrome .Breast cancer will develop in almost all young women with the disease. Germ line (inherited) mutations in p53 account for 1% of breast cancers among women under the age of

40 years. Somatic p53 mutations are common in sporadic breast cancers (Allered DC et al 1993)[28].

Clinical Presentation in Breast Cancers: Breast cancer often discovered by the woman or her physician is a deceptively discrete, solitary, painless, and movable mass typically 2 to 3 cm in size at diagnosis. Involvement of the regional axillary group lymph nodes is already present in about 45% patients. With mammography, these tumours are more frequently detected before they become palpable. The average invasive carcinoma found by screening is around 1 cm in size and only 15% of these have nodal metastases.

Clinical Prognostication of Breast Cancers

Prognosis is influenced by the following factors as emulated by the American Joint Committee on Cancer. American Joint Committee On Cancer: AJCC Cancer Staging Manual ,6th edition. New York, Springer (2002)

1. ***Size of Primary Tumour***. Invasive carcinomas smaller than 1 cm have an excellent prognosis in the absence of lymph node metastases and may not require systemic therapy.
2. ***Lymph node involvement***. With no axillary node involvement, the 5-year survival rate is close to 90%. The survival rate decreases with each involved lymph node and is less than 50% with 16 or more involved nodes. Sentinel node biopsy introduced as an alternative is less morbid than a full axillary dissection; is identified by using a dye or a radioactive tracer and is highly predictive of the absence of metastatic carcinoma in the remaining lymph

nodes. However, the clinical significance of the finding of micrometastases measuring less than 0.2 cm is still unclear.

3. ***Distant metastases***. Patients who develop hematogenous spread are rarely curable, although chemotherapy may prolong survival.
4. ***Tumour Grading***. The most common grading system for breast cancer evaluates tubule formation, nuclear grade, and mitotic rate to divide carcinomas into three groups.
5. ***Histological Typing***. All specialized types of breast carcinoma (tubular, medullary, cribriform, adenoid cystic, and mucinous) have a better prognosis than carcinomas of no special type ("ductal carcinomas").
6. ***Receptor Studies***. The presence of hormone receptors confers a slightly better prognosis and predicts response to therapy. The highest rate of response (80%) to anti-estrogen therapy like oophorectomy or tamoxifen is seen in women with both estrogen and progesterone receptors. Lowest rates of response (10%) is seen if both are absent.
7. ***Proliferative Index***. Proliferation is measured as mitotic counts which are usually included in the grading systems or by flow cytometry or through immunohistochemical markers. High proliferative rates are associated with poor prognosis.
8. ***Aneuploidy***. Abnormal DNA content have a worse prognosis.
9. ***Overexpression of HER2/NEU***. Overexpression of this membrane-bound protein, usually determined by immunohistochemistry or by fluorescence in situ hybridization is associated with a poorer prognosis.

Based on the AJCC guidelines (Tavassoli FA and Devilee P,eds loc.cit 2001), Breast carcinomas are divided into clinical stages as follows:

Stage 0. Ductal carcinoma in situ or Lobular carcinoma in situ (5-year survival rate: 92%).

Stage I. Invasive carcinoma 2 cm or less in diameter (including carcinoma in situ with microinvasion) without nodal involvement (or only metastases < 0.02 cm in diameter) (5-year survival rate: 87%).

Stage II. Invasive carcinoma 5 cm or less in diameter with up to three involved axillary nodes or invasive carcinoma greater than 5 cm without nodal involvement (5-year survival rate: 75%).

Stage III. Invasive carcinoma 5 cm or less in diameter with four or more involved axillary nodes; invasive carcinoma greater than 5 cm in diameter with nodal involvement; invasive carcinoma with 10 or more involved axillary nodes; invasive carcinoma with involvement of the ipsilateral internal mammary lymph nodes or invasive carcinoma with skin involvement (oedema, ulceration or satellite skin nodules), chest wall fixation, or clinical inflammatory carcinoma (5-year survival rate: 46%).

Stage IV. Any breast cancer with distant metastases (5-year survival rate: 13%)

In quintessence, the prognosis of the patient depends on whether the tumour has spread or not.

The most common cause of death in patients with breast carcinoma is metastatic disease (Panabieres CA et al 2007)[29]. Despite the numerous prognostic indicators currently in use, it is not fully possible to predict the outcome of treatment in most patients. This can be attributed in part to the metastatic behaviour of the tumour and the milieu intern of the patient. Though macrometastasis is not evident, the existence or absence of a minimal residual disease within the patient is not taken into account in the existing prognostic systems. The prediction of recurrences & relapses depend upon the presence of micro-metastatic foci in the patient at primary diagnosis (Catherine CA et al.,loc. Cit 2007).

Micrometastasis in Breast Cancer

Stephan Braun et al., loc.cit (2005) in a pooled analysis has assessed the prognostic significance of the presence of bone marrow micrometastasis. The presence of micrometastasis was a significant prognostic factor with respect to poor overall survival and breast cancer specific survival. Hence a clear understanding of the tumour biology and tumour behaviour is essential to evaluate the patient responses and their outcome.

Biology of Tumour Metastasis is the process by which discrete tumours form in a new site, often remote from the original parent neoplasm by seeding of tumour cells which arrive by active locomotion or passive transport through body fluids and subsequently grow progressively (Recamier JCA et al1829)[30]. Invasive breast cancer spreads primarily through the lymphatic and hematogenous spread. Sugarbaker EV set al (1982)[31] have demonstrated that there is good statistical

correlation between the size of the primary tumour and the incidence of metastasis for a number of common tumours including carcinomas of breast, colon, lung and malignant melanoma. However there are a significant number of patients in all of these tumour groups who either have small or clinically undetectable primaries with widespread large metastases or who have massive tumours and no secondaries because cell migration alone will not result in true metastasis. A number of steps are required for malignant cells to establish metastasis namely – (1) Invasion of the basement membrane underlying the tumour (2) Movement through the extracellular matrix (3) Penetration of vascular or lymphatic channels (4) Survival and arrest within the circulating blood or lymph (5) Exit from the circulation into a new tissue site (6) Survival and growth as a metastasis, a process that involves angiogenesis. The entire metastatic sequence from the initial binding of the tumour cells to the underlying extracellular matrix to the growth in a distant location depends on the expression of numerous molecules by the malignant cells like integrins, ICAM-1, MUC18,VCAM-1,E-cadherin, catenin(α,β), growth factors and cytokines.

Autocrine motility factor (AMF), a tumour cell cytokine stimulates motility via a receptor mediated signalling pathway. Matrix metalloproteinases, a zinc-dependent endopeptidase which under normal circumstances is susceptible to tissue inhibitors of matrix metalloproteinases and the balance is strictly regulated. In contrast, metastatic phenotypes of cancer cells have a dysregulation of this balance leading to matrix metalloproteinases induced basement membrane and extracellular matrix degradation followed by invasion and spread to distant sites by

vascular invasion or by local growth. Paget S et al (1889)[32] reviewed the autopsy records of 735 patients dying of breast cancer at the Middlesex hospital and he drew the analogy that disseminating cancer cells are like seeds, which after being scattered on the wind grow only in sites (soil) that are congenial and this therefore came to be known as his seed and soil hypothesis of metastatic spread. LeeY.T.N et al (1983)[33], Paget S et al loc.cit (1889) found that the incidence of deposits in bone in patients with breast cancer was approximately 70% compared with a figure of about 20% for other malignant tumours.

Cancer dormancy is an apparent tumour free or latent state in which disseminated malignant cells are present but are under growth control by biological mechanisms. Withdrawal or inhibition of these restraining mechanisms lead to overt tumour progression. Dormancy may be induced in disseminated tumour cells by lack of primary tumour microenvironment such as absence of stimulating growth factors, presence of growth inhibiting cytokines control by inhibition of immune containment or by stimulation of protumourigenic mechanisms (Yuhas JM et al 1978)[34], (Korah Ret al 2004)[35]. Transition from dormancy to progressive disease involves number of factors including genetic alterations in the cancer cells which enhance its survival and replication ability, immune escape capability and angiogenic potential of the tumour cells the relative ability to stimulate angiogenesis. According to Pierga JY et al (2005)[36], UPAR (urokinase type plasminogen activator receptor) expression by bonemarrow disseminated tumour cells has been linked to a poor prognosis in a population of patients with micrometastasi.

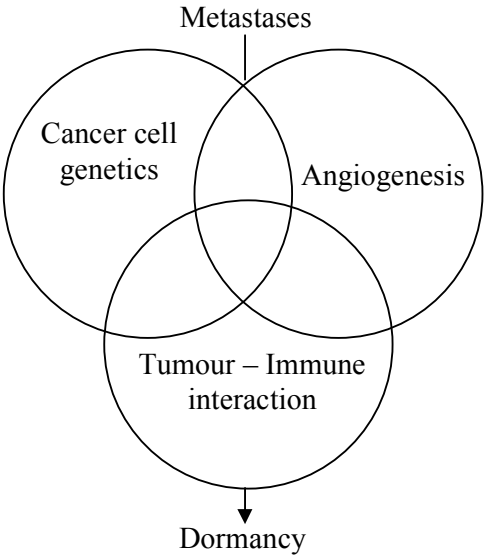


Fig.1. Cancer dormancy

Bone Marrow Micro-Metastasis: Those metastatic stem cells with the capability to detach from the primary growth unlike most other cells within the primary tumour travel through the extracellular matrix and basement membrane into the circulation and exit by attaching and extravasating through the endothelium at another distant site i.e. Bone Marrow (Duffy MJ et al 1998)[37]. Bone marrow is a common homing organ for such tumour cells derived from various epithelial tumours including breast, prostate, lung and colon cancer (Zach O et al 2004)[38]. It is also reported that these tumour cells detected in the bonemarrow of patients need not preferably metastasize to the bone (Fidler U et al 2003)[39]. Bone marrow might hence be an important reservoir of such tumour cells from where they may recirculate to other distant organs such as liver or lungs where better growth condition may exist. This view is consistent with Paget S et al loc.cit (1889) famous seed and soil concept. Tumour cells expressing CXC chemokine receptors on their cell membrane use these molecules to home to

specific distant sites such as lung, liver and bonemarrow. CXCR4 is a G protein – coupled receptor, which plays a role in the chemo taxis of breast cancer cells. This cellular response is attributed to activation of the PI3K/PTEN/AKT/mTOR signalling pathway rather than the MAP/ERK pathway (Peng SB et al 2005)[40]. This pathway might be responsible for early spread of breast cancers as circulating cancer cells have been detected in the blood. Epithelial-mesenchymal transition and primary tumour micro vessel density may also be a factor in such spread of the tumour cells (Willipinski Stapelfeldt B et al 2005)[41] . In addition, hypoxia is also discussed as a driving force that enables cells to leave the primary tumour. Hypoxia results in secondary processes (E.g. proliferation, angiogenesis, and cell death) like release of hypoxia-inducible factor-1α which is significantly up regulated in bone marrow positive breast tumours (Woelfle U et al 2003)[42] . These cells are suitably labelled as Disseminated Tumour Cell (DTCs').

Disseminated Tumour Cells and Bone Marrow: Bone marrow is reported as a common homing site for DTC derived from breast cancer even in the absence of Lymph Node metastases or clinical signs of overt distant metastases (Pantel K & Brakenhoff RH 2004)[43]. It is also reported in various literature that disseminated tumour cells are present in bone marrow samples of 20-40% of patients even in the absence of lymph node metastases (stageN0). The detection rate of DTC in Bone Marrow in breast cancer patients with no evident metastasis has been reported to be in the range from 0% (Fetsch et al 2000)[44] to 100% (Slade MJ et al. 2005)[45]. This illustrates the variability of results obtained by the use of different techniques or marker genes. In a recent large (more than 3500 cases) study of stages I–III

breast cancer patients, the incidence of DTC in bone marrow detected by immunocytochemistry (ICC) ranged from 13 to 43% (Braun S loc.cit 2005). The presence of DTC in Bone Marrow may be useful not only in predicting the development of bone metastases, but also in predicting the development of metastases in other distant organs such as lung and liver. To date, however, it remains unknown whether bone marrow is a reservoir that allows for DTC to adapt and disseminate later into other organs or whether the presence of DTC in bone marrow might reflect the general propensity of these cells to disseminate and survive in organs, rather than just in the Bone Marrow. Until methods are developed to detect the presence of DTC in organs, such as the lung or liver, it will not be possible to distinguish between these two possibilities. That bone marrow could serve as a reservoir in breast cancer is supported by the presence of epithelial (cytokeratin positive) cells in the peripheral blood of patients with overt distant metastases years after the removal of the primary tumour. This suggests that tumour cells could break from bone marrow metastases to re circulate and disseminate to secondary tissues (Pantel K & Brakenhoff RH loc.cit 2004). This 'two-step' metastasis model could explain why the DTC in patients with overt metastases closely resemble each other genetically (Klein CA et al 2002)[46]. According to Al-Hajj et al (2003)[47] and Ginestier C et al (2007)[48] breast cancers are initiated and maintained by a small fraction of self renewing highly tumourigenic cells called "Cancer Stem Cells". In a study, Balic M et al (2006)[49] concluded that bone marrow micro metastatic breast cancer cells exhibits a stem cell like Immunohistochemical phenotype. The hypothesis that stem cells may

reside in the bone marrow is further strengthened by observations that most DTCs' are non proliferating and resistant to Chemotherapy (Reya T et al 2001)[50], (Zohu S et al 2001)[51]. According to Ring A et al (2004)[52], in studies using antibody-based cytometric assays, cells with the characteristics of tumour cells have been shown in the peripheral blood of 0 to 100% of patients with operable (stages I–IIIa) breast cancer and 3–100% in patients with metastatic disease. The variability of results obtained in DTCs' detection results from dramatic variations in methodology.

Circulating Tumour Cells(CTC) in the blood stream is an important intermediate event in metastasis. While bone marrow aspirates are usually accessible, obtaining them can be both time consuming and invasive. Detection of CTC with the cells search system detects CTC using Ep-CAM coated beads for enrichment followed pan CK staining and provide significant prognostic information before and after initiation of chemotherapy in patients with metastatic breast cancer (Cristofanalli M et al 2004)[53]. Two immunocytochemical studies conducted by Muller V et al (2005)[54],Pierga J V et al loc. cit (2005), showed that bone marrow was more frequently positive than blood CTC.

Factors that may influence the variability in data in DTC detection include:

1) **Heterogeneity of the studied populations** according to the: **(a)** Stage: The number of positive patients and the absolute numbers of DTC per patient rise as clinical stage rises (Ben Hsieh H et al 2006)[55.] **(b)** Interval of time: separating surgery from the obtaining of DTC. Surgery may increase the number of breast cancer DTC (from 0 to 8000 cells/ml) in the peripheral blood,

which persist for varying length of times in different patients (Hu XC et al 2003)[56]. **(c)** Metastases location. The division of populations into those with early and metastatic breast cancer is probably simplistic. Moreover, metastasis sites could be missed when DTC are obtained leading to a misclassification of the patient in the 'early breast cancer' category.

2) **Sample Handling and Preparation**: (a) Delay between collection and analysis. (b) Conditions of sample storage (c) Contamination with normal epithelial cells. The introduction of skin cells into a sample at the time of puncture could lead to false positive results. Many investigators advocate that the first few millilitres of sample are to be discarded to avoid such contamination. It has also been suggested recently that false positivity of single lymph node could result from iatrogenic displacement and transport of benign epithelial cells in patients with breast carcinoma (Bleiweiss IJ et al 2006)[57].
3) **Criteria/Threshold of positivity**: (a) Number of cells analysed. (b) Evaluation or not of the apoptotic status of analysed cells.
4) **Analytical and preanalytical (enrichment) techniques**
5) **Markers**. A number of different markers have been used. They may considerably vary the levels of sensitivity and specificity.

Techniques in Detection of DTCs':

The methods to identify DTCs must distinguish between epithelial and other (mainly hematopoietic) cells. Secondarily, it may be desirable although not necessarily essential to distinguish between cancer and normal epithelial cells.

The most conventional technique has been focussed on staining of previously embedded tissue in paraffin wax with two dyes, hematoxylin and eosin (H&E). It is likely that very small amounts of DTC present cannot be detected by this technique. An increase in sensitivity can be achieved by serial sectioning and histopathologic examination of an extensive number of sections. However, this approach is time consuming which hampers its routine application. More sensitive approaches have been developed. Immunohistochemistry (IHC), using antibodies that bind to more or less specific breast cancer cell marker(s) with ability to detect regions of metastases undetected by H&E staining is a well reported methodology (Cote RJ et al 1999)[58]. However, IHC has several drawbacks: it is a labour intensive and time consuming method particularly because at least 100000 cells need to be available for a reliable assessment of the presence of tumour cells (Silva J M et al 2001)[59]. Moreover, IHC requires a trained cytologist to confirm the identity of the stained cells. Although IHC has been previously applied to bone marrow smears, this technique is unable to make an accurate measurement of the frequently low DTC load within BoneMarrow (Gilbey et al 2004)[60]. Two major approaches involve antibody- and nucleic acid-based techniques to identify DTC in marrow smears.

1. **Antibody-based techniques:** Approaches by fluorescence microscopy, Immunocytochemistry and flow cytometry analysis aim to isolate and enumerate individual tumour cells. Immunocytochemistry is still a gold standard for DTC detection, and most of the available clinical data have been gathered by immunocytochemistry screening, especially in Bone Marrow (Zieglschmid et al 2005)[61].

An advantage of this approach is that it may allow further characterization of the cells at a molecular level, in terms of expression of key biological markers, such as ERBB2 (ERBB2 gene amplification estimated by FISH analysis) and morphological cell analysis. However, identification of intracellular targets, such as cytokeratins, by antibodies needs cell permeabilization. As a consequence, cell viability is lost making the important discrimination of dead and viable DTC impossible. Since only viable cells might lead to metastasis, this valuable information cannot be assessed (Zieglschmid et al loc.cit 2005).Like Immunocytochemistry, fluorescence microscopy and Flow cytometry are labour intensive and time consuming, making these techniques too expensive for routine implementation. When compared with conventional techniques, qualitative fluorescence microscopy and Immunocytochemistry, Flow cytometry offers the advantage of a fully automated technique allowing quantitative measurements with high sensitivity, good resolution, speed, reproducibility and statistical reliability. For breast tumours, the most used targets for antibody-based techniques are the cytokeratins, ERBB2, MUC1 and TACSTD1. Many of the antibodies directed at epithelial and breast cancer cells are known to also stain haematopoietic cells also. Nonspecific staining of plasma cells can also occur due to alkaline phosphatase reaction against the kappa and lambda light chains on the cell surface (Smerage &Hayes et al 2006)[62]. According to the antibody used, a false positive detection rate of 1–3% can be expected (Zieglschmid et al loc.cit 2005). Since tumour and epithelial specific cell marker antigens are expressed differentially in DTC, the use of a panel of monoclonal antibodies may help to enrich DTC and facilitate their detection, as notably shown by Hager et al. (2005)[63].

2. **Nucleic acid-based techniques:** Polymerase chain reaction(PCR), either qualitative or quantitative, has been used to identify and characterize DTC through the detection of genetic (allele-specific expression, microsatellite instability, loss of heterozygosity) and epigenetic alterations (methylation status) that are specifically associated with cancer cells (Sidransky D et al 1997)[64]. This includes the search for tumour-associated point mutations in oncogenes or tumour suppressors. This latter PCR approach, however, is complicated by the substantial degree of genetic variability between tumours. For instance, TP53, the gene coding for p53 is mutated in about 25% of breast tumours, however, more than 1400 different mutations of this gene have been observed (Lacroix M et al 2006)[65.]Of note, PCR has been used to detect free DNA within plasma. For instance, the analysis of DNA methylation status of specific genes (ESR1, APC, HSD17B4, HIC1,RASSF1A) in serum of breast cancer patients has been shown to be of prognostic value (Muller V et al 2003)[66.] The PCR-based measurement of RASSF1A methylation has been used for monitoring efficacy of adjuvant tamoxifen therapy (Fiegl et al 2005)[67]. However, this use of PCR is limited by poor specificity. This is due in part to the high stability of DNA in plasma when compared with mRNA (Silva JM et al loc.cit 2002). As a result, it is unclear whether the free DNA that is amplified from plasma is from DTC present in plasma or if the DNA is being shed from primary tumours, metastatic tumours or from normal tissue (Ring A et al loc.cit 2004).

Challenges with Detection of DTC

This issue remains open for debate even today.

1) **Are These Definitely Cancer Cells?** There are no criteria to unequivocally define cells as malignant, however the evidence strongly supports them as being malignant. Many detection methods depend on identification of epithelial specific markers. However, not all cells that stain positive with epithelial anti-cytokeratins can be unequivocally defined as malignant. Support for these cells as tumour cells is provided by molecular analyses and clinical studies showing their prognostic significance (Stathopoulou A et al 2002)[68]; (Xenidis N et al 2006)[69]; (Ignatiadis M et al 2008)[70].
2) **Are All Breast Cancer Cells Detected?** To be clinically useful, DTC detection must identify all breast cancer cell types. This is difficult, as there is great diversity in breast cancer biology coupled with great diversity in detection platforms. Variation in detection rates between techniques raises questions about whether different platforms are detecting different cells. Cytokeratins have become a widely used protein marker for the detection of epithelial tumour cells. With IHC, results depend on both the pattern of CK expression and the CK panel employed by the particular assay. Different antibodies may give different results for the same patient (Effenberger KE et al 2010)[71]. A false negative result may result from loss of cytokeratin expression (e.g., loss of CK19 expression by cells that have undergone epithelial mesenchymal transition or treatment induced changes in CK expression in which lack of expression may be incorrectly interpreted as the elimination of

disease). A recent preclinical study specifically assessed the ability of CellSearch to detect recognized breast cancer subtypes (Sieuwerts AM et al 2009)[72]. In vitro genomic profiling confirmed the molecular subtype of 10 breast cancer cell lines. Two cell lines were selected from each of five following subtypes: luminal A, luminal B, normal-like, basal, and HER2. From each cell line, 50–150 cells were injected into normal blood. CellSearch did not detect the normal-like subtype. These findings were not confirmed in a subsequent exploratory clinical study involving a limited number of patients (n = 58), in which CTC detection was reported in all subtypes, including normal-like (CTC in 2 of 7 patients) (Bidard F et al 2009)[73].Normal-like disease may be aggressive with expression of cell markers, particularly with high CD44 and high TWIST.1 expression and low CD24 expression. This is suggestive of their breast cancer stem cell like properties. Tests that recognize all breast cancer subtypes are required and further studies will address CTC detection across defined subtypes.

3) **Are DTC and CTC the Same Cells?** It is unclear if DTC and CTC are the same cells. If CTC measurements could replace DTC, this would be ideal, due to ease of collection of peripheral blood compared with invasive bone marrow sampling. Two studies, in which patients had both DTC and CTC assessment by IHC, showed a correlation between the two, but a higher rate of detection of DTC (Pierga JY et al 2004)[74]. This may be explained by the more constant presence of DTC in the bone marrow with sporadic shedding of CTC.

4) **Are These Cells Viable?** A critical question is whether these cells are apoptotic cells or they are viable cells capable of self-renewal and systemic metastases. If they are viable, with self-renewal properties and the capacity to deposit systemically and progress, they may provide important clinical information in the adjuvant setting. If they are non-viable, their detection may have limited clinical meaning in early breast cancer. In contrast, in metastatic disease, the presence of CTC, viable or not, is shown to predict metastatic disease prognosis (Cristofanilli M et al 2005)[75.]

Applications of Disseminated Tumour Cell Detection in Breast Cancers

1) **Prognostication of Breast Cancers:** Bonemarrow as the host organ of these DTCs' is very accessible for tissue harvest and further analysis of Cohorts of breast cancer patients who had their initial surgery and in some patients adjuvant chemotherapy or hormonal therapy were followed with subsequent bone marrow sampling after surgery and prior to any further diagnosis of relapse. [1] In a study conducted by Mansi JL et al (1989)[76] micro metastatic disease was identified in only 2-3% of cases where no evidence of relapse had previously been detected. In patients who subsequently developed either local or distant relapse, bone marrow metastasis had been identified in 19 and 30% respectively and in all 10 patients who developed bone metastasis disease. [2]. This lead to a conclusion that the increasing incidence of bone marrow metastasis in cases of women who subsequently developed recurrent local or distant disease is as a consequence of cells shedding from the original tumour and from aggressive dissemination of tumour cells in a metastatic fashion.

According to studies conducted by Braun S (2000)[77], Molino A et al (1999)[78], Braun S et al loc cit (1999), pretreatment levels of bone marrow positivity were much higher with 49-51% of cases showing bone marrow metastasis. This level do not change significantly after chemotherapy (44.1 %). The presence of DTCs' after treatment was found to be an adverse independent prognostic factor. Persistence or disappearance of DTC after systemic treatment could therefore used as a surrogate marker of treatment response (Lacroix M et al loc.cit 2006). Studies have shown that adjuvant chemotherapy has no effect on the elimination of single dormant tumour cells in the bone marrow of high risk breast cancer patients (Quintela-Fandino M et al 2006)[79].

2) **Adjuvant Therapy in Breast Cancers**: Diel and coworkers (2006)[80], randomized 302 patients known to have bone marrow metastasis to receive bisphosphonate clodronate (1600 mg daily for 2 years). A statistically significant lower number of relapses were seen in the clodronate group . The number of deaths were also lower in the clodronate group 6 versus 22 in control group (p= 0.001). This result was also consistent with a large study done by Powels.T et al (2006)[81], involving 1069 patients with operable stage 1-3 breast cancer. This blinded study randomized patients to receive clodronate or placebo and found that clodronate significantly improved the 5 year relapse free survival by 31%. Zoledronic acid ,a more potent bisphosphonate than clodronate is under study. New agents –denosumab (AMG 162) ,which is a fully human monoclonal antibody to Receptor activator of nuclear factor- kappa B ligand (RANKL- which is essential for the

differentiation ,function and survival of osteoclast) is in clinical trial in women with breast cancer (Body JJ et al 2006)[82]. A therapeutic approach to bone marrow metastasis was utilised by Braun S and co-workers loc.cit(2005) who demonstrated that monoclonal antibody Edrecolomab or Traztuzumab directed against HER2 and EPCAM –positive cells in the bone marrow of breast cancer patients (Bozionelloli V et al 2004)[83].

Prognostic Importance of Disseminated Tumour Cells in Breast Cancers:

In the usual clinical setting following a definitive surgery for early stage breast cancer, patients are free of overt disease. The aim of adjuvant therapy is eradication of residual microscopic malignancy based on the concept that residual disease is the source of subsequent incurable systemic relapse. However with current tools, the presence of minimal residual disease is presumed not measured. Our current approach for determining adjuvant systemic therapy is to assess the primary tumour using traditional clinical and pathological features (e.g., patient age, tumour grade, tumour size, lymph node involvement, hormone receptor (HR) expression, and expression of HER2) or more recent molecular profiling tools (e.g., 21-gene Oncotype Dx and 70-gene Mamma-print). Based on assessment of the primary tumour, risk of disease recurrence is estimated. The estimates are based on breast cancer relapse and overall survival data from prior clinical trials. Furthermore, following the presumption of the presence of residual disease, subsequent treatment decisions are based on characteristics of the primary tumour (e.g., HR, HER2) with the presumption that biological characteristics and treatment sensitivity are consistent between the primary tumour and

micrometastases. There are no current tools in the adjuvant setting to assess treatment efficacy once a treatment has commenced (Oakman C et al 2010)[84]. Current adjuvant treatment is based on the following factors [1] Presence of residual disease [2] Consistent biological characteristics between residual disease and the primary tumour [3] Consistent treatment sensitivity between residual disease and the primary tumour. Using such an approach has its limitations. Some individuals with low risk disease develop recurrence despite treatment. Some individuals assessed as high risk remain relapse free in the long term without any systemic adjuvant therapy (Bonadonna G et al 2005)[85].Such risk overestimation or underestimation by traditional methods is exemplified by the pivotal adjuvant chemotherapy trial in which 386 patients assessed as high risk, based on nodal involvement were randomized to either a surgery plus cyclophosphamide, methotrexate, and 5-fluorouracil trial (CMF) or to surgery alone. Thirty year follow up data confirms a sustained survival benefit in the Chemotherapy & Surgery over Surgery alone with a relapse free survival (RFS): 29% for the former vs. 22% for the later.

It is hence important to note that 22% of patients had an excellent outcome despite apparent high-risk disease and no systemic treatment. Similarly, in a 20 year follow up of 90 patients, with estrogen receptor(ER) negative, node-negative disease, randomized to surgery plus CMF versus surgery alone, chemotherapy was associated with sustained benefit with RFS: 65% vs. 45% for the later (Bonadonna G et al loc.cit 2005). Again, substantial RFS was seen in a substantial number of patients treated with surgery alone. It may be thought that such risk overestimation may be overcome by recent molecular profiling tools.

However, these tools are also shown to overestimate risk in a substantial number of women. The 21-gene Oncotype Dx was assessed in 355 placebo treated patients from the NSABP-B14 trial, all with node-negative, ER positive disease (Paik S et al 2005)[86]. Ten year distant-recurrence free survival (DRFS) for these patients treated with surgery alone was 86%, 62% and 69% for low, intermediate, and high recurrence scores respectively. For women with a high recurrence score, it would be reasonable based on current data to offer adjuvant systemic chemotherapy in addition to endocrine therapy. However, nearly 70% of women with a high recurrence score had long-term DRFS without any adjuvant intervention (Paik S et al loc.cit 2005).

Similarly, one study from the Netherlands Cancer Institute Tissue Bank applied the 70-gene Mamma-print to 151 lymph node-negative patients, only 10 of whom received any adjuvant therapy. The research showed a 10-year distant metastases free survival of 87% for good signature patients and 44% for the poor prognosis cohort. A striking feature of these current assessment tools in the adjuvant setting is overestimation of risk of disease recurrence. Some individuals, despite apparent 'high risk' disease, clearly have excellent long-term prognosis. Refined assessment, with identification of these individuals, would spare them from unnecessary and potentially toxic chemotherapy (van de Vijver MJ et al 2002)[87].

In this setting, assessment of micrometastases is a promising alternative to presumption of residual disease. We now have tools to identify disseminated tumour cells (DTC) in the bone marrow. Emerging evidence for the clinical

potential of micro-metastases in breast cancer is reflected in their consideration within recent guidelines. Both the American Society of Clinical Oncology 2007 Recommendations for the use of Tumour Markers in Breast Cancer and the San Gallen Consensus of 2009 reviewed evidence for micro-metastases (Harris L et al 2007)[88]. While recognizing available data, especially for the strong prognostic role of DTC and acknowledging the future potential of these tools, the guidelines advise that available evidence and methodology are insufficient to currently support a routine role in the management of patients with breast cancer. Detection of micrometastases may have future value in early breast cancer for refining prognosis, monitoring treatment efficacy, and allowing bio-characterization of residual disease. Potential roles for micrometastases in Breast carcinoma management include: [1] Refinement of prognosis [2] Serial detection to assess efficacy of adjuvant therapy and [3] Bio-characterization of residual disease, with potential therapeutic implications (Goldhirsch A et al 2009)[89].

Recent guidelines recommend that a positive DTC result is the presence of at least one CK positive cell in the bone marrow which meets morphological criteria for malignancy. These morphological features include large cells with a large nucleus, nuclear granulation or stippling, a large nucleolus, strong or irregular staining for cytokeratin, cytokeratin filaments, a high nuclear to cytoplasmic ratio, and the presence of cell clusters. (Fehm. T et al 2006)[90]. Morphological assessment distinguishes CK+ cells as DTC, haemopoietic, squamous, or normal epithelial cells. Whilst epithelial cells are rarely found in the bone marrow, they have been reported in 1–2% of normal volunteers. With

morphology, DTC rates are approximately 13–15%. Other methods with the potential for increased accuracy of DTC detection include antibody-linked immunomagnetic enrichment, flowcytometry, PCR, and RT-PCR assays (Braun S et al loc.cit 2000). Detection of DTC at the time of surgery for the primary tumour in stage I-III breast cancer is an independent prognostic factor for poor outcome. Many studies show correlations between DTC and tumour size, tumour grade, and lymph node status. Several small studies have shown the poor prognostic significance of DTC in univariate analyses. The largest analysis of DTC in the bone marrow came from a pooled analysis of 4703 patients from 9 prospective studies with 10-year follow-up (Braun S et al loc.cit 2005). The pooled analysis was strengthened by the use of individual patient data.

The presence of micrometastases was a significant and independent prognostic factor for poor overall survival, breast cancer specific survival, disease-free survival, and distant disease-free survival. In multivariable analysis of death from breast cancer, DTC were an independent predictor of poor outcome. The median follow up among survivors was 62 months. For overall survival and breast cancer specific survival, the mortality ratios for micrometastases vs. no micro-metastases were 2.44 (95% CI 2.08–2.86; $p < 0.001$) and 2.15 (95% CI 1.87–2.47, $p < 0.001$). Despite their clinical validity, there are concerns regarding analytical validity. Assays are heterogeneous and are not clearly standardized or reproducible. The clinical utility of DTC is not clear. Due to the high correlation between DTC and high grade, large tumour size, and nodal involvement, many patients would already be considered candidates for systemic therapy without

considering bone marrow status. Conversely, for a patient with an apparently good prognosis with a small, node-negative, low grade tumour, it is unclear that a positive bone marrow result is sufficient to warrant differential recommendations for adjuvant therapy (Cote RJ et al loc.cit 1991). A comparative analysis of the various international studies on detection of DTC and the prognostication of Breast cancers is tabulated herewith.

Table 3. Studies Evaluating the Prognostic Relevance of DTC in Bone Marrow from Breast Cancer Patients at Primary Diagnosis

Reference	No. of patients	Detection rate (%)	Antibody/target antigen	Material	Prognostic relevance
Coombes et al (1986)[91]	269	23	E29	Aspirate	DFS
Schlimok et al (1987)[92]	155	18	CK18	Aspirate	DDFS
Porro et al (1988)[93]	159	16	Mbr1	Biopsy	—
Salvadori et al (1990)[94]	121	17	Mbr1	Biopsy	—
Mathieu et al (1990)[95]	93	1	KL1	Biopsy	—
Dearnaley et al (1991)[96]	37	33	EMA	Aspirate	DFS, OS
Cote et al loc.cit (1991)	49	37	T16, C26, AE-1	Aspirate	DFS, OS
Wiedswang et al (2003)[97]	817	13	AE-1, AE-3	Aspirate	DDFS, OS[*]
Harbeck et al (1994)[98]	100	38	E29, 12H12	Aspirate	DFS, OS[*]
Diel et al (1998)[99]	727	43	TAG 12/2E11	Aspirate	DFS, OS[*]
Funke et al (1996)[100]	234	38	CK18	Aspirate	ND
Mansi et al (1999)[101]	350	25	E29	Aspirate	DFS, OS

Braun.S. et al loc.cit (2000)	552	36	A45-B/B3	Aspirate	DDFS, OS[*]
Gerber et al (2001)[102]	554	31	CK8, CK18, CK19	Aspirate	DFS, OS[*]
Gebauer et al (20010[103]	393	42	CK/EMA	Aspirate	DFS,[*] OS
Braun.S. et al loc.cit (2005) (pooled analysis)	4703	31	CK, mucin	Aspirate	DDFS, OS

DFS *indicates disease-free survival (significant correlation between positive bone marrow status and DFS); CK, cytokeratin; DDFS, distant DFS (significant correlation between positive bone marrow status and DDFS); EMA, epithelial membrane antigen; OS, overall survival (significant correlation between positive bone marrow status and OS); RT-PCR, reverse-transcriptase polymerase chain reaction; ND, not done.*

MATERIALS & METHODS

This study was proposed and conducted in the Tirunelveli Medical College Hospital. A pilot study was done and approval of the ethical committee of the Tirunelveli Medical College & Hospital was obtained.

Patients who presented to the outpatient department of the Departments of Surgery and Oncology at Tirunelveli Medical College Hospital, during the period January 2009 to September 2010 with complaints of lumps in the breast were evaluated. Of the 385 patients examined 123 patients were found malignant on FNAC. Of these, 50 patients were selected for our study based on a set of inclusion and exclusion criteria.

Inclusion Criteria

1. Patients with FNAC positive for malignancy.
2. Biopsy / Mastectomy proven Breast Carcinoma.
3. Patients with no Radiological Bony Lesions.
4. Patients with or without lymphatic spread.

Exclusion Criteria

1. Patients with carcinoma in situ.
2. Patients with proven secondary metastasis.
3. Patients with radiological bone lesions suspected as metastasis.
4. Patients undergoing or have underwent chemotherapy / radiotherapy.
5. History / existence of other cancers.

FNAC was done with standard procedures and processd. Of these 123 patients who were diagnosed as having a malignancy were evaluated in the respective departments using a standard proforma **(Appendix 1).** Of the 123 patients with FNAC proven malignancies, 63 patients were subjected to surgical treatment namely simple mastectomy / modified radical mastectomy / segmental mastectomy. The specimens were processed and reported at the Department of Pathology. These 63 patients were evaluated for macrometastatic disease and lymph node spread. Of these, 50 patients who had FNAC and Histopathology proven breast malignancies and no obvious radiological lesion were taken up for the study. 13 patients were not included in the study – 3 cases had Non Epithelial Tumours, 2 cases had secondary liver metastasis at diagnosis, 6 cases had already undergone adjuvant chemotherapy and or radiotherapy, 2 cases refused marrow evaluation.

Written consent was obtained from the patient or her nearest relative after proper counselling. Bone Marrow aspirate was obtained from the 50 patients selected for the study. Bone Marrow aspirate was obtained by direct puncture of bilateral posterior iliac crests under local anaesthesia with strict aseptic precautions. 2.5 ml of marrow was obtained. 10 Marrow Smears were prepared and fixed in methanol, of which 3 smears were stained with Leishman stain and 3 with Haematoxylin and Eosin. 1 ml of the marrow was centrifuged using a Wintrobes tube and the gray column was aspirated and smears were prepared and stained similarly. The remaining material was allowed to clot in controlled temperature and fixed in 10% buffered formalin for 24 hrs and later processed

using routine schedules and the serial sections stained with H&E were obtained and studied. **(Appendix 2)**

The cases were evaluated for disseminated tumour cells using usual cytological parameters namely (1) cell size (2) cytoplasmic borders (3) nuclear size & nuclear cytoplasmic ratio (4) nuclear membrane irregularity (5) presence of nucleolus (6) hyperchromasia. Based on this evaluation, the patients were divided into two groups (1) DTC negative and (2) DTC positive. The Bone Marrow smears of the DTC positive patients were further evaluated using Pan Cytokeratin Stain (CK18). **(Appendix 3)**

Cytomorphologic Features of DTC Detected by Immunocytochemical Staining using Cytokeratin Antibody: (Tanja Fehm, Stephan Braun et al loc.cit 2006.)

1. Cell clusters
2. Nuclear size clearly enlarged
3. Strong and/or irregular cytoplasmic staining for cytokeratin
4. Cytokeratin filaments may be seen
5. Staining partially covers nucleus
6. Large nucleoli may be seen
7. High nuclear to cytoplasmic ratio
8. Nucleus often granular or stippled

The bonemarrow slides are interpreted as positive when Disseminated tumour cells are present in bone marrow and absence of cells with disseminated tumour cell morphology in corresponding negative controls and it is interpreted as negative when no disseminated tumour cells or presence of cells with disseminated

tumour cell morphology in both anticytokeratin stained slides and in corresponding negative control slides

The Mastectomy specimens obtained from these patients were studied according to existing pathology evaluation protocols and the prognostic parameters were tabulated. The tissue samples of the patients were also subjected to hormone receptor evaluation.

The results and observations were tabulated, analysed and statistically evaluated for their significance.

RESULTS AND OBSERVATION

Three hundred and Eight five patients were referred to our FNAC OP for Breast Masses during the period January 2009 to September 2010. Of these, 123 patients were diagnosed by FNAC as having a malignancy. Of these 63 patients were subjected to surgical treatment namely simple mastectomy, modified radical mastectomy and or segmental mastectomy. (Table1)

TABLE 1: GENERAL STATISTICS

TOTAL NUMBER OF CASES REFERRED FOR BREAST FNAC	385	
TOTAL NUMBER OF CASES OF BREAST FNAC FOUND MALIGNANT	123	31.9%
TOTAL NUMBER OF CASES WITH FNAC PROVEN BREAST MALIGNANCY WHICH WERE OPERATED	63	
NO OF EPITHELIAL MALIGNANCIES	60	95.2%
NO OF NON EPITHELIAL MALIGNANCIES	3	

Of the 60 patients with primary epithelial malignancies of the breast 10 patients were not included in further study- 2 cases had secondary liver metastasis at diagnosis, 6 cases had already undergone adjuvant chemotherapy and or radiotherapy, 2 cases refused marrow evaluation. (Table 2)

TABLE 2: THE STUDY GROUP

NO OF PRIMARY EPITHELIAL MALIGNANCIES OF BREAST DIAGNOSED WITH HISTOPATHOLOGY	60
NO OF CASES SELECTED FOR STUDY	50
NO OF CASES NOT SELECTED FOR STUDY	10

Of the total 50 patients included in this study, all the 50 patients were studied using both marrow concentrate smears and marrow clot sections as

referred to in the materials and methods of this study. Of the 50 patients studied with marrow concentrate smears 16 patients (32%) were positive for Disseminated Tumour cells while only 1 patient (2%) was positive in clot sections.(Table 3)

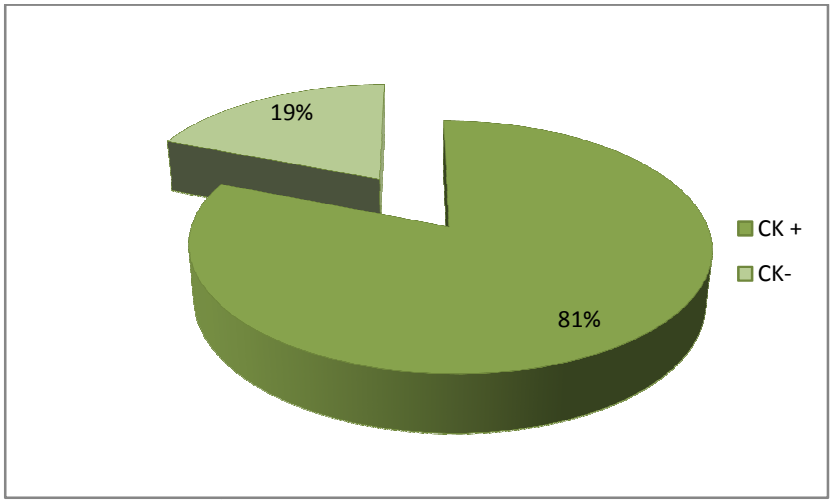
TABLE:3 METHODS OF DETECTION OF DISSEMINATED TUMOUR CELLS

	TOTAL	**DTC +**	**DTC -**
SMEAR	50	16	34
CLOT SECTION	50	1	49

The diagnosis of DTC in the marrow preparation in 16 cases were reviewed with cytokeratin staining, of which 13 patients (81%) were positive.(Table 4)

TABLE:4 CYTOKERATIN STAINING & DISSEMINATED TUMOUR CELLS

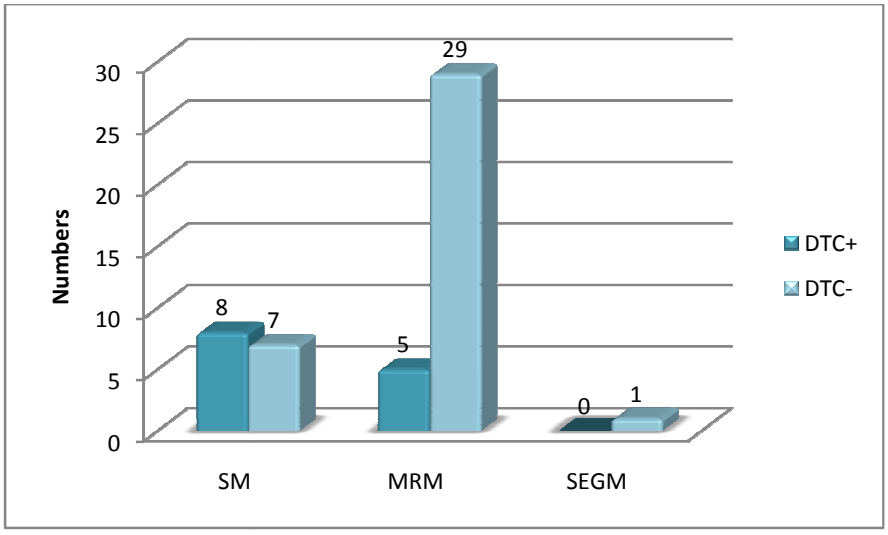
CYTOKERATIN IN DTC	TOTAL	%
TOTAL	16	-
CK +	13	81%
CK-	3	19%



Of the 50 patients included in this study 15 patients underwent simple mastectomy, while 34 patients underwent Modified radical mastectomy and 1 patient had a segmental mastectomy done. Of the 15 patients who had Simple Mastectomy done 8 patients (53.3%) were positive for DTC. Of the 34 patients who had modified Radical Mastectomy 5 patients(14.7%) were positive for DTC. 1 patient who had segmental mastectomy done was negative for DTC. (Table 5)

TABLE:5 TYPE OF SURGERY & DISSEMINATED TUMOUR CELLS

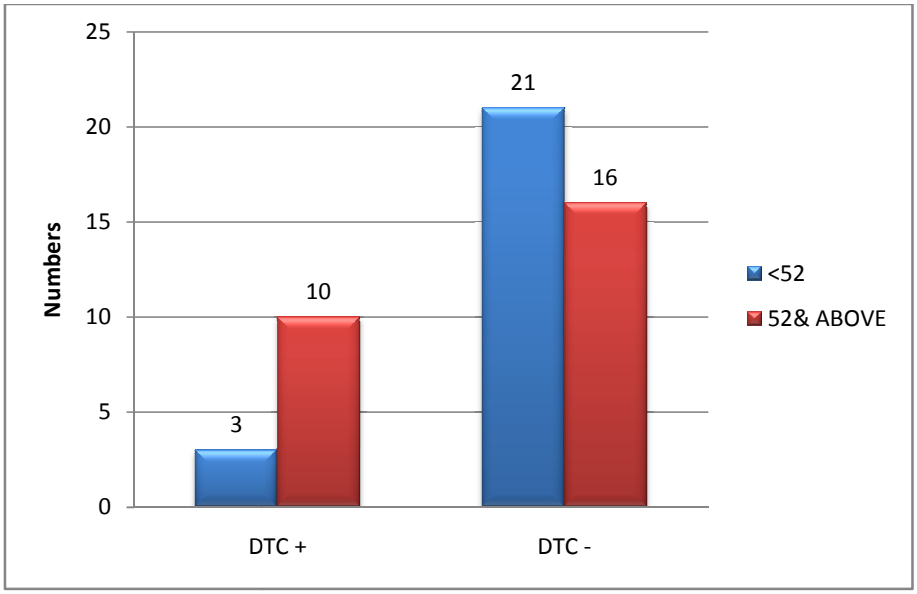
TYPE OF OPERATION	TOTAL	DTC+	DTC-
SM	15	8	7
MRM	34	5	29
SEGM	1	0	1
TOTAL	50	13	37



Of these 50 patients taken for the study, all 50 patients were subjected to bone marrow evaluation for Disseminated Tumour Cells. Of these 50 patients, 24 patients were less than the median age of 52yrs and of which 3 patients were positive for DTC. 26 patients were above the median age ,of which 10 patients were positive for DTC. (Table 6)

TABLE 6: AGE OF PATIENTS & DISSEMINATED TUMOUR CELLS

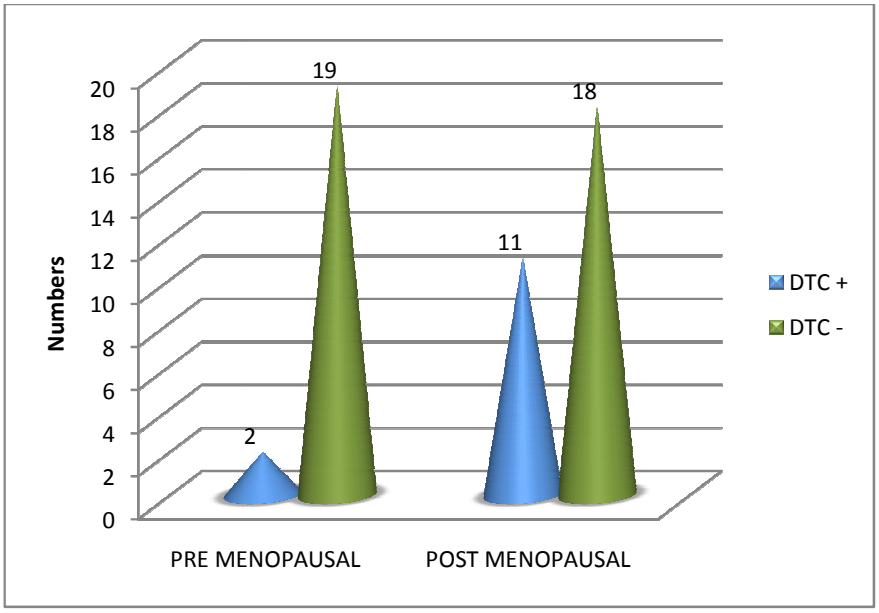
AGE(IN YRS)	TOTAL	DTC +	DTC -
<52	24	3	21
52& ABOVE	26	10	16
TOTAL	50	13	37



Of the 50 patients studied 29 patients were post menopausal (58%) while 21 were premenopausal (42%). Of the post menopausal group 11(37.9%) patients were DTC positive while only 2(9.5%) were positive in the premenopausal group.(Table 7)

TABLE 7: MENOPAUSAL STATUS & DISSEMINATED TUMOUR CELLS

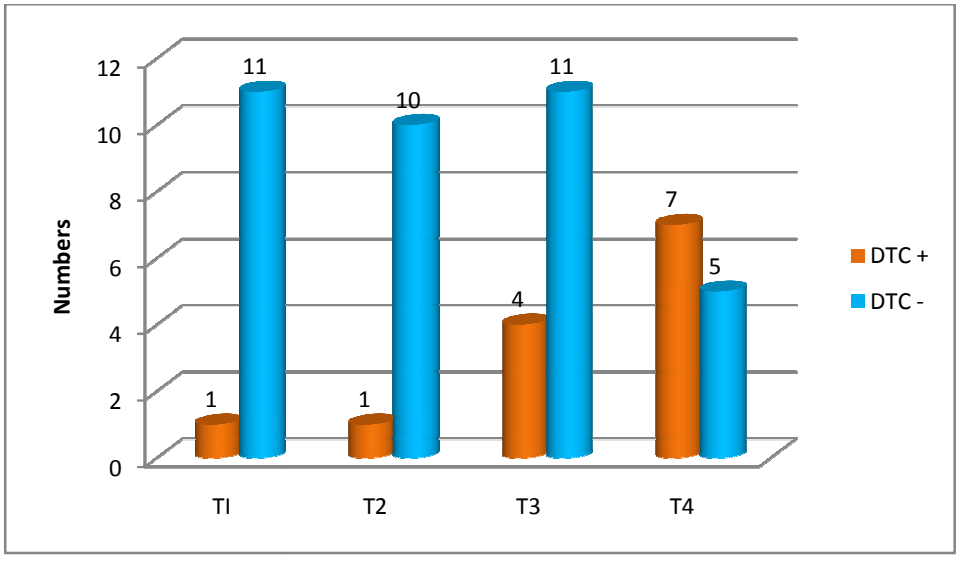
MENOPAUSAL STATUS	**TOTAL**	**DTC +**	**DTC -**
PRE MENOPAUSAL	21	2	19
POST MENOPAUSAL	29	11	18
TOTAL	50	13	37



The mastectomy specimen examined, and it was correlated that of the 50 patients studied 12patients (24%) had a primary tumour size of less than 2cms **(T1)** of which 1 patient(8.3%) had DTC, 11 patients(22%) had a primary tumour size of more than 2cms but less than 5cms **(T2)** of which 1 patient(9.1%) had DTC, 15 patients (30%) had a primary tumour size of more than 5cms **(T3) of** which 4 patients (26.7%) had DTC and 12 patients (24%) had a primary tumour size of variable sizes with skin or chest wall infiltrates **(T4)** of which 7 patients (58.3%) had DTC.(Table 8)

TABLE 8: TUMOUR SIZE & DISSEMINATED TUMOUR CELLS

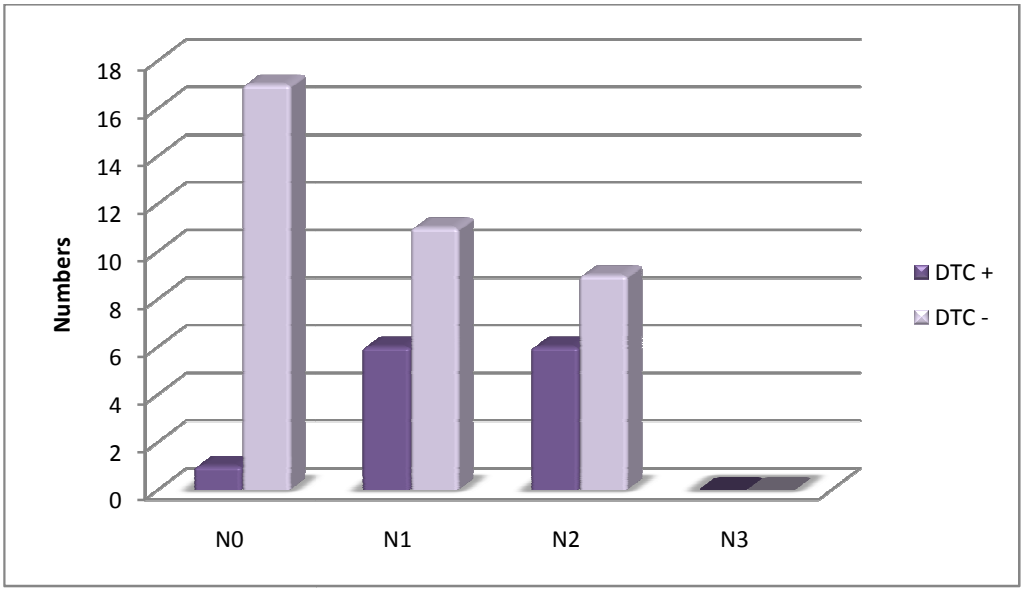
TUMOR SIZE	TOTAL	DTC +	DTC -
TI – LESS THAN 2cm	12	1	11
T2—2- 5cm	11	1	10
T3- >5cm	15	4	11
T4- INFILTRATION OF SKIN &CHEST WALL	12	7	5
TOTAL	50	13	37



The mastectomy specimen examined, and correlated in the 50 patients studied shows that 18 patients (36%) had no lymphnode metastasis of which 1 patient (5.6%) had DTC. 17patients (34%) had N1 level lymphnode metastasis of which, 6patients(35.3%) had DTC and 15 patients (30%) had N2 level lymphnode metastasis of which, 6patients (40%) had DTC. There were no N3 level lymphnode metastasis in our study group. (Table 9)

TABLE 9: LYMPHNODAL STATUS & DISSEMINATED TUMOUR CELLS

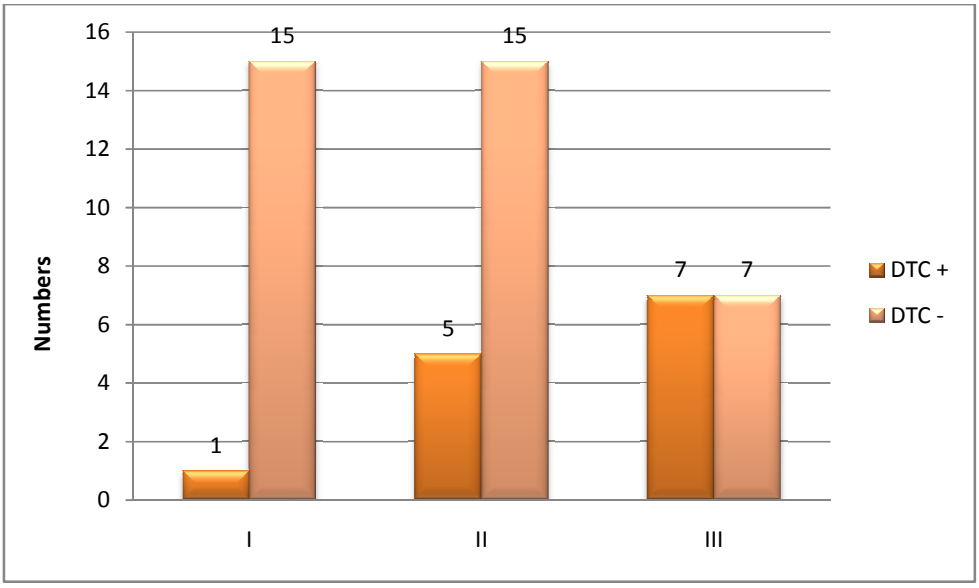
LYMPHNODE METASTASIS	TOTAL	DTC +	DTC -
N0- NO LYMPH NODE METASTASIS	18	1	17
N1- 1-3 NODES	17	6	11
N2- 4-9 NODES	15	6	9
N3- > 10 NODES	0	0	0
TOTAL	50	13	37



Of the 50 patients studied all 50 patients (100%) had Infiltrating Ductal Carcinoma of Breast – Not Other wise Specified type. These cases were graded using the modified Bloom and Richardsons System of Grading (Elston CW et al 1991)[104].Of the 16 patients(32%) with grade I tumor, 1 patient (10%) had DTC, , while of the 20 patients (40%) with Grade II tumours, 5 patients (24%) had DTC, and of the 14 patients (28%) with Grade III tumours, 7 patients (66%) had DTC.(Table 10)

TABLE 10: HISTOLOGY GRADING & DISSEMINATED TUMOUR CELLS

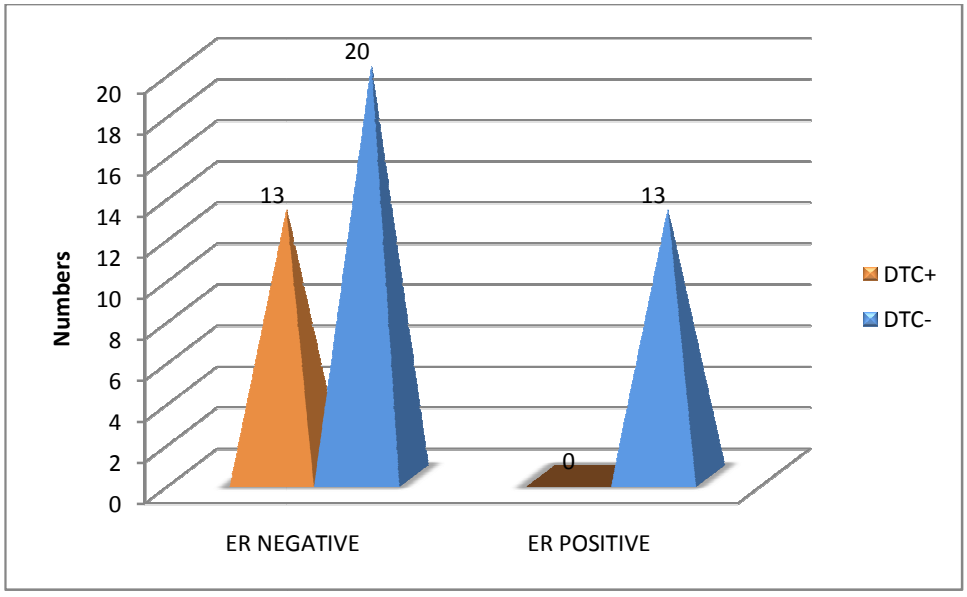
HISTOLOGY GRADING	TOTAL	DTC +	DTC -
I	16	1	15
II	20	5	15
III	14	7	7
TOTAL	50	13	37



Of the 50 mastectomy specimens 46 were subjected to hormonal receptor status evaluation. Of the 33 patients who were ER negative, 13 patients (39.39%) were positive for DTC. Of the 13 patients who were ER positive, no DTC was detected.(Table 11a)

TABLE 11a: ESTROGEN RECEPTOR STATUS & DISSEMINATED TUMOUR CELLS

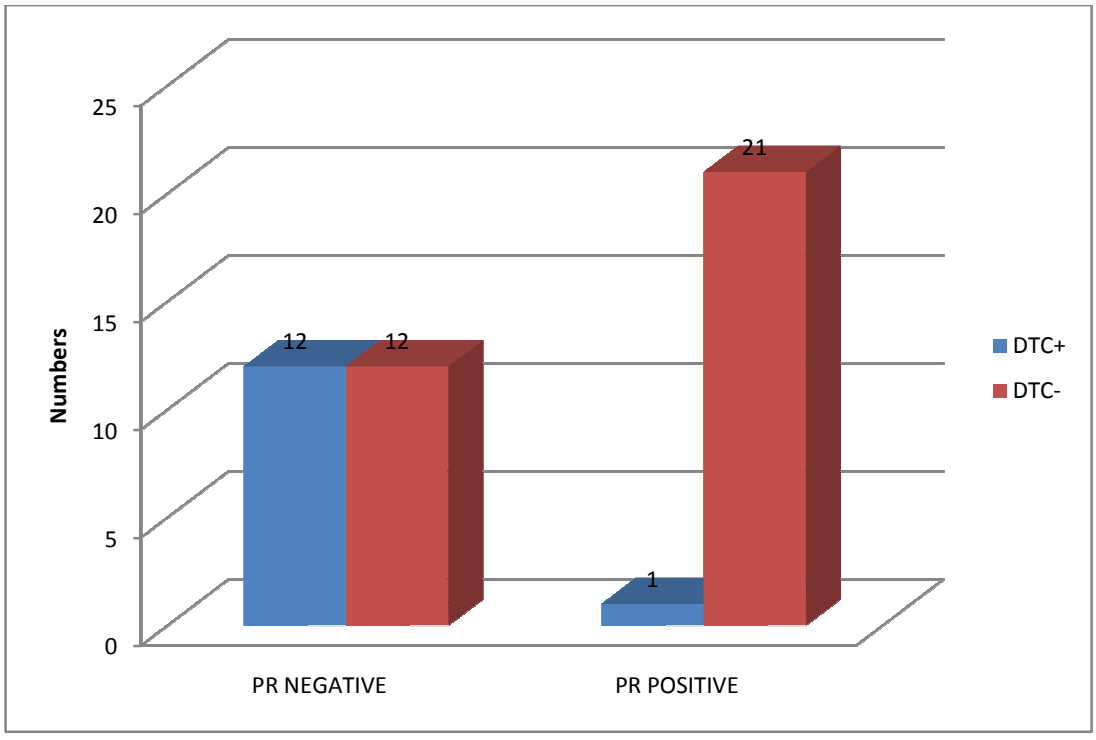
ESTROGEN RECEPTOR STATUS	TOTAL	DTC+	DTC-
ER NEGATIVE	33	13	20
ER POSITIVE	13	0	13
TOTAL	46	13	33



Of the 50 mastectomy specimens 46 were subjected to hormonal receptor status evaluation. Of the 24 patients who were PR negative,12 patients (50%) were positive for DTC. Of the 22 patients who were PR positive, 1 patient (4.54%) was positive for DTC.(Table 11b).

TABLE 11b: PROGESTERONE RECEPTOR STATUS & DISSEMINATED TUMOUR CELLS

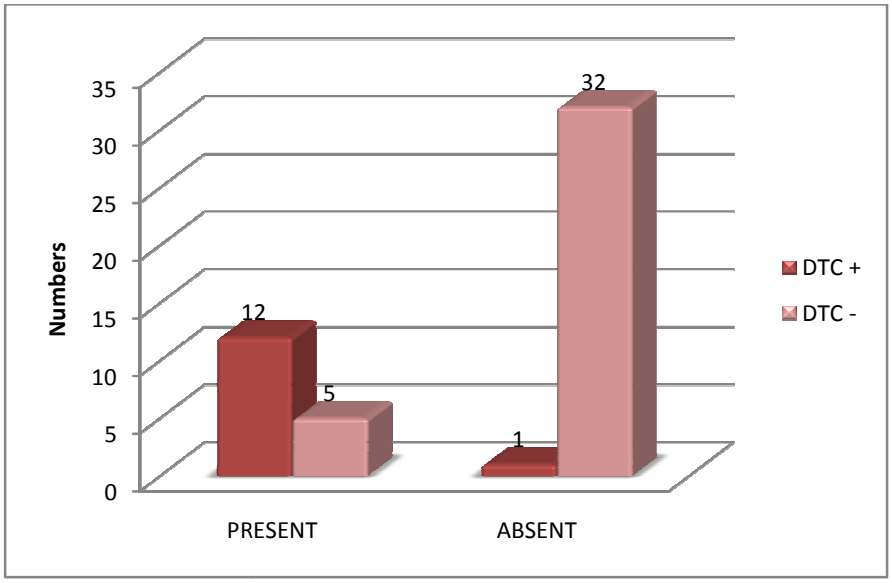
PROGESTERONE RECEPTOR STATUS	**TOTAL**	**DTC+**	**DTC-**
PR NEGATIVE	24	12	12
PR POSITIVE	22	1	21
TOTAL	46	13	33



All 50 Mastectomy specimens were screened for lymphovascular permeation and correlated with DTC status of the patients, Of the 17 patients(34%) who showed obvious lymphovascular permeation, DTC was positive in 12 patients (70.6%), while 1 patient (3%) was positive for DTC but did not show any obvious lymphovascular permeation.(Table 12)

TABLE 12: LYMPHOVASCULAR PERMEATION & DISSEMINATED TUMOUR CELLS

VASCULAR INVASION	TOTAL	DTC +	DTC -
PRESENT	17	12	5
ABSENT	33	1	32
TOTAL	50	13	37



The clinico-pathological characteristics and their correlation was studied and statistically analysed in terms of percentages and their differences were interpreted by Chi Square Test. The above statistical procedures were performed by S.P.S.S (13.0). The p Value of less than 0.05 was considered as significant.

DISCUSSION

The incidence of breast cancer is gradually increasing over the past 20 years due to valuable and accurate diagnostic methodologies and increased awareness. But the morbidity and mortality have not fallen as expected, even in the presence of newer detection methods and better therapeutic options. Currently, one in eight American women may be expected to develop breast cancer, one quarter of whom will die of the disease. Many risk factors have been identified namely geographic factors which vary in different areas. The risk of breast cancer increases with age after the 3rd decade, while a family history of breast cancer increases the risk by 1.2-3.0 fold in the first degree relatives. There is also an 8.5-9.0 fold increase in risk in premenopausal women, while early menarche (<12yrs) and late menopause (>55yrs) also increases the risk considerably. The presence of benign breast diseases like proliferative diseases without atypia have a lesser risk of 1.6 times while Lobular carcinoma in situ have increased risks of 6.9-12.0 times. (Bilimoria MM Morrow M et al loc.cit 1995)

The most common cause of death in patients with breast carcinoma is metastatic disease (Panabieres CA et al loc.cit 2007). Despite the numerous prognostic indicators currently in use, it is not possible to predict accurately the outcome of treatment in most of such patients. This can be attributed in part to the metastatic behaviour of the tumour and the milieu-intern of the patient. Though metastasis is not evident, the existence or absence of a minimal residual disease within the patient is not taken into account in the existing prognostic systems. The prediction of recurrences & relapses depend upon the presence of micro-metastatic

foci in the patient at the time of primary diagnosis. (Panabieres CA et al loc.cit 2007). Thus the existence of micro-metastatic foci in the bone marrow was studied widely and various authors have come up with variable analyses and conclusions.

Weidswang.G et al 2003 **(Table A)** in their prospective analysis of the clinical relevance of detection of isolated tumor cells in breast cancer patients have studied a total of 817 patients. Of these 817 patients, 108 (13.21%) were positive for DTC. Of the 817 patients, 231 (28.3%) were in the premenopausal age and 503 (61.6%) were in the postmenopausal age while in 83 (10.1%) menopausal status was not known. Of the pre-menopausal patients 31 (13.4%) were positive for DTC, while 68 (13.5%) patients who were postmenopausal were positive for DTC. The P value was 0.964 hence reported insignificant. In this study, 513 of the patients (62.8%) had no lymphnode metastasis of whom 50 (9.7%) had DTC. 172 patients (21%) had N1 level lymphnode metastasis of whom 26 patients(15%) had DTC and 105 patients (12.8%) had N2&N3 level lymphnode metastasis of whom 30 (28.5%) had DTC. In 28 patients (3.4%) the lymphnode status was not known of whom 2 patients (7.1%) had DTC. The P value was 0.001 which was reported significant and hence had a positive correlation in this study. In this study, 496 patients had T1 tumour status of whom 55 (11.1%) had DTC, 252 patients (30.8%) had T2 tumour status of whom 37 (14.7%) had DTC and 47 had a combined tumour status of T3&T4 of whom 11 (23.4%) were positive for DTC. Of the 22 patients (2.7%) in whom tumour status was not known, 5 were positive for DTC. The P value was 0.011 and hence reported to have a positive correlation in this study. While correlating the histological grading, 617 patients had grade I of which

78 (12.6%) were positive for DTC, 156 patients had grade II of which 26 (16.7%) were positive for DTC, 203 patients had grade III of which 29 (14.3%) were positive for DTC while in 16 patients the histological grade was not known, of whom 2 (12.5%) were positive for DTC. The P value was 0.164 and hence was reported as not significant. Of the 143 patients (17.5%) who had lymphovascular permeation, DTC was positive in 25 (17.5%), while 29 (17.5%) were positive for DTC without lymphovascular permeation. Of the 198 patients who were ER negative, 33 patients (16.7%) were positive for DTC, while in contrast of the 582 patients who were ER positive, 69 patients (11.9%) were positive for DTC. The P value was 0.083 which is not significant. The relation between DTC & Progesterone receptor status was reported as not significant. But it is still important to note that DTC was positive in patients with ER & PR negativity.

In this study the authors have highlighted the following intricacies stressing the importance of DTC in Breast Cancer prognosis. The combination of tumor, hormone receptor status, and Bone Marrow status can categorize node-positive patients into a low risk group and a high-risk group for predicting early disease progression. By combining these, approximately 30% of the node-positive patients could be placed into low risk group. Meanwhile tumor size greater than 2 cm, receptor negativity, and/or BM-positive status predict a high systemic relapse rate. This study also confirms that the occurrence of DTC in Bone Marrow predicts the future systemic relapses and death from breast cancer, and hence the detection of these cells is to be considered as an independent prognostic factor for systemic relapses and breast cancer specific deaths. The proportion of positive Bone

Table A: CLINICOPATHOLOGICAL CORRELATION OF THE PATIENT AS IN THE STUDY OF WIEDSWANG.G et al 2003						
CHARACTERISTIC		**TOTAL**		**BM-DTC**		**p-VALUE**
		NO	**%**	**NO**	**%**	
MENOPAUSAL STATUS	PRE	231	28.3	31	13.4	0.964
	POST	503	61.6	68	13.5	
	UNKNOWN	83	10.1	9	10.8	
LYMPHNODE STATUS	N0	513	62.8	50	9.7	<0.001
	N1	172	21	26	15	
	N2&N3	105	12.8	30	28.5	
	NX	28	3.4	2	7.1	
TUMOUR STATUS	T1	496	60.7	55	11.1	0.011
	T2	252	30.8	37	14.7	
	T3&T4	47	5.8	11	23.4	
	TX	22	2.7	5	22.7	
HISTOLOGY	DUCTAL	617	75.5	78	12.6	0.188
	LOBULAR	156	19.1	26	16.7	
	OTHERS	44	5.4	4	9.1	
HISTOLOGICAL GRADE	I	199	24.4	19	9.5	0.164
	II	399	48.8	58	14.5	
	III	203	24.8	29	14.3	
	UNKNOWN	16	2	2	12.5	
ER STATUS	POSITIVE	582	71.2	69	11.9	0.083
	NEGATIVE	198	24.2	33	16.7	
	UNKNOWN	37	4.5	6	16.2	
PR STATUS	POSITIVE	461	56.4	46	10	0.191
	NEGATIVE	313	38.3	53	16.9	
	UNKNOWN	43	5.3	9	21	
HORMONAL STATUS	POSITIVE	622	76.1	73	11.7	0.051
	NEGATIVE	166	20.3	29	17.5	
	UNKNOWN	29	3.5	6	21	
VASCULAR INVASION	POSITIVE	143	17.5	25	17.5	0.103
	NEGATIVE	569	69.5	70	12.3	
	UNKNOWN	105	12.9	13	12.4	
TYPE OF SURGERY	CONSERVATIVE	252	30.8	22	8.7	0.042
	MASTECTOMY	546	66.8	83	15.2	

Weidswang.G, Borgen.E, Karesen.R etal Detection of Isolated Tumour Cells in Bone Marrow is an independent prognostic factor in Breast Cancer; JClin Oncol 2003; 21: 3469-3478

Marrow in this report is lower than in most other studies. This is explained by a different stage distribution of the patient population, methodological aspects and traditional markers for aggressiveness such as ER/PgR negativity and histologic grade 3, were less frequently registered in this study. Differences in the use of negative controls might explain some of the variations in the reported rate of Bone Marrow positives. The choice of monoclonal antibodies for detection and the morphologic criteria used for the scoring of tumor cells may also influence the results and also short follow up times as being an important limiting factor for exploring the clinical value of DTC detection. This study concluded that DTC had an independent prognostic value in breast cancer management.

Braun.S et al 2005 **(Table B)** had done a pooled analysis of Bone Marrow micrometastasis in Breast Cancer**.** A total of 4703 patients were studied of which 1438 patients (13.21%) were positive for DTC. Of the 224 patients(4.76%) in the age group of 20-35yrs, 78(34.8%) had DTC, of 1454 patients (30.91%) in the age group of 36-50yrs, 484(33.3%) had DTC, of 1980 patients (42.1%) in the age group of 51-65yrs, 485(29.5%) had DTC and of 1045 patients(22.21%) above 65yrs, 291(27.8%) had DTC. The P value was 0.001 which was statistically significant. Of these 4703 patients, 1579 (33.5%) were in the premenopausal age and 3124 (66.5%) patients were in the postmenopausal age. Of the premenopausal group 31(13.4%) patients were positive for DTC while 921 (29.5%) patients in the postmenopausal age group were positive for DTC. The P value was 0.001 which showed a significant correlation between menopausal status and DTC.

Of the 2725 patients (58%) who had no lymphnode metastasis 719 (26.4%) had DTC while 1101patients (23.4%) with N1 level lymphnode metastasis 330 (30%) had DTC, 469 patients (9.9%) with N2 level lymphnode metastasis, 185 (39.4%) had DTC and 408 patients (8.6%) with N3 lymphnode metastasis, 294 (50%) had DTC. The P value was 0.001 which was reported as significant. Of 2507 patients with T1 tumour status, 633 patients (25.2%) had DTC, while 1706 patients (36.2%) with T2 tumour status, 568 (33.3%) had DTC, 263 patients with T3 tumour status, 100 (38%) were positive for DTC and 227 patients with T4 tumour status, 137 patients (60.4%) had DTC. The P value was <0.001, which shows an increased incidence of DTC with higher tumour status. Of the 4703 patients studied 3605 patients (76.6%) had ductal carcinoma not otherwise specified, of which 1108 patients (30.7%) were positive for DTC. Of the 4703 cases, 693 patients had grade I tumour of which 156 (22.5%) were positive for DTC, 2141 had grade II tumour of which 641 (29.9%) were positive for DTC, 1462 had grade III tumour of which 504 (34.5%) were positive for DTC. In 407 patients the histological grade was not known and those patients were negative for DTC. The P value was 0.001 which was reported statistically significant. Of the 923 patients who were negative for hormonal receptors, 979 patients (29.4%) were positive for DTC.

Of the 3326 patients who were positive for any one of the receptors, 318 patients (34.5%) were positive for DTC. The P value was 0.003 which showed a positive correlation. This study enrolled nine clinical studies and found strong evidence of the independent, adverse prognostic significance of the presence of bone marrow micrometastasis at the time of the initial diagnosis of operable breast

TABLE B: CLINICOPATHOLOGICAL CORRELATION OF THE PATIENT

AS IN THE STUDY OF - BRAUN.S. et al 2005

CHARACTERISTIC		**TOTAL**		**BM-DTC**		**p-VALUE**
		NO	**%**	**NO**	**%**	
AGE AT DIAGNOSIS	20-35	224	4.76	78	34.8	0.001
	36-50	1454	30.91	484	33.3	
	51-65	1980	42.1	485	29.5	
	>65	1045	22.21	291	27.8	
MENOPAUSAL STATUS	PRE	1579	33.5	31	13.4	0.02
	POST	3124	66.5	921	29.5	
LYMPHNODE STATUS	N0	2725	58	719	26.4	0.001
	N1	1101	23.4	330	30	
	N2	469	9.9	185	39.4	
	N3	408	8.6	294	50	
TUMOUR STATUS	T1	2507	53.3	633	25.2	<0.001
	T2	1706	36.2	568	33.3	
	T3	263	5.5	100	38	
	T4	227	4.8	137	60.4	
HISTOLOGY	DUCTAL	3605	76.6	1108	30.7	0.08
	LOBULAR	646	13.7	203	31.4	
	OTHERS	452	9.6	55	12.6	
HISTOLOGICAL GRADE	I	693	14.7	156	22.5	<0.001
	II	2141	45.5	641	29.9	
	III	1462	31	504	34.5	
	UNKNOWN	407	8.6	0	0	
HORMONAL STATUS	POSITIVE	3326	70.7	318	34.5	0.003
	NEGATIVE	923	19.6	979	29.4	
	UNKNOWN	454	9.6	0	0	

Braun.S, Florial.D, Vogel. et al A pooled Analysis of Bone Marrow Micro-metastasis in Breast Cancer; New Eng J Med 2005; 353; 793-802

cancer. In conclusion, this study also prescribed the prognostic value of the presence of bone marrow DTCs' and opined that DTC could be useful in the design of trials of the adjuvant treatment for breast cancer.

Naume B et al 2001 **(Table C)** did a prospective analysis of clinical relevance of detection of isolated tumor cells in 920 breast cancer patients. Of these 108 patients (13.21) were positive for DTC. Of these patients 266(29.5%) were premenopausal and 564(62.6%) were postmenopausal while in 71(7.8%) patients the menopausal status was not known. Of the premenopausal patient 32 (13.6%) patients were positive for DTC while 70(13.8%) in the post menopausal patients were positive for DTC. The P value was 0.906 which was reported as not significant.

Of 567 patients (40%) had no lymphnode metastasis of which 51 patients (9.9%) had DTC. Of the 185 patients (24%) with N1 level lymphnode metastasis 27 (15.6%) had DTC, 114 patients (36%) with N2&N3 level lymphnode metastasis, 31 (28.4%) had DTC while in 35 patients (3.9%) the lymphnode status was not known and they were all negative for DTC. The P value was <0.0005 which showed a significant increase in DTC presence with higher lymphnode status. Of the 530 patients with T1 tumour status 56 (11.2%) had DTC, of 267 patients (29.3%) with T2 tumour status, 38 patients(15%) had DTC and of 55 patients with a combined tumour status of T3&T4 , 12 patients(22.6%) were positive for DTC. The P value was 0.013 which showed a significant association between DTC and tumour status. Of the patients studied 662 patients(72%) had ductal carcinoma not otherwise specified, of which 81 patients (12.9%) were positive for DTC.

Of the 214 patients who had grade I tumour; 19 patients (9.5%) were positive for DTC, Of the 417 patients with grade II tumour, 59 patients (14.7%) were positive for DTC and of the 217 patients with grade III tumour, 31 patients (14.9%) were positive for DTC. The P value was 0.013 and hence a significant association between DTC and histological grade.

Of the 222 patients who were ER negative, 34 (16.8%) were positive for DTC while of the 625 patients who were ER positive, 71 (12.2%) were positive for DTC. The P value was 0.092, hence reported as statistically not significant. Of the 347 patients who were PR negative, 48 (15.1%) were positive for DTC while of the 494 patients who were PR positive, 48 (15.1%) were positive for DTC. The P value was 0.157 which is not statistically significant. Of the 159 patients(20.1%) who showed lymphovascular permeation, DTC was positive in 26 patients (17.4%), while 72 patients (12.6%) who were positive for DTC did not show any lymphovascular permeation. P value was 0.045 which shows a significant association between DTC and vascular invasion.

This study concluded that (1) In node-negative patients, a markedly higher frequency of DTCs in Bone Marrow was observed among patients with receptor-negative primary tumors, (2) The lower frequency of DTCs in ER and/or PgR positive patients could be expected because it has been observed previously that early metastatic relapse is less frequent in hormone receptor-positive breast cancer. (3) The reported incidence of DTCs in Bone Marrow in breast cancer patients has varied considerably in different studies and may be attributed to (a) number of mono nuclear cells analyzed (b) expression of antigen used for detection of DTCs, (c) specificity of the monoclonal antibody used (d) sensitivity and specificity of the

TABLE C: CLINICOPATHOLOGICAL CORRELATION OF THE PATIENT

AS IN THE STUDY OF NAUME. B et al 2001

CHARACTERISTIC		**TOTAL**		**BM-DTC**		**p-VALUE**
		NO	**%**	**NO**	**%**	
MENOPAUSAL STATUS	PRE	266	29.5	32	13.6	0.906
	POST	564	62.6	70	13.8	
	UNKNOWN	71	7.8	0	0	
LYMPHNODE STATUS	N0	567	40	51	9.9	<0.0005
	N1	185	24	27	15.6	
	N2&N3	114	36	31	28.4	
	NX	35	3.9	0	0	
TUMOUR STATUS	T1	530	58.2	56	11.2	0.013
	T2	267	29.3	38	15	
	T3&T4	55	6	12	22.6	
HISTOLOGY	DUCTAL	662	72	81	12.9	0.238
	LOBULAR	165	17.9	26	16.6	
	OTHERS	46	5	4	9.1	
HISTOLOGICAL GRADE	I	214	25.2	19	9.5	0.013
	II	417	49.2	59	14.7	
	III	217	25.6	31	14.9	
ESTROGEN RECEPTOR STATUS	POSITIVE	625	73.8	71	12.2	0.092
	NEGATIVE	222	26.2	34	16.8	
	UNKNOWN	73		0	0	
PROGESTERONE RECEPTOR STATUS	POSITIVE	494	58.7	54	11.7	0.157
	NEGATIVE	347	41.3	48	15.1	
	UNKNOWN	0	0	0	0	
HORMONAL STATUS	POSITIVE	624	78.5	75	12	0.055
	NEGATIVE	170	21.4	30	17	
	UNKNOWN	73	0	0	0	
VASCULAR INVASION	POSITIVE	159	20.1	26	17.4	0.045
	NEGATIVE	609	76.9	72	12.6	
	UNKNOWN	24	3	0	0	

Bjørn Naume,2 Elin Borgen, Gunnar Kvalheim et al.Detection of Isolated Tumor Cells in Bone Marrow in Early-StageBreast Carcinoma Patients: Comparison with Preoperative Clinical Parameters and Primary Tumor Characteristics1 Clinical Cancer Research 2001;7:4122–4129.

method (e) use of negative controls (f) accuracy of the screening process and evaluation of immunopositive cells and (g) stage distribution in the patient series.

Jean-Yves Pierga et al 2004 (Table D) studied the clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients in 114 patients of whom 67 patients (59%) were positive for DTC. Of 114 patients 75 patients' clinicopathological characteristics were correlated with DTC. Of these 43(57%) were premenopausal and 32(43%) were postmenopausal. Of the premenopausal group 26(60.5%) patients were positive for DTC while 11(34%) patients were positive for DTC in the postmenopausal age group. The P value was 0.024 which was reported as a significant association between menopausal status and DTC. Of the 36 patients (48%) who had no lymphnode metastasis, 16 (44.5%) had DTC, while of the 32patients (43%) with N1 level lymphnode metastasis, 16patients(50%) had DTC and 7 patients (9%) with N2 level lymphnode metastasis, 5 patients (71.5%) had DTC. The P value was 0.879 which was reported as not statistically significant. Of the 39 patients with T1 & T2 tumour status 13 (33%) had DTC while of the 20 patients(27%) with T3 tumour status 12 (60%) had DTC. Of 16 patients with tumour status of T4 12 (75%) were positive for DTC. The P value was 0.35 which was reported as not significant. Of 75 patients studied 65 patients (87%) had Ductal carcinoma Not Otherwise Specified. Of 12 patients with grade I tumours 5(42%) were positive for DTC, while of 27 patients with gradeII tumour 10 (37%) were positive for DTC and of 34 patients with grade III tumour 22 (65%) were positive for DTC. The P value was 0.079 which is not statistically significant.

TABLE D: CLINICOPATHOLOGICAL CORRELATION OF THE PATIENT

AS IN THE STUDY OF PIERGA.A. et al 2004

CHARACTERISTIC		TOTAL		BM-DTC		p-VALUE
		NO	%	NO	%	
MENOPAUSAL STATUS	PRE	43	57	26	60.5	0.024
	POST	32	43	11	34	
	UNKNOWN	0	0	0	0	
LYMPHNODE STATUS	N0	36	48	16	44.5	0.879
	N1	32	43	16	50	
	N2	7	9	5	71.5	
TUMOUR STATUS	T1/T2	39	52	13	33	0.35
	T3	20	27	12	60	
	T4	16	21	12	75	
HISTOLOGY	DUCTAL	65	87	33	51	0.573
	LOBULAR	9	12	4	44	
	OTHERS	1	1	0	0	
HISTOLOGICAL GRADE	I	12	16	5	42	0.079
	II	27	36	10	37	
	III	34	45	22	65	
ER STATUS	POSITIVE	44	59	17	38.6	0.026
	NEGATIVE	31	41	20	64.5	
PR STATUS	POSITIVE	14	19	6	43	0.328
	NEGATIVE	45	60	26	58	
VASCULAR INVASION	POSITIVE	33	44	21	64	0.043
	NEGATIVE	40	53	16	40	

Jean-Yves Pierga,Charlyne Bonneton,Anne Vincent-Salomon et a. lClinical Significance of Immunocytochemical Detection of Tumor Cells Using Digital Microscopy in Peripheral Blood and BoneMarrow of Breast Cancer Patients.Clinical Cancer Research.2004;10, 1392–1400.

Of the 31 patients who were ER negative, 20 (64.5%) were positive for DTC and of the 44 patients who were ER positive, 17 (38.6%) were positive for DTC. The P value was 0.026 which was reported significant. Of the 45 patients who were PR negative, 26 patients (58%) were positive for DTC. Of the 14 patients who were PR positive, 6 cases (43%) were positive for DTC.P value was 0.328 which is not statistically significant. Of the 33 patients(44%) who showed lymphovascular permeation, DTC was positive in 21 patients (64%), while 16 patients (40%) were positive for DTC but did not show any lymphovascular permeation. P value was 0.043 and hence there was significant association between vascular invasion and DTC in this study. This study detailed that the prognostic factors for disease-free survival in the series were classical i.e. tumor size; clinical nodal status; tumor emboli and Bone Marrow micrometastatic disease. Pathological nodal status was not a significant prognostic factor in this series. In conclusion, this study supported the prognostic value of the presence of bone marrow micrometastasis in breast cancer prognostication.

Diel Ingo. J. et al.,1996 (Table E) did a study on micrometastatic breast cancer cells in bone marrow at primary surgery in 727 patients of whom 315 (43.32%) were positive for DTC. Of 727 patients studied 296(40.94%) were premenopausal and 431(59%) were postmenopausal. In the premenopausal group 112(38%) were positive for DTC while 203(47%) were positive for DTC in the postmenopausal group. The P value was 0.01 reported as significant.

Of 360 patients (49.5%) without lymphnode metastasis, 112 (31%) had DTC while of 367patients (50.4%) with lymphnode metastasis 203 (55%) had

DTC. The P value was 0.001, hence reported significant. Of 258 patients with T1 tumour status, 77 (30%) had DTC, of 323 patients (44.4%) with T2 tumour status, 137 (42%) had DTC, of 69 patients with T3 tumour status, 43 (62%) had DTC and of 77 patients with T4 tumour status, 58 (75%) had DTC. The P value was <0.001 which was reported significant. Of 403 patients with grade I & II tumours 159 (39%) were positive for DTC while of 431 patients with grade III tumour 144 (52%) were positive for DTC. The P value was 0.01, which was a significant correlation.

Of 207 patients who were ER negative, 91 (44%) were positive for DTC and of 410 patients who were ER positive, 186 (45%) were positive for DTC. The P value was 0.74, hence was reported as not significant. Of 247 patients who were PR negative,119 (48%) were positive for DTC while of 341patients who were PR positive, 145 (42%) were positive for DTC. The P value was 0.17, hence was reported as not significant.

This study mainly aimed at prognostication in comparison with the nodal status and concluded that the worst prognosis was associated with the presence of tumor cells in both axillary lymph nodes and bone marrow.

The results of the study provided consistent evidence that DTC is a prognostic indicator independent of the nodal status.

TABLE E: CLINICOPATHOLOGICAL CORRELATION OF THE PATIENT_

DIEL.J.ET AL., 1996

CHARACTERISTIC		TOTAL		BM-DTC		p-VALUE
		NO	%	NO	%	
MENOPAUSAL STATUS	PRE	296	41	112	38	0.01
	POST	431	59	203	47	
LYMPHNODE STATUS	N0	360	49.5	112	31	0.001
	N+	367	50.4	203	55	
TUMOUR STATUS	T1	258	35.4	77	30	<0.001
	T2	323	44.4	137	42	
	T3	69	9.4	43	62	
	T4	77	10.5	58	75	
HISTOLOGICAL GRADE	I&II	403	48	159	39	0.01
	III	431	52	144	52	
ER STATUS	410	66.4	186	45	410	0.74
	207	33.5	91	44	207	
PR STATUS	POSITIVE	341	57.9	145	42	0.17
	NEGATIVE	247	42	119	48	

Ingo J. Diel, Manfred Kaufmann, Serban D. Costa. Et al., Micrometastatic Breast Cancer Cells in Bone Marrow at Primary Surgery: Prognostic Value in Comparison With Nodal Status.Journal of the National Cancer Institute.1996; 88:1652-1658

Our Study 2009-2010. (Table F) We did a prospective analysis of clinical relevance of detection of isolated tumor cells in the bone marrow of breast cancer patients at primary diagnosis in 50 patients. Of these 50 patients studied, 13 (26%) were positive for DTC. The mean age of patients in our study is 52 years. Of the 50 patients studied, 24 patients were below 52 years of whom 3 (12.5%) were positive for DTC while 26 patients were above 52 years of age of whom 10 patients (38.5%) were positive for DTC. The age and occurrence of DTC in the bone marrow statistically correlated (P value 0.037). This indicates that the risk of DTC is increased with the age of the patient. The study of Braun S et al (2005) made a contrasting observation that the occurrence of DTC was higher in ages younger than 51 years. This observation may be attributable to the variation in our study size and selection methodologies. The existence of a racial factor to this observation also needs to be studied.

Of the 50 patients studied, 21 patients (42%) were premenopausal while 29 patients (58%) were postmenopausal. Of the premenopausal patients, 2 (9.5%) were positive for DTC while of the postmenopausal patients 11(37.9%) were positive for DTC. Menopausal status and the presence of disseminated tumour cells revealed that there is an increased incidence of DTC in postmenopausal age (p Value 0.024). This correlated well with the study of Braun S et al 2005 and Deil IJ et al 1996 while this was in contrast with the study of Pierga A et al 2004 in which the premenopausal patients predominanted. Weidswang G et al 2003 and Naume B et al 2001 did not report any significant correlations in their studies. The South Indian rural population included in our study have to be further evaluated for unique racial and socioeconomic parameters that may be reasons for the significant increase in DTC in the postmenopausal group in our study.

Of the 50 patients studied, 18 patients (36%) had no lymphnode metastasis of which one of the patients (5.6%) had DTC. Of 17patients (34%) with N1 level lymphnode metastasis, 6patients (35.3%) had DTC and of 15 patients (30%) with N2 level lymphnode metastasis, 6 patients (40%) had DTC. There were no N3 level lymphnode metastases in our study group. The association between DTC and lymphnode status was significant (p value 0.045). This indicates that the risk of DTC increases with a higher nodal status. This correlates with the study of Braun S et al 2005, Wiedswang.G et al 2003, Naume B et al 2001 and Deil IJ et al 1996. Pierga A et al 2004 did not report any such significant correlation between DTC and lymphnode status in their study. In our study the comparison of the nodal status and presence of DTC indicated that there was an increased concordance in the presence of tumor cells in axillary lymph nodes and bone marrow with increasing incidence with a higher lymphnode status. This result of our study provides significant evidence that DTC is an independent prognostic indicator.

The mastectomy specimens were examined, and it was correlated that of the 50 patients studied, 12 patients (24%) had a primary tumour size of less than 2cms **(T1),** of whom 1 patient (8.3%) had DTC. Of the 11 patients (22%) with a primary tumour size of more than 2cms but less than 5cms **(T2),** 1 patient (9.1%) had DTC. Of the 15 patients (30%) with a primary tumour size of more than 5cms **(T3),** 4 patients (26.7%) had DTC and of 12 patients (24%) with a variable primary tumour sizes with skin or chest wall infiltrates **(T4),** 7 patients (58.3%) had DTC. The tumour size and the occurrence of DTC were correlated statistically in our study (p value 0.018). This indicates that the frequency of DTC was more

with a higher tumour size (T). This correlated well with the studies of Braun S et al 2005, Wiedswang.G et al 2003, Naume B et al 2001and Deil IJ et al 1996. Pierga A et al 2004 did not report any correlation between DTC and tumour status in their study. Our study indicates that there is a progressive increase of incidence of DTC with the increase in size of the tumour with a maximal incidence associated with T4 tumours. This compares well with existing tested prognostic indices and hence contributes to the value of DTC as a prognostic indicator.

Of the 50 patients studied all 50 patients (100%) had Infiltrating Ductal Carcinoma of Breast – Not Otherwise Specified type. These cases were graded using the modified Bloom and Richardson's System of Grading (Elston CW et al loc.cit 1991). Of the 16 patients(32%) studied with grade I tumor , 1 patient (10%) had DTC, while of the 20 patients (40%) with Grade II tumour 5 patients (24%) had DTC, and of the 14 patients (28%) with Grade III tumour 7 patients (66%) had DTC. Statistical correlation (p value 0.0u24) indicates that the risk of DTC increases with higher histological grades. This correlated well with the studies of Braun S et al 2005, Naume B et al 2001 and Deil IJ et al 1996, while this was in contrast with observation of Pierga A et al 2004 in which the grade I tumor predominated. Wiedswang.G et al 2003 did not report any significant correlation with the histological grades in their study. In our study, the progressive increase of incidence of DTC with the increase in tumour grade and a maximal association with grade III tumours was observed. This signifies that DTC has an independent prognostic value.

Of the 50 mastectomy specimens studied, 46 were subjected to hormonal receptor status evaluation. Of these, 33 patients were ER negative. In this group, 13 patients (34.4%) were positive for DTC. Of the 13 patients who were ER positive, no DTC was detected. The occurrence of DTC and ER status was statistically correlated (p value 0.011). This indicates that the risk of DTC increased in ER negative tumours. This correlated well with the study of,Wiedswang.G et al 2003, Naume B et al 2001, Pierga A et al 2004. But Diel J et al 1996 did not report any such correlation between DTC and ER status in their study.

Of the 24 patients who were PR negative, 12 patients (50%) were positive for DTC. Of the 22 patients who were PR positive, 1 patient (4.54%) was positive for DTC. The frequency of DTC and PR status was statistically correlated (p value 0.001). This indicates that the risk of DTC increased in PR negative tumours. This correlated well with the study of Wiedswang.G et al 2003, Naume B et al 2001, Pierga A et al 2004. But Diel J et al 1996 did not report any such correlation between DTC and PR status in their study. Both ER and PR hormonal receptor status was computed together. Of the 46 patients subjected to both hormonal evaluations, 13 patients (71.73%) were positive for hormonal status and none of them had DTC. Of the 33 patients (71.73%) who were negative for both hormone receptors, 13 patients (39.39%) had DTC. This is statistically significant (p value 0.011). This indicates that the risk of DTC increased with hormone negative tumours. This correlated well with the study of Wiedswang.G et al 2003, Naume B et al 2001, Pierga A et al 2004. But Diel J et al 1996 did not report any such

correlation between DTC and hormonal status in their study. The patients who was ER positive or PR positive or Positive for both ER and PR were found to have a lesser incidence of DTC in the bone marrow. This signifies that the tumour cells were more bound to the primary site with a hormone positive state and correlates with the tumour biology. In our study, significantly the absence of both or either of the receptors i.e. ER & PR was associated with increased DTC incidence in the bone marrow. This is ample evidence that the current methods of prognostication are significant while DTC adds value to their use and can act as an independent index. This observation of ours also throws light on the tumour cell growth and behaviour. The breast cancer cells with ER / PR receptors on the surface are prone to less metastasis than ER / PR negative cells. This has to be considered for further research.

Of the 17 patients (34%) who showed obvious lymphovascular permeation, DTC was positive in 12 patients (70.6%), while 1 patient (3%) was positive for DTC, but did not show any obvious lymphovascular permeation. This was statistically significant (p value 0.000) and correlated well with the studies of Naume B et al 2001, Pierga A et al 2004. But Wiedswang G et al 2003, did not report any such correlation. Braun S et al 2005 and Diel J et al 1996 did not correlate vascular invasion with DTC in their studies. In our study we considered the correlation between lymphovascular permeation and presence of DTC in the bone marrow as significant.

For all the 50 patients included in this study, we had done a bone marrow aspiration and a clot section as described. Of the 50 cases studied only 1 patient

had DTC in the clot sections, while 13 patients had DTC in the aspirate smears. This shows that the clot section had lesser predictive value compared to aspirate smears. But, with better concentration methods and use of density gradient enrichment methods of bone marrow material, the sensitivity and specificity of the clot section is bound to improve. The detection rate of DTC with Pan Cytokeratin immunocytochemical staining in our study was 26%, this correlated with the studies of Redding WH et al (1983)[105] 28%, Untch M et al (1999)[106] 28% and Coombes RJ et al loc.cit (1986) 23%. Redding used MUC, while Untch used CK18/CK2 as the immmunocytochemical marker in their studies.

The total study size of 50 patients in our study was spread over a smaller period of time. The observations and inferences drawn during the course of our study are hence limited by the study size and time. But considering the significant conclusions derived in this study and its significance in patient management, a detailed prospective study with follow up is proposed.

Our study in comparison with the study of various other authors referred to in our discussion have highlighted the following intricacies stressing the importance of DTC in Breast Cancer prognosis. The combination of tumor size / T status, Hormone receptor status in relation with ER and PR, Lymphnode status / N status, Tumour grade, Lymphovascular permeation and presence of DTC in the Bone Marrow can categorize patients into a good prognosis group and a high-risk group which can be applied for predicting early disease progression. The node positive patients (N1 or more) with a Tumor size greater than 2 cm (T2 or More), receptor negativity, and BM-positive status predict a high relapse rate.

TABLE F: CLINICOPATHOLOGICAL CORRELATION OF THE PATIENTS IN OUR STUDY

CHARACTERISTIC		TOTAL		BM-DTC		p-VALUE
		NO	%	NO	%	
AGE AT DIAGNOSIS	<52	24	48	3	12.5	0.037
	52&above	26	52	10	38.5	
MENOPAUSAL STATUS	PRE	21	42	2	9.5	0.024
	POST	29	58	11	37.9	
	UNKNOWN	0	0	0	0	
LYMPHNODE STATUS	N0	18	36	1	5.6	0.045
	N1	17	34	6	35.3	
	N2	15	30	6	40	
	N3	0	0	0	0	
	NX	0	0	0	0	
TUMOUR STATUS	T1	12	24	1	8.3	0.018
	T2	11	22	1	9.1	
	T3	15	30	4	26.7	
	T4	12	24	7	58.3	
	TX	0	0	0	0	
HISTOLOGY	DUCTAL	50	100	13	100	-
	LOBULAR	0	0	0	0	
	OTHERS	0	0	0	0	
HISTOLOGICAL GRADE	I	16	32	1	10	0.024
	II	20	40	5	24	
	III	14	28	7	66	
	UNKNOWN	0	0	0	0	
ESTROGEN RECEPTOR STATUS	POSITIVE	13	26	0	0	0.011
	NEGATIVE	33	66	13	39.39	
	UNKNOWN	4	8	0	0	
PROGESTERONE RECEPTOR STATUS	POSITIVE	22	44	1	4.54	0.001
	NEGATIVE	24	48	12	50	
	UNKNOWN	4	8.7	0	0	
HORMONAL STATUS	POSITIVE	13	26	0	0	0.011
	NEGATIVE	33	66	13	39.39	
	UNKNOWN	4	8	0	0	
VASCULAR INVASION	POSITIVE	17	34	12	70.6	0.000
	NEGATIVE	33	66	1	3	
	UNKNOWN	0	0	0	0	
TYPE OF SURGERY	SEG	1	2	0	0	0.015
	SIMPLE	15	30	8	53.3	
	MOD RAD	34	68	5	14.7	

Our study also confirms that the occurrence of DTC in bone marrow predicts the future systemic relapses and death from breast cancer in consonance with existing prognostic factors and hence the detection of these cells can be considered as an independent prognostic factor. The proportion of positive Bone Marrow DTC in our report is lower than in most other studies. This is explained by a different age and menopausal distribution of our patient population, variation in certain methodological aspects, social, regional and racial factors which were not registered in our study. The choice of monoclonal antibodies for detection and the morphologic criteria used for the scoring of tumour cells may also have influenced our results and also the small study size and the short time period may be important limiting factors in our study. This study yet confirms that the independent prognostic value of detection of DTC in Bone Marrow in breast cancer is significant and has to be studied widely in the Indian context with specific references to the regional, racial and socio-economic factors that may play a significant role.

SUMMARY AND CONCLUSION

Our study has highlighted the following important observations indicating the importance of DTC in Breast Cancer Prognosis.

1. Our Study concludes that the incidence of DTC is higher in post menopausal patients with Breast Carcinoma.
2. Our Study concludes that there is a significant concordance in the presence of tumor cells in lymph nodes and the presence of DTCs' in bone marrow and the incidence increased with increasing lymphnode status with a maximal association for N2 tumours.
3. Our Study concludes that there is a progressive increase in the incidence of DTC with increase in tumour size with a maximal association for T4 tumours.
4. Our Study concludes that there is a progressive increase of incidence of DTC in relation to tumour grade and a maximal association with histological grade III tumours.
5. Our Study concludes that the incidence of DTC increase with Hormone receptor negative tumours, while in concordance patients who were positive for Hormone receptors had a lesser incidence of DTC in the bone marrow. The relationship between breast cancer hormone receptors and intercellular adherence and hence metastasis has to be considered for further research.
6. Our Study concludes that lymphovascular permeation has a positive and significant relationship with DTC in the Bone Marrow.

7. Our Study concludes that clot sections have lesser predictive value compared to aspirate smears. But with better methodology, the sensitivity and specificity of clot sections may improve.
8. Our Study concludes that the evaluation of Bone Marrow for the disseminated tumour cells correlates with most other standard prognostic indices.
9. Our study concludes that the evaluation of bonemarrow for the disseminated tumour cells, can assist in categorization of breast cancer patients into low risk and high-risk groups, for a better prediction of early disease progression and relapse.
10. Our study also concludes that disseminated tumour cells in Bone Marrow could have an independent prognostic value and has to be studied widely in the Indian context with specific reference to the regional, racial and socioeconomic factors.

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APPENDIX -1

PROFORMA

BM No :

Name : Hospital No :

Age : Cancer OP No :

Sex : Ward / Unit

CLINICAL HISTORY

- Duration
- Nipple discharge
- Family history of breast or ovarian cancer
- Prior breast cancer diagnosis
- Prior breast surgery (Site, Diagnosis, Rx)
- History of exposure to radiation / Chemotherapy / hormonal therapy
- Current pregnancy / Lactation
- Menopausal status

CLINICAL EXAMINATION

General condition

Lump : size

Quadrant

Surface

Fixity

Lymph node : Axillary

Cervical

Other

Hepatosplenomegaly

CLINICAL STAGING

INVESTIGATIONS

Hb%	Total
TC%	Direct
DC	Indirect
RBC	SGOT
Platelets	SGPT
ESR	ALP
MCV	T. Protein
MCH	Albumin
MCHC	Globulin
P. Smear	

IMAGING STUDIES

X – ray

USG

CT

MRI

Mammography

CYTOLOGY

FNAC

Fluid cytology

BIOPSY	✓	**x**
Specimen	□	□
Lumpectomy	□	□
Simple mastectomy	□	□
Radical mastectomy	□	□

PROCEDURE		
Core biopsy / wedge biopsy	□	□
Lumpectomy	□	□
Total mastectomy	□	□

Lymph node sampling		
No lymph nodes present	□	□
Sentinel lymph node(s)	□	□
Axillary dissection	□	□
(Partial or complete)		

Specimen integrity		
Single intact specimen	□	□
Fragmented		
(Margins cannot be evaluated)	□	□

Specimen size

Greatest dimension

Specimen laterality

Right	□	□
Left	□	□
Not specified	□	□

Tumor Size

Tumor site

Upper outer quadrant	□	□
Lower outer quadrant	□	□
Upper inner quadrant	□	□
Lower inner quadrant	□	□
Central	□	□
Nipple	□	□

Tumor focality

Single focus	□	□
Multiple focus	□	□
No of foci	□	□
Sizes of individual foci.	□	□

MACROSCOPIC AND MICROSCOPIC EXTENT OF TUMOR SKIN

- Skin is not present □ □
- Invasive ca does not invade the skin □ □
- Invades the skin without skin ulceration □ □
- Invades the skin with skin ulceration □ □
- Satellite foci of skin invasion. □ □

Skeletal muscle

- No skeletal muscle present □ □
- Skeletal muscle present & is free of carcinoma □ □
- Carcinoma invades skeletal muscle □ □

Architectural pattern

Solid □ □

Papillary □ □

Micropapillary □ □

Cribriform □ □

Comedo □ □

Other (Specify) □ □

Nuclear Grade

Grade I □ □

Grade II □ □

Grade III □ □

Necrosis

Not identified □ □

Present, focal □ □

Present, central □ □

Margins

Margins cannot be assessed □ □

Margins uninvolved by ca □ □

Distance from closet margins

Margin positive for invasive ca □ □

* Specify margin

* Extent focal / minimal / extensive

Lymphovascular invasion - Not identified / present / indeterminate

Perineural invasion Not identified / Present Indeterminate

Lymph node status

No nodes ☐ ☐

If present : Total No of lymph nodes

Extra nodal extension Not identified / Present / Indeterminate.

TNM Staging

Distant metastasis Present / Not Present

Ancillary studies

ER / PR

Status of residual breast

BONE MARROW

Procedure - Iliac ☐ ☐

Biliac ☐ ☐

Sternum ☐ ☐

Amount collected

Anticoagulant used

Fixative used

Processing : Direct smear - H & E ☐ ☐

Leishman ☐ ☐

Special stain ☐ ☐

		IHC	□	□
Centrifuged Concentrate Smear	-	H & E	□	□
Stain used		leishman	□	□
		Special stain	□	□
		IHC	□	□
Clot sections	-	H & E	□	□
Stain used		Leishman	□	□
		Special stain	□	□
		IHC	□	□

RESULTS

APPENDIX -2

HAEMATOXYLIN AND EOSIN STAINING PROCEDURE

1. Dewax sections, hydrated through graded alcohols to water
2. Stained in alum haematoxylin for 7 min
3. Washed well in running tap water until sections blue for 5 minutes
4. Differentiated in acid alcohol for 5 seconds
5. Washed well in running tap water until sections blue for 5minutes
6. Stained in 1%Eosin Y for 3minutes
7. Washed in running tap water for 5minutes
8. Dehydrated through alcohols, cleared and mounted

John D.Bancroft, Alan Stevens; "Theory and Practice of Histological Techniques", 4th Ed; Churchill Livingstone; 1996 ;104.

APPENDIX -3

STANDARDS FOR DETECTION OF DISSEMINATED TUMOUR CELLS BY IMMUNOCYTOCHEMISTRY

1. A Bone Marrow sample of 5ml to 10 ml is aspirated from anterior and/or posterior iliac crests and collected in tubes containing either heparin or EDTA and stored within the range from +4°C to +20°C until further processing of the samples, preferably within 24 hours.
2. Smears were air-dried at room temperature and then immunostained. For fixation, we used acetone for 10 minutes or methanol for 15 minutes.
3. For immuno-staining, the anti-CK-antibodies A45-B/B3 or AE1/AE3, used together with the APAAP detection system. Blocking of endogenous alkaline phosphatase by using commercially available blocking kits.
4. Breast cancer cells were admixed to bone marrow samples from individuals without cancer is used for positive staining controls. Positive cells are defined as Cytokeratin-positive/immunocytochemically positive cells with disseminated tumor cell morphology. The bonemarrow slides are interpreted as positive when Disseminated tumor cells are present in bone marrow.

Tanja Fehm, Stephan Braun et al; A concept for the standardized detection of disseminated tumor cells in bone marrow from patients with primary breast cancer and its clinical implementation. Cancer. 2006;107:885-892.

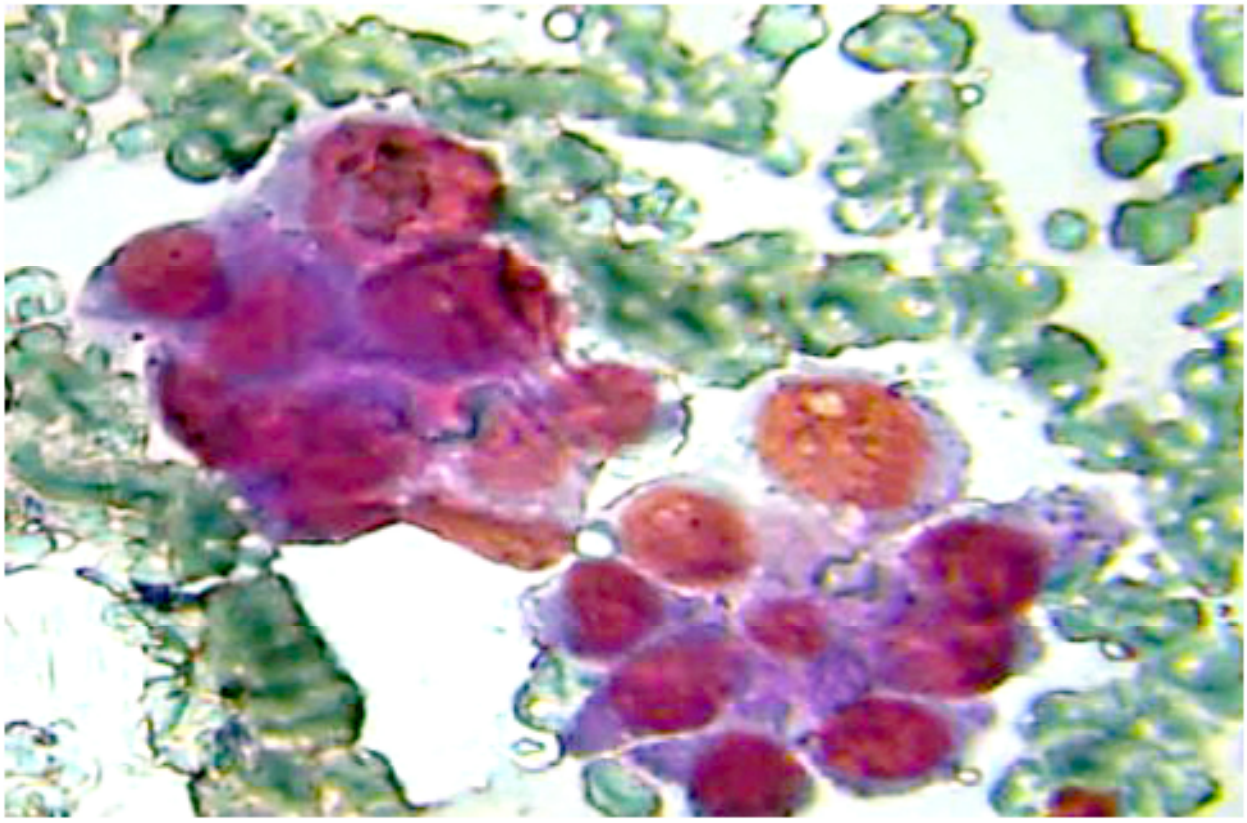

Fig :1 Photomicroscopic picture of bonemarrow smear showing disseminated tumor cells. Leishman (x100)

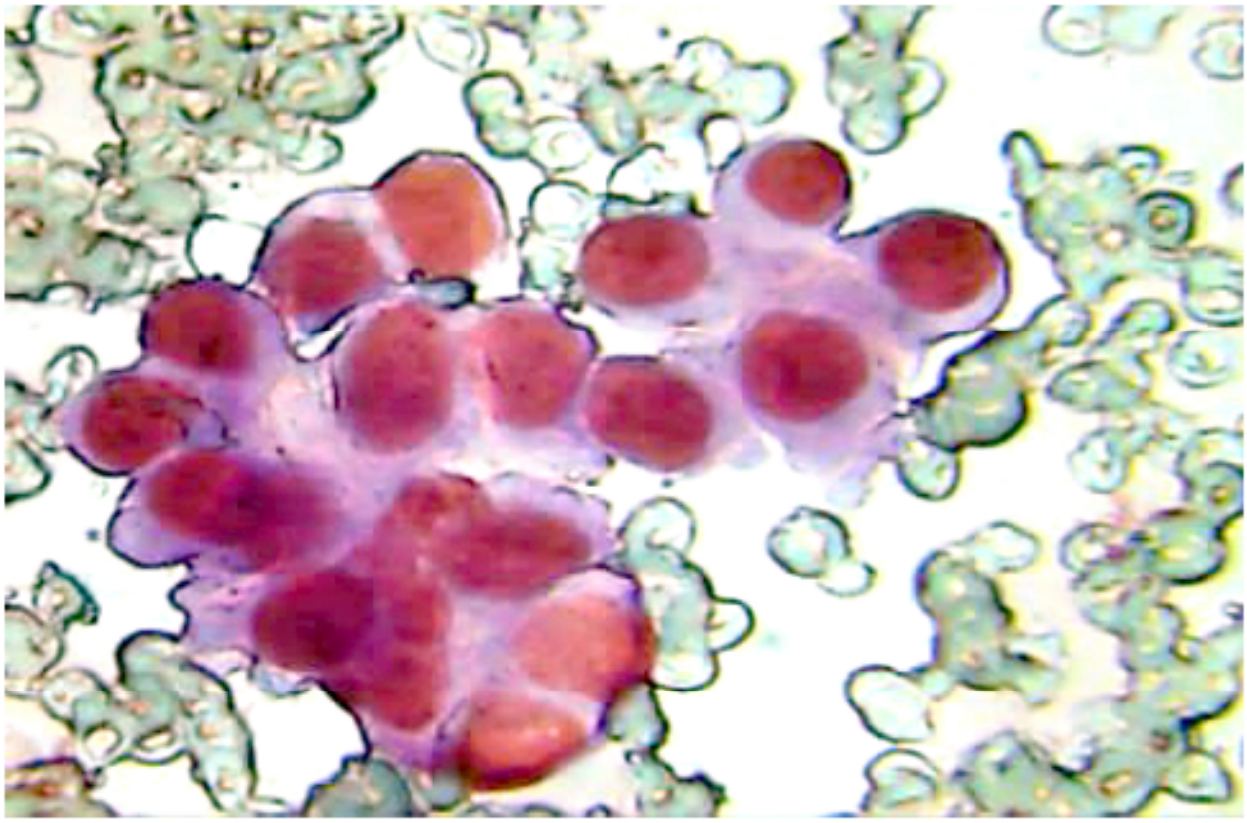

Fig :2 Photomicroscopic picture of bonemarrow smear showing disseminated tumor cells. Leishman (x100)

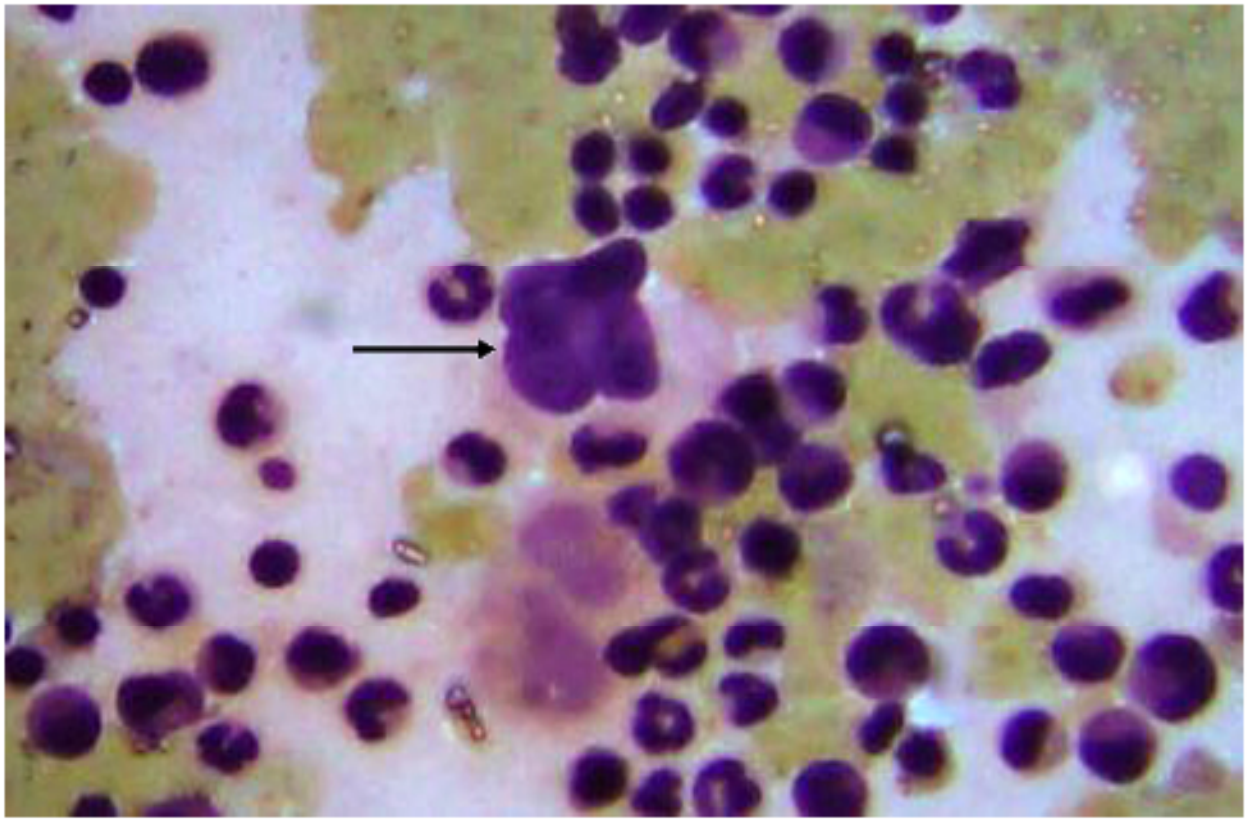

Fig :3 Photomicroscopic picture of bonemarrow smear showing isolated disseminated tumor cell(Arrow). H&E (x40)

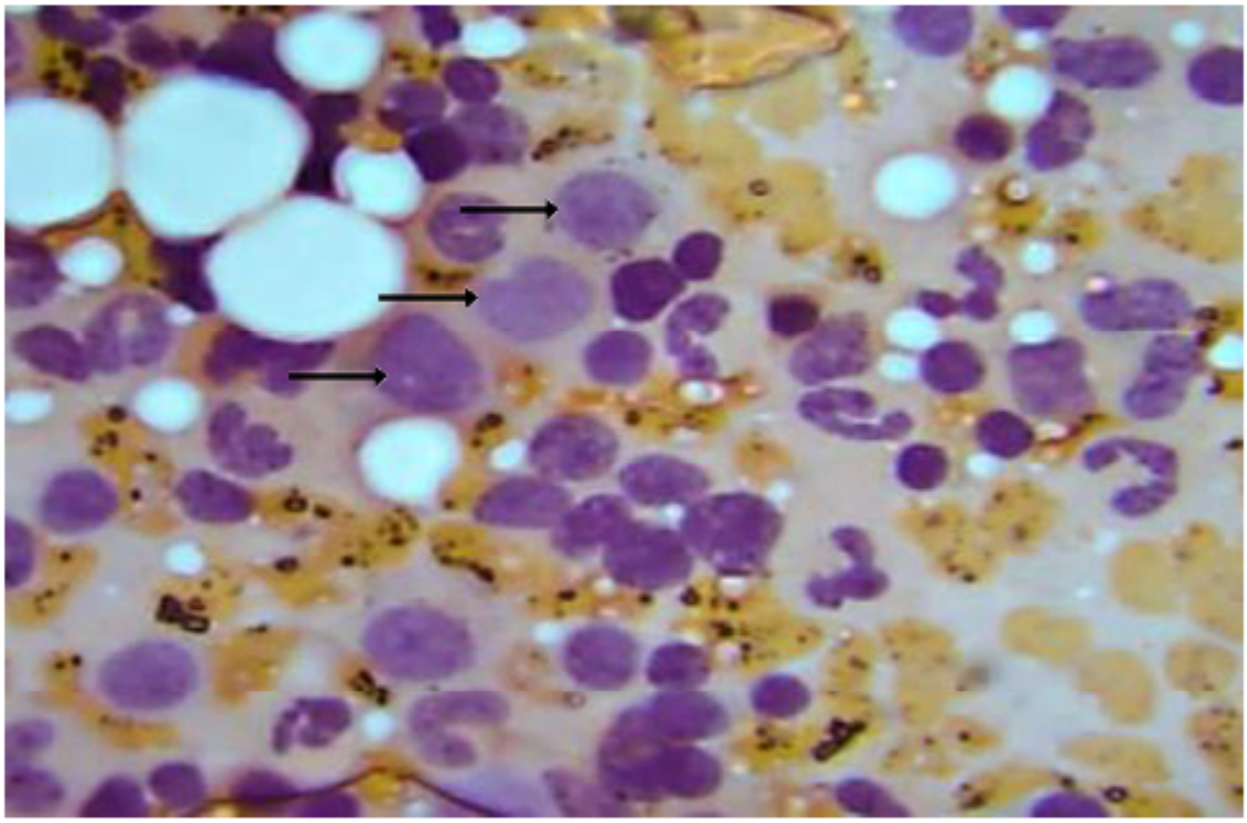

Fig :4 Photomicroscopic picture of bonemarrow smear showing disseminated tumor cells (Arrow). H&E (x40)

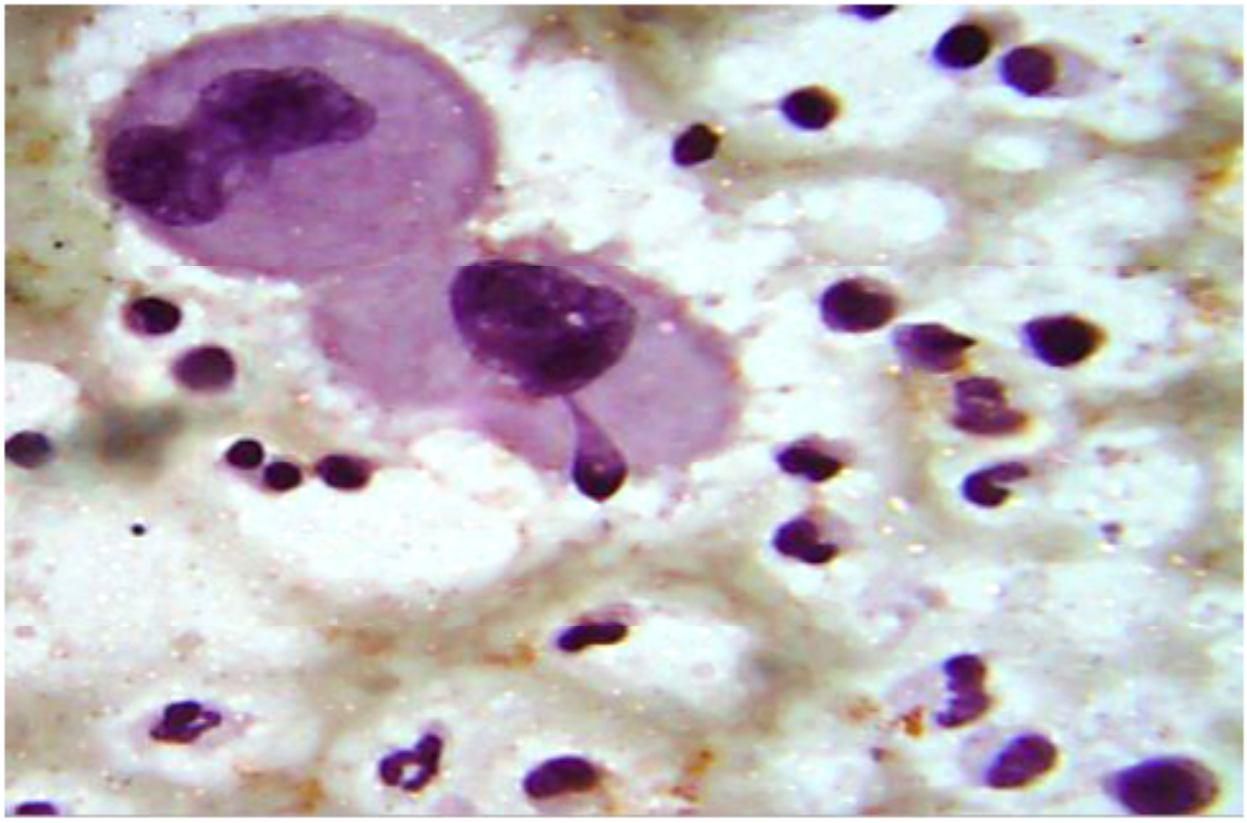

Fig :5 Photomicroscopic picture of bonemarrow smear showing disseminated tumor cell. H&E (x100)

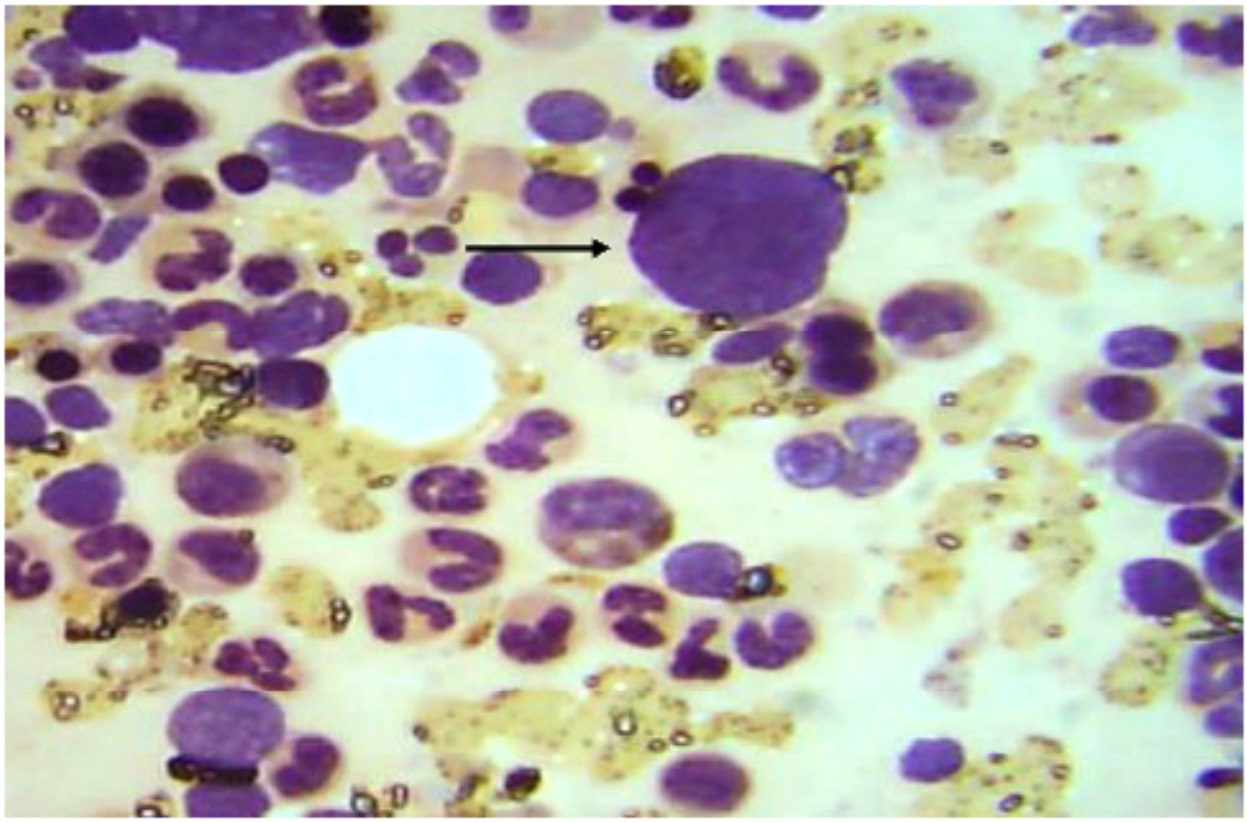

Fig :6 Photomicroscopic picture of bonemarrow smear showing isolated disseminated tumor cell (Arrow). H&E (x40)

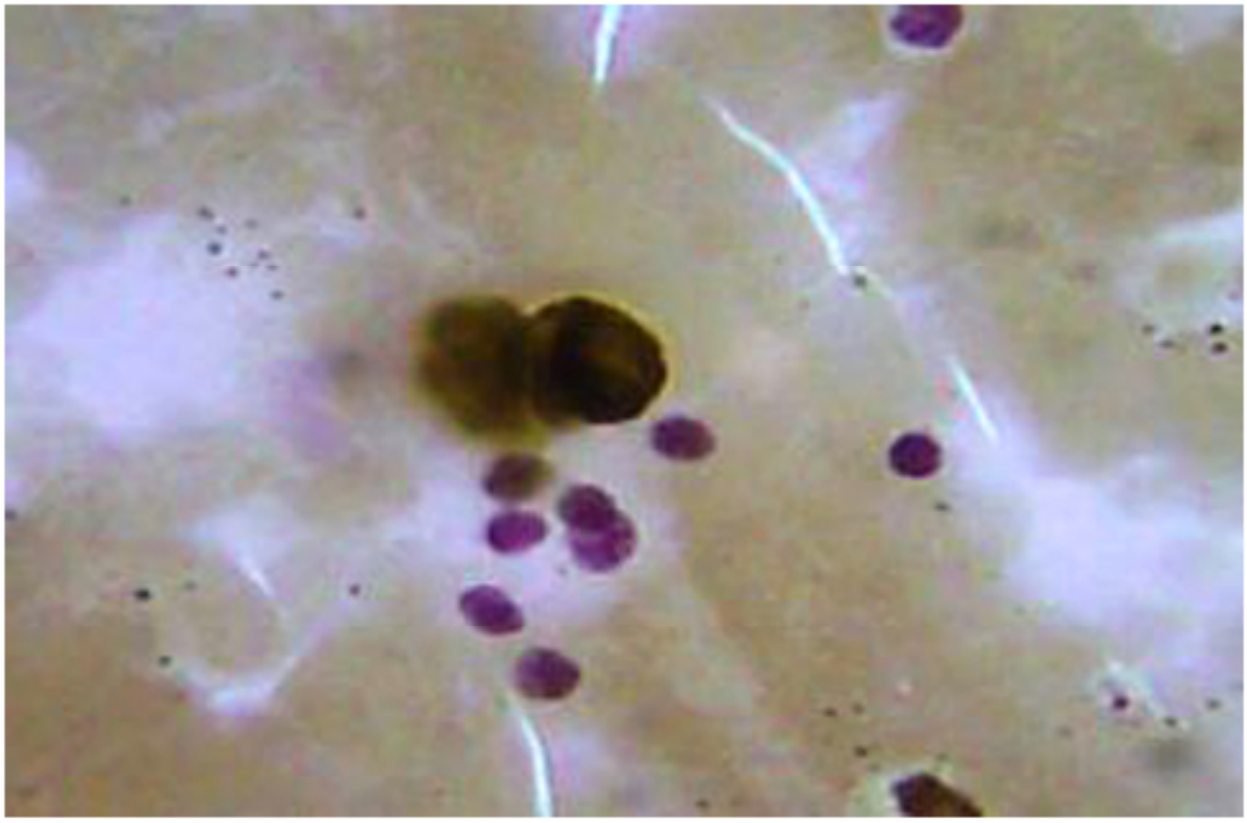

Fig :7 Photomicroscopic picture of pancytokeratin positive stain (CK18) X100

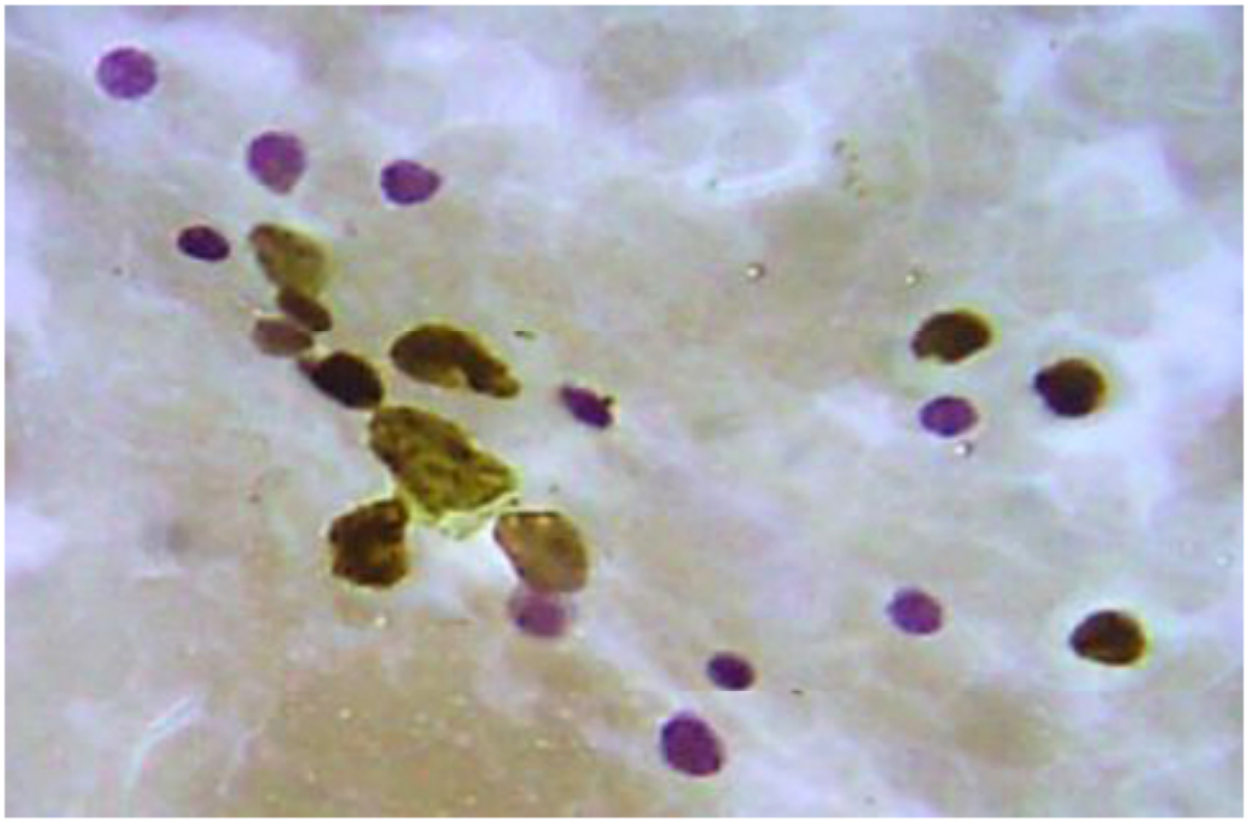

Fig :8 Photomicroscopic picture of pancytokeratin positive stain (CK18) X100

S NO	NAME	IP NO	AGE/SEX	MENOPAUSAL STATUS	TUMOR SIZE IN CM	TUMOR STATUS	NODE SIZE IN CM	LYMPH NODE STATUS	METASTASIS	HISTOLOGY	TNM STAGE	HISTOLOGY GRADE	VASCULAR INVASION	TYPE OF OPERATION	ER STATUS	PR STATUS	HORMONAL STATUS	DTC IN SMEARS	CLOT SECTION	CYTOKERATIN POSITIVE
1	LATHA	27634/09	35/F	PRE	3	T4	0	N0	M0	DUCTAL	IIIB	2	-	MRM	+	+	+	-	_	_
2	CHELLATHAI	31748/09	35/F	PRE	7	T3	5	N2	M0	DUCTAL	IIIA	2	+	MRM	-	+	-	-	_	_
3	LAKSHMIAMMAL	33643/09	65/F	POST	6	T3	4	N2	M0	DUCTAL	IIIA	2	+	SM	-	_	-	+	_	+
4	USHA	34558/09	48/F	PRE	3	T2	0	N0	M0	DUCTAL	IIA	1	-	MRM	+	+	+	-	_	-
5	ARUMUGATHAMMAL	37072/09	48/F	PRE	4	T2	1	N1	M0	DUCTAL	IIB	1	_	MRM	NA	NA	NA	-	_	-
6	MALLIKA	36904/09	39/F	PRE	8	T3	0	N0	M0	DUCTAL	IIB	3	-	SM	+	+	+	-	_	-
7	SENDU	37252/09	48/F	PRE	7	T4	5	N2	M0	DUCTAL	IIIB	1	+	MRM	-	+	-	-	_	-
8	LAKSHMIAMMAL	44698/09	50/F	PRE	4.2	T2	0	N0	M0	DUCTAL	IIA	2	-	MRM	+	_	+	-	_	-
9	MANOGARAM	41706/09	70/F	POST	5.5	T3	0	N0	M0	DUCTAL	IIB	2	-	MRM	_	_	_	+	_	+
10	PONKILI	43521/09	45/F	PRE	1.2	T1	2	N1	M0	DUCTAL	IIA	1	-	MRM	-	+	-	-	_	-
11	MARIAMMAL	45835/09	46/F	PRE	7.5	T3	3	N1	M0	DUCTAL	IIIA	2	-	MRM	+	+	+	-	_	-
12	MUTHULAKSHMI	44534/09	45/F	PRE	2	T1	0	N0	M0	DUCTAL	I	1	-	SM	-	_	-	-	_	-
13	CHELLATAI	45511/09	46/F	PRE	1.8	T1	2	N1	M0	DUCTAL	IIA	2	-	MRM	+	+	+	-	_	-
14	MARIAMMAL	45535/09	70/F	POST	6	T4	5	N2	M0	DUCTAL	IIIB	3	+	SM	-	+	-	+	_	+
15	MUTUMARI	46366/09	39/F	PRE	1.5	T1	0	N0	M0	DUCTAL	I	3	-	MRM	NA	NA	NA	-	_	-
16	CHELLAMAL	51198/09	55/F	POST	5	T4	2	N1	M0	DUCTAL	IIIB	3	+	MRM	-	_	-	+	_	+
17	MUTHUALKSHMI	48054/09	53/F	POST	2	T1	5	N2	M0	DUCTAL	IIIA	1	-	SM	-	_	-	-	_	-
18	MAKMUTHAL BEGAM	54187/09	40/F	PRE	6	T4	1	N1	M0	DUCTAL	IIIB	2	+	MRM	_	_	_	+	_	+
19	SAKINA BEGAM	47094/09	29/F	PRE	1.8	T1	0	N0	M0	DUCTAL	I	1	-	MRM	-	+	-	-	_	-
20	POOLAMMAL	55555/09	50/F	POST	6	T3	2	N1	M0	DUCTAL	IIIA	1	-	MRM	+	_	+	-	_	-
21	PARVATHI	53861/09	60/F	POST	5	T4	0	N0	M0	DUCTAL	IIIB	3	-	MRM	-	_	-	-	_	-
22	ARUMUGAM	52762/10	43/F	PRE	3	T2	0	N0	M0	DUCTAL	IIA	1	-	MRM	_	+	_	-	_	-
23	ATHILAKSHMI	19978/10	45/F	POST	1.5	T1	1	N1	M0	DUCTAL	IIA	1	+	SM	-	_	-	+	_	+
24	RAMALAKSHMI	14152/10	53/F	POST	5	T4	3	N1	M0	DUCTAL	IIIB	3	+	MRM	-	_	-	+	_	+
25	KALA	16525/10	38/F	PRE	2	T1	0	N0	M0	DUCTAL	I	2	-	MRM	-	+	-	-	_	-

S NO	NAME	IP NO	AGE/SEX	MENOPAUSAL STATUS	TUMOR SIZE IN CM	TUMOR STATUS	NODE SIZE IN CM	LYMPH NODE STATUS	METASTASIS	HISTOLOGY	TNM STAGE	HISTOLOGY GRADE	VASCULAR INVASION	TYPE OF OPERATION	ER STATUS	PR STATUS	HORMONAL STATUS	DTC IN SMEARS	CLOT SECTION	CYTOKERATIN POSITIVE
26	SUBBAMMAL	8023/10	65/F	POST	5.5	T3	7	N2	M0	DUCTAL	IIIA	3	+	MRM	+	+	+	-	_	-
27	ESAKKIAMMAL	15258/10	58/F	POST	2.8	T2	6	N2	M0	DUCTAL	IIIA	2	+	SM	-	_	-	+	+	+
28	AVUDAIAMMAL	14397/10	35/F	PRE	6	T3	2	N1	M0	DUCTAL	IIIA	2	+	SM	-	_	-	+	_	+
29	SHANMUGASUNDARAVADIVU	11190/10	61/F	POST	5	T4	6	N2	M0	DUCTAL	IIIB	3	+	SM	-	_	-	+	_	+
30	MUPIDATHY	12651/10	58/F	POST	4.5	T3	0	N0	M0	DUCTAL	IIB	3	-	MRM	+	_	+	-	__	-
31	NIRMALA	12163/10	58/F	POST	3	T2	1	N1	M0	DUCTAL	IIB	2	-	MRM	_	+	_	-	_	-
32	AYYAMMAL	14267/10	65/F	POST	6	T3	4	N2	M0	DUCTAL	IIIA	2	+	SM	-	_	-	-	_	-
33	ESAKKIAMMAL	16258/10	58/F	POST	5.5	T3	0	N0	M0	DUCTAL	IIB	1	-	MRM	-	+	-	-	_	-
34	MARIYAL	13564/10	65/F	POST	3.5	T2	3	N1	M0	DUCTAL	IIB	2	-	SM	NA	NA	NA	+	_	-
35	RAMATHILAGAM	15683/10	45/F	PRE	4	T2	3	N1	M0	DUCTAL	IIB	1	-	MRM	_	+	_	-	_	-
36	SANKARAMMAL	16386/10	55/F	POST	6	T3	4	N2	M0	DUCTAL	IIIA	2	-	SM	-	+	-	-	_	-
37	SUSAIAMMAL	16253/10	50/F	PRE	2	T1	0	N0	M0	DUCTAL	I	1	-	MRM	+	_	+	-	_	-
38	VELMAYIL	16362/10	65/F	POST	5	T4	0	N0	M0	DUCTAL	IIIB	3	-	SEGM	-	_	-	-	_	-
39	POONAMMAL	18347/10	54/F	POST	6.5	T3	3	N1	M0	DUCTAL	IIIA	2	-	MRM	+	+	+	-	_	-
40	SIVAGAMI	21564/10	61/F	POST	3.5	T2	2	N1	M0	DUCTAL	IIB	2	-	MRM	_	_	_	+	_	-
41	KUMARI	19345/10	58/F	POST	1.8	T1	0	N0	M0	DUCTAL	I	1	-	SM	NA	NA	NA	-	_	-
42	NAGOORAMMAL	20325/10	55/F	POST	4.5	T2	3	N1	M0	DUCTAL	IIB	2	-	MRM	-	+	-	-	_	-
43	PICHAMMAL	22567/10	56/F	POST	6	T3	4	N2	M0	DUCTAL	IIIA	3	+	MRM	-	_	-	+	_	+
44	YOGAMMAL	21298/10	53/F	POST	6	T4	4	N2	M0	DUCTAL	IIIB	3	+	MRM	+	+	+	-	_	-
45	MAHESWARY	21532/10	48/F	PRE	1.5	T1	0	N0	M0	DUCTAL	I	1	-	MRM	_	+	_	-	_	-
46	ESAKKIAMMAL	2199/10	57/F	POST	4.8	T4	3	N1	M0	DUCTAL	IIIB	3	+	SM	-	_	-	+	_	+
47	DIRAVIYAM	22011/10	60/F	POST	6.2	T3	5	N2	M0	DUCTAL	IIIA	2	-	MRM	-	_	-	-	_	-
48	MUTHAMMAL	22112/10	56/F	POST	1.5	T1	0	N0	M0	DUCTAL	I	1	-	MRM	+	+	+	+	_	-
49	SAKTHI	28011/10	48/F	PRE	2.5	T2	6	N2	M0	DUCTAL	IIIA	2	-	MRM	-	_	-	-	_	-
50	RAJAM	29056/10	55/F	POST	5	T4	5	N2	M0	DUCTAL	IIIB	3	+	SM	-	_	-	+	_	+