MSc Thesis

STUDY OF MARKERS OF BIOGENESIS AND RESPONSE TO THERAPY IN INFLAMMATORY AND LOCALLY ADVANCED BREAST CANCER

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This 60-credit research project thesis was submitted to the
The University of Birmingham for the degree of MSc in
Cancer Sciences

ABSTRACT

Background: One of the subtypes of locally advanced breast cancer (LABC), known as inflammatory breast cancer (IBC), is considered rare but profoundly aggressive. Traditionally, this disease was consistently fatal; nevertheless, with the induction of chemotherapy and carefully coordinated multimodality treatment, the prognosis of the patients has enhanced.

The study aims to examine the feasibility of predicting response to neoadjuvant chemotherapy by studying the expression of specific molecular markers CD151, CD68, and CD3 in IBC and LABC in clinical tissue samples and correlates the expression of these markers with disease-free survival and overall survival rate.

Method: Patients diagnosed with breast cancer regardless of the type were identified from the oncology database in the duration from January 2006 to December 2015. We also collaborated with other hospitals in the UK and outside the UK, e.g. Japan, to get reasonable IBC cases to conduct the study. Only patients who were treated primarily with neoadjuvant chemotherapy (NACT) 6-months prior to the surgery were included in this study.

Patients were divided into three groups based on the type of cancer. Group 1; included patients with locally advanced breast cancer, group 2; included patients with inflammatory breast cancer, and group 3; included the rest of the patients (neither IBC nor LABC). In our study, we focused on group 1 and group 2, aiming to predict the response of neoadjuvant chemotherapy by studying the expression of two tumour microenvironment markers: CD3 and CD68 and one marker of tetraspanins family CD151.

Statistical analyses were used to determine if the expression of the markers (CD151, CD3, and CD68) show a significant association with overall and disease-free survival, as well as response to neoadjuvant chemotherapy and other factors.

Results: From 2006 to 2015, there were 1347 patients reviewed, with a mean age of 54.5 years (range 28 to 81 years). There were 327 patients that met the inclusion criteria. The total number of final cohorts was comprised of 301 cases. Group 1; patients with a diagnosis of locally advanced breast cancer (LABCs), included 43 patients. Group 2; patients with a diagnosis of inflammatory breast cancer (IBCs), included 96 patients. Group 3, patients were neither IBC nor LABC included 162 patients, but this group was excluded from this study. In locally advanced breast cancer (group1), statistically significant findings showed a higher percentage of CD3 that was associated with complete pathological response, increased Disease-Free Survival (DFS), and HER2 positive subtype. In addition, a significant association between complete pathological response and HER2 subtype tumour was noted. On the other hand, inflammatory breast cancer (group 2) showed some potential association between increased Overall Survival (OS), complete pathological response to chemotherapy, and increased expression of CD3.

In locally advanced breast cancer (group 1), although a higher percentage of CD68 was significantly associated with a higher grade of cancer, it was noted in inflammatory breast cancer (group 2) that a higher percentage of CD68 was correlated with a better response to NACT. Also, in group 1, a significant correlation was noted between histological tumour type and response to NACT. Furthermore, invasive ductal carcinoma including (NST and micro-papillary) had a better response to neoadjuvant chemotherapy than invasive lobular carcinoma. Also, in-group 1, significant alteration in receptor markers after treatment with neoadjuvant chemotherapy was noted, thus crucially altering potential adjuvant therapy. In inflammatory breast cancer (group 2), there was a correlation with a significant value between complete pathological response and over-expression of CD151 and overall survival. In addition, there was a significant correlation between the patient's age and over-expression of CD151 and CD3 as women >50 years were found to have a higher expression of both markers compared to women <50 years.

The greater nodal burden was significantly associated with poor response to NACT and greater nodal metastasis, which was inversely correlated with HER2 positive subtype tumour in both locally advanced breast cancer and inflammatory breast cancer.

Conclusions: Identifying reliable predictive prognostic markers for the outcome is essential to developing our understanding of IBC and LABC which will improve treatment outcomes. Nevertheless, identifying reliable, informative, and uniform endpoints is a crucial first step to increasing confidence in the value of neoadjuvant chemotherapy.

ACKNOWLEDGMENT

I would like to thank my supervisors ***Dr. Abeer Shaaban*** and ***Dr. Fedor Berditchevski***, for their help during the course of my research. I would also like to thank all the staff from the Queen Elizabeth Hospital Birmingham Pathology Laboratory and Staff from the University of Birmingham Cancer Sciences Department Laboratory who helped me during my lab work. I would like to thank ***Mariam Gachechiladze*** for her help in staining and scoring. Finally, I would like to thank all my friends and family who provided moral support throughout the project.

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1.1 Breast Cancer

The most common cancer in women is breast cancer, with over 40,500 new cases in England and Wales annually. Despite a gradual decline in age-standardised mortality rates due to screening and enhanced management, the disease still causes 10,900 deaths annually in England and Wales, with a large social and emotional impact **(Harnett, 2009)**.

Breast cancer has been identified as a heterogeneous disease for a long time while many factors have been studied to categorise patients by risk and treatment options (age, parity, family history, etc..), receptor status has shown to be the most helpful in predicting both prognosis and responsiveness to treatment. The over-expression of human epidermal growth factor receptor-2 (HER2), progesterone receptor (PR) and oestrogen receptor (ER), PR can be scored by utilising immuno-histochemical (IHC) techniques. According to the presence or absence of those receptors, breast cancers are then categorised as cancers that don't have all three designated receptors resulting in triple-negative breast cancer (TNBC). Those cancers that express ER, PR, or HER2-neu are susceptive to targeted therapies directed at these receptors, however, TNBC patients are treated with standard chemotherapy **(Rouzier, 2005)**.

1.2 Intrinsic molecular subtypes

Breast cancer has been divided according to the four molecular subtypes: Luminal A, luminal B, HER2-enriched, and basal-like breast cancer (BLBC). These subtypes can be derived clinically by employing IHC determination of receptor status as follows: Luminal A derives ER and/or PR positive, and HER2 negative, luminal B derives ER and/or PR positive, and HER2 positive, HER2-enriched derives ER and PR negative,

and HER2 positive; and BLBC derives TNBC. Furthermore, a correlation between these subtypes and different prognoses was observed, with basal-like breast cancer having the worst prognosis compared with luminal A tumours having the best prognosis. Nevertheless, depending on their subtypes, patients may be categorised by treatment options. Patients with luminal A and B, and HER2-enriched subtypes are susceptible to targeted therapy, whilst patients with BLBC now are having chemotherapy as the only option **(Rouzier, 2005)**.

1.3 Locally advanced breast cancer

Locally advanced breast cancer is a rare type of breast cancer, and it occurs in about 5% of patients **(Aebi, 2022)**. Although locally advanced breast cancer (LABC) has advanced loco-regionally, it has not yet metastases outside the breast. It is a mostly non-operable type of breast cancer and requires neo-adjuvant systemic treatment followed by surgery. **(Aebi, 2022)**.

Patients with locally advanced breast cancer could be presented with large-sized tumours or diffusely infiltrated tumours without a dominant mass as in inflammatory breast cancer which is considered an aggressive subtype of locally advanced breast cancer **(Aebi, 2022; Yalcin, 2013)**.

1.3.1 Diagnosis and staging

Locally advanced breast cancer encompasses mainly stage IIIB and IIIC disease, but some clinicians consider stage IIIA as locally advanced breast cancer **(Aebi, 2022)**. It includes large breast cancer more than 5cm in microscopic size, the tumour shows a direct extension to the skin of the breast or invades the underlying muscles of the chest wall. In addition to cancer exhibits involvement of multiple regional lymph nodes. AJCC or UICC staging systems include inflammatory breast cancer which is classified as T4d in the TNM8 staging system **(Aebi, 2022)**.

In addition, guidelines for the workup of women with just recently diagnosed breast cancer have been published by the National Comprehensive Cancer Network **(Gradishar, 2020)**. The recommended assessment for women with a high-stage disease includes history and physical examination, complete blood pictures (platelets, liver enzymes, etc..), imaging including Magnetic Resonance Imaging (MRI) and pathology review with the determination of hormonal receptor status. In a borderline candidate for breast-conserving surgery, the evaluation of advisable assessment incorporates bone scans and abdominal CT scans, ultrasound, or MRI. The extent of the disease can be confirmed from these tests to guide the management plan. Core biopsy confirms the diagnosis and assesses tumour type, grade, hormonal receptors status, and testing other prognostic indicators, such as HER2, p53 and ki67. While lymph node involvement is discovered on physical examination or by ultrasound, diagnosis of axillary lymph node involvement by needle aspiration will influence management decisions **(MD, 2000)**.

1.3.2 Epidemiological features

Based on the surveillance epidemiology and end results (SEER), a database between 1992–1999 in the United States showed that the percentage of women diagnosed with locally advanced breast cancer reached 4.6% of all female breast cancers **(Yalcin, 2013)**. The mean age at diagnosis within this group was 60.6 years old. However, another assessment of the SEER database during the years 1988–2000 (n = 180,224) indicated that the age-adjusted incidence rates of LABC declined enormously from 1988 to 1999. [From 2.5 to 2.0 cases/100,000 woman-years; p = 0.0025] **(Yalcin, 2013)**.

When comparing both black and white women, black women were more at risk of being diagnosed with LABC. Moreover, this risk included presenting a larger breast mass (>2cm) found in this group, compared to patients with a smaller mass (<2.0 cm) (relative risk 5.4; 95%) **(Yalcin, 2013)**. For women in either developing countries or medically deprived populations in industrialised countries, this represents a much larger problem **(MD, 2000)**.

Haakinson *et al.* in a previous study stated that obese patients had a higher proportion of larger tumours, a greater rate of lymph node metastases and a higher proportion of cases of women aged >50 years **(Haakinson, 2012)**. Another study by **Cui** *et al.* also displayed that patients with obesity at diagnosis of breast cancer were associated with a greater proportion of advanced stage **(Cui, 2002)**. These studies showed a discrepancy with the study by **Arce-Salinas** *et al.* that showed no distinction between the clinical and pathological characteristics of patients with obesity in comparison with normal-weight patients **(Arce-Salinas, 2014)**.

1.4 Inflammatory Breast Cancer

The most aggressive subtype of locally advanced breast cancer (LABC) is inflammatory breast cancer (IBC). It is a rare subtype as it represents 1-6% of breast cancer, but can be characterised by local and distant metastases, rapid progression, as well as poor survival rate compared to other types of breast cancer **(Uden, 2015)**. Despite the consistent improvement of the overall survival of non-IBC patients, IBC patients have a poorer prognosis with most patients developing metastasis after one year and according to data from the National Cancer Institute's Surveillance, Epidemiology, and Results Database, the 5-year survival rate

was approximately 40% for women who were diagnosed with IBC between 1988 and 2001. This corresponds to about 87% of all breast cancers combined.

1.4.1 Diagnosis and staging

Inflammatory breast cancer is exclusively diagnosed clinically. The patients are often presented with diffuse erythematous and oedematous breast skin (peau d'orange), which is firm in palpation, without underlying palpable mass. Consequently, inflammatory breast cancer can be mis-diagnosed with other mimickers, which have the same clinical presentation as bacterial mastitis, resulting in a delay in diagnosis and treatment **(Rea, 2015)**. Mammography may exhibit a noticeable mass or area of calcification. It also may show skin thickening over the breast, with or without breast mass **(Rea, 2015)**. Improvements in imaging techniques have enhanced the diagnosis and staging of IBC. Ultrasound-guided breast core biopsy is considered the gold standard method in the diagnosis of breast cancer if breast mass is apparent and for evaluating the regional lymph nodes. Inflammatory breast cancer is a subtype of locally advanced breast cancer and, according to tumour-node-metastasis (TNM), IBC is classified as T4d **(Uden, 2015)**. Fundamental diagnostic features of breast MRI are oedema, diffuse skin thickening, and enhancement **(Rea, 2015)**. MRI can also assist in taking full-thickness skin punch biopsies if IBC is suspected. Although there are no diagnostic histological features, marked lymphovascular invasion of breast tissue, and the overlying skin is not essential for diagnosis. In inflammatory breast cancer, tumour cells obstruct the lymphatic flow and hinder proper drainage of lymph fluid. These changes lead to swelling of the breast and its inflammatory-like appearance **(Uden, 2015)**. For patients with suspected or palpable regional lymph nodes, an ultrasound with a guided fine needle aspiration (FNA) and/or core needle biopsy should be performed **(Rea, 2015)**. Approximately 30%

of IBC patients presented with metastasis and consequently, primary staging is critical once the diagnosis of malignancy is confirmed because it provides essential information for prognosis and management **(Uden, 2015)**. CT scanning is recommended as the initial staging tool **(Rea, 2015; Rouzier, 2005)**.

Bone scan provided a little additional value over CT staging, and it is not utilised in routine staging in UK practice. On the other hand, PET-CT is very valuable for the detection of asymptomatic metastasis **(Groheux** *et al.,* **2013)**.

1.4.2 Epidemiological features

Inflammatory breast cancer is a rare type of breast cancer that only represents 0.4-1.8% of all breast cancer in the United Kingdom **(Copson, 2018)**. It has a worse prognosis compared with other T4 breast cancer **(Andic, 2010)**. Patients with IBC are commonly younger at diagnosis compared to patients diagnosed with all different types of breast cancer (median age, 57 vs. 64 years) **(Andic, 2010)**. Despite the available multi-modality treatment choices, the median overall survival is shorter than five years. Nevertheless, increasing survival in recent years has been recorded with the advancement of chemotherapy **(Uden, 2015)**. Three studies with a large number of patients have stated that the incidence of inflammatory breast cancer in young African American women is higher and has a worse survival rate in comparison with Caucasian women **(Hance, 2005; Chang, 1998; Wingo, 2004)**. Additionally, these studies reported that a high Body Mass Index (BMI) is directly correlated with the diagnosis of IBC compared to non-IBC and the low risk of ER-positive IBC is associated with a higher level of education. The study by **Schairer** *et al.* showed that advanced age at first birth was linked with a reduced risk of ER-negative IBC **(Schairer, 2013)**.

1.4.3 LABC/ IBC molecular subgroup

Although IBC does not represent a particular molecular subgroup of breast cancer, there is a greater proportion of Her-2 enriched cancer **(Uden, 2015)**. It has been published that up to 83% of IBC tumours lack oestrogen receptor (ER) expression compared with other forms of LABC, which are mostly ER-positive **(Uden, 2015)**.

Analysis of 200 patients with IBC from the California Cancer Registry has shown that expression of ER and PR was lower among IBC patients compared to both non-T4 carcinomas (56% ER, 45% PR versus 80% ER, 68% PR) and in patients with LABC (67% ER, 54% PR) **(Uden, 2015)**. Additionally, the study by **Bertucci** *et al.* stated that five molecular phenotypes including Luminal A and B, have HER2 over-expression and basal-like phenotype defined for non-Inflammatory Breast Cancer (NIBC) as well as IBC **(Bertucci, 2005)**.

1.4.4 Management

The latest management of non-metastatic IBC/LABC includes neo-adjuvant Chemotherapy (NACT), surgery combined with axillary lymph node dissection in case of lymph node involvement, and locoregional radiotherapy but also neoadjuvant trastuzumab and adjuvant hormonal therapy in case of HER2 or ER/PR positive tumour **(Uden, 2015)**. This multimodality method in treatment has considerably improved patients' survival in recent years **(Hance, 2005)**.

2. Neo-adjuvant chemotherapy

Neoadjuvant chemotherapy is considered a primary line of treatment for operable breast cancer. Originally, it was recommended that neoadjuvant chemotherapy should only be considered for women with either operable locally advanced cancer, non-operable or inflammatory breast cancer. However, currently, neoadjuvant chemotherapy is commonly used to treat women with high-risk operable primary breast cancer. Many international groups have provided guidelines for the use of neoadjuvant chemotherapy in operable breast cancer based on clinical and histological examination, and imaging, with recommendations for patient selection and treatment regimens **(Hance, 2005)**.

Due to the advantages of neoadjuvant systemic therapy in operable breast cancer, its use is currently becoming more popular. This includes higher rates of breast-conserving surgery and the likelihood of measuring early response to systemic treatment.

However, the implementation of neoadjuvant treatment in patients, who present with operable breast cancer, demonstrates equivalent survival outcomes in comparison with adjuvant breast cancer treatment **(Rapoport, 2014)**. In addition, there is an assumption that the overall survival for aggressive breast cancer subtypes may be improved by NACT **(Pinder, 2015)**. The goals of preoperative systemic therapy in LABCs include early termination of subclinical distant micro-metastases, down-staging of the primary tumour to enable operability (including breast-conserving surgery in some cases), and *in-vivo* assessment of response to specific systemic therapies and above all prolonging survival rate and improving the quality of life **(Specht, 2009)**. The most crucial parameter for successful treatment and improved overall survival is the attainment of pathological complete response (pCR).

2.1 Definition of Pathological Complete Response:

There is no consensus on the definition of pathological complete response. Some researchers have described it as the total eradication of both invasive and in situ components following preoperative chemotherapy while others have described pCR as the only absence of invasive cancer after neoadjuvant chemotherapy regardless of the non-invasive component. Nevertheless, some studies defined pCR as the complete absence of residual invasive cancer in both breast and regional axillary lymph nodes following NACT, while others described pCR as the total elimination of invasive cancer in the breast only, regardless of the regional lymph nodes **(Untcha, 2014)**. Based on the work of the CTNeoBC consortium, the FDA has now supported two descriptions for pathological complete response (pCR) **(Cortazar, 2014)**, which are the elimination of invasive components in both breast and regional lymph nodes or the complete absence of invasive and non-invasive cancer in the breast and all examined lymph nodes following treatment with NACT. The study by **Cortazar** *et al.* reported patients who achieved pCR following NACT had better overall survival and disease-free survival (DFS) in comparison with patients who obtained pathological partial response (pPR) or Pathological No Response (pNR). Furthermore, total eradication of residual invasive cancer in both breast and regional lymph nodes was associated with demonstrated prolonged overall survival and Disease-free survival in comparison with the complete elimination of the tumour from the breast alone **(Cortazar, 2014)**.

2.2 Defining patient groups for neo-adjuvant chemotherapy:

One of the large studies by **B. Fisher** *et al.* the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-18 trial, exhibited that preoperative chemotherapy should be considered in the large-sized tumour,

more than 5cm to increase the incidence of breast conservating surgery by reducing the tumour size and decrease the rate of metastatic regional lymph nodes **(Fisher, 1997)**. Even though there was no obvious difference in overall survival or Disease-Free Survival, women younger than 50 years of age had more benefits from neoadjuvant chemotherapy compared with postoperative chemotherapy. On the other hand, older women aged 50 years and above had more favourable outcomes with postoperative chemotherapy. The study by **Rapoport** *et al.* explained the advantage of preoperative chemotherapy in younger women could be age dependent as younger women are more likely to have oestrogen receptor (ER)–negative tumours **(Rapoport, 2014)**.

International Breast Cancer Study Group data recommended early initiation of chemotherapy in pre-menopausal women with ER-negative tumours **(Colleoni, 2000)**. These results could help to explain why younger women appear to have a greater benefit from preoperative chemotherapy.

2.3 Assessment of Response to NACT

2.3.1 Clinical Assessment of Response to NACT:

The fundamental clinical examination included the palpable measurement of primarily breast lump and regional lymph nodes followed by regular assessment of tumour size after initiating neoadjuvant chemotherapy to evaluate clinical response to preoperative chemotherapy **(Romero, 2013)**. In spite of the fact that evaluation of clinical response is considered an index of response to chemotherapy, it has many errors as extensive fibrosis that occurs following chemotherapy can provide a false impression of an increase in tumour size. In addition, a large tumour which appears to have responded quite well to chemotherapy might still contain residual cancer which consequently makes patients unable to undergo breast-conserving surgery **(Sethi, 2013)**.

2.3.2 Radiological Assessment of Response to NACT:

Clinical imaging and histological evaluation of the response to neoadjuvant chemotherapy provide an accurate measurement of the effectiveness of neoadjuvant therapy. Mammograms, ultrasound-based, and MRI scans can consistently detect the breast tumour so that dynamic alterations during treatment can be evaluated. It is crucial that any abnormalities seen on imaging are carefully analysed with needle core biopsies of suspicious lesions and placement of clips in order to mark areas of malignancy before therapy can be initiated. Clips also help in surgical localisation and pathologic evaluation of the tumour bed if there is a complete response to therapy. Irrespective of the tumour size at the presentation, clips should be placed to trace the location before chemotherapy. Various methods have evolved to analyse the response to chemotherapy. The response evaluation criteria in solid tumours (RECIST) measures tumour response utilising X-ray, computerised tomography, and MRI and classifies tumour responses into four categories, namely complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD) **(Romero, 2013)**.

The decrease in the two largest diameters of the primary tumour size by more than 50% is implied as a partial remission. A noticeable change in tumour size can be recognised after 4-6 weeks (2 cycles) of chemotherapy **(Untcha, 2014)**. Early response to neoadjuvant chemotherapy increases the probability of accomplishing pCR and therefore improves the long-term prognosis **(Untcha, 2014)**.

2.3.3 Pathological assessment of response to NACT:

The pathological response was assessed in the surgery specimen (either wide local excision or mastectomy; both with additional Sentinel lymph node biopsy (SLNB) or axillary clearance). Biopsies were also

examined for the evaluation of tumour response. Histopathological examination was performed, particularly looking for chemotherapy-induced changes such as necrosis, fibrosis, inflammatory reaction, and other changes. There are other methods to assess tumour response based on varying approaches, including:

Miller and Payne classification **(Sahoo, 2009)** assessed tumour response to primary chemotherapy according to the reduction of cancer cellularity following the treatment in patients with large-sized tumours and locally advanced breast cancer. It classified the response to neoadjuvant chemotherapy into five grades: Grade-1 no change or some changes to singular malignant cells, and no decrease in overall cellularity (pNR). Grade-2 no significant decrease in tumour cellularity, up to 30% loss only (pPR). Grade-3 between an approximated 30% and 90% decrease in tumour cells (pPR). Grade-4 the presence of only small clusters of tumour cells or widely dispersed individual cells; 90% loss of tumour cells (almost pCR). Grade-5 no residual malignant cells are detected; only response to chemotherapy in the form of fibrotic stroma, macrophages but in situ component may be present (pCR). The **Symmans** *et al.* study intensified a way to assess the Residual Breast Cancer Burden (RCB) by evaluating four factors; the maximum diameters of the primary tumour bed in the resection specimen, the dimension of the primary tumour bed, the number of metastatic lymph nodes LN), and the size of the largest tumour deposits in an axillary lymph node **(Symmans, 2017)**. According to the RCB-index, categories of RCB-0 (complete response) to RCB-III (chemotherapy resistance) can be outlined **(Symmans, 2017)**. It is agreed that pathological complete response (pCR) is a robust prognostic factor following neoadjuvant chemotherapy (NACT). There are a few alternative systems for recording the response to neoadjuvant chemotherapy, which is considered the gold standard.

Chevallie Method **(Sahoo, 2009)**; this method divided the treatment response into four classes: Class 1; Complete eradication of the tumour (pCR), Class 2; No invasive carcinoma and negative lymph node (pCR), regardless of the presence of DCIS Class 3; Presence of a stromal response to the invasive breast carcinoma (pPR). Class 4; occasional alterations of the tumour appearance (pNR).

Ogston *et al.* system **(Ogston, 2003)** this system categorised the treatment response into five groups; Group 1; no decrease in cellularity, Group 2; minor destruction of tumour cells (up to 30%), Group 3; 30-90% reductions in cellularity, Group 4; >90% loss of tumour cells and Group 5; complete eradication of the invasive disease.

Sataloff system **(Sahoo, 2009)** treatment response for the lymph nodes and primary carcinoma is incorporated in this system. The category of pCR (called T-A) incorporates a ‘‘near-complete response,’’ suggesting that a tiny focus of residual tumour could be present. There are two categories of partial response (T-B and T-C). One of the categories, pNR (called T-D), exhibits no therapeutic response. LVI is not included in this system. The study by **Sunati Sahoo** *et al.* recorded that those patients with pathological; complete response had better survival of five years compared to the other three categories of patients but did not exhibit any variations in the two categories of partial response.

Sinn system **(Sinn, 1994)**: Divided the regressive changes utilising a scoring system from a scale of 0 to 4; a score of 0 means no signs of regression/no effect score, a score of 1 means minimal evidence of tumour regression, a score of 2 means focal residual invasive tumour, a score of 3 means residual non-invasive disease only and a score of 4 means a complete absence of tumour cells in the breast.

NSABP B-18 system: his study is considered one of the largest studies where the comparison between neoadjuvant and adjuvant therapy was made. Three categories of responses were identified; complete response meant no tumour cells, partial response meant the presence of scattered individual cells or small clusters of cells in desmoplastic/hyaline stroma, and no response meant that tumours are not exhibiting any changes **(Fisher, 1997)**. In the same study, **Fisher** *et al.* assessment of lymph node metastasis were recorded individually, as well as the correlation with overall survival and disease-free survival.

According to the latest recommendation of "The Royal College of pathologists"

The pathological response of tumour and lymph node neoadjuvant chemotherapy is subdivided into three categories each **(Ellis, 2016)**.

Tumour response: (i) complete pathological response is considered either no residual carcinoma or no residual invasive tumour, regardless of the presence of DCIS. (ii) Partial response to therapy is either minimal residual disease/near-total effect typically (e.g., <10% of the tumour seen present in the tumour bed as an area of residual fibrosis outlines the original tumour extent) or 10–50% of the tumour remaining or >50% of the tumour is left, comparing with the previous core biopsy sample could be beneficial. (iii) No evidence of any response to therapy.

Nodal response: (i) The first category has two definitions - either no evidence of metastatic disease or changes in the lymph nodes or metastatic tumour not identified, but there is evidence of response/down-staging. (ii) In the second category, there is a partial therapeutic effect with evidence of response, but the residual tumour is also present. (iii) The third category, metastatic cancer, is identified, and there is no evidence of a therapeutic effect.

2.4 Factors Predicting Response to Chemotherapy

2.4.1 Biological Factors

The German GeparTrio study **(Huober, 2010)** was carried out to examine the effect of NACT on clinical midcourse and pCR during surgery in various biological breast cancer types. This study exhibited the highest pCR rate observed in women with high-grade triple-negative breast cancer and below the age of 40. Tumour grading, clinical-stage, HER2-positive tumour and triple-negative tumour are considered independent factors to predict pCR within biological subtypes.

On the contrary, another study conducted by **Alvarado-Cabrero** *et al.* **(2009)** displayed no significant correlation between a patient's age and the distribution of complete and incomplete pathological response, breast tumour type, and axillary lymph node. In addition, in the study by Fisher *et al.* the age of patients was unsuccessful in predicting the clinical response of breast tumours **(Fisher, 1997)**. Although **Jacquillat** *et al.* stated that tumour response is inversely correlated to age. In their study, a complete response was obtained in 18% of patients above 50 years old in comparison to 37% of those who were less than 50 years old **(Jacquillat** *et al.*, **….)**.

2.4.2 Molecular subtypes

Seung Il Kim *et al.* investigated the correlation between molecular subtypes and the response of a tumour to NACT **(Kim, 2010)**. The ER status was utilised for the initial subdivision of molecular subtypes. The ER-positive tumours were separated into luminal A and luminal B in accordance with the HER2 status. The ER-negative tumours were subdivided into the HER2 positive and triple-negative subtypes. The study demonstrated that patients with triple-negative or HER2-positive breast cancer have a higher proportion of pCR to NACT compared to the luminal subtype. As a result, breast conservation surgery was conducted in patients with the triple-negative subtype more frequently. **Carey** *et al.* reported that

triple-negative tumour has proven to have a worse prognosis in comparison with those with ER-positive tumours among patients with residual disease **(Carey, 2007)**.

2.4.3 Tumour Size

In **2009, Alvarado-Cabrero** *et al.* reported a favourable relationship between small tumour size and the rate of a pCR to neoadjuvant chemotherapy therapy **(Alvadro-Caberero, 2009)**. **Bonadonna** *et al.* also showed a strong correlation between tumour size and response to preoperative therapy; the complete response was 50% for patients with tumours smaller than 2 cm, 38% in tumours 2 to 4 cm, and only 18% for tumours larger than 5 cm **(Bonadonna, 1976)**. Moreover, it is currently established that patients with lower-stage breast cancer are more likely to have a pCR compared to those with higher-stage breast cancer **(Alvadro-Caberero, 2009)**.

2.4.4 Tumour Histological Grade

The study by Isabel Alvarado-Cabrero *et al.* also proved a significant proportion between tumour histological grade in pre-treatment core biopsy and the pathological response to neoadjuvant chemotherapy **(Alvarado-Caberero, 2009)**. Tumours with a high nuclear grade were more likely to have a pCR compared to tumours with a low nuclear grade.

2.4.5 Tumour Histological Type

Also, the same study reported a strong association between the histological type and pathologic response to chemotherapy **(Alvarado-Caberero, 2009)**. The pathologic response to NACT is higher with invasive ductal carcinoma (IDC) in comparison with invasive lobular carcinoma (ILC). Patients with invasive ductal carcinoma (IDC) achieved 75% of pCR to NAC, whereas patients with invasive lobular carcinoma

(ILC) achieved 25% of pCR. The results of the Alvarado-Cabrero *et al.* study are comparable to that reported by **Cristofanilli** *et al.* in **2004**. They observed patients with invasive lobular carcinoma, which were less feasible to have a complete pathological response (pCR) (3% vs. 15%) than those with IDC. The low response rate of ILCs to chemotherapy may be related to their particular biological profile when compared with IDC; ILCs have higher hormone receptor levels and bcl-2 expression and a lower Ki 67 score and c-erb-B2 expression **(Cristofanilli, 2004)**.

The most impressive results of the Alvarado-Cabrero *et al.* study was that patients with invasive pure micropapillary carcinoma (PMCs) failed to achieve a pCR to therapy **(Alvadro-Caberero, 2009)**. The tumour was multifocal in the largest number of cases, and patients had extensive residual invasive carcinoma following the neoadjuvant therapy. Most PMCs were ER-positive (89%) and had a high nuclear grade (92%); this finding suggests that this specific carcinoma subtype could be an independent predictor for pCR.

2.5 Alteration in preoperative tumour marker status after NACT:

Patients treated with neoadjuvant chemotherapy could have changes in receptor and marker status following the neoadjuvant chemotherapy **(Piper, 2004)**. This study displayed a notable number of patients that had a change in receptor expression as well as a change in marker status where the suitability of hormonal therapy or the use of Herceptin was altered. Controlling this variability is vital as ER/PR and HER2 status are the fundamental components in adjuvant therapy The utilisation of tamoxifen in hormonally susceptible patients has led to improved disease-free survival and overall survival in both pre-and post-menopausal women **(Nguyen, 2008; Chen, 2015)**.

The study by **Piper** *et al.* exhibited 22% of patients undergoing a change in the receptor expression would also have a change in receptor status, thus crucially changing possible adjuvant therapy **(Piper, 2004)**.

In **2016, Gahlaut** *et al.* revealed notable changes in tumour morphology, grade, hormone receptors, and HER2 status following NACT **(Gahlaut, 2016)**. New treatment options are provided to patients when a change from negative to positive marker status occurs. The Study by **Sheng Chen** *et al.* reported the cancer cells that were detected after the primary treatment might carry different gene expression and DNA mutational signatures **(Chen, 2015)**.

2.6 Clinicopathological features of early failure of NACT

Several studies demonstrated a significant correlation between pCR to NACT and triple negative and HER2 positive subtypes in comparison to the HR-positive subtype **(Kim, 2010; Carey, 2007)**. Although TNBC subtypes exhibit higher rates of pCR, it was reported that TNBC has the worse overall survival rate and higher distant metastasis **(Carey, 2007)**. Furthermore, the study by Moon Ki Choi *et al.* stated that HER2+ tumours have the highest pCR followed by triple-negative breast cancer but ER+ tumours have the worst pCR rate **(Choi** *et al.,* **2014)**. Also, the same study recorded that the presence of lymphovascular invasion (LVI) was significantly associated with a high risk of early failure. There was a strong association between the presence of LVI and poor outcomes, even in the absence of metastatic lymph nodes and small tumour sizes **(Hanrahan, 2006; Rakha, 2012)**.

Moon Ki Choi *et al.* study reported that brain metastasis as the first site of recurrent disease was higher in TNBC and HER2+ subtype breast cancer than hormonal receptors positive tumour (HR+) **(Choi** *et al.,* **2014)**.

Over the last decade, it has been proven that many neoadjuvant chemo-therapies, including anthracyclines, taxanes, and trastuzumab, have limited influence on the increasing rate of brain metastasis because of their incapability to penetrate the blood-brain barrier. Nevertheless, they have increased systemic disease control. Therefore, CNS metastases endure a serious problem in breast cancer. The weakness in Moon Ki Choi *et al.* study is that more than half of the patients with HER2+ cancer didn't receive anti-HER2 therapy due to insurance problems.

2.7 Type of neoadjuvant chemotherapy

The predominance of chemotherapy regimens includes an anthrax-cycline regimen. One of the largest randomised controlled trials, the National Surgical Breast and Bowel (NSABP B-27), in comparison to a neoadjuvant anthracycline regimen with an anthracycline–taxane combination. 2411 patients with operable breast cancer were randomly divided into three groups: four cycles of preoperative anthracycline (AC) (arm I), four cycles of anthracycline followed by four cycles of docetaxel (D) prior to surgery (arm II), and four cycles of anthracycline (AC) followed by surgery then four cycles of docetaxel (D) (arm III). This study revealed that the addition of preoperative docetaxel was correlated with a higher pCR in breast rate (26% vs. 14%; $p<0.001$) when compared with the group of patients who received only AC followed by surgery. Nevertheless, a significant impact on disease-free survival or overall survival was not observed as a result of the addition of D to AC **(Mamounas, 1997)**.

The study by Minckwitz *et al.* reviewed if sequencing anthracycline and taxanes, as opposed to the dose-dense regimen, would improve outcomes **(Minckwitz *et al.*, 2005)**. This study stated that sequential therapy with doxorubicin and cyclophosphamide then by docetaxel is more successful in achieving pCR compared to a dose-dense doxorubicin and

docetaxel regimen (14.3% vs. 7%). Another trial by Minckwitz (GEPARTRIO) in operable breast cancer patients stated that early response to two cycles of docetaxel; doxorubicin and cyclophosphamide (TAC), accurately identify patients with a high chance of inducing a pCR **(Minckwitz** *et al.,* **2005)**. Regular or continuous chemotherapy leads to the intake of a higher dose of chemotherapy over a short period of time, this includes a weekly dose of intravenous drugs, including doxorubicin, and a daily dose of oral cyclophosphamide, which revealed an improved pathologic complete response (pCR) rate with the regular schedule **(Ellis, 2011)**.

The study by Kim *et al.* reported that both HER2 + and TNBC subtypes are more sensitive to taxane-based NACT than the luminal subtype **(Kim, 2010)**. In addition, it has been reported that patients with HER2+ breast cancer who have been treated with both anti-HER2 therapy, trastuzumab, and NACT showed better survival. The results of the NOAH study were comparable with the study by Kim *et al.* as it concluded that combined neoadjuvant chemotherapy with one year of anti=HER2 therapy (commencing as neoadjuvant and proceeding as adjuvant therapy) in patients with HER2-positive locally advanced or inflammatory breast cancer has improved the rate of pathological complete response to nearly doubled rates, disease-free survival and overall survival (OS) compared with patients who did not receive anti-HER2 therapy **(Ellis, 2011)**. The study by V. Semiglazov *et al.* showed combining anti- HER2 therapy with neoadjuvant chemotherapy improved long-term outcomes and allowed selected patients with LABC or IBC to have breast-conserving surgery instead of mastectomy **(Semiglazov, 2011)**.

2.8 Association between response to NACT and outcome:

Higher rates of pathological complete response and improvement of outcomes are related to early clinical response **(Specht, 2009)**. Additionally, it was reported that modified chemotherapy in clinically non-responders' patients (described as no noticeable reduction in tumour size by at least 50% by imaging) showed no improvement in response to chemotherapy **(Specht, 2009)**. In the same study, patients were selected randomly to undergo two different chemotherapy regimens. One regime included four more cycles of TAC, and the second one included four cycles of vinorelbine and capecitabine before surgery.

The pathological complete response rate was low in both regimes and was not improved by switching to another chemotherapy regimen **(Minckwitz, 2005)**. In another trial, reviewers in Aberdeen studied if increasing the duration of neoadjuvant chemotherapy would be beneficial in patients who were responding to this therapy **(Specht, 2009)**.

Patients who achieved complete or partial clinical response to the first four cycles of CVAP were randomised to 4 more cycles of CVAP and changed to 4 cycles of docetaxel. The pCR increased to double in the group that turned to docetaxel, and this group showed prolonged Disease-free and overall survival. The same study proved that shifted chemotherapy in patients with constant or progressive disease has no benefit in improving the outcome, indicating that tumours not responding to one chemotherapy regimen is unlikely to exhibit a noticeable response to another regimen. However, the study by Fatayer *et al.* stated switching NACT in the non-responder group on imaging exhibited improvement in response to the initial chemotherapy **(Fatayer, 2016)**. Both Aberdeen and Gepar Trio's studies recommended a treatment program should not be altered based on an early response unless there is strong evidence of disease progression.

Diverging from the planned course of therapy in non-responders has not been proved to improve clinical or pathological complete response rates or increased overall survival. Patients with obvious disease progression throughout preoperative systemic treatment should be switched to an alternative regimen. However, if the disease is unresectable, local therapy should be offered, or further investigational approaches should be carried out. The study by **Specht** *et al.* reported that the potential for improved outcomes has not been able to be used to advantage, based on altering chemotherapy, the response to specific chemotherapy drugs **(Specht, 2009)**. The study by Tulbah presented that there was no notable difference in disease-free survival (75% vs. 84%) or overall survival (81% vs. 84%) when comparing those who over-expressed HER-2 and those with negative HER-2 test **(Tulbah, 2002)**. The study by **Carey LA** *et al.* displayed a significant correlation between worse overall survival, distant recurrence and loco-regional recurrence (LLR) outcome in LABC patients with triple negative (TN) tumours **(Carey, 2007)**.

The study by Nguyen et al stated that women with HER2 positive or basal-like subtype were more prone to have a loco-regional recurrence (8.4 and 7.1%, respectively) in comparison with very low rates of loco-regional recurrence for the luminal A (0.8%) or luminal B (1.5%) subtypes **(Nguyen, 2008)**. The study by Kim *et al.* showed that patients with the residual invasive disease presented a worse prognosis no matter the tumour subtype **(Kim, 2010)**. The Study by **Sheng Chen** *et al.* explained that patients with positive hormonal receptors had direct reactions to anti-tumour treatments, which are correlated with the patient outcome. On the other hand, in patients with negative hormonal receptors and a residual tumour, the reaction to treatment may not be correlated with a survival rate **(Chen, 2015)**.

2.9 Surgery post NACT

Historically, in patients with LABC or IBC treated with mastectomy there was no support for the obligatory requirement for recommending mastectomy in these patients. Breast conservating surgery following preoperative chemotherapy can be considered after discussion in a multidisciplinary meeting by assessing the response to NACT clinically and radiologically and reviewing the pathological features of the tumour **(Rea, 2015)**.

The study by Caudle *et al.* showed that NACT could be an effective tool to promote breast conservation therapy (BCT) with cosmetically favourable results in patients who require neoadjuvant chemotherapy that provides tumour down-staging **(Caudle, 2014)**. Some of the most successful methods are treating patients with large primary and locally advanced breast cancer by preoperative therapy, which would delay BCT. The study by **Costa** *et al.* recorded that BCT was carried out in 12.9% of patients with IBC and 33.0% of patients with LABC after receiving NACT **(Costa, 2009)**. The tumour in all patients who underwent breast-conserving surgery had been evaluated by imaging procedures. Although some studies have indicated that BCT is feasible in the primary inoperable tumour with a good response to primary chemotherapy treatment, mastectomy is still considered the gold standard in these patients. For axillary management of patients with IBC or LABC treated with NACT, there is no published data that suggests these patients should be treated any differently than any other patients **(Rea, 2015)** with invasive breast cancer receiving neoadjuvant chemotherapy.

3. Molecular Markers

3.1 Tetraspanins

Tetraspanins can also be known as tetraspans or transmembrane four superfamilies (TM4SF). Members within the tetraspanin family have four domains (TM1-TM4). Two of these domains are extracellular, with one being short (known as the small extracellular domain or loop, SED/SEL or EC1) and one being longer (termed as the large extracellular domain/loop, LED/LEL or EC2), one short inner loop joining TM2 and TM3 and intracellular N- and C-termini **(Termini, 2017) (Fig. 1)**.

All tetraspanins contain a CCG (Cysteine-Cysteine –Glycine) motif within the large extracellular loop, with most containing other relatively well-conserved amino acids, including additional cysteines in the large extracellular loop. Alongside a few other features, these separate tetraspanins from other transmembrane domain proteins **(Berditchevski, 2001)**. In the tetraspanin superfamily, transmembrane proteins are involved in a various range of biological phenomena, which include cell differentiation, cell motility, and cell proliferation. The details of their function are not entirely understood, but data reveals that tetraspanin works by interacting with other transmembrane proteins, including integrins and receptor tyrosine kinases, at tetraspanin-enriched microdomains (TERMs) to bind proteins together and stabilise or activate them. Homophilic and heterophilic complexes are made through the interaction between tetraspanins and other trans-membrane proteins, which are then included in secondary interactions depending on the palmitoylation of tetraspanins so that they bind with one another heterophilically and obtain these other proteins into functional complexes. Due to many variations, it is thought that TERMs are different from other cell surface signalling platforms, for

example, lipid rafts, even though the study was carried out by Hemler, who explained that tetraspanins could interact with lipids **(Hemler, 2005)**.

As tetraspanins play an essential role in normal cellular processes, they also play a role in cancer growth and metastasis. Recently it has been proven that tetraspanins can not only be metastasis suppressors but metastasis promoters too. For example, two tetraspanins, CD151 and tetraspanin 8 are overexpressed in many human tumours and appear to promote tumour progression **(Hemler, 2013; Zöller, 2009)**. CD151 controls cell migration, often through its association with α3β1, α6β4, and matrix metalloproteinases. Tetraspanin 8 monitors cell motility and survival and plays a role in the promotion of angiogenesis **(Zöller, 2009)**. The study by **Wang** *et al.* presented the contradictory effects of CD9 in prostate cancer as CD9 expression is notably reduced and even lost throughout prostate cancer progression **(Wang, 2007)**. Furthermore, mutations and deletions of the CD9 mRNA could be correlated with the loss of protein. Another metastasis suppressor is tetraspanin CD82, which down-regulates human prostatic, lung, and pancreatic cancers **(White, 1998)**.

Fig. 1 below illustrates a schematic of the basic molecular structure of the tetraspanin which was taken from the study done by Christina M. Termini and Jennifer M. Gillette **(Gillette, 2017)**.

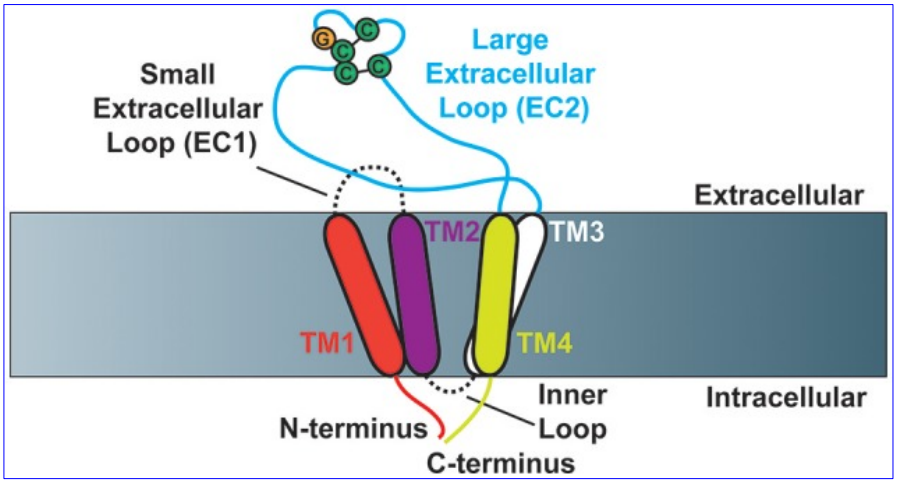

Fig. 1. Schematic of tetraspanin molecular structure (taken from the study by **Christina M. Termini and Jennifer M. Gillette, 2017**.

3.1.1 CD151 (TSPAN 24)

One of the 33 proteins in the mammalian tetraspanin protein family is CD151. This is extensively expressed on the surface of several cell types, where it links strongly with laminin-binding integrins and weakly with some additional integrins. Therefore, CD151 is positioned well to moderate integrin-dependent cell spreading, signalling adhesion strengthening, and migration **(Yang, 2008)**. It has an essential role in forming tetraspanin-enriched microdomains (TERM or TEM), which also encompass a lot of additional transmembrane receptors **(Voss, 2011)**. CD151 is expressed in nearly all cell types and tissue displaying high expression, particularly in epithelial and endothelial cells **(Kwon, 2012)**. Clinically increased expression of CD151 is associated with higher tumour grade and poor prognosis in a variety of tumours. The study by **Ang** *et al.* recorded a significant correlation between over-expression of CD151 and reduction in overall survival in prostate cancer **(Ang, 2004)**. This was compatible with the findings in lung cancer **(Tokuhara, 2001)**. In colon cancer, changes in expression of CD151 appear to be more complex as an early report has illustrated that over-expression of tetraspanin correlated with a more

advanced stage of the disease **(Zijlstra, 2008)**. However, Chien *et al.* study in 2008 has found that expression of CD151 protein is decreased in human colon cancers in comparison with surrounding normal tissue, which is strongly expressed on the lateral and basal surfaces of epithelial cells while CD151 expression in cancer cells is downregulated by hypoxia **(Chien, 2008)**. The ability of cell adhesion is reduced by decreased expression of CD151. The same study by Chien also revealed there is a higher expression of CD151 in metastasised cancer in comparison to the primary tumour. The precise mechanism of this phenomenon has not been identified. One possibility could be that CD151 is induced in the circulatory system throughout the time of migration. This was defined by the reappearance of CD151 in cancer cells after re-oxygenation. Re-expression of CD151 in circulating cancer cells retains adhesive capacity, leading to an increase in the opportunity for growth within the secondary site. Another possibility is that factors synthesised or secreted by cells within the secondary site may provoke the production of CD151, resulting in an increase in adhesive ability. On the other hand, the study by **Voss** *et al.* identified that a high level of CD151 expression in endometrial carcinoma is associated with a longer overall survival rate, inferring that endometrial carcinoma is separate from other epithelial cancers where CD151 has a suppressive role resulting in a favourable outcome **(Voss, 2011)**. One possibility could be that CD151 prevents the metastatic progression of endometrial cancer by maintaining E-cadherin based on cell-to-cell interactions as there was a strong relationship between the expression levels of E-cadherin and CD151 **(Voss, 2011)**.

CD151 in breast cancer:

In **2008, Yang XH** *et al.* displayed a notable correlation between the over-expression of CD151, tumour grade, and negative ER hormonal receptor in invasive ductal breast carcinoma **(Yang 2008)**. Triple-negative

tumours with a basal-like phenotype exhibited the highest proportion of CD151 (45%). On the contrary, ER (+)/HER2 (−) luminal-like tumours had the lowest proportion with only 17% **(Yang, 2008)**. On the other hand, the study by **Romansky and Berdichevsky** recorded no correlation between the expression level of CD151 and hormonal status (ER and PR) **(Romanska, 2010)**. Additionally, the same study demonstrated over-expression of CD151 in invasive ductal carcinoma in situ which was expressed in cells at the periphery of the ducts, and its level is significantly associated with the disease grade.

By contrast, in lobular carcinoma in situ (LCIS), there was a uniform and strong expression of CD151 by all cells of the lesion. Furthermore, increased expression of CD151 in DCIS implied that CD151 mixed with interacting molecules could also have a vital role during the early stages of the disease **(Sadej, 2013)**. The study by **MJ Kwon1** *et al.* revealed that CD151 over-expression is an independent negative predictor of overall survival rate in women with breast cancer **(Kwon, 2012)**.

Luminal A subtypes and TNBC showed the worst overall survival rate. Solely, the basal-myoepithelial cell layer that surrounds ducts and lobules, shows a normal expression of CD151, which was compatible with the results of a previous report **(Yang, 2008)**. On the contrary, CD151 showed different patterns of expression in invasive breast cancer, which ranged from complete absence to strong diffuse over-expression appearing in the membrane and/or cytoplasm of tumour cells. **Yang** *et al.* reported in 2008 a significant correlation between over-expression of CD151 and other tumour parameters, including tumour grade, ER hormonal status, and combination of ER/HER2 status **(Young, 2008)**. Additionally, inhibition of CD151 was seen to hinder the invasion, migration, and spreading of breast cancer cells, indicating that CD151 has a role in breast tumour progression **(Yang, 2008)**.

Nevertheless, the same author 2010 confirmed that in HER2-positive breast cancer, there was an adhesion between cancer cells and basement membrane laminin-5, which provides significant resistance to anti-HER2 therapy. Destruction of laminin-binding integrins or associated tetraspanin protein CD151 sensitised HER2 positive cells to anti-HER2 treatment.

3.1.2 Tetraspanin6 (TSPAN6)

There is not much known about TSPAN6. The expression of Tetraspanin6 in Breast cancer has not yet been studied. A literature review revealed no published reports or studies. A current unpublished study in our laboratory using tissue micro-arrays has explained that the expression of this protein is reduced in higher-grade breast cancer. TSPAN6 gene expression was extensively observed in human adult tissues. Amongst the tissues tested, TSPAN6 was highly expressed in the kidney, liver, pancreas, and ovary, whilst low-level expression was recognised in the skeletal muscle, brain, and lungs. The expression level of the foetal lung could be higher than in the adult as the foetal lung cDNA library used for the screening of Tetraspanin6, provides a strong signal for the gene **(Maeda, 1998)**.

TM4SF6 was the protein encoded by this gene, which is a member of the transmembrane four superfamilies. These proteins take part in the regulation of cell development, activation, growth, and motility **(HGNC, 2020)**.

3.2 Tumour microenvironment

The microenvironment of breast cancer plays a vital role in tumour initiation and supports the invasion and metastasis of malignant tumours, such as breast cancer. The tumour microenvironment of malignant tumours consists of several cell types. These include immune cells, stromal cells,

and the secretion of the active medium by the tumour cells **(Yang, 2015)**, which frequently plays a role in tumour carcinogenesis. Huang *et al.* study showed that in inflammatory breast cancer, microenvironment cells (including inflammatory cells, immune cells, matrix cells and cancer cells) produce other molecules that interact with each other, producing a complex which controls IBC growth, and progression **(Huang, 2017)**. The aberrant expression of inflammatory factors and immune factors may augment carcinogenesis, tumour cell proliferation, migration, invasion, and metastasis. A recent study by **Badr** *et al.* reported that immune cell populations, especially tumour-infiltrating lymphocytes (TILs) in breast carcinoma, microenvironment cells can change the tumour response to different neoadjuvant chemotherapy regimens and designation of TIL subtypes and locations in relation to tumour cells in breast carcinoma. This is encouraging for the advancement of more efficient choices of targeted therapy **(Badr, 2019)**.

3.2.1 CD68

The tumour-associated macrophages (TAMs) are the dominant components of the cells in the tumour microenvironment, and their presence is associated with the poor prognosis of solid tumours. The pan-macrophage marker CD68 is considered the best marker protein TAMs and is now often used to predict the prognosis in several tumours, including thyroid, hepatocellular, pancreatic, and lung cancer **(Yang, 2015)**. CD68 is expressed on monocytes/macrophages. It is also present to a minor extent on dendritic cells and peripheral blood granulocytes. It is classified as a member of the scavenger family. The role of CD68 includes the removal of cellular debris, activation of macrophages and phagocytosis. CD68 is also a member of the Lysosome-Associated Membrane Protein (LAMP) family, where CD68 mainly localises to lysosomes and endosomes, with a small particle spreading to the cell surface. Immunohistochemistry is utilised to

determine the existence of CD68, which shows a cytoplasmic expression of various blood cells and myocytes. Several cells of the macrophages, including monocytes, histiocytes, giant cells, Kupffer cells, and osteoclasts, demonstrate positive expression of CD68. It is useful in differentiating between diseases of similar appearances (which is CD68 negative) and disorders associated with an abnormality of these cells, including malignant histiocytosis, histiocytic lymphoma, and Gaucher's disease **(Leong, 2012; Manduch, 2009)**. The study by **Catharina Medrek** *et al.* stated that CD68 macrophages in tumour stroma are directly associated with tumour size and inversely correlated to luminal A breast cancer **(Medrek, 2012)**. Furthermore, CD68 in tumour stroma was an independent prognostic factor for decreased survival rate in breast cancer **(Medrek, 2012)**.

Additionally, the study by Campbell et al showed an increasing number of intertumoral macrophages that were noticeably associated with high-grade negative hormonal receptor tumours and a basal-like phenotype **(Campbell, 2010)**. The increasing number of macrophages was a significant predictor of recurrence and survival, therefore proliferating macrophages in breast cancer may be used as a prognostic indicator of poor outcome and early recurrence. Moreover, there was no association noted between the increasing number of macrophages and tumour stage, including tumour size and the number of involved lymph nodes.

3.2.2 CD3 (Tumour infiltrating lymphocytes)

Tumour infiltrating lymphocytes (TILs) are commonly found in tumours, inferring that the tumour initiates an immune response in the host. Tumour antigens facilitate this so-called tumour immunogenicity. These antigens detect the tumour from healthy cells by providing an immunological stimulus **(Gooden, ….)**. Immunohistochemical and digital assessments of TILs are at the moment restricted to the research setting

(Badr, 2019). Tumour infiltrating lymphocytes (TILs) have been deemed to play a crucial role in inhibiting tumour proliferation and metastasis in tumours and perhaps can be considered as an independent prognostic marker. The increase of infiltrating lymphocytes within the tumour cell is correlated with a good prognosis in a variety of tumours **(Rathore, 2013)**. The study by **Badr** *et al.* was in line with the previous studies as it showed a significant correlation between the increased number of TILs and good outcomes in breast cancer in patients who received NACT. The same results were also noted in inflammatory breast cancer patients who were treated with NACT, as a tumour with a stromal TIL infiltration rate of more than 10%, displayed a prolonged overall survival rate. Consequently, increased TIL is considered a predictor factor of favourable outcomes **(Badr, 2019)**. CD3 is a marker of the tumour infiltrating lymphocytes, which allows the activation of the cytotoxic T-Cell as a T-cell receptor. It contains a protein complex that is made up of four separate chains, including a CD3γ chain, a CD3δ chain, and two CD3ε chains, to enable the activation signals in T-lymphocytes. The protein complex binds to molecules, which are identified as the T-cell receptor (TCR) and the ζ-chain (zeta-chain). The TCR, ζ-chain, and CD3 molecules bind together to produce the TCR complex. These four chains are greatly associated with cell-surface proteins of the immunoglobulin superfamily, including a single extracellular immunoglobulin domain **(Call and Wucherpfennig, 2004)**.

Fig. 2 below shows a schematic of the basic structure of the TCR-CD3 complex.

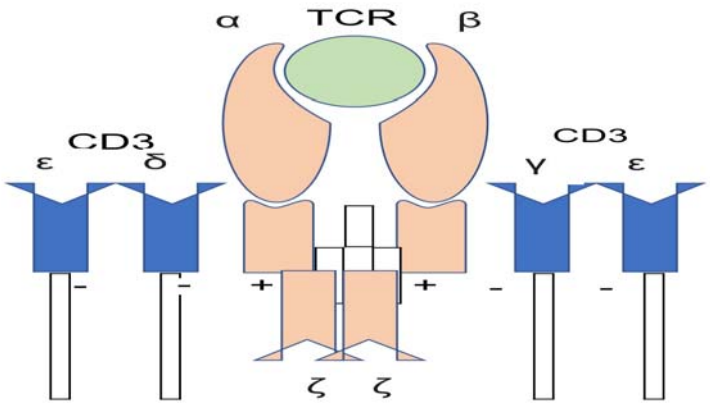

Fig. 2. A schematic of the basic structure of the TcR-CD3 complex based on Dr Collin RA Hewitt's presentation

In the cytoplasm of stem cells, CD3 is first expressed whereby T-cells appear in the thymus known as pro-thymocytes, which differentiate into common thymocytes followed by into medullary thymocytes, where CD3 antigen starts to move to the cell membrane. The antigen can be seen to be attached to the membranes of all mature T-cells but no other cell type apart from being seen in Purkinje cells in small amounts. This high specificity, along with CD3 being present throughout all stages of T-cell development, makes it an advantageous immunohistochemically marker in tissue sections for T-cells. The antigen has been detected in nearly all T-cell lymphomas and Leukaemia and can accordingly be utilised to differentiate them from similar B-cell and myeloid neoplasms **(Call and Wucherpfennig, 2004)**. A greater percentage of CD3 has been associated with favourable outcomes in oropharyngeal cancer **(Rajjoub, 2007)**, and low CD3 has been stated to predict shorter disease-free survival in colon and cervical cancer **(Sinicrope, 2009; Ancuta, 2009)**. The Study by **Rathore** *et al.* revealed CD3 positive TILs were distinguished in intra-tumoral and stromal areas of all the TNBC. Intra-tumoral CD3+ TILs were considerably associated with the clinical stage **(Rathore, 2013)**. The

association of intra-tumoral CD3 TIL was not important when correlated with age, family history, histological grade, and both lymph node and menopausal status. Likewise, the stromal CD3 TIL was also significantly linked with the clinical stage, while factors like age, menopausal status, family history, grade, and lymph node status did not display a significant association with CD3+ stromal TIL count. Furthermore, the same study explained that patients with higher intra-tumoral and stromal CD3+ T-cell infiltration led to longer disease-free survival. High CD3 is considered a good prognosis marker in TNBC. The study by De Nardo *et al.* established that after neoadjuvant chemotherapy, high CD3 could predict pathological Complete Response in triple-negative breast cancer **(DeNardo, 2011)**.

4. Objectives of the Study

- To predict the response of the patient to neo-adjuvant chemotherapy by studying the expression of CD151, CD68 and CD3 on core biopsy
- Correlate expression of these markers to patients' Disease-Free Survival and Overall Survival
- To identify any association between patients 'demography or hormonal status with response to NACT and the expression of markers (CD151, CD3 and CD68)

5. Methods and Materials

5.1 Methods Search Strategy

The MEDLINE search was done on the Ovid database and PubMed search. This search used the following keywords: 'neoplasm', 'cancer,' 'breast', 'inflammatory', 'Locally advanced', 'neoadjuvant', 'chemo-therapy', 'free-survival', 'tumour,' 'tumour,' 'infiltrating lymphocyte', 'intra-tumoral lymphocyte', 'TIL', 'tetraspanin', 'CD3', 'CD68', 'CD151', 'TSPAN6'. I utilised the subsequent MeSH terms: 'prognosis', 'mortality',

'survival', 'survival analysis', 'disease-free survival', 'lymphocytes, tumour infiltrating'. I combined each one with another, trying to find all the relevant articles. Only papers in the English language were included.

5.2 The Cohort

A retrospective review of the oncology database was conducted. This incorporated 1347 patients who were diagnosed with histologically confirmed invasive breast cancer at Queen Elizabeth Hospital Birmingham, from January 2006 to December 2015. A total of 301 consecutive patients with a diagnosis of locally advanced breast cancer (LABC), inflammatory breast cancer (IBC), and others were involved in the study. Given the fact that the total number of inflammatory breast cancer during the period of nine years was insufficient to perform the study and obtain reliable results, a decision was made to involve other centres, including City Hospital of Birmingham, St James's University Hospital in Leeds and one institution in Japan. The total number of patients with a diagnosis of IBC was 96 cases in total. All experiments were approved by the West Midlands-Black Country NRES Committee (07/Q2702/24).

Eligibility criteria: The following patients were included:

Newly diagnosed patients with invasive breast cancer who received neoadjuvant chemotherapy prior to the surgery followed by surgery with a postoperative assessment of the pathological response to NACT. We also incorporated patients who did not survive till surgery or developed metastasis before surgery. Nevertheless, patients with metastatic breast cancer at presentation or known to have breast cancer were excluded from this study or if a core biopsy was not available. A database of information was collected for all cases. It incorporated patients' demographic data, the pathological characteristics of the tumour, including the type of tumour,

histological and nuclear grade, and the receptor status of the oestrogen receptor (ER), progesterone receptor (PR) and HER2.

Applying the Quick Scoring system (Allred score), which assesses ER and PR positivity by adding the proportion of nuclear positivity and the intensity of staining. The proportion score (0-5) depends on the percentage of positive tumour cells which are, 0 (0%), 1 (<1%), 2 (1-10%), 3 (11-33%), 4 (34-66%), and 5 (67-100%). The intensity of staining for the positive nuclear staining is graded as 0, 1, 2, and 3, according to a negative, mild, moderate, and strong intensity. The total scores for ER and PR are based on both positive staining and the intensity of staining. 0-2 are considered negative scores, while 3-8 are taken as positive scores. HER2 status, grades 0 and 1 as evaluated by IHC, were described as a negative result, and grade 3 was established as a positive result. Amplification of HER2 was verified by FISH if HER2 was evaluated as 2+ by IHC. The tumours were categorised into three subtypes using immunohistochemistry. The hormone receptor-positive (HR+) group was known as ER positivity and Her2 negativity. The Her2-positive (Her2+) group was classified as Her2 positivity regardless of ER/PR hormonal status.

Triple negative breast cancer (TNBC) was identified as ER, PR, and HER2 negativity. The database also incorporated clinical information such as date of diagnosis, date of recurrence, date of last seen or date of death, as well as the type of NACT, number of cycles, and response to treatment. The pathological response to the neoadjuvant chemotherapy was investigated in the surgical specimen (either Wide Local Excision or mastectomy both with additional sentinel lymph node biopsy (SLNB) or axillary clearance). The pathological response to neoadjuvant chemo-therapy was evaluated as follows:

Pathological complete response – The category of pCR (includes a ‘‘near-total complete response/ almost complete response’ for the purpose of statistical analysis.’ The reason I lumped a tiny focus of residual invasive carcinoma as pCR was to be able to conduct the statistical analysis. This is regardless of the presence or absence of DCIS.

Pathological partial response (pPR) – Described as the decrease in tumour size or cellularity associated with chemotherapy-induced histopathologic changes like necrosis, fibrosis, inflammatory reaction, and other changes.

No response (pNR) – The category of pNR includes a ‘‘minimal pathological response”, implying no/minimal change or increase in tumour size, grade, and no localised fibrosis.

5.3 Slide Preparation

The paraffin block for the core biopsies was cut at a thickness of 3-µm using a microtome. Once cut, the tissue ribbons are conscientiously transported to a warm water bath to float on the surface and can then be carried up onto a slide set below the water levels. Slides were identified and then left to dry overnight at 37 degrees Celsius to smoothly melt the excess paraffin wax, leaving the tissue section intact. Then, they were stored at room temperature prior to staining.

For patients with locally advanced breast cancer and inflammatory breast cancer at QE hospital, the slide(s) of excision specimens containing tumours were selected by studying haematoxylin and eosin (H&E) stained slides to decide which block(s) to be collected and the areas of tumour cells were marked for tissue microarray (TMA). Where possible, four tumour areas were circled on the glass slide to be able to mark these areas on paraffin block(s).

The TMA for this write-up will be created in the future by other members of the group, as unfortunately, there was not enough time. TMA will be carried out by using small punches around 0.6mm in diameter from the area of interest in paraffin blocks and embedded in a recipient paraffin block in an accurately aligned pattern. Sections from this paraffin block will be cut using a microtome followed by positioning it onto the slide for immunohistochemistry.

5.4 Immunohistochemistry Protocol

The core biopsy slides were cut from the paraffin blocks to evaluate the expression of each marker individually, conducting the IHC technique. The positive control slides of normal breast tissue and breast cancer tissue were used in each batch to ensure that nothing went wrong according to our experimental protocol. DAKO auto-stainer machine at QEHB was used for the evaluation of CD3 and CD68 expression in breast cancer. However, over-expression of tumour markers (CD151 and TSPAN6) was performed by using a manual immunohistochemistry method on formalin-fixed and paraffin-embedded samples of breast cancer, according to the protocol outlined in the manufacturer's guide, which usually comes with the kit. Automated staining was utilised for the established antibodies. Although automated staining is more accurate and reliable, manual staining is more cost-effective.

5.4.1 DAKO Autostainer Protocol

Tissue sections were cut and dried overnight at 37°C and 65°C for 20 minutes preceding staining. Sections were then placed into Dako PT link in high pH solution (K8002) for pre-treatment at 97°C for 20 mins. Slides were then placed into buffer tanks for 5 mins. On the staining machine, sections were treated with peroxidase block for 5 mins to prevent endogenous peroxidase staining.

Slides were then washed in buffer (Dako K8002). The slides were incubated in the primary antibody and left for 30 minutes. Slides were washed in buffer followed by envisioning for 30 mins. (Dako K8002). Before the addition of DAB, the slides were washed in a buffer again. DAB chromogen was added and left for 10 mins. Slides were then rinsed in DI water before being counterstained in Mayer's hematoxylin, and blue nuclei in Scott's tap water. Finally, slides were placed into alcohol for dehydration, followed by Histoclear before the addition of coverslips.

5.4.1 Manual Staining Protocol

Firstly, paraffin was removed from the tissue sections by putting them in a Histoclear for ten minutes, this was followed by rehydration through Industrial Methylated Spirits (IMS) for another 10 minutes, followed by distilled water. Slides were then put into 0.3% of hydrogen peroxide for 15 minutes to prevent interaction with endogenous peroxidase staining. The slides were then washed in water before being added to the antigen retrieval, 0.01 mol/L sodium citrate buffer (pH 6.0). Next, the buffer was heated for 10 minutes in a microwave at higher power before the slides were placed into the buffer and heated for a further 20 minutes. Following this, the slides were cooled down to room temperature and washed with PBST before being incubated in Casein for 10 minutes. Slides were incubated in 100µl of primary antibody (anti-TAPAN6 and left for an hour) and 200µl of primary antibody (anti-CD151 and left overnight at (40 C). When reclaimed after 1 hour in TSPAN6 and the following day in CD151, slides were washed in PBST for three rounds in 5 minutes. The slides were next incubated in the secondary antibody (ImmPRESS TM universal anti-mouse/rabbit Ig) for another half an hour. The slides were then carefully washed six times for 10 minutes before the addition of DAB.

The ImmPACT™ DAB peroxidase kit was used by adding one drop of chromagen to 1ml of diluents, followed by 200 µl of the solution to each slide for up to 5 minutes.

After this, slides were rinsed in running water for 5 minutes and then stained in Haematoxylin for 3 minutes. Slides were washed again in water for 5 minutes using an alternate hot to the cold method before being placed into a higher concentration of IMS for 10 minutes to dehydrate the slides. Ultimately, slides were placed into both Histoclear 1 and 2 for 5 minutes each before the addition of coverslips using DPX.

5.5 Optimisation of the Antibody

Each antibody had to be optimised before manual staining of the slides by trying various concentrations of the primary antibody on positive external control (breast cancer tissue). Different concentrations and various incubation periods have been tried to be able to optimise the TSPAN6 antibody. Firstly, Irini tried concentration 1: 200, then 1: 150, and lastly, 1: 100 for 1 hour incubation period. The same concentrations were then tested using 2 hours incubation period as well as an overnight incubation period. The optimum concentration for anti-TSPAN6 was 1: 100 with 2 hours incubation period. This concentration was used for staining all LABCs and IBCs cases. The same method was utilised to optimise the CD151 antibody, meaning that different dilutions, including 1: 200, 1: 175, and 1: 150 were tested in various incubation periods, including one-hour, two-hours, and overnight. The optimum dilution for anti-CD151 was 1: 150 with overnight incubation.

5.6 Scoring the IHC staining

CD151

The results of immunostaining of CD151 were scored in a semi-quantitative method, including both intensities of staining and distribution, like HER2 immunohistochemistry scoring. Tumours with a complete absence of staining or membrane staining in less than 10% of the tumour cells were scored 0. A score of 1+ was given to tumours with a faint or incomplete membrane staining in more than 10% of the tumour cells, with only part of the cell membrane staining. A score of 2+ was given to tumours with a weak to moderate complete membrane staining in more than 10% of the tumour cells. A score of 3+ was given to tumours with a strong complete membrane staining in more than 10% of the tumour cells. Scores of 0 and +1 were deemed negative for expression, and scores of +2 and +3 were considered positive for expression.

Fig. 3 below displays an example of how specimens were scored.

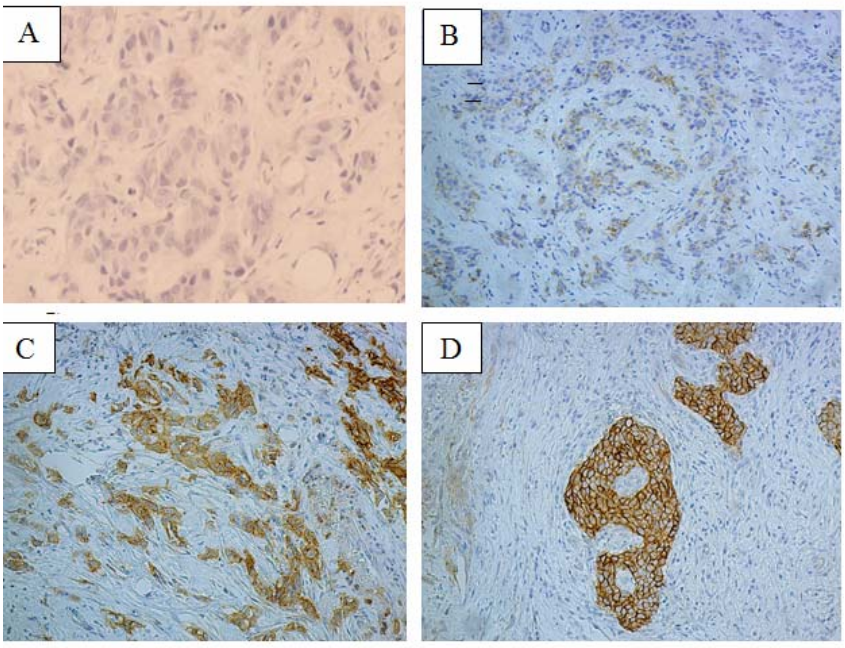

Fig. 3. CD151 immunohistochemistry scoring in IBC. This figure showed: a CD151 (0) complete absence of staining or membrane staining in less than 10% of the tumour cells (A), a CD151 (+1) faint or incomplete membrane staining in more than 10% of the tumour cells (B), a CD151 (+2) weak to moderate complete membrane staining in more than 10% of the tumour cells (C), a CD151 (3+) strong complete membrane staining in more than 10% of the tumour cells. The magnification used for the image was 20x and staining was carried out by Irini.

CD3 TIL

Two scoring methods were utilised to assess the expression of CD3

Old scoring method

In areas of high lymphoid density, CD3 positive T-lymphocytes were counted, with necrotic areas excluded. A score of zero was given to the absence of tumour infiltrating lymphocytes. A score of one was given to minimal infiltration by T lymphocytes (CD3) (less than ten positive cells in HPFx40). A score of two was given to positive T-lymphocytes that were easily identified but not in large aggregates. A score of three was given to the presence of large aggregates of positive T-lymphocytes in more than 50% of the tumour. **Fig. 4** below exhibits an example of how specimens were scored.

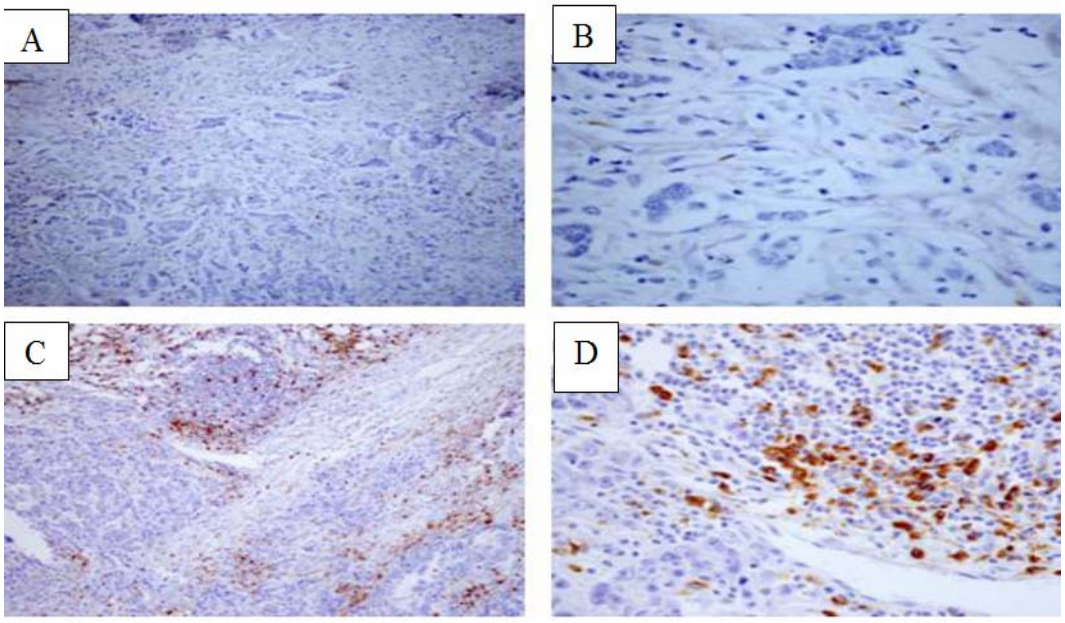

Fig. 4. CD3 immunohistochemistry scoring in LABC and IBC. This figure showed a CD3(0) complete absence of tumour infiltrating lymphocytes (A), a CD3 (+1) minimal infiltration by CD3 (less than ten positive cells (B), a CD3 (+2) positive T lymphocytes that were easily identified but not in large aggregates (C), a CD3 (+3) presence of large aggregates of positive T lymphocytes in more than 50% of the tumour (D). The magnification used for the image was 40x and staining was carried out by Mariam.

New scoring method

The results of immunostaining of T lymphocytes (CD3) have been assessed individually for both the stromal compartment (= % stromal TILs) and the tumour cell compartment (=% intra-tumoral TILs) based on the recommendations of the immune-oncology biomarker group. (TLS working group). In addition to assessing the tumour stromal element and tumour cell element, identifying "hot spots," which are defined as small areas with a significant increase in TILs, were evaluated.

According to the study done by **Salgado** *et al.* **(2014)** intra-tumoral TILs were assessed, and they were defined as the presence of lymphocytes within intraepithelial tumour nests, or in direct contact with tumour cells with no intervening stroma. While stromal TILs were dispersed in the stroma between the carcinoma cells and did not directly contact carcinoma cells', the stromal TILs were assessed in five areas in percentage, and CD3 expression was estimated by counting the number of CD3 using a light microscope x400 in five regions. From each slide, the concentrated areas were selected and the mean of 5 counts was taken. The median value was utilised to divide the groups into the high-infiltration group and the low-infiltration group based on the study by **Salgado** *et al.* **(2014)**.

CD68

Two scoring methods were utilised to assess the expression of CD68

Old scoring method

CD68+ Tumour Associated Macrophages (TAMs) were estimated by counting the number of CD68+ (granular cytoplasmic and membranous positivity) using a light microscope x400 in three regions of intensive areas from each slide, with the highest number taken. The areas of necrosis were avoided and excluded from counting. To separate the patients into TAMs high-infiltration group and the low-infiltration group, the median value of CD68+ cells was utilised **(Yang, 2015)**.

New scoring method

The same methodology used in scoring CD3 was also used in scoring CD68. CD68+ TAMs were estimated by counting the number of CD68+ TAMs using a light microscope in x400 in five regions of intensive areas from each slide, with the mean of 5 counts taken. The median value was utilised to divide the groups of all immune histochemical variables in our data. Following this, the patients were separated into TAMs high-infiltration group and the low-infiltration group.

TSPAN6

Irini has finished the manual staining for the LABC(s) cases, resulting in a total of 50 cases. She also accomplished the staining of all IBC cases, resulting in a total of 100 cases. These cases were from QEHB, Heartlands Hospital, City Hospital of Birmingham, and the St James's University of Leeds, as well as from Japan. Another student will complete TSPAN6 scoring and any further study.

6. Statistical Analysis

In this study, the primary endpoints were disease-free survival (DFS) and overall survival (OS) rates.

From the date of diagnosis to the date of death, OS was calculated. From the date of diagnosis to the date of disease recurrence, DFS was calculated. Patients with no evidence of recurrence were reviewed at the last follow-up date. In September 2018, all patients' database was updated. The Kaplan–Meier method was applied to estimate the time to recurrence or death before comparing groups with log-rank tests. Mann-Whitney-U tests and Kruskal-Wallis tests were utilised to find a relationship between markers' expression and other variables with OS and DFS.

They were also used to assess for a correlation between response to NACT and other variables with nodal burden and patient age. Univariate and multivariate analysis of time to the event was conducted adopting a Cox proportional hazards model.

The variables examined were age at diagnosis (younger than 50 years versus 50 years and above) and nodal burden (less than median versus median and above) with tumour nuclear grade (grade 3 versus grade 1–2), hormone receptor expression (positive or negative), HER-2 expression (positive or negative) and the response to NACT, which were subdivided into three categories: the category of pCR (Includes a ‘‘near-total complete response/almost complete response’, pPR and the category of pNR (Includes a ‘‘minimal pathological response” non-responders (pNR). Chi-square tests were also utilised to test if there was a significant correlation between hormonal receptors, tumour grade, and tumour type with markers’ expression and response to NACT. All statistical tests were two-sided, with p-values of 0.05, which is seen as being significant. SPSS statistical software version 26 was used to complete all calculations.

7. Results

7.1 The Cohort

The final total cohort consisted of 301 cases. The patients were separated into three groups depending on the type of cancer. Group 1 consisted of 43 patients with a diagnosis of locally advanced breast cancer (LABCs). Group 2 patients consisted of 96 patients with a diagnosis of inflammatory breast cancer (IBCs). Group 3 consisted of 162 patients with neither IBC nor LABC. Therefore, in this study, she focused on groups 1 and group 2.

7.2 Group 1 (LABCs)

From the original cohort of 52 cases, 43 satisfied the inclusion criteria. The study population included mostly Caucasians (65.11%) as well as Asians, Black, and mixed ethnicity aged between 34 and 83 years, resulting in 59 being the median age at diagnosis. The most common type seen in the study was ductal carcinoma NST; the micro-papillary change was observed in one patient after chemotherapy who was diagnosed with lobular carcinoma in the initial biopsy. Furthermore, a transformation from papillary pattern to ductal NST was observed in one case. The apocrine differentiation was seen in one patient after chemotherapy who was diagnosed with ductal carcinoma, NST, on the biopsy. Over half of the cases (53.5%) had a high histological grade (grade-3) pre-treatment. Pre-treatment ER/PR status was examined in all patients, with 58% of the patients having ER/PR positive tumour +/- HER2, and 42% of the patients having HER2 positive tumour +/- ER/PR. However, post-treatment hormonal status was also examined in 43 patients who were treated with NACT before surgery. Out of 43 patients, 11 patients achieved complete pathological response (pCR). The receptor's expression in the remaining 32 cases was analysed. A change in the strength of receptor expression was recorded in 11 of the 32 patients (34.4%). Amongst the 11 patients, a total of 17 receptor marker changes were recorded: 6 ER (6/17) (35%), 9 PR (9/17 (53%), and 2 Her2 2/17 (11.7%). Of the 11 patients whereby changes in receptor expression were recorded, four patients endured a change in receptor status including ER/PR and HER2. Two patients went from ER/PR negative to ER/PR positive, leading to them becoming new candidates for hormonal therapy. In addition to the two changes in ER/PR hormonal receptor status, there were also two changes in HER2 status whereby patients went from HER2 positive to HER2 negative. Never-theless, it was recorded that the change of hormonal receptor status was linked with poor response to neoadjuvant chemotherapy and disease

progression. All four patients displayed disease-free survival and overall survival in less than five years. Breast-conserving surgery was carried out in 32.5% of patients with LABC following the treatment with NACT. Axillary clearance was performed in 88% of patients, as 62.8% of patients presented a positive nodal status and a range of positive lymph nodes (one-20).

Table 1 exhibits patient demographics and clinic-pathologic information on locally advanced breast cancer (LABC).

Table 1. Patient demographics and clinic-pathologic information of locally advanced breast cancer

Characteristics		**No. of patients**
No. of assessable patients		43
Median age at diagnosis		59
Ethnicity	Caucasian	28
	Asian	5
	Black	6
	Mixed	2
	Unknown	2
Histology	Ductal	36
	Lobular	4
	Mixed ductal /lobular	1
	Micropapillary	1
	Missing	1
Tumor grade	1	1
	2	19
	3	23
Estrogen receptor pre-treatment	Positive	25
	Negative	18
Progesterone receptor pre-treatment	Positive	21
	Negative	22
HER2 status pretreatment	Positive	18
	Negative	25
Type of surgery WLE		14
Mastectomy		29
Axillary nodal status	Positive	27
	Negative	16

Neoadjuvant chemotherapy, number of deceased patients and recurrences rates:

Over half of the patients (53.5%) underwent only anthracycline-based chemotherapy, while 17 (39.5%) patients underwent a combination of anthracycline and taxane-based chemotherapy, with a median number of cycles being six. Most of the patients (79%) responded to NACT either in the form of complete (pCR) or partial response (pPR). Pathological complete response was observed in 25.5 % of patients, and pathological partial response was observed in 53.5% of patients, while 21% of patients had no response (pNR) following NACT.

When comparing combined anthracycline and taxane-based chemotherapy with anthracycline-based chemotherapy regimens, no notable difference was observed. Amongst surviving patients, the median followed-up period was 88 months ranging from 30 to 129 months. Locoregional recurrence (LRR) or distant metastasis (DM) developed in 17 patients (39.5%), and death occurred in 13 patients (30.5%), with distant metastasis being the cause of death in 10 cases. **Table 2** summarises the regimen of NACT, the number of cycles, and the response to chemotherapy, in addition to OS and DFS of locally advanced breast cancer (LABC). **Table 3** displays CD3 and CD68 expression in locally advanced breast cancer (LABC).

Table 2. A regimen of NACT, number of cycles, response to chemotherapy and survival in locally advanced breast cancer

		No. of patients
Number of cycles	8-10 cycles	9
	5-7 cycles	22
	2-4 cycles	5
	Unknown	7
Disease free survival months	Mean	29.8
	Range	1-77
Number of alive		28
%		65.11
Number of DM/LRR		17
%		39.5
Number of deceased		13
%		30.2
Unknown		2

Table 3. CD3 and CD68 expression in the study cohort of locally advanced breast cancer

Stained for CD3	Number	38/43
	%	88%
Stained for CD68	Number	39/43
	%	90%
CD3 new score	Median (10) or less	23
	More than median	15
	Missing	5
CD68 new score	Median (45) or less	24
	More than median	15
	Missing	4

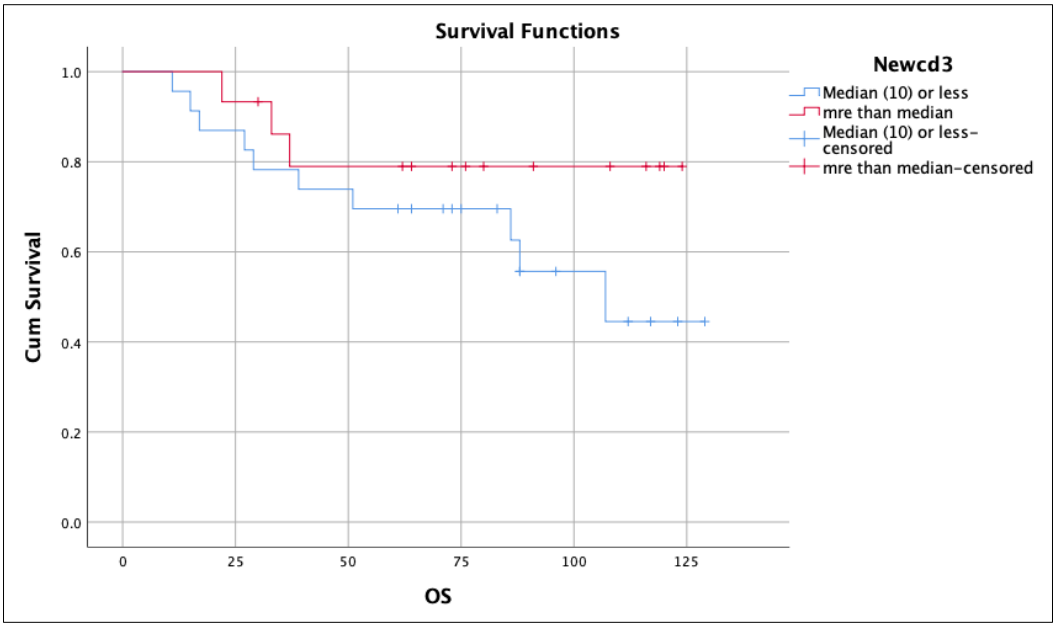

Fig. 5A. New scoring CD3 expression effect on overall survival in locally advanced breast cancer

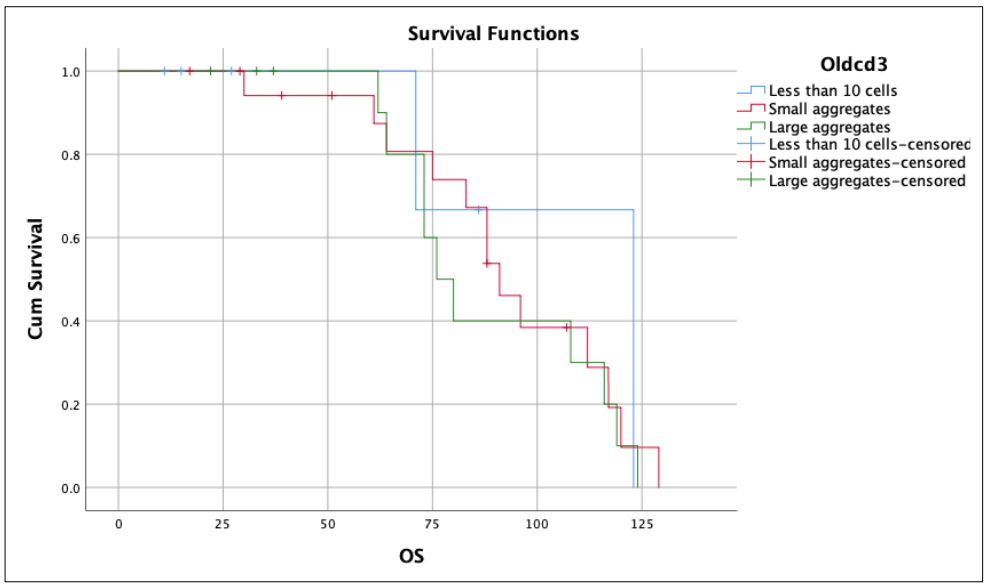

Fig. 5B. Old scoring CD3 expression effect on overall survival in locally advanced breast cancer.

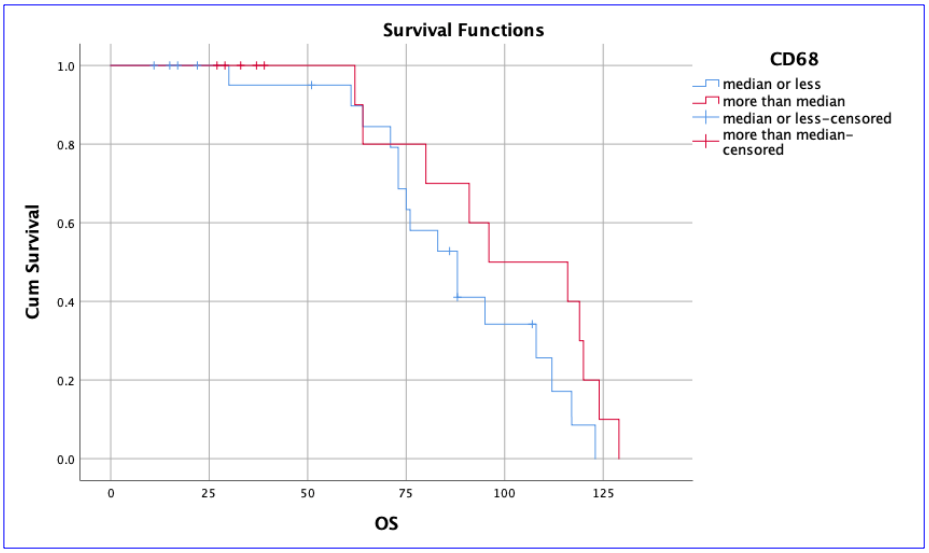

Fig. 5C. CD68 expression effect on overall survival in locally advanced breast cancer

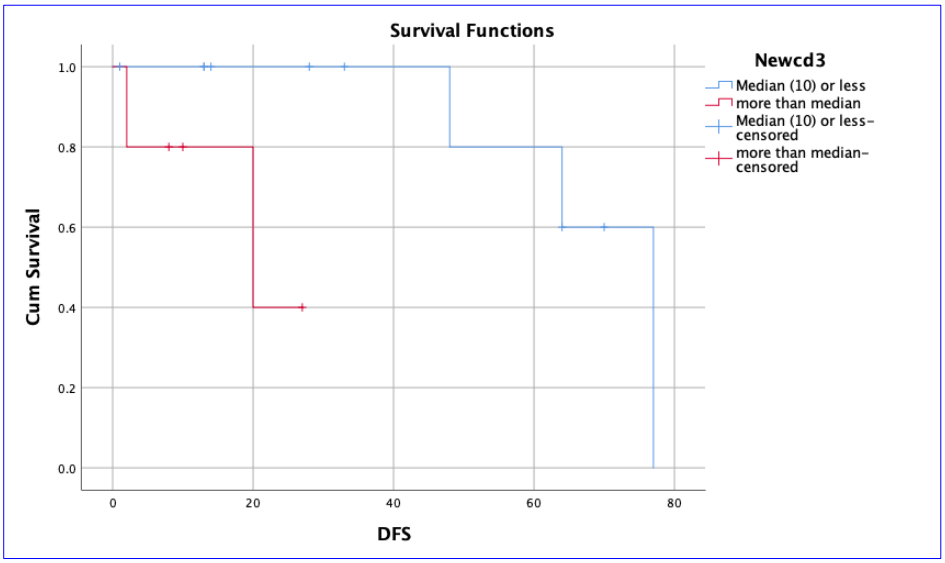

Fig. 5D. New scoring CD3 expression effect on Disease-free survival in locally advanced breast cancer

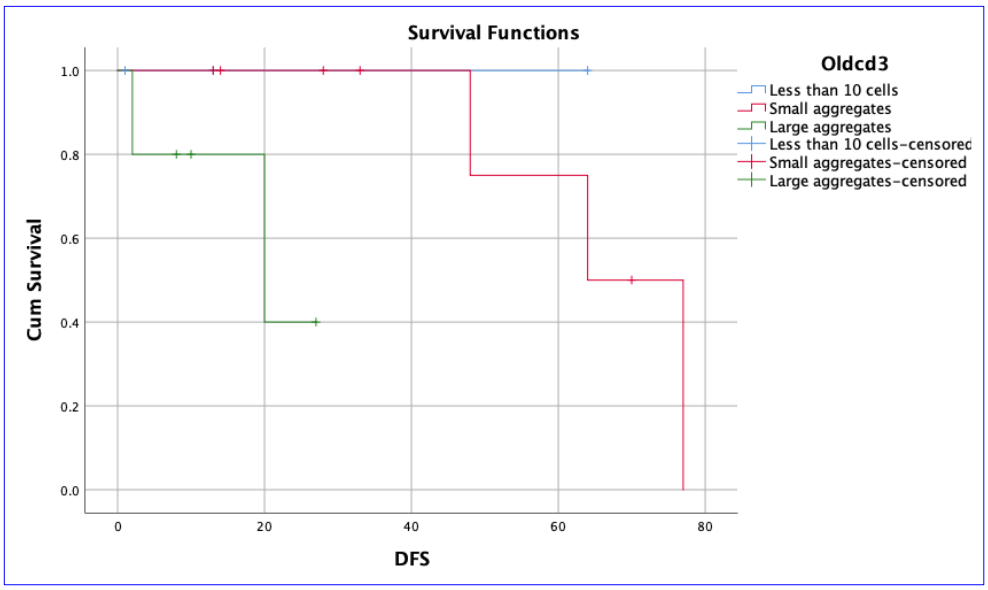

Fig. 5E. Old scoring CD3 expression effect on Disease-free survival in locally advanced breast cancer

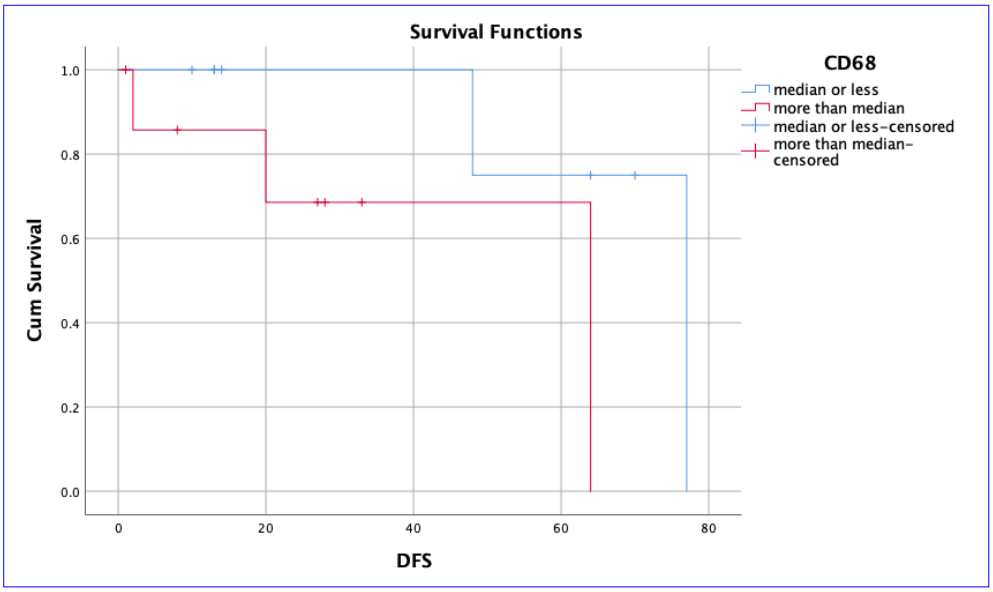

Fig. 5F. CD68 expression effect on Disease-free survival in locally advanced breast cancer

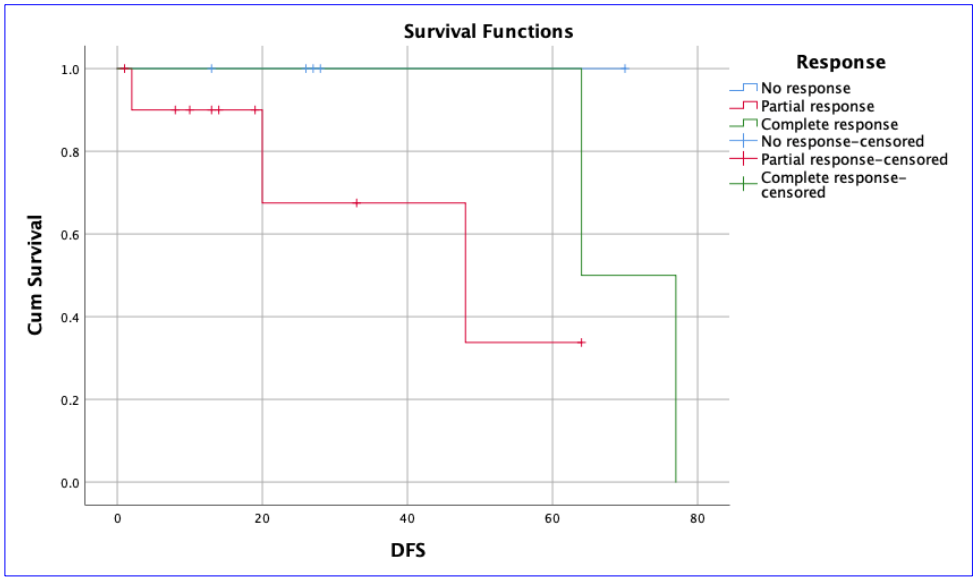

Fig. 6. Response to NACT on disease-free survival in locally advanced breast cancer

7.2.2 CD3 and CD68 expression association with other factors

The expression of those markers was also examined against other factors. No statistically significant correlation was detected between expression of CD68 and ethnicity, age at diagnosis, histological type, and ER, PR, and Her2 expression. Nevertheless, there was a correlation with a statistically significant value between the expression of CD68 and tumour grade ($p = 0.01$). The higher grade was linked with increased expression of CD68.

Additionally, there was no statistically significant correlation between expression of CD3 and ethnicity, age at diagnosis, histological type, tumour grade, and ER, PR hormonal expression. Nevertheless, an increasing number of CD3 was seen to correlate with HER2-positive tumours ($p=0.05$).

7.2.3 Other factors associated with response to chemotherapy

Other factors significantly correlated with the response to NACT included the type of the tumour, HER2 status, and greater nodal burden disease. Invasive ductal, NST, and micro-papillary carcinoma exhibited a better response to treatment compared with lobular carcinoma and other types ($p= 0.01$). Additionally, the HER2 positive phenotype tumour was correlated with a complete pathological response to NACT ($p=0.02$) and inversely associated with nodal metastasis with a significant value ($p=0.035$). Pathological complete response was inversely related to a greater nodal burden disease ($p=0.001$).

7.3 Group 2 (IBC)

The second cohort involved 96 patients. It is a multicentric study where various hospitals from Birmingham, Leeds, and Japan collected the data. Hence, the data for this group was not as comprehensive as the data

for group 1 and group 3. For example, the collecting data from City hospitals and Japan did not incorporate the patients' age, ethnicity, type of tumour, PR hormonal status, type of chemotherapy, and the number of cycles. The study population from QEHB and Leeds was 49 cases, aged 34 to 85 years, leaving 58.8 to be the mean age at diagnosis and 61 years as the median age. Most of the population was Caucasian (90%). The most common type observed was ductal carcinoma NST; Micro-papillary change was observed in two patients following chemotherapy, who were diagnosed with lobular carcinoma and invasive ductal carcinoma, NST on the initial biopsy. The apocrine differentiation was observed in one patient following chemotherapy, who was diagnosed with ductal carcinoma, NST on the biopsy. Almost half of the cohort (47%) (45/96) had a high histological grade (grade 3)

Table 4 below displays patient demographic and clinic-pathological information on inflammatory breast cancer (IBC).

Table 4. Patient demographic and clinic-pathological information in inflammatory breast cancer

Characteristics		**No. of patients**
Total patients' number		96
Treated at QEHB		25
Treated at Leeds		23
Treated at City hospital		18
Treated at Japan		28
Tumour grade	1	6
	2	42
	3	45
	Unknown	3
Oestrogen receptor pre-treatment	Positive	45
	Negative	51
HER2 status pre-treatment	Positive	27
	Negative	67
	Unknown	2
Additional data for treated patients at QEHB and Leeds	Median age at diagnosis	61
	Ethnicity	45
	Caucasian	1
	Asian	1
	Black	0
	Mixed	3
	Unknown	
Histology	Ductal	33
	Lobular	7
	Mixed ductal /lobular	2
	Micropapillary	3
	Mucinous	1
	Metaplastic	1
	Unknown	2
Type of surgery	WLE	1
	Mastectomy	46
	Unknown/ no surgery	2
Axillary nodal status	Positive	25
	Negative	22
	Unknown	2

Neoadjuvant chemotherapy and patient outcome:

Only in the Leeds and QEHB database, the type of chemotherapy and the number of cycles were able to be obtained. It was recorded that in group-1 (LABC), anthracycline-based chemotherapy regimens were utilized in over half of the patients, which is different from group 2, where over half of the patients (59%) underwent a combination of anthracycline and taxane-based chemotherapy with a median number of cycles being 6. Less than 50% of the patients (44.46%) responded to NACT either in the form of complete or partial response (PR). pCR was observed in 11.45 % of patients, and pPR was observed in 34.4% of patients. Nevertheless, 42.7% of patients had no response (pNR) following NACT.

When comparing combined anthracycline and taxane-based chemotherapy with anthracycline-based chemotherapy regimens, there was no notable difference was observed.

The median overall survival period was 28.5 months (ranging from 3 to 138 months) for the entire cohort. From the QEHB and Leeds database, locoregional recurrence (LRR) and/or distant metastasis (DM) appeared in 12 out of 49 patients (24.5%), and death occurred in 27 patients (55%). **Table 5** displays the regimen of NACT, the number of cycles, and disease-free survival for QEHB and Leeds patients, as well as the Overall Survival, response to chemotherapy, and the number of deceased for the whole cohort.

Table 6 below presents CD3, CD68, and CD151 expression in inflammatory breast cancer.

Table 5. Regimen of NACT, number of cycles and disease-free survival for QEHB and Leeds patients. Overall survival, response to chemotherapy and number of deceased for inflammatory breast cancer

Characteristics		**No. of patients**
Response to chemotherapy for whole cohort (96 cases)	pCR	11
	pPR	33
	pNR	41
	Unknown	11
Regimen of chemotherapy for Leeds and QEHB patients (49 cases)	Anthracycline-based chemotherapy	21
	Anthracycline and taxane-based chemotherapy	27
	Unknown	1
Number of Cycles for Leeds and QEHB patients (49 cases)	8-10 cycles	17
	5-7 cycles	26
	2-4 cycles	0
	Unknown	7
Overall Survival, months	Mean	37.7
	Range	3-138
Number of alive (%)	No.	44/96
	%	45.83%
Number of deceased (%)	No.	52/96
	%	54.16%

Table 6. CD3, CD68 and CD151 expression in inflammatory breast cancer

Stained for CD151	No.	83/96
	%	86.5%
Stained for CD3	No.	91/96
	%	95%
Stained for CD68	No.	86/96
	%	89.5%
CD3 score	1	38/91
	2	46/91
	3	7/91
CD68 score	Median or less	44/86
	More than median	42/86
CD151score	Positive	59/83
	Negative	24/83

CD151 Score 0 and score +1= negative
CD151 Score +2 and +3 = positive

7.3.1 CD151, CD3 and CD68 expression association with survival and response to NACT

The correlation between the overall survival and the expression of the markers is displayed in the Kaplan –Meier curves in **Figs. 7 A, B, and C**. In the entire cohort, not statistically, a significant value is seen between the survival and the expressions of three markers. On the other hand, in the QE and Leeds cohort, there is some possible correlation recorded between survival and expression of CD3. There was a relationship between the increase in survival rate and increased expression of CD3, as seen in **Fig. 8** with a p-value=0.08. Furthermore, there was no notable correlation between disease-free survival and expression of three markers in the QE

and Leeds cohort. Nevertheless, there was a correlation between overall survival and response to neoadjuvant chemotherapy (p=0.049). The complete pathological response was seen to be correlated with increased overall survival, as illustrated in **Fig. 9**. There was a correlation between pathological complete response and overexpression of CD151 and CD68 with a significant value of p=<0.001 and p=0.04. The complete pathological response was directly associated with the overexpression of CD151 and increased expression of CD68.

A trend was recorded between a complete pathological response and increased expression of CD3 (p = 0.08) by applying an old scoring system.

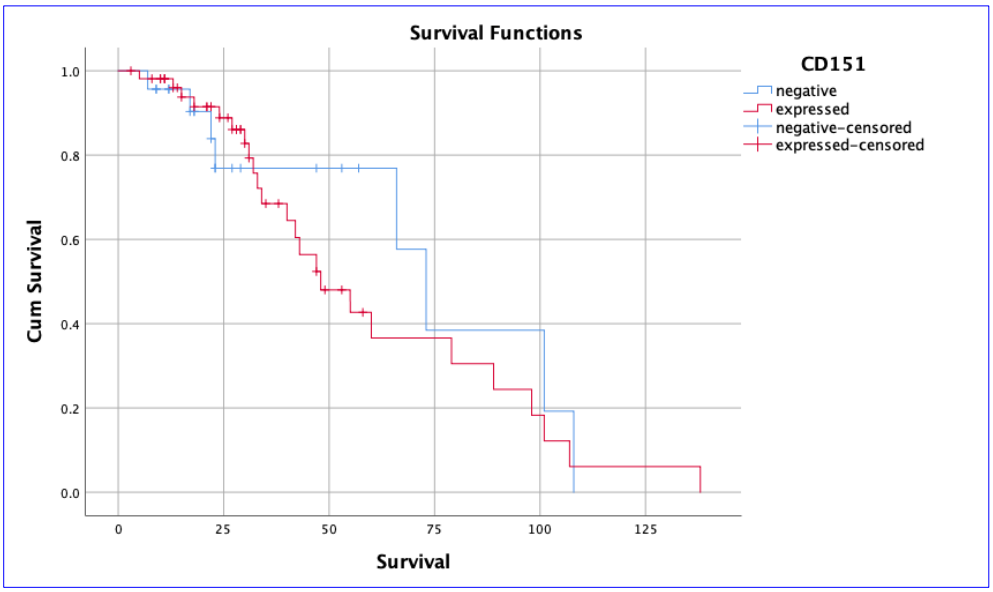

Fig. 7A. CD151 expression effect on overall survival in inflammatory breast cancer

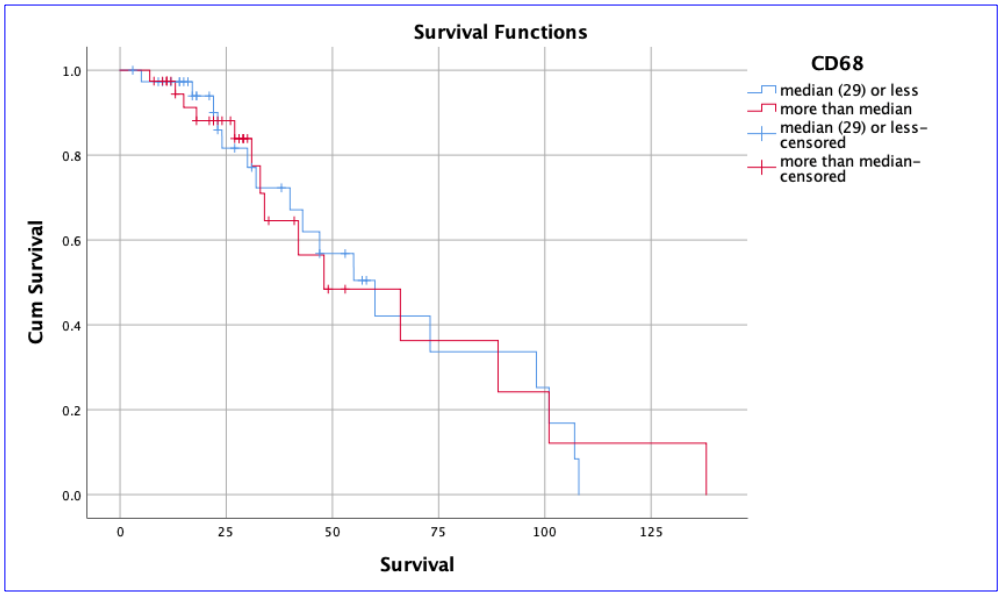

Fig. 7B. CD68 expression effect on overall survival in inflammatory breast cancer

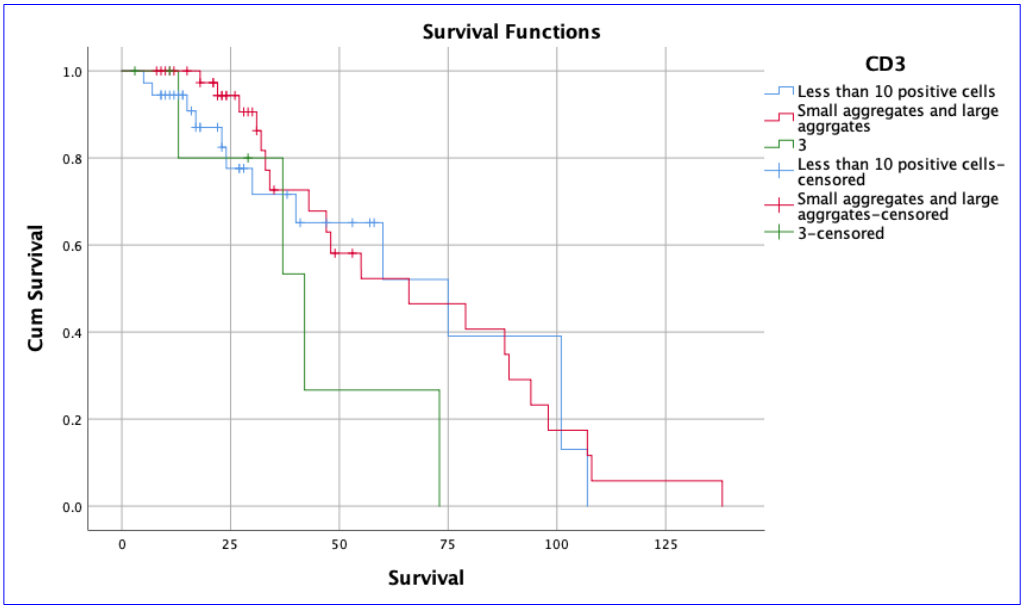

Fig. 7C. CD3 expression effect on overall survival in inflammatory breast cancer

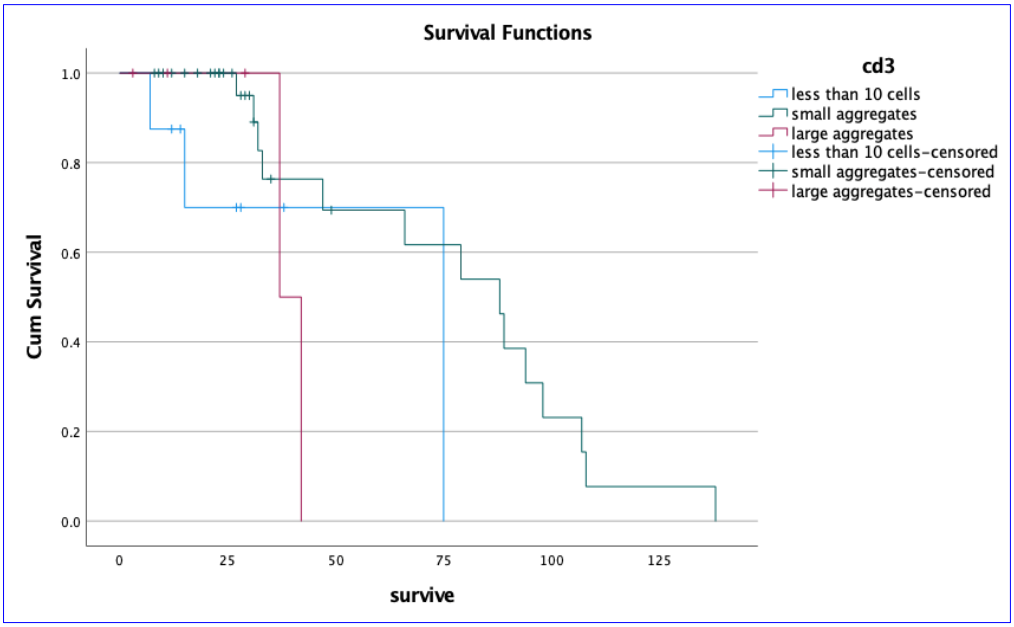

Fig. 8. CD3 expression effect on overall survival in inflammatory breast cancer of Leeds and QE cohort

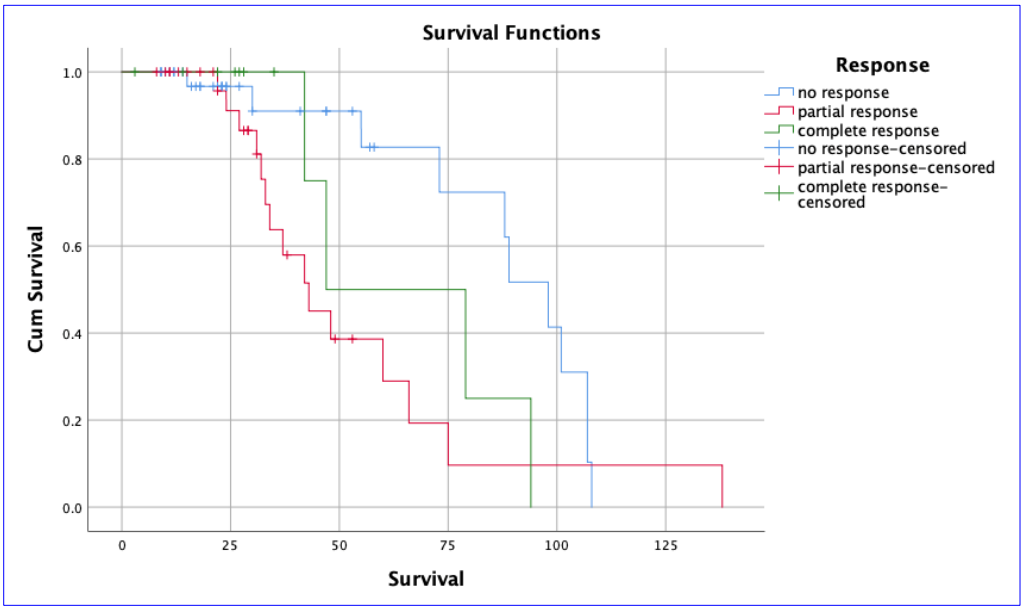

Fig. 9. Kaplan-Meier survival curves for response to NACT on disease-free survival in inflammatory breast cancer

7.3.2 CD151, CD68 and CD3 expression association with other factors

In the entire cohort, the expression of those markers was also examined against other factors. There was no statistically significant correlation between the expression of CD151, CD68, and CD3 and histological grade, ER hormonal expression, and HER2 expression.

In the QE and Leeds cohort, there was an association with a significant value between over-expression of CD3 and CD151 and increased patient age with significant values of p=0.01 and p=0.03. Patients aged 50 years and above exhibited high expression of CD3 and positive expression of CD151 compared to patients younger than 50 years. No statistically significant association was recorded between the expression of CD68 and the patient's age. There was also no statistically notable relationship between expression of the three markers and tumour type, grade and ER, PR, and HER2 expression.

7.3.3 Other factors associated with response to chemotherapy

Our result displayed a significant value between response to neoadjuvant chemotherapy and axillary node burden (p=0.01). Pathological complete response was inversely correlated to greater axillary node burden. The current study presented a significant association between nodal metastasis and HER2 over-expression (p=0.025). Greater axillary nodal metastasis was inversely correlated to HER2-positive breast cancer. There was no statistically significant correlation between response to chemotherapy and histological grade, ER, and HER2 expression in the entire cohort. The same result was also recorded in QE and Leeds cohort as there was no statistically notable association between response to chemotherapy and other factors, such as tumour type, grade, ER, PR, and Her2 expression.

8. Discussion:

Locally advanced breast cancer incorporates stage 2, stage 3, and inflammatory breast cancer patients. Patients often present with a high-stage disease, with at least stage 2. The risk of reoccurrence and death is remarkably high in patients who do not respond to NACT, which is deemed a standard treatment to treat LABC patients, aiming to improve disease-free survival and overall survival. In this study, we tested the expression of three molecular markers (CD151, CD68, and CD3) in locally advanced breast cancer (group 1) and inflammatory breast cancer cases (group 2) who have been treated with neoadjuvant chemotherapy, followed by surgery. Nevertheless, those markers' expression has been studied in breast cancer previously but has not been researched in locally advanced breast cancer cases treated with neoadjuvant chemotherapy. In the present study, 59 years old was the median age in locally advanced breast cancer patients, which is younger than the previous study by **Balogunet,** *et al.* as the median age was 61-years **(Balogun** *et al.,* **2015)**. The median age in inflammatory breast cancer patients in the QE and Leeds cohort was 61 years old, which is slightly more than the previous study by **Andic** *et al.* as 57 years was the median age **(Andic, 2010)**. The most common ethnicity in both locally advanced breast cancer and inflammatory breast cancer was Caucasian, 65% and 90%. This finding contradicted the studies by **B. Yalcin** *et al.* **(2013)** and **Hance** *et al.* **(2005)**, as both studies showed that black women had a higher risk of being diagnosed with LABC and IBC compared to Caucasian women.

8.1 Comparison between CD3 and CD68 expressions association with survival and response to NACT in LABC and IBC

The survival analysis showed no statistical significance of CD3+ TIL and CD68+ TAM expression with overall survival in both locally advanced breast cancer and the whole cohort of inflammatory breast cancer.

Nevertheless, in the QE and Leeds cohort, some potential association was noted between overall survival and expression of CD3+ TIL (p=0.08). There was an association between the increase in survival rate and high expression of CD3+ TIL.

This finding is in line with the previous study by **Zhu** *et al.* **(2017)** who stated that increasing inter-tumoral CD3 infiltration was related to increased overall survival (OS) (p =0.008).

In locally advanced breast cancer, there was an association with a statistically significant value between increased disease-free survival (DFS) and high expression of CD3+TIL by using a new scoring system (p=0.02) and a less significant value by applying the old scoring system (p=.006). This finding is compatible with previous studies, including **Ankita Singh** *et al.* **(2013), Sinicrope FA** *et al.* **(2009) and Ancuta E** *et al.* **(2009)**, which demonstrated that a higher percentage of CD3+ TIL is associated with longer disease-free survival in breast, colon, and cervical cancer. A trend was noted between the increase in disease-free survival and high expression of CD68+ TAM (p= 0.09), which contradicted the previous study **(Catharina Medrek** *et al.*, **2012)** that showed a correlation between high expression of CD68+TAM and reduced survival. However, in inflammatory breast cancer, there was no significant association between disease-free survival and expression of those two markers.

In locally advanced breast cancer, our study showed an association with a statically significant value between a complete pathological response and a high percentage of CD3+TIL expression (p= 0.035), and in inflammatory breast cancer, there was some potential association noted between a complete pathological response and the increased expression of CD3+ TIL by using an old scoring system p = 0.08).

Our finding is compatible with the previous studies by **David** *et al.* **(2011), Brown** *et al.* **(2014) and Badr** *et al.* **(2019)**, illustrating that high CD3+ TIL predicts pathological complete response (pCR) after neoadjuvant chemotherapy in breast cancer.

In inflammatory breast cancer, our study showed a significant association between high expression of CD68+TAM and complete pathological response (p=0.04). However, the correlation between the expression of CD68 and response to NACT in breast cancer has not been studied thoroughly.

Our finding contradicted a previous study by **Minna Tervahartial** *et al.* **(2017)**, which showed patients with a high number of macrophages in bladder cancer had poorer responses to NACT, and the study by **Larionova** *et al.* **(2019)** stated that a high percentage of CD68+TAM was recorded in triple-negative breast cancer, which proved to be associated with shorter overall survival and disease-free survival. It was noted that TNBC had a high risk of metastasis. In contrast, the same study by Larionova *et al.* recorded that the average percentage of CD68+TAM expression was found to be lower in cases with lymph node (LN) metastases compared to negative lymph nodes without NACT and after NACT in breast cancer patients. On the contrary, it was mentioned in the same study by **Larionova** *et al.* that high levels of CD68+ TAMs were associated with a prolonged survival rate in colon cancer **(Larionova, 2019)**.

8.2 Comparison between Survival and response to NACT in LABC and IBC

In inflammatory breast cancer, our study showed an association with a statistically significant value between overall survival and response to neoadjuvant chemotherapy (p= 0.049). The complete pathological response was found to be linked with increased overall survival.

Our finding was compatible with a previous study by **Tithi Biswas** *et al.* **(2019)**, who reported that women with inflammatory breast cancer enduring pathological complete response had a longer 5-year overall survival rate (OS) in comparison with women who achieved a partial or minimal pathological response. However, in locally advanced breast cancer, there was no association between overall survival and response to neoadjuvant chemotherapy.

8.3 Comparison between CD68 and CD3 expression association with other factors in LABC and IBC

The expression of those two markers was also tested against other factors. In locally advanced breast cancer, our study exhibited an association with a statistically significant value between expression of CD68+TAM and tumour grade (p = 0.01). The higher grade was associated with increased expression of CD68+ TAM. This finding is compatible with a previous study by **Campbell** *et al.* which showed that the presence of intertumoral proliferating macrophages was significantly correlated with high grade **(Campbell, 2010)**. Furthermore, in locally advancer breast cancer, our study displayed an association with a significant value between HER2 positive tumour and increased percentage of CD3+ TIL (p-0.05), which was compatible with a previous study by **Marinos Tsiatas** *et al.* **(2018)**, who reported that HER2 - positive tumour had higher CD3. Additionally, inflammatory breast cancer at the QE and Leeds cohort showed a significant correlation between a high percentage of CD3+ TI and

increases patient age with a significant value of p=0.01. Patients above the age of 50 years displayed a high expression of CD3 compared to patients below the age of 50 years.

This is the first study where a significant correlation was found between patient age and high expression of CD3. A previous study by **Marinos Tsiatas** *et al.* assessed the predictive value of tumour infiltrating lymphocytes expression, including CD3, CD8, and FOXP3 mRNA with early-stage breast cancer patients who received anthracycline-based adjuvant chemotherapy **(Tsiatas, 2018)**. Postmenopausal women proved to have higher mRNA expression of FOXP3 ($p<0.001$); nevertheless, no significant relationship was found between women's age and expression of CD3. In both locally advanced breast cancer and inflammatory breast cancer, there was no statistically significant association between the expression of CD68+ TAM and CD3+ TIL and ethnicity, histological type, ER, and PR hormone.

8.4 CD151 expression associated with response to NACT and other factors in IBC

Our study showed a significant correlation between a pathological complete response and over-expression of CD151 with a significant value of p= <0.001. However, previous studies by **Kwon** *et al.* **(2012) and Yang XH** *et al.* **(2010)** revealed that CD151 overexpression was an independent negative predictor of overall survival (OS) in patients with breast cancer, and there was a significant correlation between overexpression of CD151 and tumour high grade in invasive ductal carcinoma. On the contrary, a study by **Voss** *et al.* **(2011)** showed that a high level of CD151 expression in endometrial carcinoma positively correlated with improved survival, unlike other epithelial cancers where CD151 has a suppressive role.

Additionally, inflammatory breast cancer patients in QE and Leeds cohort displayed a significant correlation between the overexpression of CD151 and increased patient age with a significant value of p=0.03. Patients above the age of 50 years showed a higher expression of CD151 compared to patients below the age of 50 years. This is the first study that discovered a significant relationship between patient age and the positive expression of CD151.

No statistically significant association was noted between the expression of CD151 and ethnicity, tumour subtypes, histological grade, ER, PR, and HER2 expression.

8.5 Other factors associated with response to NACT in LABC and IBC

Our study showed other markers linked with the response to neoadjuvant chemotherapy. In locally advanced breast cancer, our study demonstrated that the histological type was significantly associated with the pathological response to neoadjuvant chemotherapy, whereas invasive ductal carcinoma (NST) showed a better response to treatment compared to lobular carcinoma (p=0.01). Patients with invasive ductal carcinoma achieved 27.8% (10 out 36) of pCR, whereas patients with invasive lobular carcinoma showed either partial response or no response to the treatment. No complete pathological response was achieved in invasive lobular carcinoma. This finding was compatible with a previous study by **Alvarado-Cabrero** *et al.* **(2009)**, who reported that patients with invasive ductal carcinoma achieved 75% of pCR, whereas a patient with invasive lobular carcinoma obtained 25% of pCR. In addition, pure micro-papillary cancer showed a complete pathological response which contradicted the study by **Alvarado-Cabrero** *et al.* **(2009)**. In their study, there was a poor response to neoadjuvant chemotherapy in pure micro-papillary cancer compared to other types. Our study showed an association with a significant value between complete pathological response to neoadjuvant

chemotherapy and the HER2 subtype of locally advanced breast cancer (p=0.02). This finding was compatible with previous studies including **Kim** *et al.* **(2010), Choi** *et al.* **(2014) and Sevcikova** *et al.* **(2013)** which demonstrated that HER2-positive patients reached a pathological complete response (pCR) to neoadjuvant chemotherapy (NACT), even in the absence of HER2-targeted therapy. The aim of utilising targeted therapy is to improve the chemosensitivity of HER2-positive breast cancer and increase the pCR rate. Clinical trial results from the last few years suggest that newer HER2-targeted agents could allow more effective inhibition of HER2 and increase benefits for the NACT regimen given with HER2-targeted therapy. However, in inflammatory breast cancer, no statistically significant association was noted between response to chemotherapy and tumour type and HER2 expression.

Both locally advanced breast cancer and inflammatory breast cancer showed no correlation between patient age and response to neoadjuvant chemotherapy, which was in line with previous studies conducted by **Alvarado-Cabrero** *et al.* **(2009) and Fisher** *et al.* **(1997)**, who revealed no significant difference in the distribution of complete and incomplete pathologic response between younger (<50 years) and older (>50 years) patients as patient age failed to predict the clinical response of breast cancer. On the contrary, the study by **Jacquillat** *et al.* **(….)**, demonstrated tumour response to be related to patient age, as, in their study, a complete response was achieved in 18% of patients younger than 50 years compared to 37% of those who were older than 50 years. On the contrary, the German Gepar Trio study **(Huober, 2010)** displayed that the highest pCR rate noted was in patients below 40 years of age with triple-negative or grade 3 tumours. There was no statistically significant association between the response to chemotherapy and ethnicity, tumour grade, ER, and PR hormonal expression.

8.6 Type of NACT in LABC and IBC

In locally advanced breast cancer, over half of the patients (53.5%) were treated by only anthracycline-based chemotherapy, and 39.5% of patients received a combination of anthracycline and taxane-based chemotherapy, with a median number of cycles being six cycles. Nevertheless, in inflammatory breast cancer patients, more than half of the patients (59%) obtained a combination of anthracycline and taxane-based chemotherapy. Locally advanced breast cancer patients demonstrated a higher response to neoadjuvant chemotherapy compared with inflammatory breast cancer patients. In inflammatory breast cancer patients, 44.46% responded to NACT, and only 11.45% showed pCR, whereas 79% of locally advanced breast cancer patients responded to NACT, and pCR was achieved in 25.5%. Our study revealed no significant difference in response when anthracycline and taxane-based chemotherapy were compared. On the contrary, in one of the largest randomised controlled trials, the National Surgical Breast and Bowel (NSABP B-27) **(Mamounas, 1997)**, compared a neoadjuvant anthracycline regimen with an anthracycline–taxane combination. This study showed that the addition of preoperative docetaxel was associated with a higher pCR in breast rate (26% vs. 14%; $p<0.001$) in comparison with the group of patients who received only anthracycline-based chemotherapy prior to surgery. However, the addition of docetaxel to anthracycline had no significant impact on Disease-free survival (DFS) or overall survival (OS).

8.7 Alteration in Preoperative Tumour Marker status after NACT in LABC

It has been reported in Piper *et al.* study those patients treated with neoadjuvant chemotherapy may have changes in the receptor and marker status after neoadjuvant chemotherapy **(Piper, 2004)**. In our study, pre-and post-treatment, the ER/PR and HER2 status were only measured in locally

advanced breast cancer patients. Due to incomplete provided information, including the hormonal status in inflammatory breast cancer patients, this area could not be assessed.

In locally advanced breast cancer patients, 11 out of 43 patients underwent complete pathological response (pCR). The receptors' expression in the remaining 32 cases was reviewed. A change in receptor expression was noted in 11 out of the 32 patients. Among the 11 patients, four patients experienced a change in receptor status. Two patients went from ER/PR negative to ER/PR positive, making them new candidates for hormonal therapy, and two patients went from HER2 positive to HER2 negative, where the role of Herceptin was altered. These findings are comparable with previous studies by **Piper** *et al.* **(2004)** and by **Chen** *et al.* **(2015)**, who showed a significant change in receptor expression as well as a change in receptor/marker status after neoadjuvant chemotherapy; this immensely modified potential adjuvant therapy. Surprisingly, in our study, it was recorded that the change in hormonal receptor status was associated with a poor response to neoadjuvant chemotherapy and disease progression. Three out of four patients showed no response to chemotherapy, and one patient showed partial response to chemotherapy. In all patients, disease-free survival (DFS) and overall survival (OS) was below five years. This finding is in line with the study by Yang *et al.,* who reported that group patients with any receptor change after neoadjuvant chemotherapy had worse disease-free survival (DFS) compared to patients in the constant receptor group **(Yang, 2018)**.

8.8 Surgery post-NACT in LABC and IBC

Breast-conserving therapy (BCS) has usually been confined to low-stage breast cancer like T1 and T2 tumours because it was almost impossible to obtain a good local control in patients with advanced breast

cancer (T3 or T4). Previous studies by **Caudle** *et al.* **(2014) and Costa** *et al.* **(2009)** exhibited that NACT could be a very useful and effective means for tumour down-staging, especially in locally advanced diseases that would allow patients who have a significant response to NACT to be treated with Breast-conserving therapy (BCS).

Our result showed that 32.5% of patients with locally advanced breast cancer underwent a wide local excision. However, 94% of patients with inflammatory breast cancer from the QE and Leeds cohort had mastectomy following NACT. The study by **Costa** *et al.* **(2009)** showed that breast-conserving surgery was performed in 33% of patients with LABC and 12.9% of patients with IBC after treatment with NACT.

Historically, axillary lymph node clearance was the standard of treatment in advanced breast cancer to provide local control and proper staging information. Sentinel lymph node biopsy is being used as a minimally invasive procedure in clinically and imaging node-negative breast cancer patients. Additionally, sentinel lymph node biopsy is associated with a lower morbidity rate compared to axillary lymph node clearance **(Ahmed, 2016)**. Axillary lymph node status endures an essential prognostic factor for breast cancer patients influencing a management plan after NACT.

For axillary management, there is no published evidence that advises patients with locally advanced diseases that are treated with neoadjuvant chemotherapy should have their axilla staged or treated separately from any other patients **(Rea, 2015)** with invasive breast cancer who obtained neoadjuvant chemotherapy. The lymph node stage remains an independent prognostic factor after NACT. The study by **Pinder** *et al.* **(2015)** reported that patients with a greater nodal burden disease have a poorer outcome than those with less disease in the axilla. Also, NSABP B-18 and B-27

trials **(Fisher, 1997; Bear, 2003)** were conducted in less heavily node-positive populations. There was a correlation between the response of primary breast carcinoma and nodal disease **(Arimappamagen, 2004; Matso, 2002)**.

In our study, 43 inflammatory breast cancer patients at the QE and Leeds cohort and 38 locally advanced breast cancer patients underwent axillary clearance (88%); 3 patients with IBC and 5 patients with LABC had sentinel lymph node biopsy, and 3 patients of IBC had palliative mastectomy only. More than half of the inflammatory breast cancer and locally advanced breast cancer patients showed metastatic lymph nodes after neoadjuvant chemotherapy.

This finding is comparable with the previous study by **Cox** *et al.* **(2013)**, who reported that 68% of patients with LABC that underwent NACT had evidence of lymph node metastases as the effectiveness of NACT appeared to be less when eradicating the disease in the lymph nodes compared to in the primary tumour. The National Surgical Adjuvant Breast and Bowel Project study B-27 demonstrated that 15.5% of patients with a pathologic complete response (pCR) in the breast still had cancer in the axillary lymph nodes. **Kuerer** *et al.* **(1999)** also stated that 41% (78 of 191) of patients with a pCR in the breast still had positive axillary nodes following NACT. Our study showed a significant association between the response to NACT and a greater nodal burden disease in locally advanced breast cancer and inflammatory breast cancer ($p=<0.001$ and $p=0.01$). The finding can be compared with the previous studies by **Pinder** *et al.* **and Cox** *et al.* **(2013)** as a complete pathological response was inversely related to greater axillary node burden. Additionally, our study showed a significant association between nodal status and HER2 expression in locally advanced breast cancer and inflammatory breast cancer ($p=0.035$

and p=0.025). Greater nodal metastasis was inversely associated with HER2-positive breast cancer. On the contrary, previous studies by **Ahmed *et al.* (2016) and Domagoj Kustic (2019)** reported a significant correlation between HER2 over-expression and a marked increase in the metastatic potential of breast cancer which could be an indicator of both regional and local metastatic disease.

Also, this finding can be compared with a previous study by Del *et al.* **(Prete, 2019)**.

8.9 Summary

In locally advanced breast cancer, there was an association with a statistically significant value between increased disease-free survival, complete pathological response, HER2 positive subtype, and a high percentage of CD3 expression. In addition, there was an association with a significant value between complete pathological response to neoadjuvant chemotherapy and HER2 subtype tumour. Some potential association was noted between an increase in overall survival, complete pathological response, and increased expression of CD3 in inflammatory breast cancer.

In locally advanced breast cancer, although a higher percentage of CD68 was significantly associated with a higher grade of cancer, it was noted in inflammatory breast cancer that a higher percentage of CD68 was correlated with a better response to NACT. Also, a trend was noted between the increase in disease-free survival (DFS) and the high expression of CD68 in locally advanced breast cancer.

In locally advanced breast cancer, there was a significant correlation between the histological type and pathological response to neoadjuvant chemotherapy as invasive ductal carcinoma (NST and pure micropapillary carcinoma) showed a better response to treatment compared with lobular carcinoma.

In locally advanced breast cancer, a change in receptors expression and the receptors /markers status was noted after neoadjuvant chemotherapy, whereby the eligibility of hormonal therapy and the role of Herceptin was amended. In inflammatory breast cancer, there was an association with a statistically significant value between increased overall survival, overexpression of CD151, and complete pathological response to neoadjuvant chemotherapy.

In Inflammatory breast cancer, a significant association was noted between patients above the age of 50 years and high expression of CD3 and CD151.

A greater nodal burden was significantly associated with poor response to NACT and greater nodal metastasis, which was inversely correlated with HER2 positive subtype tumour in both locally advanced breast cancer and inflammatory breast cancer.

9. Future Direction

The size of the cohort in locally advanced breast cancer and inflammatory breast cancer is highly inconstant. Therefore, the multicentre approach is crucial in order to increase the number of cases.

A limited number of studies explored the role of tumour-associated macrophages (TAM) in breast cancer after undergoing NACT. Additional studies are demanded to fully understand their prognostic role in response to chemotherapy.

Additional studies are required to assess the relationship between the molecular phenotype of breast cancer, tumour subtypes, and localisation of tumour infiltrating lymphocytes (TIL), in order to clarify their prognostic and predictive value in response to chemotherapy.

Tumour microarrays (TMAs) of the resection specimens will be conducted to evaluate if the treatment has an impact on the level of expression of CD151, CD3, and CD68 markers.

Currently, a further study on TSPAN6 is being carried out in our laboratory to identify the prognostic and predictive value of TSPAN6 in locally advanced breast cancer and inflammatory breast cancer.

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