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Exploring the Effect of Peri-Operative Inflammation on Systemic and Local Breast Cancer Biomarkers

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For the Degree of **Doctor of Medicine (MD)**

University College Cork
School of Medicine and Health

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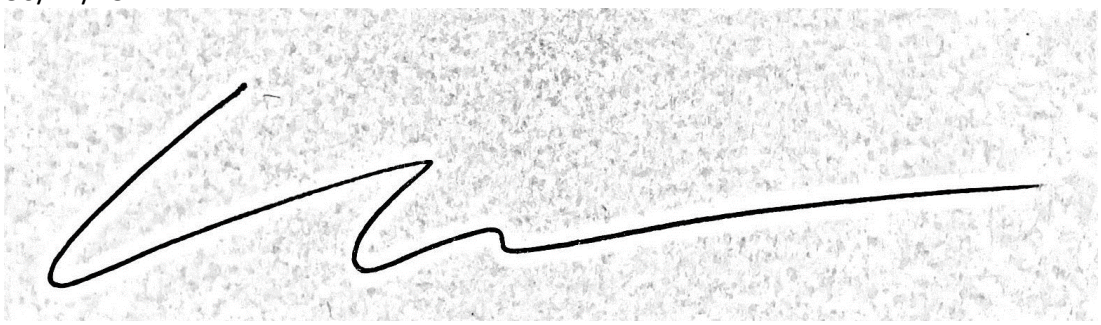
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Thesis Declaration

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Signed: Carolyn Cullinane
30/12/23



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Thesis Abstract

Background

Despite curative surgery and optimal treatment, distant metastatic disease can occur in up to 25% of patients with breast cancer. Pre-clinical research has suggested that the surgical insult can promote the growth of new metastases and facilitate seeding of micrometastases into the circulation. The exact mechanism underlying the pro-metastatic potential of the peri-operative period is yet to be fully determined. This thesis aimed to explore the effect of peri-operative inflammation on local and systemic biomarker expression.

Hypothesis

The peri-operative period can alter local and systemic mediators of breast cancer inflammation and influence circulating tumour DNA (ctDNA)release which may predict clinical and oncological outcomes.

Aims

1. To understand the relationship between peri-operative systemic inflammation and breast tumour characteristics, surgical intervention and 30-day morbidity in non-metastatic breast cancer
2. To define the perioperative dynamics of cell free DNA (cfDNA) in breast cancer and explore its prognostic potential.
3. To determine the perioperative dynamics of circulating tumour DNA (ctDNA) in breast cancer and define its prognostic potential.

Methods

This study is a sub-study of the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity study, and sample collection is within the remit of the biospecimen protocol (NCT01840293). Patients presenting for elective breast cancer surgery, with non-metastatic disease, aged 18-85 years of age were screened for inclusion prospectively. Blood samples were phlebotomised peri- operatively from patients at the following time-points– pre-operatively the morning of surgery (pre-op), 3-5 hours post-operatively (early post-op), 10-14 days post-operatively (late post-op). Tumour and normal adjacent tumour was collected within 20minutes of surgical resection and cut up into at least 3 pieces (minimum size 2-3mm^2), and snap frozen in liquid nitrogen separate cryovials. Statistical analysis was performed using IBM SPSS software, version 28 and GraphPad PRISM, version 8.4.2. Graphs were generated using GraphPad PRISM, version 8.4.2. For demographic and clinicopathological data, categorical data were described by their counts and percentages in each. Descriptive analysis was used to depict baseline characteristics. Mann–Whitney U test and Fisher's test were used to calculate *p* value in group-comparison analysis. An analysis of variance (ANOVA) was used to compare mean cytokine values peri-operatively followed by multiple comparison analysis using Bonferroni. Student T test or Mann Whitney tests were used to determine differences in peri-operative ctDNA and cfDNA detection. Chi-squared or Fisher's exact test (ranksum) were carried out to assess associations between ctDNA/cfDNA detection and clinical variables. Binary logistic regression analysis was performed to predict the likelihood of ctDNA positivity based on dichotomous clinical outcomes. Correlation between continuous variables was analysed using Pearson correlation. Correlations were graphically represented using Manhattan plots. The association between cell-free DNA (cfDNA) and DFS were analysed using Mantel Cox log-rank test and Kaplan–Meier curves generated. For all of the above analyses, statistical significance was observed at $p < 0.5$.

Results

Blood samples were collected from 64 patient's peri-operatively. There were 38 patients who had their systemic inflammatory mediators analysed peri-operatively. IL-6, IL-8 and IL-10 levels peaked early post-operatively and returned to below baseline levels at 2 weeks post-operatively ($p < 0.05$). Early post-op IL-6 levels were lower in patients who had breast conserving surgery compared to patients who had a mastectomy ($p = <0.01$). A significant positive correlation was identified between age and peri-operative IL-6 levels at all time points. Early post-op IL-6 correlated with increasing tumour stage ($p = 0.048$). When *IL6* expression and other *IL6* related genes were analysed in normal adjacent to tumour (NAT) and tumour tissue samples, *IL6*, Leptin, JAK1, CD38, FABP4 and Adiponectin gene expression were significantly higher in NAT compared to matched tumour samples.

Pre-operatively the mean cfDNA concentration was 3.82ng/µL (SEM = 0.74). This increased significantly early post-op in keeping with surgical insult to 17.59 ng/µL (SEM = 5.75). A significant linear relationship was observed between early post-op cfDNA concentration and early post-op IL-8 levels ($r = 0.430$, $p = 0.009$). Forty-five pre- and post-operative patient samples were suitable for ctDNA analysis. The number of pre-operative ctDNA variants detected correlated positively with pre-operative cfDNA concentration ($r = 0.314$,$p = 0.035$). Two patients experienced disease recurrence within the follow up period (median 22 months) and ctDNA was detectable in both their pre- and post-op samples. Kaplan Meier estimates of disease-free survival stratified by the presence of ctDNA did not illustrate any significant difference in DFS. Patients who underwent Axillary Lymph Node Dissection (ALND) had a significant increase in post-op ctDNA variant detection compared to those who underwent SLNB (OR 21.71, $p = 0.0063$).

Conclusion

Peri-operative cytokine release appears to correlate with tumour characteristics and the magnitude of surgery. Results from this study demonstrate that cfDNA and ctDNA can be detected peri-operatively using a commercially available ctDNA assay. An

increase in post-operative ctDNA burden was evident in patients who underwent more extensive axillary surgery. Longer term follow up is required to determine the prognostic potential of peri-operative inflammatory and ctDNA variant dynamics and if modulation of these parameters might improve patient outcomes.

Chapter 1: Thesis Overview

1.1 Thesis Overview

Despite excellent advances in breast cancer prevention, worldwide, breast cancer accounts for about 30% of female cancers, and has a mortality-to-incidence ratio of 15% [1]. Breast surgery is the cornerstone of breast cancer treatment and the type of surgery depends on tumour size, nodal positivity, and genetic predisposition[2]. Despite curative surgery and optimal treatment, distant metastatic disease can occur in up to 25% of patients with breast cancer [3]. Pre-clinical research has suggested that the surgical insult can promote the growth of new metastases and facilitate seeding of micrometastases into the circulation [4, 5]. The exact mechanism underlying the pro-metastatic potential of the peri-operative period is yet to be fully determined but the effect seems to be exacerbated by stress inflammatory responses to surgery [6]. The interactions between the inflammatory cascade, mutational cancer burden and endocrine response during surgery are yet to be defined. A greater understanding of the complexities underpinning the peri-operative period would provide opportunities for prognostic biomarker development and identification of therapeutic targets to counteract their deleterious effect [7, 8]. The integration of germline mutation testing is well established in clinical practice in Ireland however the application of massively parallel or next-generation sequencing (NGS) to define the mutational landscape of breast cancer is vastly unexplored. Sporadic breast cancers account for 90-95% of cases and are characterised by a dynamically evolving process with a versatile somatic mutational profile, of which some genetic aberrations will be crucial for cancer progression [9]. This thesis explored the peri-operative dynamics of inflammatory cytokines, cell-free DNA and circulating tumour DNA in relation to breast cancer characteristics and oncological outcomes.

1.2 Aims and Objectives

Aim 1: To understand the relationship between peri-operative systemic inflammation and breast tumour characteristics, surgical intervention and 30-day morbidity in non-metastatic breast cancer.

Objective 1: Define the peri-operative trend of systemic mediators of inflammation (White Cell Count, Neutrophil/Lymphocyte Ratio, IL-6, IL-8, IL-10, TNF-α and IL-1 β) in breast cancer patients and association with 3-week morbidity.

Objective 2: Investigate the relationship between systemic inflammatory mediators and tumour characteristics.

Objective 3: Analyse the relationship between inflammatory mediator expression and specific surgical interventions (breast conserving surgery versus mastectomy).

Aim 2: To define the peri-operative dynamics of cell free DNA (cfDNA) in breast cancer and explore its prognostic potential.

Objective 1: Define the peri-operative dynamics of cfDNA in breast cancer.

Objective 2: Analyse the relationship between peri-operative cfDNA levels and pro-oncogenic inflammatory mediators, in particular IL-6, TNF-α and IL-8, and predominantly 'protective' cytokines IL-10.

Objective 3: Investigate the association between peri-operative cfDNA and prognostic markers in breast cancer (size, grade, lymph node positivity and presence of lymphovascular invasion) and subsequent cancer outcomes (early recurrence within two years).

Aim 3: To determine the clinical utility of ctDNA to predict disease free survival in breast cancer using published studies.

Objective 1: To perform a systematic review and meta-analysis to;

- identify the clinical utility of ctDNA in the metastatic versus early disease population.
- to determine whether pre-treatment ctDNA sampling or sampling during treatment (surgery or chemotherapy) could predict oncological outcomes.
- To analyse ctDNA post Neoadjuvant chemotherapy as a potential predictive biomarker.

Objective 2: To examine the association between peri-operative ctDNA and oncological outcomes.

Aim 4: To analyse the peri-operative release of ctDNA in patients with non-metastatic breast cancer undergoing curative surgery and examine the utility of post -operative ctDNA in predicting disease free survival (DFS).

Objective 1: To determine the peri-operative dynamics of circulating tumour DNA (ctDNA) in breast cancer and define its prognostic potential.

1.3 Scope

The focus of this thesis was to define peri-operative inflammation and ctDNA dynamics in a cohort of women with non-metastatic breast cancer. The mechanisms by which peri-operative inflammation and ctDNA can impact on clinical and cancer outcomes following breast cancer surgery was also explored. This thesis is composed of nine chapters including an introductory chapter, background chapter, methodology chapter, five results chapters and a concluding chapter to summarise and contextualise its findings.

Chapter 1 introduces the clinical problem of how inflammation in the peri-operative period can impact on outcomes in cancer (recurrence and survival) including evidence to support this concept along with gaps in the current literature. The

concept of breast cancer ctDNA is also explored with an appreciation of the lack of data in an Irish cohort. It also outlines the aims, objectives and overarching scope of this thesis along with a summary of its contribution to the current evidence base.

Chapter 2 summarises all relevant background to the concepts and findings described in this thesis. It provides an overview of current breast cancer epidemiology and changing population trends. It discusses the evidence for peri-operative inflammation and how if up regulated it can negatively impact on cancer outcomes. This is discussed with particular reference to emerging large population-based studies suggesting that more extensive surgical approaches have poorer oncological outcomes. Liquid biopsy in the peri-operative period is discussed and the role of peri-operative cfDNA and ctDNA as a novel method for improving early identification of cancer recurrence. It includes an introduction to somatic cancer mutations and their clinical significance in the management of breast cancer.

Chapter 3 describes the materials and methodologies utilised to perform the scientific and analytical work included in this thesis, sub-grouped as follows: study design, participant recruitment; sample biobanking; inflammatory level measurement; MSD Multiplex ELISA, RNA isolation techniques, RNA quantification, cell free DNA (cfDNA) analysis; ctDNA analysis via Next Generation Sequencing.

Chapter 4 investigates the relationship between peri-operative pro-oncogenic inflammatory cytokines and breast cancer characteristics.

Chapter 5 analyses the immune expression in breast tumour tissue compared to the normal adjacent to tumour tissue environment. Data available from the Cancer Genome Atlas (TCGA) was analysed to generate hypotheses and conceptualise the tumour microenvironment. The ICORG 09-07 Biobank samples (N=10) were then utilised to validate the findings from TCGA.

Chapter 6 presents, the dynamics of breast cancer cell free DNA (cfDNA) in the immediate peri-operative period and the association of cfDNA with clinicopathological features.

Chapter 7 reports a systematic review and meta-analyses of published ctDNA studies outline the predictive potential of ctDNA in breast cancer management.

Chapter 8 defines peri-operative ctDNA expression pre and post curative breast cancer surgery. It also explores the association between ctDNA and breast cancer tumour characteristics and oncological outcomes. Chapter 8 also outlines the presence of somatic mutations in a cohort of Irish women with non-metastatic breast cancer. The association between specific mutations and tumour characteristics was also explored.

Chapter 9: Synthesises the thesis findings and summarises how these themes are intertwined and relevant to clinical breast cancer surgery.

Chapter 2: Introduction

2.1 Breast Cancer Worldwide

Globally, there were 2.3 million women diagnosed with breast cancer and 685 000 breast cancer deaths in 2020. At the end of 2020, there were 7.8 million women alive who were diagnosed with breast cancer in the past 5 years, making it the world's most prevalent cancer [10]. Genetic predisposition or family history is a risk factor in approximately 10% of all cases of breast cancer, with variances by country and ethnicity. The most common germline mutations associated with breast cancer are in the BRCA1 and BRCA2 genes, with an average cumulative lifetime risk of about 70% [11]. National guidelines for genetic testing provide recommendations for genetic testing after personal and family history taking, risk assessment, and genetic counselling[12]. Approximately 50% of breast cancers develop in women with no identifiable risk factors other than gender(female) and age (over 40)[10]. Other risk factors for developing breast cancer include hormonal therapy, lifestyle factors and reproductive factors. Even if all of the potentially modifiable risk factors could be controlled, this would only reduce the risk of developing breast cancer by at most 30% [13].

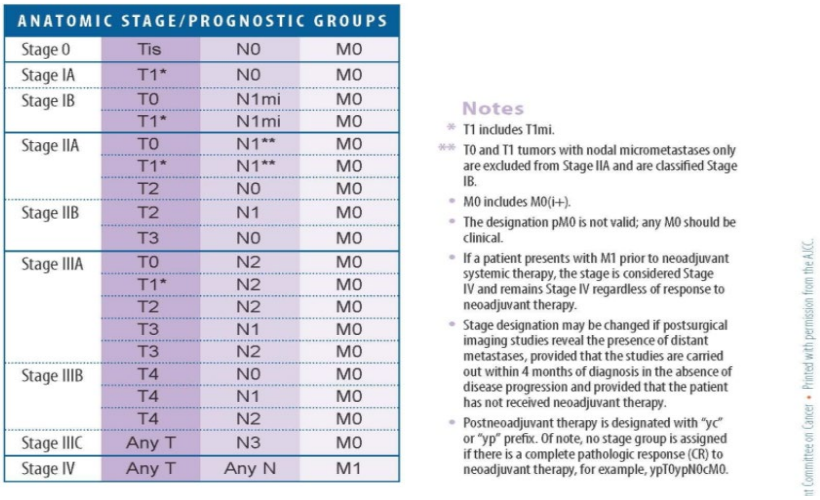
2.2 Breast Cancer in Ireland

In Ireland, an estimated one in nine women will develop breast cancer in their lifetime. Annually, 3,700 cases of breast cancer are diagnosed in Ireland. Of those diagnosed, 23% of women are aged between 23-50 years [14]. The most commonly presenting features include breast lumps, skin changes or distortion, axillary swelling or nipple changes [14]. BreastCheck Ireland offers breast cancer screening via mammography to women aged 50-69 every two years. Globally, eight randomised clinical trials have shown that breast cancer screening mammography may reduce breast cancer mortality by at least 20%. Screening detection can be improved with the use of digital breast tomosynthesis [15]. In 2019, 7 breast cancers per 1,000 women screened were detected [16].

2.3 Breast Cancer: Subtypes & Staging

Breast cancer is a heterogeneous malignancy, and clinically divided into three main subtypes by hormone receptor (ER and PR) and HER2 (ERBB2) status: luminal A/B, HER2-positive; and triple-negative breast cancer (TNBC)[17]. Standardised diagnostic evaluation of hormone receptors (ER and PR) and HER2 based on international guidelines is essential for the determination of these subtypes[18]. In clinical practice, breast and axillary tissue is usually obtained by fine-needle aspiration, core biopsy, or surgical excision. A diagnostic challenge for pathologists is the distinction between closely related diseases, such as atypical ductal hyperplasia and in situ disease, in situ disease and microinvasion, or ductal cancer and lobular cancer. Patients with clinical and/or radiological lymph node negative disease at presentation are eligible to have a sentinel lymph node biopsy. One of the major technical advances in breast surgery was the introduction of sentinel lymph node biopsy (SLNB) to replace the conventional axillary node dissection described by Giuliano et al. in 1994 [19].

Histologically, the most common breast cancer subtype is invasive ductal carcinoma (IDC) followed by invasive lobular carcinoma (ILC)[20]. ILC is characterised by epithelial cadherin (CDH1) mutations and tends to have a discohesive growth pattern[2]. Physical examination, mammography, and ultrasound are usually sufficient for local-regional staging for the diagnostic work-up of a patient with newly diagnosed breast cancer. MRI may provide additional clinical information, especially when a patient is younger, a genetic mutation or multifocal disease is suspected, or a mammogram or ultrasound yields indeterminate findings [21]. The National Comprehensive Cancer Network guidelines recommend staging with CT thorax, abdomen and pelvis for suspected IIIB/C or IV disease. Women with stage I/II/IIIa do not routinely have systemic radiological staging unless there are specific symptoms of metastatic disease [20].



ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Figure 1A. American Joint Committee on Breast Cancer Staging 7th Edition

2.4 Breast Cancer Treatment

The primary means of local and regional breast cancer treatment remains surgical intervention. Critical treatment decisions are made based on hormone expression assays, cancer staging and lymph node involvement. Neoadjuvant therapy has been widely accepted as a standard of care for patients with HER2-positive breast cancer and TNBC, even when the disease is operable[22]. The general concept is to use the same systemic therapy as would be given post-operatively before surgery, followed by surgery and irradiation and further post-neoadjuvant systemic therapy, if required. Patients who achieve a pathological complete response have significantly better disease-free survival and overall survival than patients with residual disease and this has led to studies examining the use of additional systemic therapy in patients without pathological complete response[23]. Women with early hormone receptor positive breast cancer usually undergo up front surgical intervention. Breast conservation surgery (BCS) was pioneered by Fischer et al.[24] and Veronesi et al. [25], who reported that survival with lumpectomy and radiation was equivalent to that with mastectomy in the treatment of early breast cancer. Mastectomy is usually reserved for patients who have had radiation to the affected side (for prior breast cancer or Hodgkin lymphoma) or for women with a relatively small breast in the

setting of a large primary breast cancer, extensive calcifications, patient choice or multicentric disease[20]. Women with germline mutations and increased susceptibility of breast cancer may also opt to have a mastectomy with or without reconstruction. The surgical management of the axilla has deescalated substantially since publication of the large Z0011 randomized controlled clinical trial (American College of Surgeons Oncology Group Z0011 trial). It is now widely accepted that completion axillary lymph node clearance is not required in women who have no palpable adenopathy, 1 or 2 positive sentinel nodes, and no gross extranodal extension [26]. Adjuvant chemotherapy after definitive surgery is generally recommended for patients with disease at high risk of recurrence. The following clinicopathologic characteristics may be indications for chemotherapy: ER-, PR-, and HER2-negative; HER2-positive; larger tumour size; and positive lymph nodes. For pre-menopausal patients with ER-positive tumours and lymph node negative disease (or post-menopausal women with 1-3 nodes positive), RNA-based genomic testing can be used to better estimate the risk of a distant recurrence as well as to identify patients who will benefit most from chemotherapy (e.g Oncotype DX Score)[27]. Breast conserving surgery and radiotherapy is the standard of care for locoregional recurrence prevention[28]. Endocrine therapy for 5–10 years is the usual treatment for women with ER-positive early breast cancer. For postmenopausal women, options include tamoxifen or a steroidal (exemestane) or non-steroidal (letrozole or anastrozole) aromatase inhibitor[2]. Treatment of early breast cancer is complex and involves combination of local modalities [surgery, radiotherapy (RT)], systemic anticancer treatments (Chemotherapy, Endocrine therapy, molecularly targeted therapies) and supportive measures, are delivered in diverse sequences. The use of predictive biomarkers such as ER, PR, HER2 and Ki67 and approved genomic signatures is well established to help in determining the treatment of choice.

2.5 Breast Cancer Follow Up Post Surgery

The aims of follow-up are[29]:

• to detect early local recurrences or contralateral breast cancer;

•to evaluate and treat therapy-related complications (such as menopausal symptoms, osteoporosis and second cancers);

•to motivate patients continuing adjuvant endocrine therapy;

•to provide psychological support and information in order to enable a return to normal life after breast cancer.

Despite the fact that no randomised data exist to support any particular follow-up sequence or protocol, balancing patient needs, follow-up costs and burden is necessary. Every visit should include a thorough history with a focus on symptomology of breast disease, and a physical examination. Apart from routine mammography ± breast US, an MRI of the breast may be indicated for young patients, especially in cases of dense breast tissue and genetic or familial predispositions. US can also be considered in the follow-up of lobular invasive carcinomas. In asymptomatic patients, there are no data to indicate that other laboratory or imaging tests [29].

2.6 Breast Cancer Survival

Locoregional recurrence rates after breast-conserving surgery followed by radiotherapy are approximately 2–3% at 10 years for ER-positive and HER2-positive tumours and 5% for triple negative breast cancer (TNBC), and do not differ significantly from those seen after mastectomy [3, 30]. Post-mastectomy radiotherapy is usually reserved for women with regional lymph node disease. In a meta-analysis by the EBCTCG, which included 8135 women from 22 randomised trials, postmastectomy radiotherapy for patients with involved axillary lymph nodes reduced the 10 year recurrence rate by 10·6%, leading to an 8·1% reduction in breast cancer mortality after 20 years [31]. Endocrine therapy with aromatase inhibitors or tamoxifen for a duration of 5 to 10 years is the standard treatment for women with early-stage ER-positive breast cancers. Several studies have compared different

endocrine therapy regimens and the sequential approach of endocrine treatments amongst pre- and post-menopausal cohorts. The BIG 1-98 trial demonstrated that aromatase inhibitors resulted in a significant absolute risk reduction of recurrence at 10 years of 3·6% and in an increase in overall survival of 2·1% compared with tamoxifen [32]. Aromatase inhibitor therapy has been shown to provide greater benefit in patients with advanced stage (II–III), high-grade, HER2-positive, or highly proliferative disease[32]. Five years of endocrine treatment is advocated for stage I disease. Extended therapy with 10 years endocrine treatment has been investigated in several trials and data suggests that continuation of endocrine therapy reduces the risk of recurrence in patients at high risk (node-positive, high genomic score)[33-35]. Results from the Phase 3 RxPONDER trial (NCT01272037) reported that pre-menopausal women with node positive disease had an improved 5 year invasive disease-free survival and overall survival due to combination endocrine and chemotherapy treatment irrespective of low biological risk by multigene testing [36]. Patients with stage 2 or higher HER2 positive disease receive neoadjuvant trastuzumab plus pertuzumab, in addition to doxorubicin–cyclophosphamide or epirubicin–cyclophosphamide followed by a taxane, or in addition to docetaxel–carboplatin, which is associated with higher toxicity. This regimen has a pathological complete response rate of about 65–70% and has led to an improvement in event-free survival and disease-free survival [37].

2.7 Inflammation & Cancer

The symbiotic relationship between inflammation and cancer is well established [38]. Rudolf Virchow first observed the presence of leukocytes within tumours in the 19th century and provided the first indication of a possible link between inflammation and cancer [38]. Only 5-10% of cancers are caused by germline mutations, the remaining 90% of cases are linked to somatic mutations and environmental factors [39]. Many environmental risk factors are caused by exposure to chronic inflammation. Up to 20% of cancers are linked to chronic infections, 30% can be attributed to tobacco smoking and inhaled pollutants, and 35% can be attributed to dietary factors (20% of cancer burden is linked to obesity which induces a low grade inflammatory state)

[39]. There are several recognised infections that promote cancer development including Helicobacter pylori infection causing gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma, Hepatitis B and C virus and hepatocellular carcinoma risk, and Human Papilloma Virus incurring an increased risk of developing cervical cancer [40]. The inflammatory response triggered by infection precedes tumour development and is a part of the normal host defence mechanism, whose goal is pathogen elimination. Inflammation associated with tumour development is a different entity. Solid malignancies trigger an intrinsic inflammatory response that builds up a pro-tumorigenic microenvironment [41]. Certain oncogenes can promote cell autonomous proliferation and recruitment of leukocytes, tumour promoting chemokines and cytokines and angiogenic factors into the tumour microenvironment [42]. As solid malignancies progress, they outgrow their blood supply resulting in necrotic cell death at the tumour's core and the release of proinflammatory mediators, such as IL-1 [43]. These inflammatory mediators promote neoangiogenesis and provides surviving cancer cells with additional growth factors, produced by newly recruited inflammatory and immune cells[44].

2.8 Inflammation & Breast Cancer

Post-menopausal obesity is associated with a significantly higher risk of ER+/progesterone receptor-positive (PR+) breast cancer, attributable in part to increased local oestrogen production from the breast adipose tissue increased breast cancer risk [45]. Obesity causes chronic low-grade inflammation via several mechanisms. The large expansion of adipose tissue is associated with endoplasmic reticulum stress, adipose tissue fibrosis and localised hypoxia, this causes adipocyte cell death and initiation of the inflammatory cells [46]. In contrast to lean adipose tissue, which predominantly contains activated M2 macrophages, obese adipose tissue is characterised by the infiltration of classically activated M1 macrophages which promote inflammation, generate reactive oxygen species and release cytokines such as tumour necrosis factor-a (TNFa) and interleukin 6 (IL-6)[47]. The adipokine leptin, which is elevated with obesity, also stimulates the production of TNFa, IL-6 and other cytokines, and promotes monocyte phagocytic function [48].

Cancer-associated systemic inflammation is strongly associated with poor oncological outcomes in patients with cancer [49]. Aaltomaa et al. defined inflammatory infiltrate as a prognostic marker in breast cancer for the first time in 1992[50]. For most human epithelial tumour types, high systemic neutrophil-to-lymphocyte ratios are associated with poor overall survival, and several studies have demonstrated a causal relationship between neutrophils and metastasis [51, 52]. Pre-clinical studies have uncovered p53 as a key promoter of pro-metastatic neutrophils. Wellenstein and colleagues discovered that p53 loss in cancer cells induced secretion of Wnt ligands that stimulate IL-1β production by tumour-associated macrophages, which drives systemic inflammation[53]. Genetic and epigenetic modifications are some of the driving forces behind tumorigenesis. Tumour promoting and tumour suppressing miRNAs associated with inflammatory pathways can influence breast cancer progression and metastasis [54]. Various cytokines such as tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and interleukins (ILs) play an important role in immune activation and are overexpressed under pathological conditions. These cytokines are recognised as key factors in breast cancer development and metastasis through regulating the inflammatory tumour microenvironment[55].

2.9 Oncogenic Cytokines

Cytokine overexpression is associated with poorer oncological outcomes in breast cancer[56]. Numerous cytokines, including interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α), have been implicated in the initiation and progression of ductal carcinoma[57-59]. IL-6 is a pro-inflammatory cytokine that has an important role in tumour progression, as it can inhibit the apoptosis of cancer cells and stimulate tumour angiogenesis [60]. IL-6 also plays a critical role in metastasis through decreasing E-cadherin expression and breast cell adhesion [61]. IL-8 was originally identified as a potent neutrophil activator and chemotactic factor secreted by activated monocytes and macrophages [62]. Many other cell types found within the tumour microenvironment - including fibroblasts, lymphocytes, neutrophils, endothelial cells, and a variety of normal and malignant epithelial cells- have since

been shown to secrete IL-8[63-66]. IL-8 and ER+ breast cancer exhibit a complex interaction. Initially IL-8 levels were thought to correlate closely with ER- breast cancers however elevated levels of IL-8 in association with ER positivity was associated with a much shorter median relapse free survival time in a cohort of 91 women with postmenopausal breast cancer [59]. The biological effects of IL-8 are mediated via two class A, rhodopsin-like guanine-protein-coupled receptors (GPCRs): CXCR1 (IL-8RA) and CXCR2 (IL-8RB) [67]. Breast cancer cells are reported to express CXCR1/2 and secrete IL-8, and rare genetic polymorphisms of CXCR2 and IL-8 are associated with an increased risk of developing large, high-grade, lymph node-positive breast cancers [68, 69]. The tumour micro-environment enriched with inflammatory cells and pro-inflammatory cytokines, promotes breast cancer progression [70].

Two important pro-inflammatory cytokines are tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β). In triple negative breast cancer (TNBC), serum-profiling and immunohistochemistry provided initial evidence that high expression of the two cytokines and their signalling components promotes metastasis in patients with TNBC [71, 72]. The role of the innate immune response and inflammasome formation also appear to play a role in tumour pathogenesis. Inflammasomes are large intracellular multi-protein signalling complexes that are formed in the cytosolic compartment as an inflammatory immune response to endogenous danger signals[73]. The most notable inflammasome complex is Nod Like Receptor Protein 3 (NLRP3) which via activation of cysteine protease procaspase-1 gives rise to the proteolytic activation of pro-interleukin(IL)-1β and pro-IL-18 cytokines[74]. IL-1β is up regulated in breast neoplasm development [120] and IL-1β variations have been reported to induce breast tumorigenesis[75, 76]. IL-10, initially termed 'cytokine inhibitory factor' due to its inhibitory action on cytokine production by T helper cells, is produced by almost all leukocytes, and a variety of normal epithelial cells [77]. Both IL-6 and IL-10 signal through the signal transducer and activator of transcription 3 (STAT3). Even though they both signal mainly through STAT3, IL-6 is pro-inflammatory whereas IL-10 is anti-inflammatory and suppresses the expression of other cytokines by immune cells [78]. Elevated IL-10 expression has been previously

associated with favourable oncological outcomes in patients with colorectal cancer and breast cancer[79, 80].

2.10 Peri-Operative Period

The pro-metastatic potential of the peri-operative period was first described by Paget and Halsted at the turn of the century who found that patients who underwent radical resection of their breast cancer did not survive as long as those managed conservatively [81]. Surgery and tissue trauma ensues a complex multifaceted inflammatory response. Systemic and local activation of the innate immune response is an essential response to promote tissue healing and elimination of immunogenic pathogens however may inadvertently create an environment which promotes circulating tumour cells and growth of micrometastatic disease [82]. Surgical trauma and anaesthesia is also believed to induce a state of immunosuppression which may lead individuals susceptible to infection and residual tumour growth post operatively[82] [83]. Indeed, handling of the tumour can result in at least a 10-fold rise in circulating tumour cells [84]. IL-6 levels increase after surgery due to tissue insult and post-operative serum IL-6 elevations that are proportional to the magnitude of the surgical stress have been described [85]. Previous studies comparing the physiological stress response of open versus laparoscopic surgery reported significantly higher post-operative IL-6 concentrations were noted in the open surgery group [86, 87]. Whilst perioperative morbidity associated with a more minimally invasive approach is favourable, this has not translated into better oncological outcomes in colorectal cancer. The COlonic cancer Laparoscopic or Open Resection (COLOR) trial did not report a significant difference in disease free survival and overall survival between the two cohorts [88]. In contrast, a recent large retrospective cohort study of 48,986 women who underwent breast surgery displayed varying oncological results according to surgical intervention. Despite adjustment for confounders, breast conserving surgery with radiotherapy yielded better survival outcomes than mastectomy irrespective of radiotherapy [89]. Whether breast conserving surgery induces a diminished cytokine response and less favourable tumour microenvironment is yet to be determined.

Several translational studies have identified the peri-operative period as an opportunity to therapeutically manipulate the inflammatory process with promising results[90, 91]. More recently, several clinical trials targeting peri-operative inhibition of B-adrenergic and COX2 signalling pathways in breast cancer and colorectal cancer have been reported [92, 93]. This research group employed a similar methodological approach to both randomised controlled trials (RCT). The same dosage of B-blockade and COX-2 inhibition was initiated five days pre-op and continued for eleven consecutive peri-operative days in the breast cancer trial and twenty peri-operative days in the colorectal cancer trial. The studies differed in their outcome measures and clinical endpoints. The breast cancer trial measured Ki-67 expression, serum cytokine levels and peripheral blood mononuclear cells transcriptome and found IL-6 levels markedly increased day one post op and there was a significant reduction in IL-6 for the drug treated group [93]. On the other hand, transcriptional activity analysis was used to measure assessment of outcomes in the Colorectal trial. Blood samples were collected at 4 different timepoints, 1) before commencing treatment T1, 2) the morning of surgery T2, 3) the morning after surgery T3 and 4) at least two days after stopping treatment T4. There were 18 patients randomised to the control and treatment group in the breast RCT. However, two of the patients in the control group included a diagnosis of DCIS which may have influenced the IL-6 levels as patients with invasive cancer have a higher baseline level of IL-6 [94]. Similarly, there was no indication whether any of the patients had positive margins post-operatively which could influence results. There were also differences in the types of surgery performed, fifteen patients had breast conserving surgery (BCS) and sentinel lymph node biopsy (SLNB) performed in the treatment group, whilst 13 patients had BCS and SLNB in the control group. The remaining patients in both groups had varying types of more extensive operations which may also have influenced the surgical stress dynamics. Peri-operative COX2 and B-adrenergic blockade treatment resulted in a significant improvement of tumour molecular markers of malignancy and metastatic potential [92]. What remains to be established is whether these effects on peri-operative biomarkers will lead to better oncological clinical outcomes.

From our own institute, the results of the SURGUVANT trial was published in 2018. This multicentre randomised control trial compared serum IL-6 levels between patients with colorectal cancer who received taurolodine (IL-6 inhibitor) versus placebo[7] . The first dose of taurolodine was administered at anaesthesia induction and continued for four days post-operatively. IL-6 levels were significantly attenuated over the seven-day post-operative period in the Taurolidine group compared to placebo ($p = 0.04$). In addition, IL-6 levels were significantly lower at day seven in the Taurolidine group ($p = 0.04$). The study was not powered for a formal survival analysis, so it is not possible to determine whether IL-6 attenuation will have a clinical impact on oncological outcomes.

These studies provide a useful insight into the peri-operative dynamics of the inflammatory and stress response. What remains to be elicited is the normal peri-operative inflammatory response in breast cancer, how this differs between surgical approach and subtypes and the oncological outcomes associated with elevated circulating cytokines.

2.11 Cancer Genetics

Clinical genetics and genomics are becoming an integral component of the multi-disciplinary approach to treating breast cancer and supporting a personalised approach to breast cancer management. Genetics refers to the study of individual genes and their role in inheritance whilst genomics describes the study of all of a person's genes (the genome), including interactions of those genes with each other and environmental factors [95]. Cancer is a disease largely caused by genomic aberrations [96]. The application of massively parallel or next-generation sequencing (NGS) to large-scale cancer genomics discovery projects has revealed extraordinary new information about the underlying genomic drivers of cancer development and progression. Genomics-based assays are increasingly being used to guide the selection of the most appropriate targeted therapies for patients according to the genomes of their tumours[97]. Such assays have been incorporated into the routine

management of breast cancer to better inform the clinical care of patients and provide invaluable prognostic information[98].

An estimated 5-10% of breast cancer patients will have an underlying germline genetic predisposition and it appears the thresholds required to perform germline genetic testing in breast cancer are becoming lower all the time [99]. The other 90-95% of sporadic breast cancers are characterised by a dynamically evolving process with a versatile somatic mutational profile, of which some genetic aberrations will be crucial for cancer progression [9]. Liquid biopsy is emerging as an exciting tool to detect fragments of circulating breast tumour DNA for diagnostic, prognostic, and therapeutic purposes [100, 101]. Breast cancer tumour heterogeneity results in varying responses to therapeutic regimes[102]. Detection of circulating somatic mutations enables individualised genetic profiling and provides an opportunity to target driver mutations for a more personalised therapeutic approach [101, 103]. Alpesilib, the Phosphoinositide 3-kinase (PI3K) inhibitor and Everolimus, the mechanistic target of rapamycin (mTOR) inhibitor are examples of approved targeted therapies that have revolutionised the management of metastatic breast cancer [103, 104].

Cancer genomics also carries implications for the surgical oncologist. Surgical strategy and sequencing of treatment may be influenced by the presence of a specific pathogenic mutations. Additionally, serial analyses of circulating tumour DNA also provides real time monitoring of response to therapy and reflects residual disease burden [105]. Further understanding of the peri-operative dynamics of circulating tumour DNA is required to elucidate the baseline value for post-operative breast cancer surveillance.

2.12 Genetic Assays

Breast cancer is a heterogenous disease generally comprised of five molecular subtypes: luminal A, luminal B, normal-like, HER-2 enriched, and basal-like [106]. In recent times, breast cancer intra and inter - tumour heterogeneity has gained much appreciation due to the implications on therapeutic response to treatment [107].

Traditionally, therapeutic options for breast cancer weighed heavily on morphological assessment, subtype, hormone receptor status and lymph node burden however this process of treatment selection is limited due to a significant variation in treatment response within breast cancer subgroups. Oestrogen sensitive (ER+) breast cancer account for approximately 75% of breast cancer cases and vary considerably in their response to treatment and clinical outcomes [107]. A possible explanation for this disparity in response is individual tumour mutational burden and genomics. Breast carcinogenesis is a multifactorial event that arises from acquisition of multiple mutations and immune dysregulation[108]. Individual tumour biology and activity determines the propensity to metastasize, response to therapy and overall prognosis. The impact of tumour biological activity is not appreciated in the Nottingham Prognostic Index and online tool PREDICT, therefore more sophisticated genomic profiling was required to determine the cohort of patients with breast cancer likely to response to systemic treatment[109].

Oncotype DX is a successful example of a multigene prognostic and predictive assay that can be performed on primary Fixed Formalin Paraffin Embedded tumour samples [110]. The Oncotype DX genomic assay (Genomic Health, Inc., Redwood City, CA, USA) is a clinically validated assay that can be used to predict likelihood of recurrence of early-stage breast cancer, and can be used therefore, in decision making with respect to systemic therapy. The Oncotype DX-automated algorithm assigns a score to the expression of 16 cancer-related genes multiplied by a factor specific to the group to which each individual gene is allocated, relative to the mean expression of five reference genes to generate an overall recurrence score [111]. The highest level of prospective evidence supporting the predictive utility of the OncotypeDX originates from the TAILORx trial in women aged 18–75 years with HR-positive/HER2-negative, LN-negative, invasive breast cancer [112]. Recurrence Score (RS) results of ≤10, 11–25 and ≥26 were defined as low, intermediate and high, respectively. Patients with a low RS were allocated to received endocrine therapy alone whilst patients with a high RS received chemo-endocrine therapy, both groups had promising nine-year disease-free survival (DFS) rates (84% and 75.7% respectively). Among patients with a Recurrence Score of 11–25, endocrine therapy

was noninferior to chemo-endocrine therapy for invasive disease-free survival. Several other multigene assays have been developed, including the 7-gene Breast Cancer Index®, 12-gene EndoPredict®, 20-gene Prosigna®, and 70-gene MammaPrint® assays [113] however OncotypeDX is the only multigene assay validated for prediction of chemotherapy benefit, as well as for prognosis [110].

2.13 Cell-free DNA & Breast Cancer

Image-based screening provides an estimated 20% reduction in breast cancer mortality [114] however mammography incurs several downsides including false positive rates, limited sensitivity and patient discomfort [115]. Reliable breast cancer blood-based biomarkers are limited in clinical practice. The most widely used blood-based tumour marker CA-15 lacks sensitivity and is hindered by a long half-life in circulation reducing the potential to monitor treatment response [116]. Liquid biopsy techniques have emerged as an advantageous alternative to tumour marker analysis as it allows for serial monitoring of disease status. Tumour initiation and progression causes the release of various components into the circulation via apoptosis, necrosis or active secretion including Cell Free DNA (cfDNA), Circulating Tumour Cells (CTC's) and Circulating Tumour DNA (ctDNA) [117]. CfDNA was first discovered by Mandel and Métais in 1948 and is detectable in serum, plasma, urine, saliva and pleural fluid of healthy individuals [118]. In certain physiological states or pathological conditions such as pregnancy, organ transplantation or malignancy, additional DNA is shed into the circulation suggesting that cfDNA detection in the blood could signify abnormalities in a non-invasive manner [119]. The estimated concentration of cfDNA in a healthy population is between 0 and 100ng/ml compared to the concentration of cfDNA in patients with cancer which can range between 0 to 1000ng/ml [120]. The predictive utility of cfDNA serial analysis is gaining traction as a novel biomarker in predicting oncological outcomes in patients with breast cancer. As the half-life of cfDNA in the circulation is estimated between 16 minutes to 2.5 hours, cfDNA is an attractive "real-time" snapshot of disease burden[121]. Panagopoulou et al. analysed circulating cfDNA in a cohort of patients with metastatic breast cancer and demonstrated that elevated cfDNA levels correlated with treatment resistance,

shorter progression- free survival and poorer survival outcomes[122]. The prognostic utility of cfDNA has shown promise in several other cancer fields. Park et al demonstrated that cfDNA levels correlated with treatment outcome in patients with hepatocellular carcinoma treated with radiotherapy [123]. Similarly, quantitative high measures of cfDNA were associated with poorer oncological outcomes in patients with metastatic colorectal cancer treated with FOLFIRI treatment [124] and patients with advanced Non-Small Cell Lung Cancer (NSCLC) treated with the Epidermal Growth Factor Receptor (EGFR) tyrosine kinase inhibitor Osimertinib [125]. Furthermore, cfDNA serial monitoring has shown promise in the early detection of disease relapse. Fleming and colleagues' research centred on cell-free DNA (cfDNA) dynamics peri-operatively and discovered that cfDNA 48 hours post-surgery served as an important biomarker to identify those at risk of early recurrence in colon cancer [126]. These findings were corroborated by Chen et al who performed the only study to investigate the precise peri-operative dynamic changes in circulating tumour DNA (ctDNA) in patients with early-stage lung cancer. Elevated ctDNA detection on the third day after R0 resection was reported as the baseline value for postoperative lung cancer surveillance[127]. The peri-operative dynamics of cfDNA in breast cancer surgery and the prognostic significance of such is yet to be elicited.

2.14 Circulating Tumour DNA (ctDNA) & Breast Cancer

Liquid biopsy is a non-invasive technique which can yield important diagnostic, therapeutic and prognostic information in many types of cancer [128] . A component of liquid biopsy known as circulating tumour DNA (ctDNA) has shown promise in the detection of breast cancer and predicting clinical and cancer outcomes[100, 129]. ctDNA is a sub-group of cell-free DNA (cfDNA) which is present in healthy individuals and is released from non-malignant cells [130]. ctDNA is found within the circulation of patients with cancer owing to the continuous release of fragmented DNA from necrotic and apoptotic cancer cells, and it accounts for between 0.01% to as much

as 50% of total cfDNA in cancer patients[131, 132]. These fragments have a length of approximately 166, 332, or 498 base pairs and exhibit tumor-specific alterations, including point mutations, chromosomal rearrangement, copy-number variation (CNV), and DNA methylation[133, 134]. ctDNA levels are influenced by tumour size and burden therefore serial measurement of ctDNA can provide real time non-invasive monitoring of response to treatment and early detection of recurrence [135].

Personalised medicine and precision oncology emerged as an exciting concept in the late 90's with the advent of Trastuzumab for HER2+ breast tumours and Imatinib for BCR-ABL Chronic Myeloid Leaukaemia [136]. Around the same time, independent studies reported the presence of specific KRAS and NRAS point mutations in ctDNA from pancreatic adenocarcinoma [137]. The objective is to provide the most accurate and effective oncological treatment to patients based on individuals' genetic profile [130]. ctDNA can not only be used as a blood-based biomarker of response to treatment, but it can also indicate tumour sensitivity to specific chemotherapy regimens [138]. Phosphoinositide 3-kinase (PIK3CA) and BRCA1 are common breast cancer mutations and significant research has been invested into targeting these mutations. The Poly Adenosine Diphosphate-Ribose Polymerase (PARP) inhibitor Olaparib is more effective in the presence of BRCA1 mutation whilst PI3K3CA aberration suggests sensitivity to the mechanistic target of rapamycin (mTOR) inhibitor Everolimus and PIK3CA inhibitor Alpesilib [101, 103]. Genomics-based analyses of tumour biology and circulating tumour cells have revealed many new mechanisms of acquired resistance to breast cancer treatment. ctDNA is advantageous as a biomarker as it has a short half-life in the bloodstream, and this characteristic makes it a very important tool in analysing real-time situation of mutation dynamics and tumour burden after surgery or systemic treatment. ctDNA is also emerging as an important adjunct in screening certain types of cancer. Detection of certain genes, including APC, BRAF, KRAS and CTNNB1 in circulating plasma is highly associated with the presence of colorectal cancer or advanced adenomas in colorectal cancer screening[139]. Monitoring ctDNA levels has the potential to gauge response to treatment and aid in early detection of disease

progression or recurrence. Detection of driver mutations associated with acquired resistance provides a window of opportunity to identify treatment resistant disease early and change the therapeutic approach accordingly. Undoubtedly, genomic profiling will provide a critical role in the future management of patients with cancer, and it is imperative that cancer care providers familiarise themselves with this new evolving practice.

2.15 Liquid Biopsy & Detection Techniques

In order to demystify cancer genomics and quantify the expression of driver genes in different breast cancer subtypes, the Catalogue of Somatic Mutations in Cancer (COSMIC) and The Cancer Genome Atlas (TCGA) are continuing to curate the somatic mutational landscape of all cancer types [140, 141] . These vast databases are ever expanding in line with advances in detection and quantification of circulating tumour DNA and mutation-driver genes. Circulating tumour DNA is found within the blood of patients with cancer owing to the continuous release of fragmented DNA from necrotic and apoptotic cancer cells, accounting for as little as 0.01% of the total circulating DNA (cell-free DNA) with a very short half-life (less than 2 hours) [142]. Pathfinder is a prospective investigational multi-centre early detection test using GRAIL Galleri liquid biopsy cancer detection test (*ClinicalTrials.gov identifier (NCT number): NCT04241796)***.** This novel study has reached its accrual and aims to analyse cell free DNA and RNA levels in a high risk and healthy cohort to determine whether cancer can be detectable in the circulation before it is clinically apparent. Detection of ctDNA is a component of liquid biopsy and can provide real-time non-invasive monitoring of response to treatment and early detection of recurrence [135]. Liquid biopsy can overcome the challenges of tissue biopsies that are often not representative of the entire tumour due to tumour heterogeneity. Additionally, advanced detection techniques can isolate specific mutation-driver genes and provide opportunities for therapeutic manipulation and clinical cancer trials [143]. Traditionally, polymerase chain reaction (PCR)–based techniques have been adopted to detect ctDNA in various cancers. One of the practical limitations of PCR is that knowledge of the specific genetic variation is required before it can be quantified.

Tumour analysis is necessary before gene variation alteration measurement unless the target is well established, such as TP53, PIK3CA, and ERBB2 in breast cancer [144].

Detection of ctDNA by next-generation sequencing (NGS)–based techniques is an advancement in tumour prognostics, as this technique sequences millions of DNA templates in parallel, enabling rapid identification of tumour-specific alterations in ctDNA [145]. Unlike with PCR-based techniques, prior knowledge of the underlying gene variation is not required in order to quantify genomic alterations using NGS. Furthermore, NGS-based techniques allow for detection of several variations in multiple genes simultaneously. The minute fraction of tumour derived DNA in circulating plasma is substantially lower than that present in tumour tissue requiring much deeper sequence coverage to achieve sufficient sensitivity for low-frequency mutations that might approach a frequency similar to that of background sequencing errors. This is particularly significant in the setting of early-stage disease with a small disease volume [146]. Technological improvements using methods to suppress base line errors and provide ultra-high-depth sequencing are some of the techniques adopted to improve sensitivity however this comes with a cost. A sophisticated bioinformatics pipeline along with advanced computational data analysis is vital in order to make sense of the data. With the influx of more sequencing platforms onto the market, this will hopefully lead to more competitive pricing and enable accessibility to all patients. It is imperative that access to genomics-based techniques in the diagnosis and treatment of breast cancer is available to all.

The major advances in sequencing technologies followed by the development of computational tools have enabled analyses like whole-exome sequencing (WES), RNA-sequencing (RNA-seq) and whole-genome sequencing (WGS) to be implemented into routine clinical oncology. Targeted sequencing and gene-panels are widely used when predefined genes are to be sequenced and if coverage needs to be high to identify low-frequency tumour-drivers i.e PIK3CA. These panels can be custom designed to create a breast cancer specific panel derived from the most common mutations outlined in the COSMIC and TCGA databases. Alternatively, large “off the shelf” gene panels can be utilised to detect common cancer mutations in a variety of malignancies [147]. A brief outline of the most clinically relevant

somatic mutations in breast cancer and the clinical implications of their discovery will be explored.

2.16 Somatic Mutations

2.16.1 TP53

TP53 is mutated in approximately 30% of breast cancers and associated with more aggressive subtypes such as HER2-enriched and triple negative breast cancer [148] [149]. The TP53 protein is best characterized as a DNA-binding transcription factor with potential to bind to several hundred different promoter elements in the genome, hence regulating expression of hundreds of genes[150]. *TP53* initiates transcription of genes involved in processes such as cell cycle arrest, apoptosis, metabolism, DNA repair, and cellular regression in response to stressors like radiation and chemotherapy [151]. *TP53* mutations can be accurately detected in breast cancer by DNA sequencing [152]. Most mutations in *TP53* occur in exons 5–8 of the gene and many studies demonstrate that patients with *TP53* mutant tumours have worse survival than patients with *TP53* wild type tumours [153, 154]. Due to inconsistencies in the literature Ungerleider et al utilized large datasets to examine breast cancer oncological outcomes according to *TP53* mutation status. In patients receiving chemotherapy and no hormone therapy, wild-type *TP53* status conferred remarkably poor overall survival. The inferior survival outcome is consistent with p53 inducing arrest/senescence instead of apoptosis. Addition of hormone therapy to chemotherapy improved survival notably in patients with *TP53* wild-type tumours, but not mutant, suggesting hormone therapy could eradicate arrested/senescent cells [155]. Li-Fraumeni syndrome (LFS) is a complex hereditary cancer syndrome associated with germline p53 mutations. The syndrome shows an autosomal dominant pattern of inheritance[156]. Despite the intrafamilial and interfamilial heterogeneous clinical presentation of tumours in LFS, five “core” cancers are most observed: breast cancer, soft tissue sarcoma, brain tumours, adrenocortical carcinomas (ACC) and bone sarcomas. Whilst brain tumours, bone/soft tissue sarcomas, and ACC commonly occur within the first or second decade of life, breast cancer occurrence is usually somewhat later [157].

2.16.2 PIK3CA

After the TP53 gene, the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene is the most frequently mutated gene in Breast Cancer [158]. The frequency of PIK3CA mutations in breast cancer cases ranges from 16.4 to 45% and is more frequently associated with hormone receptor positive breast cancer [159]. The PI3K/AKT/mTOR pathway is an intracellular signalling pathway important in cell-cycle regulation. Gain of function PIK3CA mutations result in downstream activation of the AKT/mTOR pathway which promotes angiogenesis, lymphogenesis, proliferation and migration [160, 161]. There are 3 mutation ‘‘hotspots’’ in the PIK3CA gene that constitute almost 80% of PIK3CA mutations: E542K, E545K at exon 9 (helix domain) and H1047R at exon 20 (kinase domain)[162]. *PIK3CA* mutation has different clinical implications in metastatic breast cancer compared with early breast cancer. Zardavas et al conducted the largest pooled analysis of data from 10,319 patients in 19 studies of patients with early breast cancer. *PIK3CA* mutations were significantly associated with advanced age, oestrogen receptor positivity, lower grade, and smaller tumours. In multivariate analysis, *PIK3CA* mutations remained significant for better invasive disease-free survival, but not for distant disease-free survival or overall survival (OS)[163]. A similar meta-analysis by Signorovitch et al examined PIK3CA mutation in patients with metastatic breast cancer. They concluded that *PIK3CA* mutation was consistently associated with significantly shorter PFS for patients with ER+/HER2− metastatic breast cancer who received non-PI3K inhibitor therapy [164].

The function of PIK3CA is particularly significant in endocrine resistant advanced breast cancer. Therapeutic response with single agent endocrine therapy is achieved in approximately 20-40% of patients with advanced hormone receptor positive breast cancer with a median duration of response (DOR) of approximately 8–14 months [165]. However, many patients with hormone sensitive breast cancer develop resistance, either de nova or acquired, to endocrine therapy which poses a significant therapeutic challenge [165, 166]. One of the mechanisms underlying endocrine resistance involves dysregulation of the cyclin D-cyclin-dependent kinase (CDK)- retinoblastoma pathway, hence a number of CDK inhibitors in combination

with endocrine therapy have been approved for advanced hormone receptor positive breast cancer [167]. Aberrations in the PIK3CA signalling pathway is another key regulator of endocrine resistance. In vitro data indicate that endocrine-resistant cells rely on PI3K/mTOR signalling for growth and are extremely sensitive to inhibition of this pathway[168]. This is supported by the results of the phase III BOLERO-2 trial which reported that the Mtor inhibitor Everolimus combined with an aromatase inhibitor improved progression-free survival in patients with hormone-receptor–positive advanced breast cancer previously treated with nonsteroidal aromatase inhibitors [169]. Similarly, the PI3Kα-specific inhibitor Alpelisib combined with Fulvestrant prolonged progression-free survival among patients with *PIK3CA*-mutated, HR-positive, HER2-negative advanced breast cancer who had received endocrine therapy previously in a trial of 572 patients who underwent randomisation [104]. The advent of these novel therapeutic options justifies PIK3CA mutation testing using tumour tissue or ctDNA in all patients with HR+/HER2– advanced breast cancer.

2.16.3 CDH1

Invasive Lobular Carcinoma (ILC) is composed of discohesive tumour cells arranged in linear strands. This characteristic phenotype is caused by inactivation of E-cadherin (ECAD), a calcium dependent transmembrane protein and member of the cadherin-catenin complex that facilitates cell adhesion [170, 171]. Inactivation of ECAD is driven by genomic alterations targeting the CDH1 gene, located on chromosome 16q22.1. [172]. Loss of the ECAD protein can be detected by immunohistochemistry, which shows absent membranous staining which is the distinguishing feature between Invasive Ductal Carcinoma(IDC) and ILC in clinical practice [173]. Germline CDH1 mutations are not only associated with ILC but also with the development of diffuse gastric cancer in the context of the so-called hereditary diffuse gastric syndrome [174]. Criteria for CDH1 genetic testing were revised in 2010. The updated guidelines suggest CDH1 testing for (1) families with two or more patients with gastric cancer at any age, one confirmed DGC; (2) individuals with DGC before the age of 40 and (3) families with diagnoses of both DGC

and LBC (one diagnosis before the age of 50). Additionally, *CDH1* testing could be considered in patients with bilateral or familial LBC before the age of 50, patients with DGC and cleft lip/palate, and those with precursor lesions for signet ring cell carcinoma. Breast cancer surveillance with annual breast MRI starting at age 30 for women with a *CDH1* mutation is recommended. [175].

In a multi-cohort study conducted by Griffith et al, 10.5% of 1128 Oestrogen receptor positive breast tumours were found to have somatic mutations in CDH1[176]. A Secondary Analysis of the BIG 1-98 Randomized Clinical Trial revealed that 12.2% of oestrogen receptor positive HER2 negative breast cancers harboured a CDH1 mutation. The incidence of CDH1 mutation was similar between the pre-menopausal and post-menopausal cohort however CDH1 alterations were significantly associated with smaller, low-grade breast tumours [177]. Whilst an increasing number of somatic driver alterations per tumour was associated with worse outcomes in both univariate (HR, 1.1.8; 95% CI, 1.11-1.25; $P < .001$) and multivariable (HR, 1.11; 95% CI, 1.04-1.19; $P = .002$) analysis, the presence of CDH1 mutation was not independently associated with an increased risk of distant recurrence [177].

2.16.4 GATA3

In the mammary gland, GATA3 is expressed in the differentiated luminal epithelial cells lining the breast ductal structures, where it is estimated to be the most highly expressed transcription factor [178]. Genomic analyses of breast cancer have identified GATA3 as a high frequency target of mutation in this disease with mutations in GATA3 identified in approximately 10% of tumours [148, 179]. GATA3 mutations are present in 14% of Luminal A breast cancers and approximately 15% of Luminal B breast cancers [180]. Loss of GATA3 expression is associated with breast tumour types with a propensity for invasive growth and poor prognosis [181]. The malignant potential of aberrant GATA3 expression is believed to stem from two mechanisms. First, GATA3 and ER-α participate in a positive feedback loop, each stimulating expression of the other [182]. Secondly, GATA3 is proposedly a tumour

suppressor gene downregulating expression of factors critical to the malignant epithelial to mesenchymal transition and metastasis [183]. The initial publication by the Cancer Genome Atlas project identified 55 somatic GATA3 mutations (a total of 10.7% of all patients) in their cohort of 512 sequenced tumours, almost all mutations were found to impact exons 5 and 6 of the GATA3 protein [148]. GATA3 whole gene deletion or mutations throughout the open reading frame are associated with the rare haploinsufficiency disease known as Barakat syndrome**,** also known as HDR syndrome, this condition is characterised by Hypoparathyroidism, deafness and renal dysgenesis. Initially the loss of function of GATA3 gene involved in HDR syndrome was thought to represent a similar process to breast cancer tumorigenesis however a recent animal study by Emmanuel et al suggests that GATA3 encode active transcription factors, which elicit proliferative phenotypes in normal mammary epithelium and promote the growth of ER-positive breast cancer cell lines [180].

2.16.5 ESR1

Approximately 3-3.5% of patients with primary hormone sensitive breast cancer have ESR1 mutant-bearing tumours in contrast to 13.6% of metastatic tumours [184, 185]. Furthermore, ESR1 mutations are found in ER-positive samples (including ER positive/HER2 positive patients to a lesser extent), but not triple negative breast cancers [185]. Current standard of care to effectively treat and prevent recurrence of ER-positive breast cancer is with endocrine therapy (ET) which targets the ER through hormone deprivation or antagonistic binding of the receptor [186]. However, endocrine therapy only incurs benefit in approximately 30% of patients with metastatic breast cancer due to de novo or acquired Aromatase Inhibitor (AI) resistance [187]. Acquisition of ESR1 mutations, predominantly in the ER ligand-binding domain (LBD) are believed to be the most common mechanism of acquired endocrine resistance resulting in worse overall survival [188]. There is considerable evidence demonstrating that metastatic tumour cells with ESR1 mutations are most frequently acquired following treatment with aromatase inhibitor therapy [189].

Some authors believe ESR1 mutant cells represent a more “aggressive phenotype” and initiate sub clonal cell population in circulating tumour cells and metastatic sites [190, 191]. The recent PADA-1, randomised Phase 3 trial presented at The San Antonio Breast Cancer Symposium December 2021 demonstrated the effectiveness of ESR1mutation monitoring in blood to allow optimisation of the endocrine therapy partner of CDK4/6 inhibitors in patients with metastatic hormone sensitive breast cancer. Upon ESR1 mutation detection in the blood, the median Progression Free Survival (PFS) was doubled by the switch from AI and Palbociclib to Fulvestrant and Pablociclib[192]. Therefore, monitoring the rise of resistance-associated mutations opens up new opportunities for cancer therapy.

2.16.6 MAP3K1

MAP3K1, belonging to the mitogen-activated protein kinase 1 (MAP3K) family, activates the extracellular signal-regulated kinase (ERK) family involved in cell proliferation, growth, differentiation, and cell survival [193]. However, *MAP3K1* also activates the apoptotic JNK pathway through *MAP2K4 stimulation and exerts its effect via loss of function mutations* [148]*.* MAP3K1 mutant tumours disrupt these cell signalling pathways and appears to play a critical role in balancing cell fate decisions [194]. In contrast to TP53 mutations which infer a more unfavourable prognosis, the presence of MAP3K1 is associated with a more indolent presentation sensitive to endocrine therapy [195]. Similarly to PIK3CA mutations, *MAP3K1* are more commonly found in Luminal A breast cancers occurring in 13–20% of luminal A breast cancers [179]. The more favourable biology and low proliferation rate of MAP3K1 mutant tumours have been observed in several studies. [176]. MAP3K1 mutations are frequently present concurrently with PIK3CA mutant tumours inciting researchers to study potential crosstalk between PI3Kα and MAP3K1 signal transduction pathways. Avivar-Vanderas et al demonstrated that depletion of *MAP3K1* gene expression in the context of *PIK3CA* breast cancer cell lines cause resistance to PI3K pathway inhibition by PI3K pathway inhibitors *in vitro*. [196]. This

work provides important insights into how the mutational status of *PIK3CA* and *MAP3K1* genes could predict response to PI3K pathway inhibitors. Furthermore, this seminal study highlights the clinical relevance of tumour mutational status and the importance of understanding the implications of signal transduction crosstalk.

With the advent of advances in next generation sequencing and the availability of targeted therapies for certain somatic mutations, it is likely that ctDNA analysis will become an integral part of the oncological armamentarium in the future. The role of breast cancer ctDNA analysis in the peri-operative space is vastly underexplored and is the central theme of this thesis.

Chapter 3: Methodology

3.1 Patient Recruitment

Patients undergoing breast cancer surgery and treatment at our institute are being actively recruited to the ethically approved ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study. This study is sponsored by Cancer Trials Ireland (NCT01840293) and open across Irish centres. This thesis is a sub-study of the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity, and sample collection is within the remit of the biospecimen protocol (Appendix 1 & 2).

Inclusion/Exclusion Criteria for Chapter 4: Investigating the peri-operative trend of inflammatory mediators in breast cancer and association between differential inflammatory mediators, and tumour characteristics.

Patients presenting for elective breast cancer surgery, with non-metastatic disease, aged 18-85 years of age were screened for inclusion prospectively. An extensive exclusion criteria was applied when investigating the peri-operative trend of inflammatory mediators in breast cancer to reduce the effect of confounders as follows: received neoadjuvant chemotherapy or hormonal therapy, undergoing reconstructive breast surgery, pregnant or lactating; evidence of underlying liver disease (abnormal LFT's (> x2 normal), INR > 1.5); evidence of underlying renal disease (creatinine >180 for women, >150 for men); blood dyscrasia (neutropenia < 1500 cells /cm3, thrombocytopenia < 100,000 cells/cm3); metastases, morbid obesity (body mass index >40 kg/m2); American Society of Anaesthesiologists (ASA) physical status classification system score >III; coexisting active inflammatory disorder (including active Rheumatoid Arthritis, Inflammatory Bowel Disease, Systemic Lupus Erythematosus); corticosteroids usage; immunosuppressive drugs; previous diagnosis of HIV; chronic active Hepatitis B or C; active infection at the time of surgical intervention.

Inclusion/Exclusion Criteria for Chapter 5/6: Defining Peri-operative Cell Free DNA (cfDNA) and Circulating Tumour DNA (ctDNA) Dynamics in Breast Cancer Surgery.

Patients presenting for elective breast cancer surgery, with non-metastatic disease, aged 18-85 years of age were screened for inclusion prospectively. Exclusion criteria were applied as follows: received neoadjuvant chemotherapy or hormonal therapy, active infection at the time of surgical intervention. For ctDNA analysis, only patients with ER+ disease had downstream next generation sequencing.

Inclusion/Exclusion Criteria for Chapter 7: Characterising Inflammatory Mediators and Adipokine Expression in the Tumour Microenvironment.

Patients were selected retrospectively from a prospectively maintained breast biobank. Inclusion criteria included patients having breast cancer surgery for non-metastatic ER+/HER2- breast cancer aged 18-85 with tumour tissue and normal adjacent to tumour tissue available. Patients were excluded if they had neoadjuvant chemotherapy or had invasive cancer/DCIS <2mm from the specimen margin.

All patients with breast cancer were discussed at the regional Multi-Disciplinary Meeting (MDM) and managed in keeping with National Cancer Control Programme (NCCP) recommendations. In this institution all patients with breast cancer are routinely screened for axillary nodal disease using ultrasonography +/- fine needle aspiration (FNA) of indeterminate or suspicious nodes. Axillary lymph node dissection is recommended to patients with positive axillary nodal disease receiving upfront surgery. Image guided breast conserving surgery is performed using radiological placed guide wires or a Radiofrequency Identification Device (RFID) tag localisation. Tumour phenotype was recorded from final histopathological reports. Oestrogen receptor/progesterone receptor were considered positive when >10% of cells showed positive staining. HER2 status was assessed by immunohistochemistry (IHC) and Fluorescent in Situ Hybridization (FISH) confirmation in cases with 2+ by IHC. HER2 positivity was defined as either 3+ with IHC and/or amplified with FISH.

3.2 Sample Biobanking & Transfer

A dedicated research nurse is employed to assist with consenting patients for study participation and facilitating sample collection at timepoints outlined in the protocol. Blood samples were phlebotomised using a single needle puncture with a butterfly needle peri- operatively from patients at the following time-points– pre-operatively the morning of surgery, 3-5 hours post-operatively, 10-14 days post-operatively (Figure 3A). Up to 40mls of blood could be obtained as per protocol. Patients with positive invasive margins or large volume nodal disease requiring further surgery had their 2-week post op sample deferred until 2 weeks after surgery is complete. Plasma samples were stored in K3EDTA bottles and centrifuged at 2500 rpm for 15 mins within 1 hour of phlebotomy. Alternatively, samples were stored in Streck tubes and centrifuged at 2500rpm for 15 mins within 48hours of phlebotomy. Plasma samples were aliquoted into collection tubes (500 uL and 1500 uL), labelled with trial codes and stored at -80 degree Celsius.

Tumour and normal adjacent tumour was collected within 20minutes of surgical resection and cut up into at least 3 pieces (minimum size 2-3mm^2), and snap frozen in liquid nitrogen separate cryovials. Tissue samples were labelled with trial codes and stored at -80 degree Celsius.

Trial identification codes and patient clinicopathological data were stored on a password protected database on a site-specific Cancer Trials Ireland trial laptop. This central database is housed in a custom-made Access database. It allows for no free text data entry and ensures data minimisation.

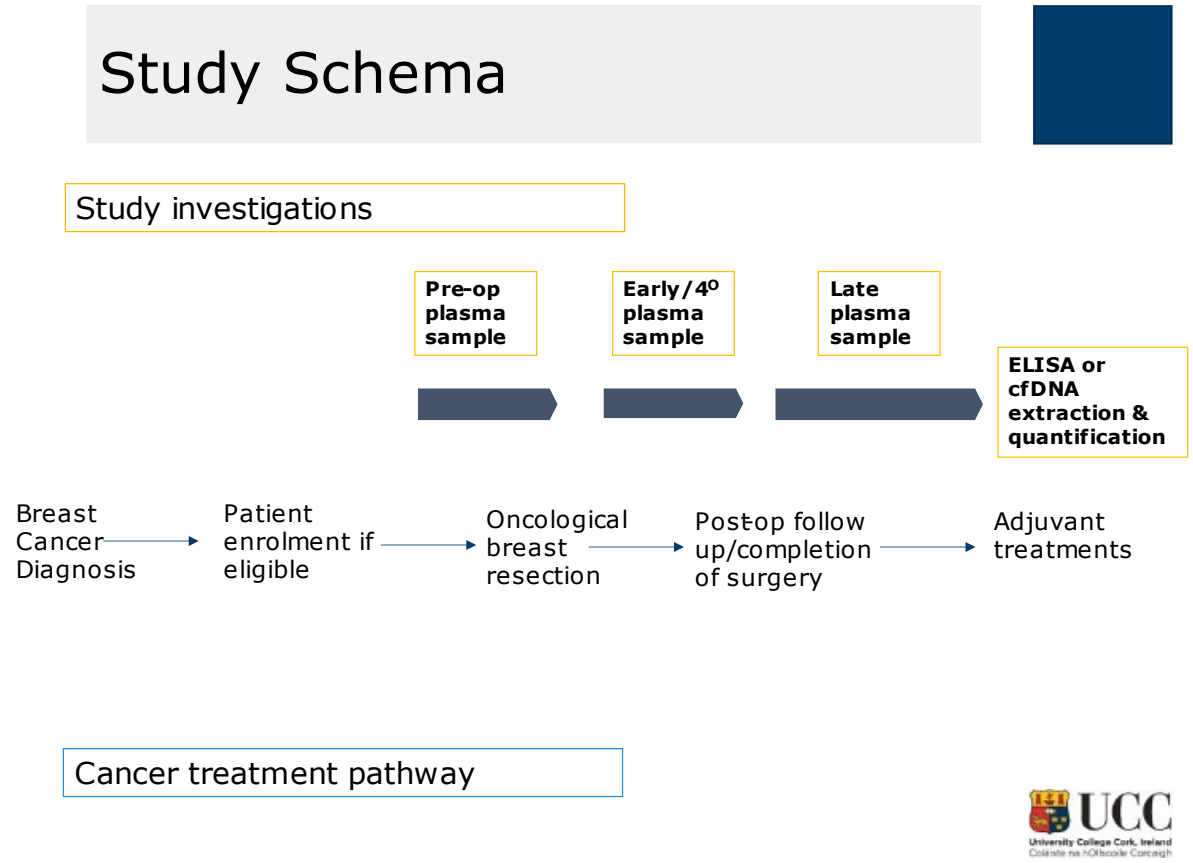


Figure 3A: Study Schema

3.3 Sample Storage

Plasma and tissue samples were stored at -80°C in a dedicated biobank freezer housed in a well-ventilated room containing generator backup power. Freezers were monitored 24/7 using internal and external temperature probes, to ensure the safe preservation of samples and secure alarm system. An automatic emergency call is made to one of three full-time laboratory staff members if a freezers temperature rises above -75°C so that freezer problems can be assessed and any problems rectified. Aliquots were stored in 500µL and 1500 µL vials and labelled with time/date and trial code. Freezer opening time was restricted to prevent samples being exposed to freeze/thaw cycles. Once extracted, cfDNA samples were stored in -20°C dedicated freezer.

3.4 Systemic Inflammatory Mediator Analysis

IL-6, IL-8, TNF- α, and IL1 β were measured using electrochemiluminescence with the Human Pro-Inflammatory Panel I multispot assay manufactured by MSD (Meso Scale Discovery)® (Gaithersburg, Maryland, USA) using the steps outlined in Figure 3B. A panel of 5 cytokines, IL-6, IL-8, TNFα, IL-10 and IL1-β, were measured in blood plasma samples following manufactures instructions. Antibodies were incubated with unique Linkers at room temperature for 30 min and at 4 °C overnight. U-PLEX 96-well plates were coated with the Linker-coupled Antibodies and incubated at room temperature for 1 hr while shaking at 1000 rpm. Plates were washed three times with PBS-T (PBS with 0.05% Tween-20). A volume of 25 µL of undiluted plasma samples, calibrators, and dilution buffer (blank), each in duplicate, were added to individual wells on the sample plate which were pre-filled with 25 µL of dilution buffer and incubated at room temperature for 1 h while shaking at 1000 rpm. Each plate was washed three times with PBS-T and reconstituted with read buffer prior to analysing on the MSD Sector Imager. The MSD Discovery Workbench 4.0 12 software (LSR_4_0_12) produced a curve using a four-parameter logistic regression model with 1/y2 weighting to determine cytokine concentration. An inter-assay analysis was performed to ensure inter-assay plate variability was <15%.

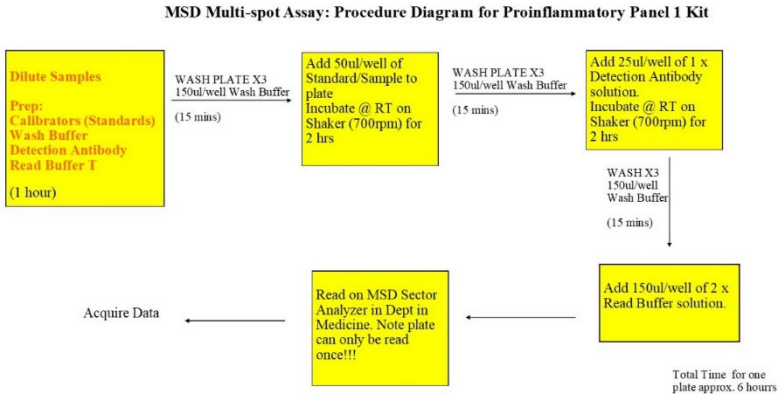


Figure 3B: Procedure Diagram for MSD Multi-Spot Assay

3.5 RNA Purification from Tumour and Normal Adjacent to Tumour Matched Samples

RNA purification was performed using the Qiagen RNEasy Mini protocol. The RNeasy kits are designed to purify RNA from small amounts of starting material. Samples are **first lysed and homogenised** in the presence of a highly denaturing guanidine-thiocyanate containing buffer, which immediately inactivates RNAses to ensure purification of intact RNA. **Ethanol is added to provide appropriate binding** conditions and the sample is then applied to an RNeasy mini spin column where the **total RNA binds to the membrane** and contaminants are washed away. **RNA is then eluted in 30-100ul of water**. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified. The procedure provides an enrichment for messenger RNA since most RNAs<200 nucleotides are selectively excluded. Figure 3C.

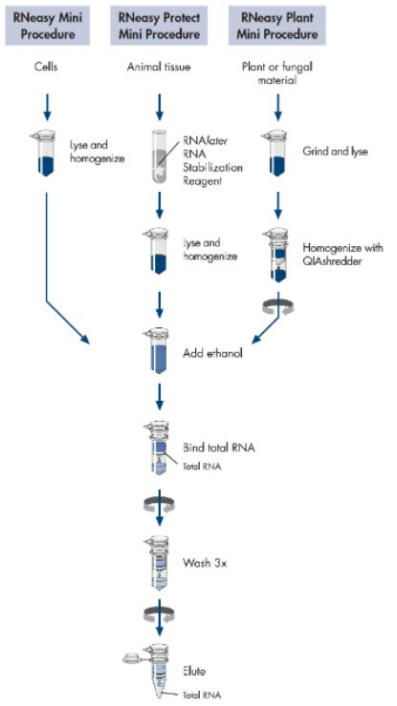


Figure 1. RNeasy Mini, RNeasy Protect Mini, and RNeasy Plant Mini procedures.

Figure 3C: Qiagen RNEasy Purification Protocol

RNEasy Protocol: Purification of Total RNA from Frozen Human Tissues

1. Disruption and homogenisation using a rotar-stator homogeniser
2. If using the entire portion of tissue, place into a suitably sized vessel for disruption (15ml tube)
3. Add the appropriate volume of **Buffer RLT** (See table below)

Table 8. Volumes of Buffer RLT for Tissue Disruption and Homogenization

Amount of starting material (mg)	Volume of Buffer RLT (µl)
<20	350 or 600*
20–30	600

* Use 600 µl Buffer RLT for tissues stabilized in RNAprotect Tissue Reagent or for difficult-to-lyse tissues.

4. Disrupt and homogenise using a conventional rotor-stator homogeniser until it is uniformly homogenous (usually 20-40secomds)
5. Centrifuge the lysate for 3 mins at full speed at >8000xg(>10,000rpm).
6. Carefully remove the supernatant by pipetting and transfer to a new microcentrifuge tube.
7. **Add 1 volume of 70% ethanol to the lysate** and mix immediately by pipetting. **1 volume= equal quantity to the volume of the sample!**
8. **Transfer upto 700 ul** (volumes may be less than 350ul due to homogenisation) of the sample including any precipitate that may have formed to an RNeasy spin column and place in a 2ml collection tube. Close lid and centrifuge for **15s** at >8000xg(>10,000rpm). Discard the follow through.
9. Reuse collection tube. **Add 700ul Buffer RW**1 to the RNeasy spin columns. Close lid and centrifuge as above for **15s.** Discard follow through
10. **Add 500ul Buffer RPE** to spin column. Close the lid and centrifuge centrifuge as above for **2 minutes**. Discard follow through
11. Place the RNeasy spin columns in a fresh 2ml collection tube and centrifuge for 1 minute.
12. Place the RNAeasy spin column in a new 1.5ml collection tube. Add 50ul of RNAase free water directly to spin column. Centrifuge for 1 min to elute the RNA.
13. If the expected RNA yield is >30ug repeat step 12 using another 20-50ul RNAse free water. Re-use the same collection tube.
14. Quantify using spectrophotometer and record 260/280 values.

3.6 Whole Transcriptome Sequencing and Gene Set Enrichment Analysis(GSEA):

Total RNA was isolated from tumour and tumour adjacent (normal) tissue from 12 patients (24 samples) with breast cancer using the Qiagen RNEasy Mini kit as per manufacturer's instructions. RNA integrity was analysed using the Agilent Bioanalyzer 2100 and only RNA samples with an RNA Integrity Number >/= 7 were

included in downstream analysis, this totalled to 19 samples. This quality control process was conducted by Biomarker Technologies (BMKGENE). RNA sequencing preparation involved using the Hieff NGS® C143P1 Ultima Dual-mode mRNA Library protocol. Samples were sequenced on the Illumina NovaSeq 6000 platform (San Diego) next generation sequencing platform with 150-bp paired-end reads to an average depth of 20 Million pair reads (6Gb data) per sample. The version of the reference genome was GRCh38 (release95) and HISAT2 which uses a Burrows-Wheeler Transform and Ferragina-Manzini(FM) index-based search was used to align reads to the genome.

Bioinformatical support was supplied by BMKGENE using the DeSeq2 differential expression workflow. The DeSeq2 RNAseq differential expression workflow starts with FASTQ files and aligns these reads to the reference genome. For experiments with biological replicates, differential expression analysis was processed using DESeq2 software package with a sequencing depth of 10-30M reads per library**.** The DESEQ2 software uses raw integer read counts to normalise the count data correcting for differences in library size and sequencing depth. Gene expression values are then prepared as a count table by counting the sequenced fragments and gene expression analysis is performed. Differences in gene expression were presented as Log2 Fold Change (Log2FC) representing the difference of two values [197]. Statistical difference was expressed as Fold Change(FC)≥2 and False Discovery Rate (FDR)<0.01.

FastQ files were analysed and verified by Dr Cathriona Foley using the EdgeR software package. The Molecular Signatures Database Hallmark adipogenesis gene set (“Hallmark_adipogenesis”) of gene set variant analysis was used to quantify adipogenesis using the Gene Set Variation Analysis Bioconductor package. In the GSEA, statistical significance was defined as false discover rate (FDR) less than 25%, recommended by GSEA software. GSEA was performed by Dr Cathriona Foley.

3.6.1 Inflammatory mediator analysis

In Chapter four systemic IL6 and IL10, were measured using the Biolegend ELISA. Samples were analysed in duplicates. An inter-assay analysis was performed to ensure inter-assay plate variability was <15%. Correlation analysis was performed using Pearson correlation calculations to assess the direct relationship between plasma IL6/IL10 levels and IL6/IL10 normalised RNA seq counts using FPKM (Fragments per kilo base per million mapped) in tumour and NAT. For each analysis, a correlation coefficient, r, was calculated (with 95% confidence intervals and p-value). r values were categorised as follows: r=1, a perfect correlation; r= 0-1 variables change in the same direction; r=0, no relationship between variation in each variable; r=-1-0, an inverse relationship exists; r=-1, a perfect inverse relationship exists.

3.6.2 Statistical Analysis

Statistical analysis was performed using SPSS, version 28 and GraphPad PRISM, version 8.4.2. Graphs were generated using GraphPad PRISM, version 8.4.2. For demographic and clinicopathological data, categorical data were described by their counts and percentages in each. Descriptive analysis was used to depict baseline characteristics. Mann–Whitney U test and Fisher's test were used to calculate *p* value in group-comparison analysis. An analysis of variance (ANOVA) was used to compare mean cytokine values peri-operatively followed by multiple comparison analysis using Bonferroni. ANOVA and multiple comparison analysis was also used to compare mean cytokine values between breast conserving surgery and mastectomy cohorts. Student T test or Mann Whitney tests were used to determine differences in peri-op ctDNA and cfDNA detection. Chi-squared or Fisher's exact test (ranksum) were carried out to assess associations between ctDNA/cfDNA detection and clinical variables. Binary logistic regression analysis was performed to predict the likelihood of ctDNA positivity based on dichotomous clinical outcomes including LVI, lymph node positivity, tumour grade 3 and tumour size >20mm. Correlation between continuous variables was analysed using Pearson correlation. For each analysis, a correlation coefficient, r, was calculated (with 95% confidence intervals and p-value).

r values were categorised as follows: r=1, a perfect correlation; r= 0-1 variables change in the same direction; r=0, no relationship between variation in each variable; r=-1-0, an inverse relationship exists; r=-1, a perfect inverse relationship exists. Correlations were graphically represented using Manhattan plots. Linear regression models were produced to generate the line of best-fit following correlation analyses. The association between cell-free DNA (cfDNA) and DFS were analysed using Mantel Cox log-rank test and Kaplan–Meier curves generated. For all of the above analyses, statistical significance was observed at $p < 0.5$.

3.7 Cell Free DNA Extraction

Cell-free DNA (cfDNA) was extracted using the QIAamp Circulating Nucleic Acid kit (Qiagen, Cat. No. / ID: 55114) via the four processes of lysis, binding, washing and elution (Figure 3D).

1. Lysis: Free-circulating nucleic acids in biological fluids are usually bound to proteins or enveloped in vesicles, requiring an efficient lysis step to release nucleic acids for selective binding to the QIAamp Mini column. Hence, samples are lysed under highly denaturing conditions at elevated temperatures in the presence of proteinase K and Buffer ACL, which together ensure inactivation of DNases and RNases and complete release of nucleic acids from bound proteins, lipids, and vesicles.
2. Binding: To ensure optimal binding of the circulating nucleic acids to the silica membrane (the QIAamp Mini membrane), buffer ACB is added to the lysate. The lysate is then transferred onto a QIAamp Mini column, and circulating nucleic acids are adsorbed from a large volume onto the small silica membrane as the lysate is drawn through by vacuum pressure. Salt and pH conditions ensure that proteins and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp Mini membrane. A vacuum manifold (e.g., the QIAvac 24 Plus with the QIAvac Connecting System) and a vacuum pump capable of producing a vacuum of –800 to –900 mbar (e.g., QIAGEN Vacuum Pump) are required for

the protocol. A vacuum regulator should be used for easy monitoring of vacuum pressures and convenient vacuum release.

3. Washing: cfDNA remain bound to the membrane, while contaminants are efficiently washed away during 3 wash steps.
4. Elution: In a single step, highly pure circulating nucleic acids are eluted in Buffer AVE, equilibrated to room temperature. Elution of pure nucleic acids Elution is performed using 100ul of Buffer AVE.

The Protocol "Purification of Circulating Nucleic Acids from 5 mls of Plasma" was utilised. Samples were mixed with PBS to bring the total sample volume to 5mls if required. 500ul of proteinase K and 4mls of buffer ACL (containing 1 ug carrier RNA) were added to the samples and incubated at 60degree Celsius for 30 minutes. 9mls of buffer ACB was added to the lysate and incubated on ice for 5 mins. The samples were then transferred into QIAamp Mini columns via tube extenders which allowed the lysate to pass through the QIAamp Mini Column Silica membrane via vacuum. Buffer ACW1, buffer ACW2 and ethanol were added to the QIAamp mini columns sequentially. The mini columns were centrifuged at 20,000 g for 3 minutes and then incubated at 56degree Celsius on a heat block for 10 minutes to dry the membranes completely. CfDNA was finally eluted with 100Ul of AVE buffer, labelled and stored in -20degree Celsius freezer. CfDNA samples were exposed to one freeze thaw cycle for quantification purposes.

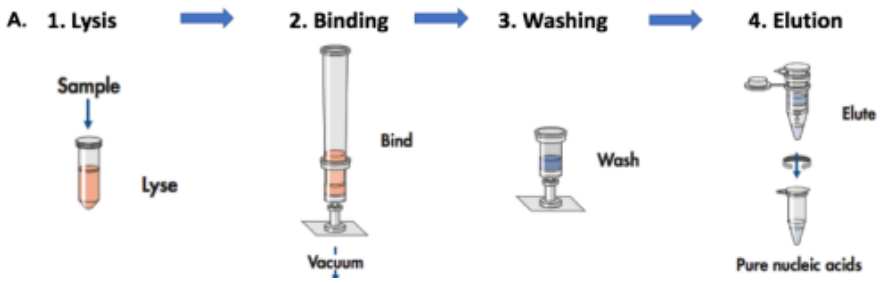


Figure 3D: Cell Free DNA Extraction Work Flow

3.8 CfDNA Quantification

Traditionally cfDNA was quantified using a spectrophotometer. This technology is based on Beer-Lambert Law which is the concept that biomolecules absorb light at characteristic wavelengths. Measurement of light absorption, by a spectrometer, is used to detect compounds and to measure their concentration in solution. Accuracy is a major concern in spectrophotometry as dsDNA, ssDNA, RNA, free nucleotides and primers cannot be discriminated from each other in a sample**.** RNA contamination in a DNA sample leads to overestimation of DNA concentration by UV spectrophotometry[198].

Fluorometry or spectrofluorometry is another type of electromagnetic spectroscopy which measures the light emitted from fluorogenic molecules called fluorophores. Fluorescence based quantitation methods take advantage of this mechanism by employing dye molecules that have been designed to preferentially bind to selected nucleic acids. This type of assay requires a dilution series of known concentration to create a standard curve. Compared to UV spectrophotometric methods, fluorescence is more specific as it is less affected by other components present in samples[198].

CfDNA samples were quantified using the Denovix DS 11-FX Spectrofluorometer (Figure 5) and the DeNovix dsDNA High Sensitivity Quantification Assay. This assay creates a two point-standard curve which covers a range of 250 pg/µL to 1500 ng/ µL. CfDNA samples were brought to room temp and then vortexed briefly to minimize reagent loss on the cap. To prepare the working solution 10mls of the assay buffer was added with 100µl of the dye.

For each standard and unknown sample, 190µl of the working solution was added to a labelled tube. 10ul of standards and 10µl of unknown samples were added to the tubes and mixed well. After being incubates at room temperature for 5 mins. Standards were measured from low concentrations to high and a standard curve was created. Once the standard curve was complete unknown samples were measured

in triplicate. A mean of the three values was then calculated as a final concentration (ng/µl).



Figure 3E: Denovix DS 11-FX Spectrofluorometer

3.9 Next Generation Sequencing

Next Generation Sequencing (NGS) of the extracted cfDNA samples was outsourced to NovoGene for paired sequencing using the Illumina Novaseq 6000 platform, employing NGS technology with the NovoPM 2.0 targeted gene panel. This panel comprises 484 genes, including the 324 genes present in the FDA-approved FoundationOne CDx gene panel, among which are the breast cancer genes of interest (Novogene Co, 2023). Targeted NGS is a powerful and highly efficient technique in the field of genomics. Unlike whole-genome sequencing, which involves sequencing an individual's entire genetic code, targeted NGS focuses on specific regions of interest, such as genes or genomic regions associated with cancer. By honing in on these specific targets it offers several advantages, including reduced sequencing costs, faster turnaround times, and the ability to detect genetic variants with high precision[199]. Figure 3F.

3.10 Library Preparation

Library preparation is a crucial step in the process of NGS, as it involves the conversion of DNA or RNA samples into a format suitable for high-throughput sequencing. This intricate process begins with fragmentation of the input genetic material, followed by the addition of unique DNA adapters to each fragment. These adapters serve as barcodes and sequencing primers, allowing for the identification of individual DNA fragments and their subsequent amplification[200]. Through various enzymatic reactions, the library preparation workflow also involves end repair, A-tailing, adapter ligation, and PCR amplification, ultimately generating a pool of DNA fragments ready for sequencing. Proper library preparation is an essential, labour-intensive step in the NGS workflow to ensure accurate, reproducible, and high-quality sequencing results. The quality of the library preparation process significantly impacts the success of downstream applications, including variant calling, gene expression analysis, and genome assembly[201].

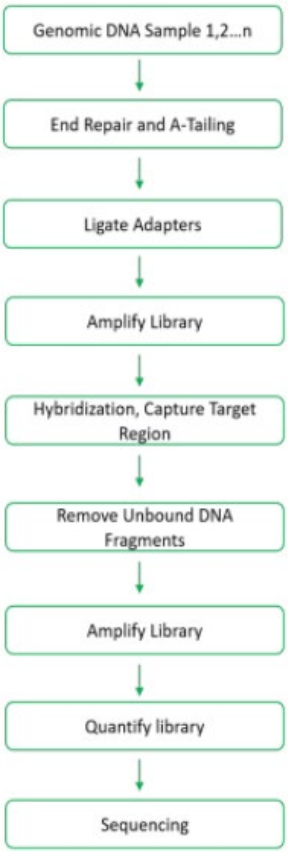


Figure 3F: Library Construction and Sequencing Workflow of NovoPM V2

3.11 Bioinformatics

Bioinformatical support was provided by Sandra Martínez Badal who analysed the data as part of her MSc in Bioinformatics and Computational Biology with UCC. The raw data files were provided by Novogene containing cfDNA sequences in FASTQ format. These files consisted of 184 raw data files, corresponding to the forward and reverse read of each patient sample at both timepoints. These raw data files served as the foundation for subsequent bioinformatic analyses, which followed a tumour-independent approach. The bioinformatic analysis pipeline involved 6 primary stages including quality control, data trimming, alignment to the Human Reference Genome GRCh38, Deduplication, Variant calling (SNP's and Indel identification) and Variant Annotation[200]. The software required for each analytical stage is outlined in Figure 3G. The variant calling step is a critical step in DNA variant discovery and this involves comparing read sequences aligned to the genome to identify variations in base pairs. The software required for this (GATK:Mutect2) specifically identifies Single Nucleotide Variants (SNV's) and small insertions and deletions (INDELs). The next important step in deciphering clinically relevant variants and evaluating their potential pathogenicity involves variant annotation using a combination of biological annotation sources including GENCODE, ClinVar and the Catalogue of Somatic Mutations in Cancer (COSMIC). When an allele frequency threshold >1% was established across the three separate population databases within the ClinVar dataset the sample was considered ctDNA positive.

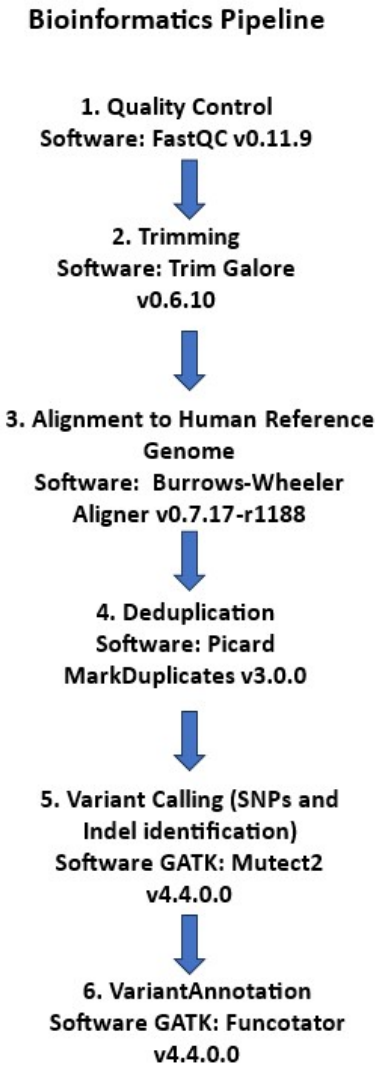


Figure 3G: Bioinformatics pipeline

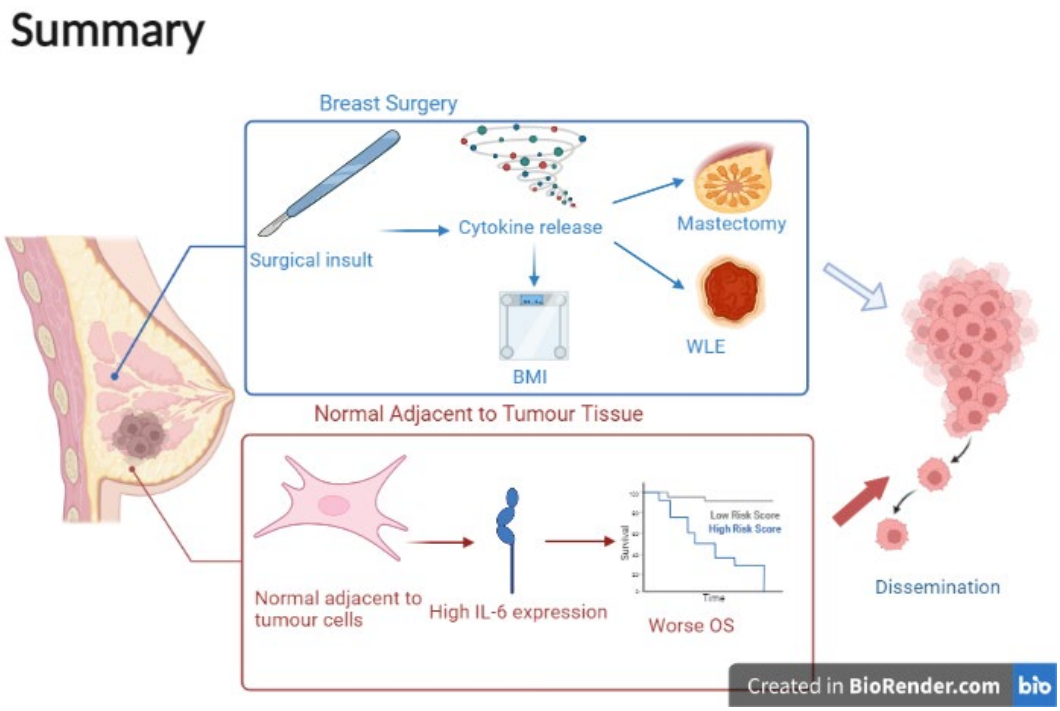


Figure 3H: Summary of study schema for chapter 4 and chapter 5.

Chapter 4: Investigating the Peri-Operative Trend of Inflammatory Mediators in Breast Cancer and Association with Clinicopathological Features

4.1 Abstract: Investigating the Peri-Operative Trend of Inflammatory Mediators in Breast Cancer and Association with Clinicopathological Features

Introduction

Pre-clinical research has suggested that the surgical insult can promote the growth of new metastases and facilitate seeding of micrometastases into the circulation. Cytokine level elevation proportional to the magnitude of surgical stress has been described. The aim of this study was to define the peri-operative dynamics of trend of IL-6, IL-8, TNF- α, IL1 β and IL-10 in patients with early-stage breast cancer and their association with tumour characteristics, surgical intervention and post-operative morbidity.

Methods

Patients presenting for non-reconstructive breast cancer surgery, with non-metastatic disease, aged 18-85 years of age were screened for inclusion. Extensive exclusion criteria were applied to exclude patients with underlying inflammatory disorders, active infection or undergoing neoadjuvant chemotherapy. Plasma samples were drawn pre-operatively, 4 hours post operatively and 2 weeks post operatively. Statistical analysis was performed using SPSS, version 28 and GraphPad PRISM, version 8.4.2.

Results

Samples from thirty-eight patients were included in the analysis. Median age was 61(range 43-79). All patients had stage I-III disease and 89% (n=34) of patients had invasive ductal carcinoma (IDC). IL-6, IL-8 and IL-10 levels peaked early post-operatively and returned to near baseline levels at 2 weeks post-operatively ($p = <0.05$). Early post-operative IL-6 levels were lower in patients who had breast conserving surgery compared to patients who had a mastectomy ($p = <0.01$). A significant positive correlation was identified between age and peri-operative IL-6

levels at all time points. Early post-operative IL-6 correlated with increasing tumour stage ($p = 0.048$). There was also a correlation between post-operative IL-8 with lobular or mixed (lobular/ductal) breast cancer subtypes. A significant linear relationship was found between BMI and early and late post-operative IL-6 levels. Only pre-operative TNF-α values correlated positively with BMI ($p = 0.027$). An inverse relationship was observed between pre-operative IL-10 and lymph node positivity ($r=-0.642$, $p = 0.033$)

Conclusion

This is the first study to define the trend of peri-operative systemic inflammatory mediators in breast cancer surgery. Cytokine elevation appears to correlate with tumour characteristics and the magnitude of surgery.

4.2 Introduction

Despite excellent advances in cancer treatment approaches, breast cancer remains the most common cause of cancer mortality amongst women globally [202]. Patients with breast cancer are considered to have a significant impairment in their immune system possibly due to inhibitory cytokines from the tumour microenvironment enabling tumour cells to escape immune surveillance and evade apoptosis [203, 204]. These systemic mediators of inflammation are also believed to confer resistance to chemo-radio therapy[205]. Cytokines are secreted principally by lymphocytes and macrophages and act by altering the function of target cells. Cytokines have several common structural and functional characteristics: (a) they tend to be glycosylated polypeptides; (b) they are biologically active at very low concentrations (pg/mL–ng/mL; (c) they exert their effects by binding to receptors on the target cell surface; (d) they are pleiotropic; and (e) their effects are additive, synergistic or antagonistic [55]. In addition to their effects on the immune system, cytokines have also been reported to influence aromatase activity and oestrogen synthesis in the tumour microenvironment and hence promote breast cancer tumour growth and aggressiveness[206]. Cytokines, chemokines and pro-angiogenic factors in the tumour microenvironment (TME) are mainly secreted by tumour cells themselves and/or other stromal cells[207].

Breast surgery is still considered the cornerstone of breast cancer treatment and the type of surgery depends on tumour size, nodal positivity, and genetic predisposition[2]. Despite curative surgery and optimal treatment, distant metastatic disease can occur in up to 25% of patients with breast cancer [3]. Pre-clinical research has suggested that the surgical insult can promote the growth of new metastases and facilitate seeding of micrometastases into the circulation [4, 5]. The exact mechanism underlying the pro-metastatic potential of the peri-operative period is yet to be fully determined but the effect seems to be exacerbated by stress inflammatory responses to surgery [6]. Cytokines released into the circulation in response to tissue injury may perpetuate the dissemination of tumour cells therefore a thorough understanding of the key inflammatory mediators in the peri-operative period is paramount to facilitate future therapeutic interventions.

IL-8 is an important inflammatory chemokine with autocrine and/or paracrine tumour-promoting potential. Clinical studies have shown that increased serum IL-8 levels are associated with positive lymph node status and higher-stage breast tumours [208]. TNF-α is one of the essential pro-inflammatory cytokines found in the TME of breast cancer patients, being secreted both by stromal cells, such tumour-associated macrophages (TAM), and by the cancer cells themselves [209]. A recent review reported a positive correlation between TNF-α expression (in the tumour or in plasma/serum) and poor oncological outcomes in patients with oestrogen receptor positive and triple negative breast cancer subtypes [210]. Similarly, IL1 β orchestrates tumour-promoting inflammation in breast cancer and is associated with advanced disease stage in breast cancer [211]. IL-10 on the other hand is a unique pleiotropic cytokine with both immunomodulatory and antiangiogenic functions [212]. IL-10 is known as the cytokine synthesis inhibitory factor due to its inhibitory role on IL-1α, IL-1β, IL-6, IL-8, IL-12, IL-18 and IL-10 itself, as well as TNF-α and granulocyte macrophage-colony-stimulating factor in T cells and macrophages[213]. However, IL-10 is also able to convert tumour associated macrophages (TAMs) to polarized type 2 (M2 macrophages) that promote cancer progression [214]. In essence, IL-10 has both anti-tumour effects via inhibition of tumour promoting inflammatory mediators and tumorigenic effects by promoting tumour angiogenesis[215, 216].

One of the most abundant cytokines is the pro-inflammatory Interleukin-6(IL-6) released by macrophages, B cells, T cells and fibroblasts in the tumour microenvironment and plays a critical role in the expansion and differentiation of tumour cells[217]. The neoplastic potential of IL-6 is suggested by elevated levels observed in the development and progression of many cancers including breast, pancreas, gastrointestinal tract, lung, ovary, prostate, and others [218]. Indeed, the concentration of serum IL-6 seems to correlate closely with breast cancer stage, tumour burden and the extent of tissue trauma/injury severity [219] [220]. IL-6 levels increase after surgery due to tissue insult and postoperative serum IL-6 elevations that are proportional to the magnitude of the surgical stress have been described [85].

Here, we review the peri-operative trend of some the more clinically relevant cytokines including IL-6, IL-8, TNF- α, IL1 β and IL-10 in patients with breast cancer and their association with tumour characteristics and surgical intervention.

4.3 Aims & Objectives

In this study, we aimed to better understand the relationship between peri-operative systemic inflammation and breast tumour characteristics, surgical intervention, and 30-day morbidity.

Objective 1: Define the peri-operative trend of systemic mediators of inflammation (IL-6, IL-8, TNF- α, IL1 β and IL-10) in breast cancer patients.

Objective 2: Investigate associations between peri-operative inflammatory mediators and clinicopathological features, wound infection rates and 30-day post-operative morbidity.

4.4 Methodology

4.4.1 Patient Recruitment, Treatment & Follow-up

To analyse the relationship between peri-operative systemic inflammation and breast tumour characteristics, a homogenous group of patients without a diagnosis of underlying acute/chronic inflammatory disorders or current/recent anti-inflammatory/immunomodulator use was recruited. Patients undergoing breast cancer surgery and treatment at our institute are being actively recruited to the ethically approved ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study. This study is sponsored by Cancer Trials Ireland (NCT01840293) and open across Irish centres. This study is a sub-study of the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity, and sample collection is within the remit of the biospecimen protocol.

Patients presenting for elective breast cancer surgery, without known metastatic disease, aged 18-85 years of age were screened for inclusion. Extensive exclusion criteria were applied as follows: received neoadjuvant chemotherapy or hormonal therapy, undergoing reconstructive breast surgery, pregnant or lactating; evidence of underlying liver disease (abnormal LFT's (> x2 normal), INR > 1.5); evidence of underlying renal disease (creatinine >180 for women, >150 for men); blood dyscrasia (neutropenia < 1500 cells /cm3, thrombocytopenia < 100,000 cells/cm3); metastases, morbid obesity (body mass index >40 kg/m2); American Society of Anaesthesiologists (ASA) physical status classification system score >III; coexisting active inflammatory disorder (including active Rheumatoid Arthritis, Inflammatory Bowel Disease, Systemic Lupus Erythematosus); corticosteroids usage; immunosuppressive drugs; previous diagnosis of HIV; chronic active Hepatitis B or C; active infection at the time of surgical intervention. Breastcheck screening patients were not included as they have their post-op review off site.

All patients with breast cancer were discussed at the regional Multi-Disciplinary Meeting (MDM) and managed in keeping with National Cancer Control Programme (NCCP) recommendations. In this institution all patients with breast cancer are routinely screened for axillary nodal disease using ultrasonography +/- fine needle aspiration (FNA) of indeterminate or suspicious nodes. Axillary lymph node dissection is recommended to patients with positive axillary nodal disease receiving upfront surgery. All patients with radiologically/clinically negative axillary lymph node disease have a sentinel lymph node biopsy procedure with Tehnetium - 99m sulfur colloid injected the morning of surgery. Image guided breast conserving surgery is performed using radiological placed guide wires inserted the morning of surgery or a Radiofrequency Identification Device (RFID) tag localisation placed 1 to 2 weeks prior to surgery. Tumour phenotype was recorded from final histopathological reports. Oestrogen receptor/progesterone receptor were considered positive when >10% of cells showed positive staining. HER2 status was assessed by immunohistochemistry (IHC) and Fluorescent in Situ Hybridization (FISH) confirmation in cases with 2+ by IHC. HER2 positivity was defined as either 3+ with IHC and/or amplified with FISH.

4.4.2 Sample Biobanking & Transfer

Blood samples were phlebotomised peri- operatively from patients at the following time-points– pre-operatively the morning of surgery (pre-op), 3-5 hours post-operatively (early post-op), 10-14 days post-operatively (late post-op) (Figure 1). These time points were selected with patient convenience in mind as they coincided with usual clinic appointment dates. Patients with positive invasive margins or large volume nodal disease requiring further surgery had their 2-week post op sample deferred until 2 weeks after surgery is complete. Plasma samples were stored in K3EDTA bottles and centrifuged at 2500 rpm for 15 mins within 1 hour of phlebotomy. Alternatively, samples were stored in Streck tubes and centrifuged at 2500rpm for 15 mins within 48hours of phlebotomy. Plasma samples were aliquoted into collection tubes (500 uL and 1500 uL), labelled with trial codes and stored at -80 degree Celsius.

4.4.3 Inflammatory Mediator Analysis

IL-6, IL-8, TNF- α, and IL1 β were measured using electrochemiluminescence with the Human Pro-Inflammatory Panel I multispot assay manufactured by MSD (Meso Scale Discovery)® (Gaithersburg, Maryland, USA) using the steps outlined in Figure 1. A panel of 5 cytokines, IL-6, IL-8, TNFα, IL-10 and IL1-β, were measured in blood plasma samples following manufactures instructions. Antibodies were incubated with unique Linkers at room temperature for 30 min and at 4 °C overnight. U-PLEX 96-well plates were coated with the Linker-coupled Antibodies and incubated at room temperature for 1 hr while shaking at 1000 rpm. Plates were washed three times with PBS-T (PBS with 0.05% Tween-20). A volume of 25 µL of undiluted plasma samples, calibrators and dilution buffer (blank), each in duplicate, were added to individual wells on the sample plate which were pre-filled with 25 µL of dilution buffer and incubated at room temperature for 1 h while shaking at 1000 rpm. Each plate was washed three times with PBS-T and reconstituted with read buffer prior to analysing on the MSD Sector Imager. The MSD Discovery Workbench 4.0 12 software

(LSR_4_0_12) produced a curve using a four-parameter logistic regression model with 1/y2 weighting to determine cytokine concentration. An inter-assay analysis was performed to ensure inter-assay plate variability was <15%.

4.4.4 Outcome Analysis

Statistical analysis was performed using SPSS, version 28 and GraphPad PRISM, version 8.4.2. Graphs were generated using GraphPad PRISM, version 8.4.2. For demographic and clinicopathological data, categorical data were described by their counts and percentages in each. Continuous variables were described by their medians and interquartile ranges.

4.4.5 Associations between Systemic Mediators of Inflammation, Tumour Characteristics and Surgical Intervention

An analysis of variance (ANOVA) was used to compare mean cytokine values peri-operatively followed by multiple comparison analysis using Bonferroni. ANOVA and multiple comparison analysis was also used to compare mean cytokine values between breast conserving surgery and mastectomy cohorts. Correlation analysis was performed using Pearson correlation calculations to assess the direct relationship between tumours characteristics (ie lymphovascular invasion, grade, lymph node status, subtype) and inflammatory mediators. For each analysis, a correlation coefficient, r, was calculated (with 95% confidence intervals and p-value). r values were categorised as follows: r=1, a perfect correlation; r= 0-1 variables change in the same direction; r=0, no relationship between variation in each variable; r=-1-0, an inverse relationship exists; r=-1, a perfect inverse relationship exists. Correlations were graphically represented using Manhattan plots. Linear regression models were produced to generate the line of best-fit following correlation analyses. For all of the above analyses, statistical significance was observed at p <0.5.

4.4.6 Clinical & Oncological Outcomes

The association between inflammatory mediators and 30-day post-operative wound complication rates were reported using odds ratios (OR), 95% confidence intervals and p-values generated with chi-square (χ^2) testing. **Wound complication referred to any wound complication that required post-operative intervention ie haematoma, wound dehiscence, antibiotics, or infected seroma.** Post-operative wound infection referred to any positive wound swab/ fluid culture. This criterion was adopted due to practical limitations in collecting surgical site infection rates defined by the centre for disease control and prevention that did not require specific interventions during the covid pandemic, as many patients avoided hospital and preferentially sought care from their general practitioner.

4.5 Results

4.5.1 Demographics & Clinicopathological Data

Thirty-eight patients who underwent breast cancer surgery and fulfilled the inclusion criteria over a 14-month period were analysed. Sample collection occurred during the Covid-19 pandemic. The patient demographic, clinical and tumour characteristics are outlined in Table 1. Median age was 61 (IQR 25.5). Twenty-three patients (61%) had Wide Local Excision (WLE) and Sentinel Lymph Node Biopsy (SLNB), three patients (8%) had WLE and Axillary Lymph Node Dissection (ALND), eight patients (21%) had mastectomy and SLNB, and four patients (10%) had mastectomy and ALND. The majority of patients had Invasive Ductal Carcinoma (IDC) breast cancer subtype (89%), three patients had Invasive Lobular Carcinoma (ILC) (8%) and one patient had a mixed lobular/ductal subtype. Half of the patient cohort had lymph node positive disease (including micro or macro-metastatic lymph node involvement). There were 34% (n=13) who had T1 stage tumours, 55% (n=21) had T2 stage tumours and 11%(n=4) had a T3 stage tumour. Regarding tumour hormone expression, 80% (n=30) were ER+/HER2-, three patients had ER+/HER2+ tumours (8%), 1 patient (2%) had a ER-/HER2+ tumour and 10% (n=4) had triple negative breast cancer. Three patients had grade 1 tumours (8%), 50% (n=19) had a grade 2 tumour and 42% (n=16) had

grade 3 tumours. Lymphovascular invasion (LVI) was present in 45% (n=17) of tumours and absent in 55% (n=21). The median BMI for all patients was 28 (IQR 7).

Table 1: Patient Demographics and Cinicopathological Data of Included Patients

Patient Demographic, Clinical and Tumour Characteristics	**N=38**
Age	Median 61 (range 43-79, IQR 25.5)
Surgery	N (%)
Wide Local Excision & Sentinel Lymph Node Biopsy	23 (61%)
Wide Local Excision & Axillary Lymph Node Dissection	3 (8%)
Mastectomy & Sentinel Lymph Node Biopsy	8 (21%)
Mastectomy & Axillary Lymph Node Dissection	4 (10%)
Subtype	
Invasive Ductal Carcinoma (IDC)	34 (89%)
Invasive Lobular Carcinoma (ILC)	3 (8%)
Mixed	1 (3%)
Lymph node status	
Positive	19 (50%)
Negative	19 (50%)
Tumour Size	
T1	13 (34%)
T2	21 (55%)
T3	4 (11%)
T4	0

Hormone expression	
ER+/HER2 -	30 (80%)
ER+/HER2+	3 (8%)
ER-/Her2 +	1 (2%)
TNBC	4 (10%)
Grade	
1	3 (8%)
2	19 (50%)
3	16 (42%)
LVI	
Present	17 (45%)
Absent	21(55%)
BMI	Median 28 (IQR 7)

WLE= Wide Local Excision, SLNB = Sentinel Lymph Node Biopsy, ALND = Axillary Lymph Node Dissection, IDC = Invasive Ductal Carcinoma, ILC= Invasive Lobular Carcinoma, ER = Oestrogen Receptor, HER2 = Human Epidermal Growth Factor Receptor 2, TNBC = Triple Negative Breast Cancer, LVI = Lymphovascular Invasion, BMI = Body Mass Index, IQR = InterQuartile Range

4.5.2 Peri-Operative Trend of Inflammatory Mediators

Peri-operative systemic mediators of inflammation were normally distributed using visual inspection of the Q-Q plots and rejection of the null hypothesis using Kolmogorov-Smirnov test. Peri-operative IL-6, IL-8, and TNF-α values were analysed in thirty-eight patients. Median IL-6 levels peaked from a baseline of 0.2283 pg/mL to 6.206 pg/mL four hours post-op and returned to above baseline levels at 2 weeks post op (median value 0.486 pg/mL, p = <0.001). Similarly, IL-8 levels significantly changed peri-operatively with a peak seen early post-operatively (median value 6.752 pg/mL, p = <0.05). There was no statistical change in TNF-α levels peri-operatively. IL-

1β was analysed in 75 samples (N=25) and was below the detection range in 73 samples. As IL-1β was poorly detectable peri-operatively a custom assay with IL-10 was used to measure IL-10 trend peri-operatively (N=13). IL-10 values increased significantly early post-op (3.716pg/mL) and returned to baseline late post-op (p = <0.5). Figures 4A-E.

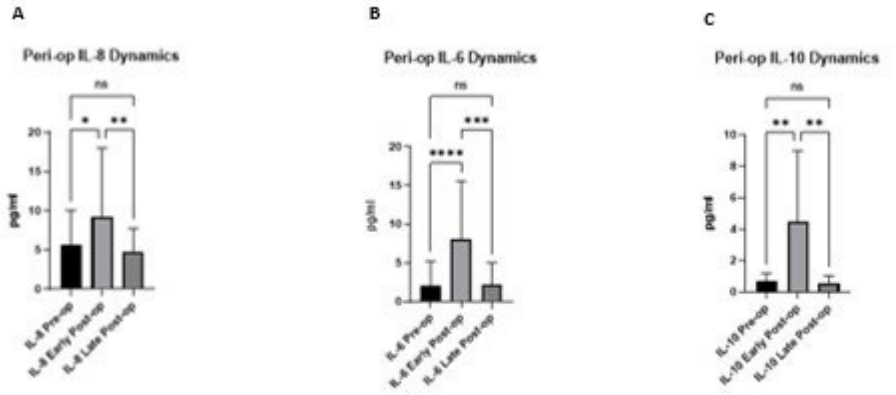


Figure 4D

Figure 4E

Figure 4A-E: Peri-Operative Systemic Cytokine Dynamics A. IL-8 B. 1L-6 C. IL-10 D. TNFa E. IL1b ***p<0.001, **p<0.01, *p<0.05

4.5.3 Surgical Approach & Inflammatory Mediators

Early post-operative mean IL-6 values were significantly higher in patients who had a mastectomy/SLNB and compared to patients who had a WLE/SLNB (p = <0.01, Figure 4F). Similarly, IL-10 levels were significantly higher early post-op in patients who had a mastectomy compared to WLE (p = <0.001, Figure 4I). There was no significant

difference in peri-operative IL-8, IL-1β and TNF-α values according to surgical approach (Figures 4G-H).

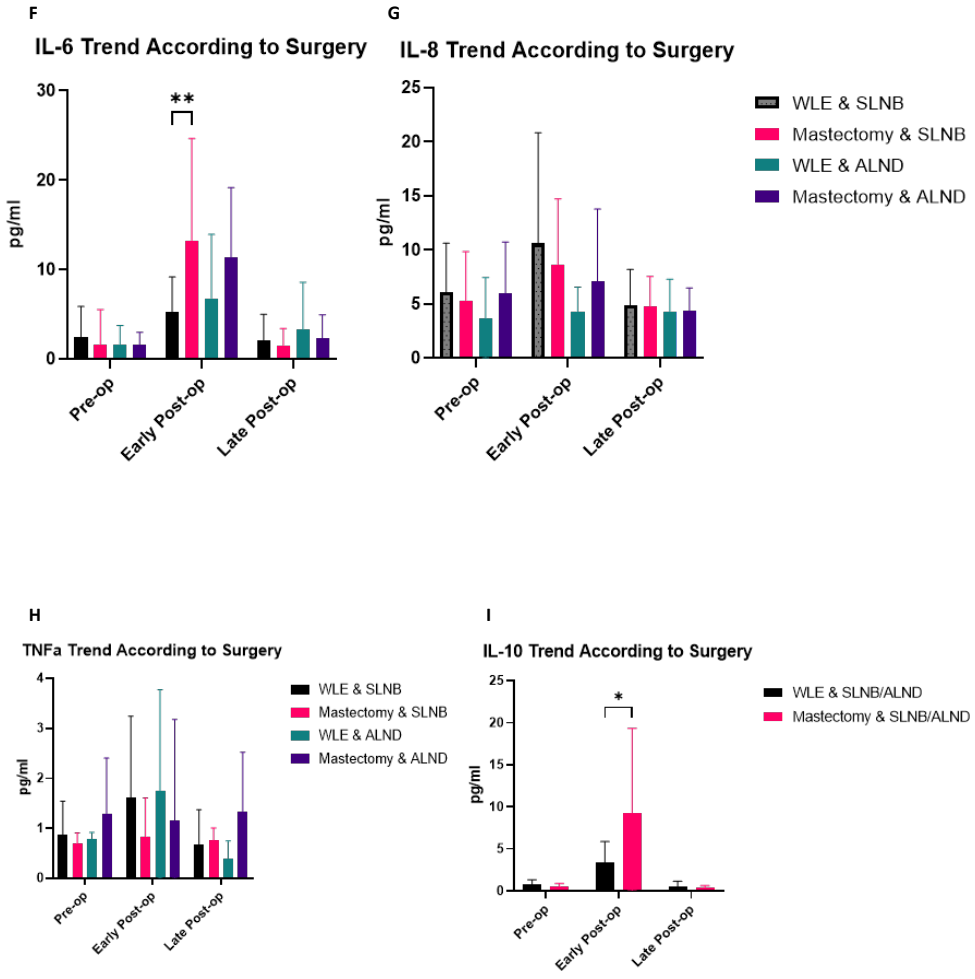


Figure 4F-I: Peri-Operative Trend According to Type of Surgery **F.** IL-6 trend peri -operatively **G.** IL-8 trend peri -operatively **H.** TNFa trend peri -operatively **I.** IL-10 trend peri -operatively. Wide local excision (WLE), Sentinel lymph node biopsy (SLNB), Axillary lymph node dissection (ALND)

***p<0.001, **p<0.01, *p<0.05

4.5.4 Inflammatory Mediators & Clinicopathological Characteristics

There was a positive correlation detected between age and late post-operative IL-6 levels (r = 0.462, p = 0.028). Early post-operative IL-6 values correlated positively with increasing tumour T stage (r=0.335, p=0.05). A significant positive relationship was found between BMI and both early and late post-operative IL-6 levels (p=0.019, p=0.009). Only pre-operative TNF-α values correlated positively with BMI (r=0.417, p=0.027). A positive correlation between post-operative IL-8 levels and lobular or mixed (lobular/ductal) breast cancer subtypes was found (r=0.5410, p=0.001). Pre-operative IL-10 levels were inversely related to lymph node positivity (r=-0.642, p=0.033). Similarly, post-operative IL-10 levels were significantly lower in patients who had DCIS associated with their primary tumour (r=-0.789, p=0.007). Pre-operative WCC and NLR values positively correlated with the presence of LVI (p=0.008, p=0.044).

Table 2:Correlations Between Peri-Operative Inflammatory Markers & Pathological Features

	Pre-op IL6	Early-PO IL6	Late-PO IL6	Pre-op IL8	Early PO IL8	Late PO IL8	Pre-op TNF	Early PO TNF	Late PO TNF	Pre-op IL1b	Early PO IL1b	Pre-op IL10	Early PO IL10	Late PO IL10	Pre-op WCC	Pre-op NLR
Age	R=0.3045 P=0.1394	R=0.1738 P=0.3256	R=0.4662 P=0.0288	R=0.1476 P=0.397	R=0.0026 P=0.9880	R=-0.0026 P=0.9880	R=0.1902 P=0.03617	R=0.0609 P=0.7823	R=-0.3068 P=0.1358	R=0.3285 P=0.2515	R=-0.00366 P=0.9915	R=0.322 P=0.335	R=-0.3705 P=0.2621	R=-0.090 P=0.833	R=-0.005 P=0.976	R=0.086 P=0.608
T stage	R=0.223 P=0.253	R=0.335 P=0.05	R=0.1558 P=0.457	R=0.1082 P=0.5360	R=0.1467 P=0.4003	R=-0.2789 P=0.1047	R=-0.072 P=0.714	R=0.1690 P=0.3899	R=-0.149 P=0.4650	R=-0.287 P=0.174	R=-0.294 P=0.222	R=-0.217 P=0.521	R=0.361 P=0.275	R=-0.370 P=0.293	R=-0.232 P=0.160	R=-0.147 P=0.379
Lymph node positivity	R=-0.070 P=0.722	R=-0.072 P=0.685	R=-0.020 P=0.923	R=-0.187 P=0.283	R=-0.316 P=0.065	R=-0.287 P=0.080	R=-0.258 P=0.185	R=-0.152 P=0.457	R=0.130 P=0.509	R=-0.227 P=0.287	R=-0.266 P=0.271	R=-0.642 P=0.033	R=0.347 P=0.296	R=0.254 P=0.478	R=0.108 P=0.519	R=0.064 P=0.703
Lymph node burden	R=-0.079 P=0.689	R=0.049 P=0.782	R=0.251 P=0.225	R=-0.096 P=0.583	R=-0.293 P=0.088	R=-0.134 P=0.423	R=-0.077 P=0.697	R=0.028 P=0.892	R=0.061 P=0.759	R=-0.165 P=0.440	R=-0.191 P=0.433	R=-0.289 P=0.388	R=0.324 P=0.331	R=-0.133 P=0.715	R=0.087 P=0.603	R=0.149 P=0.373
Grade	R=0.008 P=0.968	R=0.170 P=0.335	R=0.252 P=0.224	R=-0.135 P=0.439	R=0.049 P=0.780	R=-0.094 P=0.575	R=0.054 P=0.786	R=-0.023 P=0.913	R=-0.200 P=0.307	R=0.196 P=0.358	R=0.220 P=0.366	R=0.4188 P=0.1543	R=0.005 P=0.989	R=-0.527 P=0.118	R=0.207 P=0.213	R=0.048 P=0.777
Hormone Expression	R=-0.067 P=0.735	R=0.268 P=0.125	R=-0.001 P=0.996	R=-0.059 P=0.737	R=-0.087 P=0.619	R=-0.030 P=0.858	R=-0.040 P=0.841	R=0.159 P=0.437	R=-0.139 P=0.482	R=-0.100 P=0.641	R=-0.108 P=0.661	R=0.4929 P=0.0870	R=-0.119 P=0.729	R=-0.291 P=0.415	R=0.127 P=0.447	R=0.081 P=0.630
Subtype	R=0.149 P=0.451	R=0.052 P=0.771	R=0.134 P=0.522	R=0.198 P=0.255	R=0.068 P=0.696	R=0.5541 P=0.0005	R=0.160 P=0.417	R=-0.057 P=0.784	R=-0.064 P=0.748	R=-0.043 P=0.840	R=-0.059 P=0.812	R=0.584 P=0.051	R=0.195 P=0.566	R=0.120 P=0.740	R=0.063 P=0.707	R=0.208 P=0.210
LVI	R=-0.305 P=0.115	R=0.286 P=0.101	R=0.005 P=0.982	R=-0.009 P=0.961	R=0.123 P=0.480	R=0.3305 P=0.063	R=0.167 P=0.397	R=-0.041 P=0.842	R=0.304 P=0.115	R=0.227 P=0.287	R=0.084 P=0.691	R=0.074 P=0.8112	R=0.553 P=0.078	R=-0.190 P=0.599	R=0.4255 P=0.008	R=0.3299 P=0.044
Associated DCIS	R=-0.045 P=0.819	R=0.134 P=0.451	R=0.106 P=0.613	R=0.098 P=0.573	R=0.090 P=0.606	R=-0.025 P=0.882	R=0.069 P=0.728	R=0.154 P=0.453	R=-0.053 P=0.789	R=0.079 P=0.714	R=0.085 P=0.728	R=-0.324 P=0.331	R=0.095 P=-.782	R=-0.789 P=0.007	R=0.232 P=0.162	R=0.024 P=0.887
BMI	R=0.1621 P=0.4289	R=0.3738 P=0.0294	R=0.5190 P=0.0133	R=0.262 P=0.128	R=0.2077 P=0.2313	R=0.088 P=0.61	R=0.4173 P=0.0271	R=0.2397 P=0.2382	R=0.3652 P=0.0560	R=-0.2675 P=0.3552	R=-0.02590 P=0.9397	R=-0.132 P=0.6977	R=0.5096 P=0.1093	R=-0.09222 P=0.8281	R=-0.023 P=0.889	R=-0.138 P=0.408

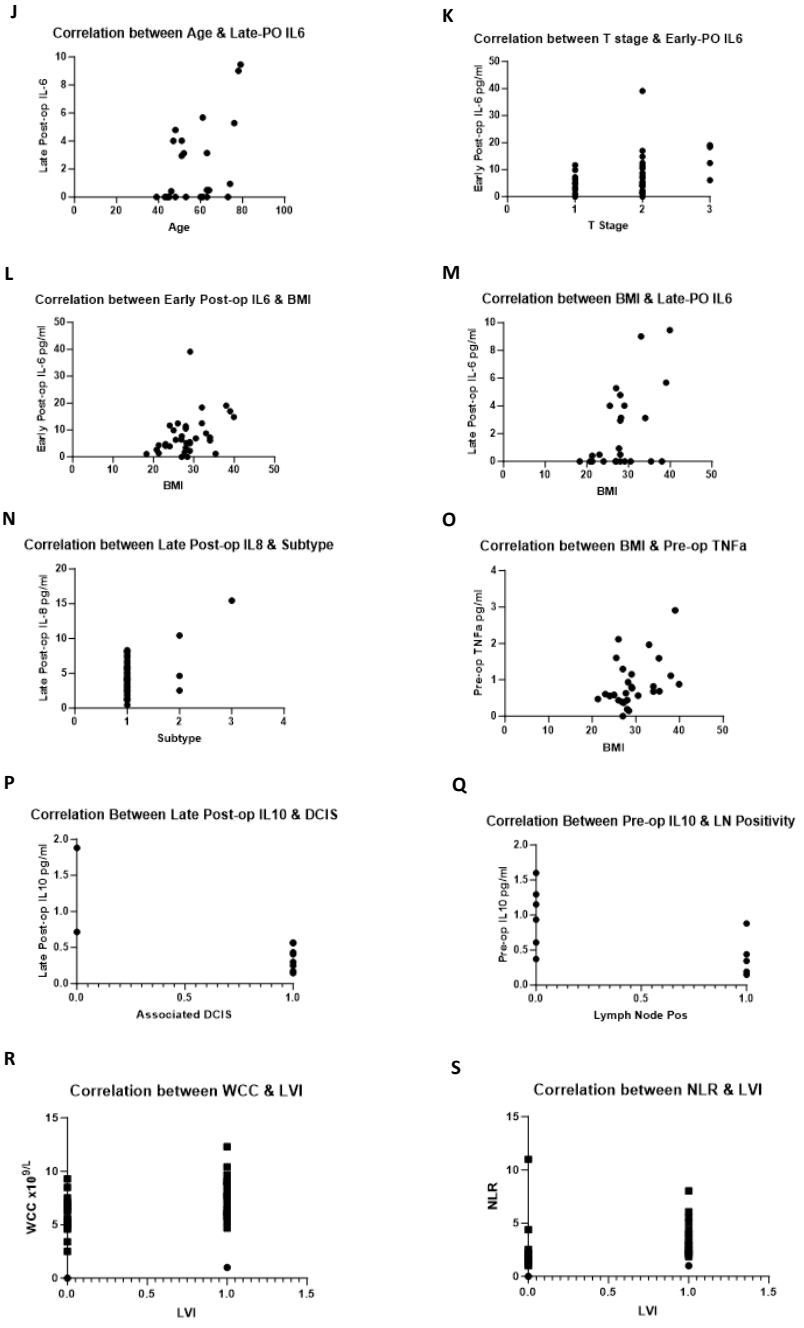


Figure 4 J-S : J. Positive correlation between age and late post -op IL-6, **K.** positive correlation between T stage and early post-op IL-6, **L.** positive correlation between early post-op IL-6 and BMI, **M.** positive correlation between late post-op IL-6 and BMI, **N.** positive correlation between late post-op IL-8 and subtype , **O.** positive correlation between late pre-op TNF-α and body mass index (BMI), **P.** negative correlation between pre -op IL-10 and lymph node positivity, **Q.** negative correlation between late post -op IL-10 and ductal carcinoma in situ (DCIS), **R.** positive correlation between pre -op white cell count (WCC) and lymphovascular invasion (LVI), **S.** positive correlation between pre -op Neutrophil to lymphocyte ratio (NLR) and LVI

4.5.5 Clinical Outcomes

4.5.5.1 Post-operative wound complications

There were six wound complications overall, one patient had a haematoma that required radiological drainage, four patients had symptomatic seromas that required radiological drainage and one patient had a superficial wound infection that required antibiotics. Of the five patients who had a seroma or superficial wound infection, four patients had positive wound/seroma fluid growth (Table 3). A median and interquartile range value was calculated for individual systemic mediators pre-operatively and early post-op to analyse whether elevated mediators of systemic levels were associated with increased risk of developing post-operative wound complications (Table 4). There was no significant association between individual mediators of inflammation and post-operative wound complications. Five of the six patients that developed post-operative wound complications had an elevated BMI however this was not statistically significant (OR 9.55, p = 0.065).

Table 3: 30-day post-operative wound complications

Post-op Wound Complication N=6					
Age	Surgery	BMI	Complication	Intervention	Culture
81	WLE & SLNB	29	Haematoma	Drainage	NA
47	WLE & SLNB	25.5	Wound infection	Antibiotics	Staph A
53	WLE & ALND	35.4	Infected seroma	Drainage & Antibiotics	Mixed growth
51	Mastectomy & SLNB	29	Infected seroma	Drainage & Antibiotics	Staph A
63	Mastectomy & SLNB	30.5	Infected seroma	Drainage & Antibiotics	Staph A
63	Mastectomy & SLNB	29	Infected seroma	Drainage & Antibiotics	No growth

Table 4: Systemic inflammatory mediators median values and interquartile range (IQR)

Pre-op IL8	Median	5.064
	IQR	(6.2)
	High	N =18
	Low	N=17
Pre-op IL6	Median	0.2283
	IQR	(3.1)
	High	N=14
	Low	N=14
Pre-op TNFa	Median	0.6814
	IQR	(0.74)
	High	N=14
	Low	N=14
Pre-op IL10	Median	0.6067
	IQR	(0.8)
	High	N=6
	Low	N=5
Pre-op IL1b	Median	0.000
	IQR	(1.719)
	High	NA
	Low	NA
Early Post-op IL8	Median	6.752
	IQR	(11.5)
	High	N=18
	Low	N=17
Early Post-op IL6	Median	6.206
	IQR	(8.3)
	High	N=17
	Low	N=17
Early Post-op TNF	Median	0.7691
	IQR	(2)
	High	N=14
	Low	N=12
Early Post-op IL10	Median	3.716
	IQR	(3)
	High	N=6
	Low	N=5
Early Post-op IL1b	Median	0
	IQR	(0)
	High	N=NA
	Low	N=NA
Pre-op WCC	Median	6.4
	IQR	(2.35)
	High	N=21

	Low	N=17
Pre-op NLR	Median	2.153
	IQR	(1.67)
	High	N=19
	Low	N=19
BMI	Median	28
	IQR	(7)
	High	N=16
	Low	N=22

IQR= interquartile range, IL6= interleukin 6, IL8= interleukin 8, TNFa= tumour necrosis factor a, IL10= interleukin 10, IL1b= interleukin 1b, WCC = white cell count, NLR = neutrophil to lymphocyte ratio, BMI = body mass index

Table 5: Association between post-operative wound complications and systemic inflammatory mediators

	30 day wound complication OR (95% CI) p value	**Infective wound complication OR (95% CI) p value**
High Pre-op IL8	0.141 (0.015-1.37) 0.091	0.275 (0.03-2.9) 0.285
High Early PO IL8	0.933 (0.085-4.3) 0.93	0.938 (0.117-7.5) 0.952
High Pre-op IL6	0.611 (0.16-1.48) 0.624	2.167 (0.173-27) 0.548
High Early Post-op IL6	0.376 (0.068-2.761) 0.660	1.00 (0.127-8.05) 1.00
High Pre-op TNFa	2.40 (0.361-2.40) 0.365	1 (0.120-8.3) 1
High Early Post-op TNFa	1.36 (0.188-9.9) 0.759	0.385 (0.030-4.86) 0.461
High Pre-op IL10	NA	NA
High Early Post-op IL10	0.30 (0.018-4.9) 0.398	NA
High Pre-op WCC	1.765 (0.282-11.04) 0.672	0.789 (0.99-6.28) 1.00
High Pre-op NLR	1.00 (0.175-5.72) 1.00	1.00 (0.126-7.94) 1.00
High BMI	9.55 (0.989-92.17) 0.065	4.85 (0.455-51.66) 0.291

IL6= interleukin 6, IL8= interleukin 8, TNFa= tumour necrosis factor a, IL10= interleukin 10, IL1b= interleukin 1b, WCC = white cell count, NLR = neutrophil to lymphocyte ratio, BMI = body mass index

4.6 Discussion

Findings from this study indicate that the systemic peri-operative immune response in breast cancer surgery is dynamic with an observed proinflammatory cytokine release and a compensatory anti-inflammatory response. The notion that surgical trauma induces a state of immunological imbalance is well recognised and was previously described by Walton B in 1979[221]. This paper outlined the mechanisms causing depression of non-specific resistance mechanisms (innate immune system) and the specific immune response and suggested that whilst various anaesthetic agents, antibiotics and steroids appear to depress immune responses, the hormonal aspects of the stress response to surgery has the most influential impact on the peri-operative immune suppression [221]. Since then, more comprehensive consequential patterns of immune activation following surgical insult have been described. The primary site of surgical incision and surrounding tissues initiates a pro-inflammatory cytokine response from macrophages, monocytes and stromal tissue as part of the acute phase response. These cytokines include IL-1β and TNF-α which stimulate the release of other pro-inflammatory cytokines including IL-6[222, 223].

IL-6 is responsible for the downstream production of many acute-phase proteins, including anti-proteinases and fibrinogen, which have a role in nonspecific and specific immunity as inflammatory mediator scavengers and protease inhibitors [223]. Although IL-6 is primarily a proinflammatory cytokine, it also functions as part of the counter inflammatory response by attenuating the action of IL-1β and TNF-α[224]. Furthermore, IL-6 induces macrophages to produce prostaglandin E2 which stimulates the release of the powerful anti-inflammatory cytokine IL-10[225, 226]. These mechanisms underpin the physiological proinflammatory anti-inflammatory balance disturbance imposed with surgery and rationalise the findings from this study. *In our cohort of thirty-eight patients, IL-6, IL-8 and IL-10 levels increased significantly early post-operatively and returned to near baseline two weeks post-surgery. Systemic TNF-α levels did not significantly change peri-operatively which may be due to the inhibitory role of IL-6 on IL-1β and TNF-α via the release of IL-1Ra and the soluble TNF-α receptors [227].*

One of the first studies to examine peri-operative systemic cytokine expression detected a significant rise in IL-1β levels between 1 and 4hours and a peak in IL-6 levels between 4-48 hours post aortic aneurysm repair surgery [222]. When compared to patients undergoing inguinal hernia repair, the IL-6 peak in those undergoing aneurysmal repair was significantly higher suggesting that cytokine response corresponds to the magnitude of surgery [222]. This concept has been well documented in several surgical fields. Previous studies comparing the physiological stress response of open versus laparoscopic surgery reported significantly higher postoperative IL-6 concentrations were noted in the open surgery group [86, 87]. Whilst perioperative morbidity associated with a more minimally invasive approach is favourable, this has not translated into better oncological outcomes in colorectal cancer. The COlonic cancer Laparoscopic or Open Resection (COLOR) trial significant did not report a significant difference in disease free survival and overall survival between the two cohorts [88]. Several animal studies have suggested that minimally invasive surgery tends to reduce the rate of tumour progression compared with laparotomy however this has not translated to clinically meaningful outcomes for patients [228] [229]. This is the first study to examine the relationship between breast surgery peri-operative systemic inflammatory response and surgical approach. In this study, early post-operative IL-6 values were significantly higher in patients who had a mastectomy compared to patients who had a wide local excision (WLE) ($p = <0.01$). Similarly, IL-10 levels were significantly higher early post-op in patients who had a mastectomy compared to WLE ($p=<0.001$). These findings might explain why the type of breast surgery intervention influenced oncological outcomes in a recent large retrospective cohort study of 48,986 women published by de Boniface et al in 2021. Despite adjustment for confounders, breast conserving surgery with radiotherapy yielded better survival outcomes than mastectomy irrespective of radiotherapy and socioeconomic factors [89]. Whether breast conserving surgery induces a diminished cytokine response and less favourable tumour microenvironment is yet to be determined.

Systemic inflammatory mediators appeared to correlate with several different tumour characteristics in this study. A correlation between late post-

operative IL-6 levels and increasing age was observed. This age dependent increase in IL-6 levels is a trend that was also echoed by Puzianowska-Kuznicka et al in their analysis of 3496 individuals [230]. Chronic low-grade inflammation and immunosenescence, conceptually referred to as “inflammaging”, are characteristic changes associated with human aging that are reflected in circulating IL-6 levels [231]. Previous studies have indicated that pre-operative IL-6 correlates with clinical disease stage [220] [94] and whilst this was not evident in the present cohort of patients, a positive association between early post-operative IL-6 and tumour T stage was observed however. This suggests that surgical insult potentiates the release of cancer-related inflammatory mediators such as IL-6 from the surrounding tumour microenvironment (TME) composed of stromal cells and inflammatory cells and the degree of elevation corresponds to the size of the TME [232]. The metabolic association between BMI and systemic mediators of inflammation is well recognised. Pre-operative TNF-α correlated significantly with BMI in this study, an observation that concurred with findings reported by Nyström et al [233]. Many studies have also reported a positive correlation between IL-6 and adiposity and this study corroborated with these findings as BMI positively associated with peri-operative IL-6 levels at each time point [234]. Adipocytes secrete high levels of IL-6 into the circulation [235] and breasts are composed of up to 56% adipose tissue harbouring a rich adipocyte supply. One explanation for the significant correlation between post-op IL-6 and BMI could centre on the concept that surgical insult initiates surrounding adipocytes of the breast and subcutaneous tissue to release IL-6 in a manner proportionate to the individuals BMI [236].

Significant associations between peri-operative systemic inflammatory mediators and tumour characteristics were detected in this study. Invasive lobular carcinoma (ILC) accounts for 10-15% of all breast cancer cases [237] and has a distinct biological profile with non-cohesive epithelial cells dispersed individually in a single file linear pattern in a fibrous stroma with loss of expression of the cell adhesion molecule E-cadherin (CDH1) [238]. In this study, late post-operative IL-8 levels correlated positively with the ILC/mixed breast cancer subtype suggesting that the pro-inflammatory cytokine IL-8 is persistently elevated post-operatively in patients

with ILC/mixed breast cancer subtype. Elevated IL-8 levels are a poor prognostic indicator in patients with breast cancer[58, 59] and as ILC confers worse long-term overall survival outcomes, the association between breast cancer subtype and certain peri-op cytokines may have prognostic implications. As only 11% (N=4) of the study population had either invasive lobular carcinoma or a mixed lobular/ductal subtype, it is not possible to deduce any firm conclusions regarding the clinical significance of this correlation. Systemically, IL-10 is a pleotropic cytokine with both pro-tumour and anti-tumour effects[215]. Previous studies suggest that IL-10 is secreted at a higher rate in response to advanced disease to down-regulate the inflammatory response of cell mediated immunity however the contrary was evident in this study as IL-10 was inversely associated with lymph node positivity[239]. The finding that systemic pre-operative IL-10 levels are lower in patients with lymph node positivity is significant IL-10 has protective immunomodulatory effects via inhibition of the synthesis of a variety of inflammatory cytokines to convey an anti-tumour effect [240]. What remains to be proven is whether the peri-operative dynamics of systemic inflammatory mediators has any prognostic significance regarding long term breast cancer oncological outcomes.

4.7 Strengths & Limitations

There were several strengths to this prospective observational cohort study. To reduce the effect of confounders we had a strict exclusion criterion and excluded any patients who had NACT or any inflammatory conditions. Samples were collected and strictly managed in accordance with best practice pre-analytical processing guidelines. An inter-assay analysis was performed to ensure inter-assay plate variability was <15%. Due to the novel, proof-of-concept nature of this study, it was not possible to formulate a formal power calculation. This body of work was hypothesis generating and an initial step towards potential future larger studies. Other limitations include, the small sample size, single institution and short follow-up duration.

4.8 Conclusion

To the best of our knowledge this is the first study to define the trend of peri-operative systemic inflammatory mediators in breast cancer surgery. Systemic peri-operative IL-6, IL-8 and IL-10 levels are dynamic and appears to reflect the magnitude of breast cancer surgery. This study showed established clinical correlations between age, BMI, T tumour size and peri-operative inflammatory mediators. Significant novel correlations between tumour characteristics and peri-operative inflammatory mediators were also detected, the clinical significance of which remains to be elucidated.

Chapter 5: Immune Expression Analysis in the Breast Tumour Microenvironment

5.1 Abstract: Immune Expression Analysis in the Breast Tumour Microenvironment

Background

The tumour microenvironment (TME) drives tumorigenesis and adipocytes via secretion of adipocytokines, such as IL-6, play a significant role in breast cancer homeostasis. In this study, we aimed to analyse and compare breast cancer tumour and normal adjacent to tumour (NAT) differential gene expression of *IL6, IL10, leptin, adiponectin, CD36, FABP4* and *IL6* related genes using data available from The Cancer Genome Atlas (TCGA) and a cohort of patients with matched tumour and normal adjacent to tumour (NAT) tissue samples.

Methods

Breast tissue specimens were collected after surgical resection. RNA integrity was analysed using the Agilent Bioanalyzer 2100. Whole transcriptome sequencing was performed using an Illumina NGS platform. Differential Gene Expression was analysed using the DeSeq2 statistical package and bioinformatical support was provided by BMKGene. Statistical difference was expressed as Fold Change(FC)≥2 and False Discovery Rate (FDR)<0.01.

Results

RNA Seq data from 54 patients with matched normal and primary tumour tissue samples comparable to our tissue cohort (ER+/HER2-, Stage I-III, IDC/ILC) available on TCGA were analysed. When *IL6* expression was analysed in normal and tumour tissue samples, *IL6* expression was significantly higher in the NAT with a FDR <0.1. From our own patient cohort, 10 patients with matched tumour and normal adjacent to tumour (NAT) tissue samples were available for gene expression analysis. *IL6, Leptin, JAK1, CD38, FABP4* and *Adiponectin* gene expression were significantly higher in NAT compared to matched tumour samples.

Conclusion

Pro-inflammatory signals from the NAT may stimulate an inflammatory response in the TME but what remains to be elucidated is the prognostic implications of this immune phenotype.

5.2 Introduction

Paget, one of the founding fathers of cancer research, referenced the significance of the tumour microenvironment in 1889 by proposing that cancer cells only induce tumorigenesis in favourable microenvironments in his famous “seed and soil” theory [241]. The breast tumour microenvironment (TME) consists of immune cells, fibroblasts, adipocytes, mesenchymal stem cells and extracellular matrix. Macrophages or tumour associated macrophages are key components of the tumour microenvironment responsible for enabling tumour cell evasion and metastatic spread [242]. Tumour associated macrophages account for more than 50% of the tumour bulk in many human solid tumours [243]. Macrophages are broadly classified into two major phenotypes; the M1 phenotype with pro-inflammatory and anti-tumour potential and the M2 subgroup which is anti-inflammatory and tumour-promoting [244]. Macrophages polarise towards the type 1 pathway in response to Th1 derived cytokines such as interferon gamma or toll like receptor, resulting in tumoricidal activity via reactive oxygen species release [245]. Conversely, M2 macrophages induce their immunosuppressive effects in response to TH2 derived cytokines such as IL-4, IL-10 and IL-13. M2 macrophages are believed to lose their antigen presenting function in favour of supporting angiogenesis and expression of immunosuppressive molecules such as IL-10 and programmed death ligand 1 (PD-L1) [246]. This oversimplified theory of macrophage function fails to account for the plethora of distinct TAM populations found within the TME, often displaying features of both M1 and M2 subtypes [247]. It is highly likely that the cytokine profile of the TME orchestrates and integrates a dynamic range of both pro-tumorigenic and anti-tumorigenic functions[248]. Tumour associated macrophages facilitate tumour immune evasion, tumour expansion and neovascularisation which are key steps in the benign-to-malignant transition [249]. The penultimate step enabling metastasis is degradation of the epithelial basement membrane. A specialised subset of TAM releases a cytokine concoction (IL-6, IL-1) to enable circulating tumour cells to engraft the secondary site [250].

IL-6 is secreted by cancer cells, stromal cells and TAM’s to induce proliferation, angiogenesis and metastasis via activation of the STAT-3 pathway

[251]. Recent studies have demonstrated the role of TAM's derived IL-6 in cancer stem cell initiation and tumour progression in breast cancer [252]. Whilst the role of tumour associated macrophages and stromal cells in cancer potentiation have been extensively studied in breast cancer, the fat-rich connective tissue comprising the mammary fat pad has been vastly overlooked [253]. Adipocytes constitute the major cellular component of the mammary fat pad and occupies up to 56% of non-lactating breast tissue and 35% of lactating breast tissue [254, 255]. Mammary adipose tissue is characteristically different to its subcutaneous adipose tissue counterpart, undergoing cyclical changes to hormonal manipulation and interacting with ductal epithelium to enable mammary ductal morphogenesis [256]. Adipocytes involved in breast tumour progression are known as cancer-associated adipocytes and believed to exert their downstream effects via release of soluble factors called adipokines including leptin, adiponectin, IL-6, TNF-α, and hepatocyte growth factor (HGF)[257]. Leptin signalling has been shown to regulate important molecules in breast cancer proliferation, adhesion, invasion, migration and angiogenesis leading to elevated leptin levels being associated with breast cancer aggressiveness and bad prognosis in epidemiological studies[258, 259]. Leptin is a mediator of tumour-stromal interactions by initiating crosstalk between breast cancer cells and TAM by stimulating IL-8 production to promote tumorigenesis and metastasis [260]. Furthermore, adipocyte-secreted IL-6 and leptin reportedly induces and regulates epithelial–mesenchymal transition (EMT) in cancer cells [261]. In contrast, adiponectin exerts different biological functions by decreasing pro-inflammatory cytokine production (IL-6), preventing monocyte migration affecting vascular epithelium and inhibiting angiogenesis [262]. Several case-control studies associated low circulating levels of adiponectin with an increased breast cancer risk and the development of a more aggressive phenotype in post-menopausal women, regardless of BMI, leptin, and Insulin Like Growth Factor (IGF-I) levels [263].

Cancer metabolomics and energy metabolism is an exciting emerging theme in the literature. Breast cancer risk is twofold higher in patients with obesity and patients with obesity present with larger tumours and have worse survival outcomes compared to patients without obesity[264]. Adipocytes release metabolites and

biomolecules, which remodel tumour cell metabolism to enhance tumour growth. CD36 is a key biomolecule involved in importing adipocyte-released fatty acid into breast cancer cells. A recent study showed that CD36 directly interacts with FABP4 to regulate fatty acid import, transport, and metabolism. Furthermore, CD36 blockade was proposed as a therapeutic target as CD36 and FABP4 inhibition induces apoptosis in tumour cells [265]. It is becoming increasingly apparent that the tumour microenvironment is key to tumorigenesis and adipocytes via secretion of adipocytokines play a significant role in cancer homeostasis. However, there is a general lack of consensus on the extent of the tumour microenvironment. Histologically normal tissue adjacent to the tumour (NAT) is commonly used as a control in cancer studies despite this tissue having many morphological and phenotypical differences to non-tumour-bearing healthy tissue, including pH levels, allelic imbalance and telomere length, stromal behaviour, and transcriptomic and epigenetic aberrations [266, 267]. Using integrated data from the Genotype-Tissue Expression project and The Cancer Genome Atlas, Aran et al demonstrated that NAT presents a unique intermediate state between healthy and tumour tissue with differential protein-protein interaction and gene expression profiles [268]. The authors concluded that pro-inflammatory signals from the tumour stimulates an inflammatory response in the adjacent endothelium but what remains to be elucidated is the prognostic implications this immune phenotype [268].

Our previous work in Chapter 4 suggests that circulating systemic IL-6, IL-8 and IL-10 levels increase early post-operatively in response to surgical trauma. The focus of this work was on examining cytokine and adipocyte related gene expression from the surrounding local tissue environment using a cohort of 10 patients with matched tumour (T) and normal adjacent to tumour (NAT) tissue samples available for gene expression analysis.

5.3 Methodology

5.3.1 Aims & Objectives

A prior unpublished analysis using data available from The Cancer Genome Atlas (TCGA) by Dr Cathriona Foley demonstrated that IL-6 was significantly elevated in NAT compared to tumour tissue and furthermore, elevated IL-6 in the NAT correlated with worse overall survival.

In this study, we aimed to analyse and compare breast cancer tumour and NAT differential gene expression of IL-6, (Adipogenesis related genes) leptin, adiponectin, CD36, FABP4 and IL-6 related genes (JAK, STAT3) in a cohort of patients from the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study biobank determine if the preliminary findings from TCGA could be replicated in our cohort of patients.

Objective 1: Perform RNA sequencing on matched breast tumour and NAT samples to perform differential gene expression analysis of IL-6, and other adipokines.

Objective 2: Compare differential gene expression analysis of NAT to circulating IL-6 and IL-10 levels.

5.3.2 Patient Recruitment

Patients undergoing breast cancer surgery and treatment at our institute are being actively recruited to the ethically approved ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study. This study is sponsored by Cancer Trials Ireland (NCT01840293) and open across Irish centres. These studies are sub-studies of the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity, and sample collection is within the remit of the biospecimen protocol.

Patients were selected retrospectively from a prospectively maintained breast biobank. Inclusion criteria included patients having breast cancer surgery for non-metastatic ER+/HER2- breast cancer aged 18-85 with tumour tissue and normal adjacent to tumour tissue available. Patients were excluded if they had neoadjuvant chemotherapy or had invasive cancer/DCIS <2mm from the specimen margin.

All patients with breast cancer were discussed at the regional Multi-Disciplinary Meeting (MDM) and managed in keeping with National Cancer Control Programme (NCCP) recommendations. In this institution all patients with breast cancer are routinely screened for axillary nodal disease using ultrasonography +/- fine needle aspiration (FNA) of indeterminate or suspicious nodes. Axillary lymph node dissection is recommended to patients with positive axillary nodal disease receiving upfront surgery. Image guided breast conserving surgery is performed using radiological placed guide wires or a Radiofrequency Identification Device (RFID) tag localisation. Tumour phenotype was recorded from final histopathological reports. Oestrogen receptor/progesterone receptor were considered positive when >10% of cells showed positive staining. HER2 status was assessed by immunohistochemistry (IHC) and Fluorescent in Situ Hybridization (FISH) confirmation in cases with 2+ by IHC. HER2 positivity was defined as either 3+ with IHC and/or amplified with FISH.

5.3.2 Sample Biobanking

Breast tissue specimens were collected after surgical resection. A larger breast tumour tissue piece was cut up into at least 3 pieces (minimum size 2-3mm 2) by pathology technicians, and snap frozen in separate cryovials. Similarly, a NAT tissue piece was cut up into at least 3 pieces (minimum size 2-3mm 2) by pathology technicians, and snap frozen in separate cryovials. Specimens were stored in -80degree freezer. Local biospecimen sampling protocol restricts biobanking of tumours less than 2cm in size therefore the tumours analysed were all greater than 2cm in size.

Blood samples were collected pre-operatively. Plasma samples were stored in K3EDTA bottles and centrifuged at 2500 rpm for 15 mins within 1 hour of phlebotomy. Plasma samples were aliquoted into collection tubes (500 uL and 1500 uL), labelled with trial codes and stored at -80 degree Celsius.

5.3.3 Inclusion Criteria

Our Inclusion criteria included patients having breast cancer surgery for non-metastatic ER+/HER2- breast cancer aged 18-85 with tumour tissue and normal adjacent to tumour tissue available. Patients were excluded if they had neoadjuvant chemotherapy or had invasive cancer/DCIS <2mm from the specimen margin. Tissue specimens were selected retrospectively from a prospectively maintained biobank with samples dating from 2019-2021. RNA was extracted from 12 patients (24 samples) and following QA processes 19 samples had a RIN value suitable for downstream whole transcriptome sequencing using an illumina NGS platform.

5.3.4 RNA Purification from Tumour & Normal Adjacent to Tumour Matched Samples

RNA purification was performed using the Qiagen RNEasy Mini protocol. The RNeasy kits are designed to purify RNA from small amounts of starting material. Samples are **first lysed and homogenised** in the presence of a highly denaturing guanidine-thiocyanate containing buffer, which immediately inactivates RNAses to ensure purification of intact RNA. **Ethanol is added to provide appropriate binding** conditions and the sample is then applied to an RNeasy mini spin column where the **total RNA binds to the membrane** and contaminants are washed away. **RNA is then eluted in 30-100ul of water**. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified. The procedure provides an enrichment for messenger RNA since most RNAs<200 nucleotides are selectively excluded. Figure 5A.

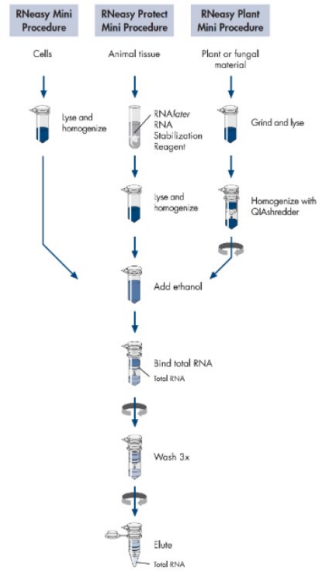


Figure 1. RNeasy Mini, RNeasy Protect Mini, and RNeasy Plant Mini procedures.

Figure 5A: QIAGEN RNeasy Workflow

RNEasy Protocol: Purification of total RNA from frozen animal/human tissues

1. Disruption and homogenisation using a rotar-stator homogeniser
2. If using the entire portion of tissue, place into a suitably sized vessel for disruption (15ml tube)
3. Add the appropriate volume of **Buffer RLT** (See table below)

Table 8. Volumes of Buffer RLT for Tissue Disruption and Homogenization

Amount of starting material (mg)	Volume of Buffer RLT (µl)
<20	350 or 600*
20–30	600

* Use 600 µl Buffer RLT for tissues stabilized in RNAprotect Tissue Reagent or for difficult-to-lyse tissues.

4.
5. Disrupt and homogenise using a conventional rotor-stator homogeniser until it is uniformly homogenous (usually 20-40secomds)
6. Centrifuge the lysate for 3 mins at full speed at >8000xg(>10,000rpm).
7. Carefully remove the supernatant by pipetting and transfer to a new microcentrifuge tube.
8. **Add 1 volume of 70% ethanol to the lysate** and mix immediately by pipetting. **1 volume= equal quantity to the volume of the sample.**

9. **Transfer upto 700** u**l** (volumes may be less than 350ul due to homogenisation) of the sample including any precipitate that may have formed to an RNeasy spin column and place in a 2ml collection tube. Close lid and centrifuge for **15s** at >8000xg(>10,000rpm). Discard the follow through.
10. Reuse collection tube. **Add 700**u**l Buffer RW**1 to the RNeasy spin columns. Close lid and centrifuge as above for **15s.** Discard follow through
11. **Add 500**u**l Buffer RPE** to spin column. Close the lid and centrifuge centrifuge as above for **2 minutes**. Discard follow through
12. Place the RNeasy spin columns in a fresh 2ml collection tube and centrifuge for 1 minute.
13. Place the RNAeasy spin column in a new 1.5ml collection tube. Add 50ul of RNAase free water directly to spin column. Centrifuge for 1 min to elute the RNA.
14. If the expected RNA yield is >30ug repeat step 12 using another 20-50ul RNAse free water. Re-use the same collection tube.
15. Quantify using spectrophotometer and record 260/280 values.

5.3.5 Whole Transcriptome Sequencing & Gene Set Enrichment Analysis(GSEA)

Total RNA was isolated from tumour and tumour adjacent (normal) tissue from 12 patients (24 samples) with breast cancer using the Qiagen RNEasy Mini kit as per manufacturer's instructions. RNA integrity was analysed using the Agilent Bioanalyzer 2100 and only RNA samples with a RNA Integrity Number >/= 7 were included in downstream analysis, this totalled to 19 samples. This quality control process was conducted by Biomarker Technologies (BMKGENE). RNA sequencing preparation involved using the Hieff NGS® C143P1 Ultima Dual-mode mRNA Library protocol. Samples were sequenced on the Illumina NovaSeq 6000 platform (San Diego) next generation sequencing platform with 150-bp paired-end reads to an average depth of 20 Million pair reads (6Gb data) per sample. The version of the reference genome was GRCh38 (release95) and HISAT2 which uses a Burrows-Wheeler Transform and Ferragina-Manzini(FM) index-based search was used to align reads to the genome.

Bioinformatical support was supplied by BMKGENE using the DeSeq2 differential expression workflow. The DeSeq2 RNAseq differential expression workflow starts with FASTQ files and aligns these reads to the reference genome. For experiments with biological replicates, differential expression analysis was processed using DESeq2 software package with a sequencing depth of 10-30M reads per library**.** The DESEQ2 software uses raw integer read counts to normalise the count data correcting for differences in library size and sequencing depth. Gene expression values are then prepared as a count table by counting the sequenced fragments and gene expression analysis is performed. Differences in gene expression were presented as Log2 Fold Change (Log2FC) representing the difference of two values [197]. Statistical difference was expressed as Fold Change(FC)≥2 and False Discovery Rate (FDR)<0.01.

FastQ files were analysed and verified by Dr Cathriona Foley using the EdgeR software package. The Molecular Signatures Database Hallmark adipogenesis gene set ("Hallmark_adipogenesis") of gene set variant analysis was used to quantify adipogenesis using the Gene Set Variation Analysis Bioconductor package. In the GSEA, statistical significance was defined as false discover rate (FDR) less than 25%, recommended by GSEA software. GSEA was performed by Dr Cathriona Foley.

5.3.6 Inflammatory Mediator Analysis

Systemic IL-6 and IL-10, were measured using the Biolegend ELISA. Samples were analysed in duplicates. An inter-assay analysis was performed to ensure inter-assay plate variability was <15%. Correlation analysis was performed using Pearson correlation calculations to assess the direct relationship between plasma IL6/IL10 levels and IL-6/IL-10 normalised RNA seq counts using FPKM (Fragments per kilo base per million mapped) in tumour and NAT. For each analysis, a correlation coefficient, r, was calculated (with 95% confidence intervals and p-value). r values were categorised as follows: r=1, a perfect correlation; r= 0-1 variables change in the same direction; r=0, no relationship between variation in each variable; r=-1-0, an inverse relationship exists; r=-1, a perfect inverse relationship exists.

5.3.7 Statistical Analysis

Bioinformatical support was provided by BMKGENE. R software was used for data analysis. Mann–Whitney U test and Fisher's test were used to calculate *p* value in group-comparison analysis. Kaplan–Meier curve with log-rank test was used to survival analysis. Pearson correlation calculations were used to assess the relationship between plasma IL-6/IL-10 levels and IL-6/IL-10 normalised RNA seq counts using FPKM using PRISMA software.

5.4 Results

5.4.1 ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study biobank

Ten patients with stage I-III ER+/HER2-, IDC were included. Characteritics of included patients are outlined in Table 1 . Nine patients had matched tumour and NAT RNA samples that had RIN value =/>7, suitable for downstream RNA sequencing (18 samples). One patient only had tumour tissue suitable for analysis as the NAT RIN value was not sufficient for RNA sequencing giving a total of 19 samples.

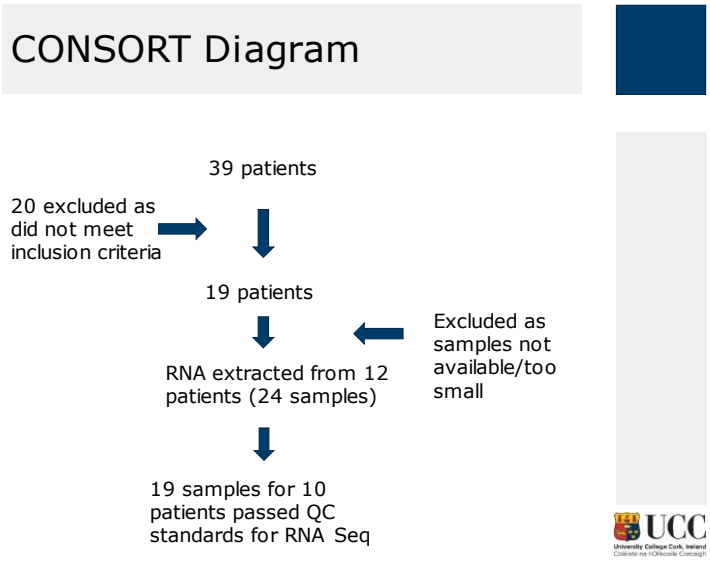


Figure 5B: Consort Diagram for ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study Biobank

Table 1: Patient Cohort Clinicopathological Characteristics N=10

Patient Demographic, Clinical and Tumour Characteristics	N=10
Age	Median 61 (IQR 25.5)
Lymph node status	
Positive	9
Negative	1
Tumour Size	
T1	0
T2	7
T3	2
T4	1
Grade	
1	0
2	3
3	7
Lymphovascular Invasion	
Present	9
Absent	1

5.4.2 *IL6 & IL6* Related Genes

Comparing TCGA data to the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study biobank, the results were comparable with *IL6* gene expression higher in NAT (Figure 5C). Similarly, the *IL6* related genes CD38, FABP4, ADIPOQ, JAK1 and Leptin were also expressed higher in NAT compared to tumour tissue. There was no statistical difference in STAT3 expression between tumour and NAT tissues in the department of surgery biobank (Figure 5D).

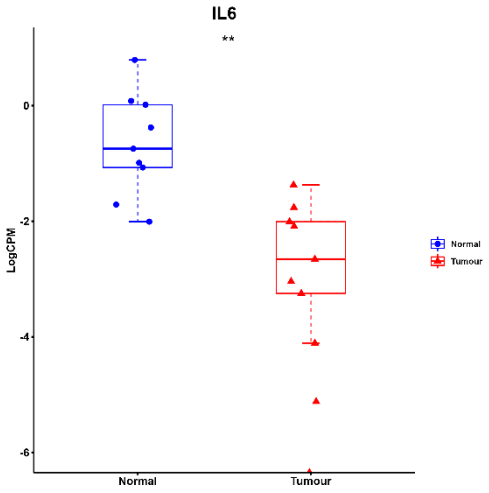


Figure 5C: Differential Gene Expression of IL6 in the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study biobank

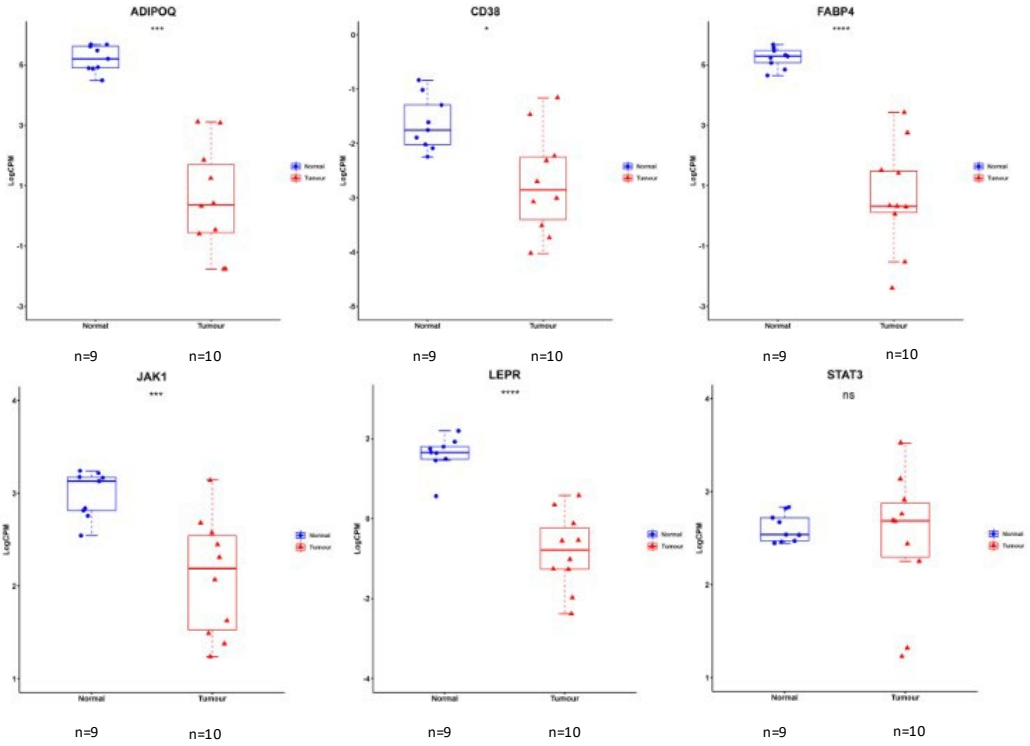


Figure 5D: Differential Gene Expression of IL6 Related Genes in the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study biobank

5.4.3 Gene Set Enrichment Analysis(GSEA) of the Adipogenesis Pathway

GSEA was performed by BMKGene and validated by Dr Foley to interrogate the gene set involved in the adipogenesis pathway in tumour vs NAT in both TCGA and ICORG 09-07 datasets. Examining the barcode, each line represents a gene. Tumour data is to the right and red and NAT is to the left and blue. The lines that are closely spaced together suggests that these genes are enriched. The green line represents the enrichment score and it climbs as it encounters genes that are highly expressed. In essence what both these graphs show is that genes involved in adipogenesis are enriched in the NAT compared to tumour in both TCGA and ICORG 09-07 cohorts (Figure 5E).

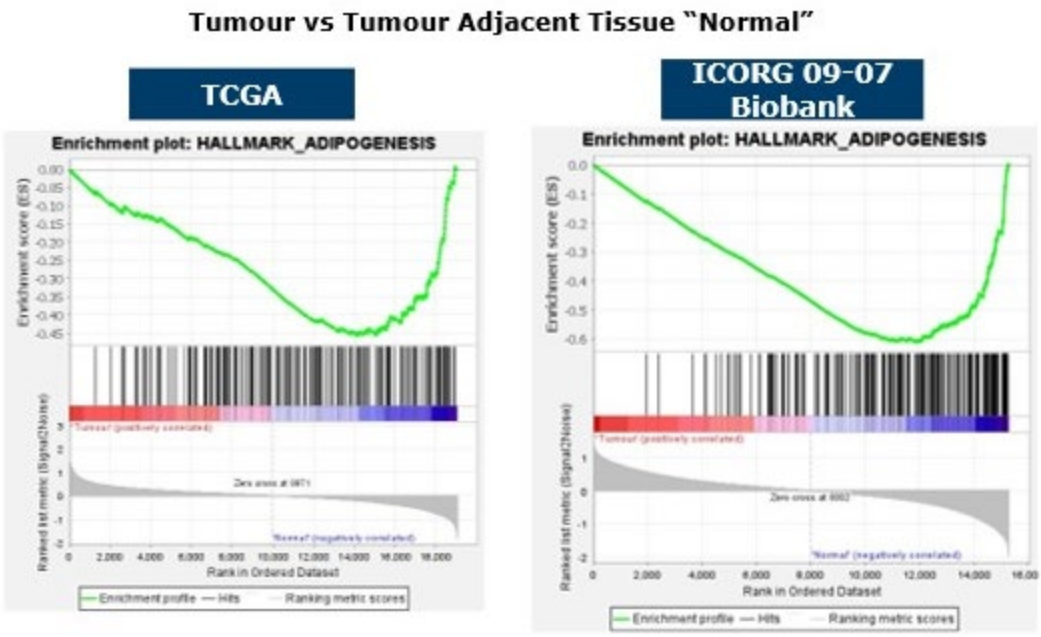


Figure 5E: Gene Set Enrichment Analysis

5.4.4 Circulating IL-6/IL-10 Levels & Tumour/NAT IL-6/IL-10 Expression

All ten patients had pre-operative blood plasma available for analysis. There was no stastically significant correlation between circulating IL-6/IL-10 plasma levels and IL-6/IL-10 normalised RNA seq counts using FPKM (Fragments per kilo base per million mapped) in tumour and NAT tissues using the Pearson correlation test. This may be due to the small sample size (N=10).

5.5 Discussion

Our results show that gene experession profiling in ER+ breast cancer tumour tissues is significantly different to NAT tissues. The morphological features evident in the NAT could be justified using Slaughters' "field cancerization" theory describing a cumulative process of carcinogenesis via acquisition of step-wise genetic alterations, resulting in the NAT tissue composed of morphologically normal but molecularly

altered cells[269]. Transcriptomic and epigenetic aberrations suspend the NAT in an intermediate, pre-neoplastic state susceptible to tumorogenic transformation[268]. Potentially, certain individuals are at an increased risk of developing cancer and cancer recurrences because of gene expression in their histologically normal breast epithelium[270].

There is emerging evidence available on the significance of gene expression and pathway enrichment of NAT. We found that NAT represents a distinct immune phenotype with potential prognostic value in ER+ breast cancer. These findings corroborate with those described by Casbas-Hernandez et al, who reported that gene expression of cancer-adjacent tissues in triple-negative tumours exhibit increased expression of genes involved in inflammation and immune response. Furthermore, the authors concluded that luminal breast cancers adjacent tissues were more commonly associated with oestrogen-response pathways involved in DNA synthesis and cell proliferation [271]. Obesity is associated with breast cancer carcinogenesis, cancer progression and poor clinical outcomes in ER+ breast cancer and as adipocytes are key players in breast cancer progression[272] we sought to interrogate the gene set involved in adipogenesis in both the tumour and NAT in oestrogen positive breast cancers. Using Gene Set Enrichment Analysis (GSEA), genes involved in adipogenesis were more enriched in the NAT compared to tumour in both the TCGA and Department of Surgery cohorts. Oshi et al investigated the role of intra-tumoural adipogenesis and cancer-associated adipocytes in breast cancer using data available from TCGA and GSE96058[273]. A high adipogenesis score in TNBC tumour specimens conferred worse Overall Survival (OS) and disease-free survival (DFS) in TNBC subtypes in the TCGA and GSE96085 cohorts. This study also reported that a high adipogenesis score was significantly associated with worse OS in ER+/HER2-negative breast cancers in the GSE96085 cohort but not the TCGA patient group. It is not reported whether patients in the GSE96085 and TCGA received adjuvant hormonal therapy which may confound the data[273].

Breast tissue is composed of 90% adipose tissue with crosstalk between immune cells, metabolites, and adipocytes [274]. Adipocytes make up one-third of the fraction of adipose tissue and are the primary cellular components comprising

the breast cancer TME[264, 272]. Normal adipocytes are driven into cancer-associated adipocytes (CAA's) by tumour cells and subsequently secrete inflammatory cytokines such as IL-6 and TNF-α[275]. These adipokines promote cancer inflammation resulting in tumorigenesis, tumour migration and activation of several metastatic pathways including epithelial-mesenchymal transition[272, 276]. Previous studies have reported that intra-tumoural adipocytes are associated with metastasis and inflammation but better patient survival outcomes using transcriptomic data from large breast cancer cohorts[277]. Tokumara et al theorised that quantity of intra-tumoural adipocytes inversely reflects cancer cell density of tumour bulk and therefore infers a less aggressive cancer[277]. In contrast, adipogenesis which is the generation of adipocytes as opposed to the quantity of adipocytes, is associated with an unfavourable tumour microenvironment and worse survival outcomes in patients with breast cancer[273]. Findings from the present study show that adipogenesis genes are significantly enriched in NAT compared to tumour tissues in ER+ breast cancers. The NAT is the breast tissue surrounding the tumour and may not be entirely resected at the time of surgery and could be immunologically and metabolically primed to promote tumorigenesis of mammary epithelium and effect patient survival outcomes.

We demonstrated that the expression of adipogenesis-related genes such as *CD36, FABP4, Leptin and ADIPOQ* are up-related in ER+ NAT breast tissues compared to tumour tissue. These findings conflict with those reported by Wang et al who used Quantitative real-time PCR to analyse gene expression profiling in mammary fat from 60 Chinese women. mRNA expression of adipogenesis-related genes such as HOXC8, HOXC9 and FABP in adipose tissue adjacent to malignancy breast tumours were significantly down-regulated [278]. The contrasting results between our findings and those reported by Wang et al could be attributed to differences in body composition and metabolomic pathways as all subjects in their study had stable weight with fluctuations of not more than 2% of their body weight for at least three months prior to surgery[278]. Asia-Pacific populations have different body fat distribution and to address this variation a different BMI range was introduced in 2000 with a normal BMI range of 18.5-22.9 kg/m^2 and the obesity class I defined as a BMI of 25-29.9

kg/m^2 [279]. Similar to the findings reported in our study, inflammatory cytokine gene expression such as TNF-α was up-regulated in tissues adjacent to malignant breast tissues [278]. As inflammatory cytokines such as IL-6 has the potential to reverse the powerful tumour suppressive mechanism of cellular senescence, upregulated neighbouring *IL6* genes may create a tumour permissive microenvironment and potentiate breast cancer tumour recurrence [280]. Elevated IL6 gene expression in NAT ER+ breast tumours is not universal to other cancers as Zeng et al demonstrated that expression of IL6 was significantly increased in colorectal cancer tissues compared to normal mucosal tissues ($p<0.001$) [281]. The differing surrounding immune environment is significant considering that the colorectal cancer recurrence rate is 16% and breast cancer recurrences occur in up to 30% of patients [282, 283].

The differential gene expression of *Leptin* and *ADIPOQ* were significantly higher in NAT compared to tumour tissue in the present study. *Leptin*, the first described adipokine, initiates cell proliferation, activation of immune cells and energy homeostasis via the JAK2/STAT3 and PI3K/AKT/MTOR pathways[284, 285]. There appears to be a link between Leptin and metabolism of oestrogens, such as aromatase expression, resulting in proliferation of breast tissue and breast carcinogenesis[286, 287]. Elevated circulating *Leptin* levels is associated with more aggressive breast cancer phenotypes and worse pathological outcomes in multiple large cohort studies [288, 289]. Furthermore, *Leptin* has been proposed as a potential biomarker to predict patients likely to experience disease recurrence[290]. Elevated gene expression of *Leptin* in NAT can stimulate aromatase transcript expression in adipose stromal tissue and potentially activate multiple oncogenic pathways[291]. In contrast, *ADIPOQ* exerts a negative regulatory function in relation to breast carcinogenesis and low circulating *ADIPOQ* levels are associated with larger tumours and worse pathological outcomes. *ADIPOQ* has an antagonistic effect on the expression and activity of *Leptin* via release of the inhibitor of *Leptin* signalling protein PTP1B[292]. Therefore, elevated gene expression of *ADIPOQ* detected in the NAT could be a compensatory response to elevated *Leptin*. Due to the antagonist effects of leptin and ADIPOQ, some studies have suggested that the *Leptin/ADIPOQ*

ratio may be more useful than individual levels for assessing an individual's breast cancer risk profile[293].

5.6 Limitations

The main limitation of this study is the small sample size due to few samples meeting the necessary quality assurance standards for whole transcriptome analysis. Furthermore, though we have demonstrated that the gene expression in NAT and tumour tissue is significantly different it is not possible to determine whether this translated into higher functional expression of these proteins in the circulation. We did analyse circulating plasma IL6 levels and looked for an association with gene expression in the NAT and tumour tissue however the findings were insignificant due to the small sample size. A sample of breast tissue from the contralateral breast could have also provided immunological profiling of the individuals' baseline inflammatory profile.

5.7 Conclusion

Findings from this study supports other studies that suggest NAT represents a distinct immune phenotype compared with tumour tissue in ER+ breast cancer. Pro-inflammatory and pro-adipogenesis signals from the NAT may stimulate an inflammatory response in the TME but what remains to be elucidated is the prognostic implications of this immune phenotype.

Chapter 6: Peri-operative Cell Free DNA (cfDNA) Dynamics in Breast Cancer Surgery

6.1 Abstract: Peri-operative Cell Free DNA (cfDNA) Dynamics in Breast Cancer Surgery

Background

Liquid biopsy techniques have emerged as an advantageous alternative to tissue biopsy analysis as it allows for serial non-invasive monitoring of disease status and overcomes the issue of intra-tumour heterogeneity. One such liquid biopsy method namely cell-free DNA (cfDNA) is gaining traction as a novel biomarker in predicting oncological outcomes in patients with breast cancer. The aim of this study was to define peri-operative plasma cfDNA dynamics in patients with non-metastatic breast cancer and cfDNA association with certain tumour characteristics.

Methods

Blood samples were phlebotomised peri- operatively from patients undergoing elective breast cancer surgery, with non-metastatic disease at the following time-points; pre-operatively the morning of surgery (pre-op), 3-5 hours post-operatively (early post-op), 10-14 days post-operatively (late post-op). This study is a sub-study of the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity, and sample collection is within the remit of the biospecimen protocol. Statistical analysis was performed using SPSS version 28 and graphs generated using GraphPad PRISM, version 8.4.2.

Results

Sixty-three patients (178 cfDNA samples) were included in the study with a median age of 61(IQR 23.5). Pre-operatively the mean cfDNA concentration was 3.82ng/µl (SEM = 0.74). This increased significantly early post-op in keeping with surgical insult to 17.59 ng/µl (SEM = 5.75). Late post-op there was a significant decrease in cfDNA to near baseline with a mean value of 3.78ng/µl (SEM = 0.62). Patients with associated ductal carcinoma in situ (DCIS) and lymphovascular invasion (LVI) in their

tumour had statistically higher cfDNA concentrations early post-operatively. A significant linear relationship was observed between early post-operative cfDNA concentration and early post-operative IL-8 levels ($r = 0.430$, $p = 0.009$).

Conclusion

Peri-operative changes in breast cancer cfDNA are dynamic with a significant rise in cfDNA concentration detected four hours post-surgery and a return to below baseline two weeks post-operatively. Early post-operative cfDNA release appeared to correlate positively with both IL-8 release and the presence of tumour LVI. These clinical correlates suggest the presence of peri-operative immunological and genomic crosstalk. Immunomodulation in the peri-operative space could provide an opportunity to suppress tumour-promoting inflammation post breast cancer surgery.

6.2 Introduction

Cancer initiation and progression causes the release of various components into the circulation including Cell Free DNA (cfDNA), Circulating Tumour Cells (CTC's) and Circulating Tumour DNA (ctDNA) [117]. Detection of these components in the systemic circulation is referred to as "liquid biopsy". Liquid biopsy techniques have emerged as an advantageous alternative to tissue biopsy analysis as it allows for serial non-invasive monitoring of disease status and overcomes the variable results of tissue biopsy owing to intra-tumour heterogeneity [294]. CfDNA was first discovered by Mandel and Métais in 1948 and is detectable in serum, plasma, urine, saliva and pleural fluid of healthy individuals [118]. In certain physiological states or pathological conditions such as pregnancy, organ transplantation or malignancy, additional DNA is shed into the circulation suggesting that cfDNA detection in the blood could signify abnormalities in a non-invasive manner [119]. The estimated concentration of cfDNA in a healthy population is between 0 and 100ng/ml compared to the concentration of cfDNA in patients with cancer which can range between 0 to 1000ng/ml [120].

The mechanisms of cfDNA release into the circulation is via cell death processes (including apoptosis, necrosis or NETosis) or active transport[295]. Apoptosis refers to programmed cell death involving the formation of apoptotic bodies and microparticles with fragments of DNA inside and/or on the membrane surface. Necrosis on the other hand is a disorganised process resulting in loss of membrane integrity and the release of intracellular contents, including long-stranded DNA, into the circulation[295]. In NETosis, activated neutrophils release neutrophil extracellular traps (NETs), which are webs of chromatin and mitochondrial DNA coated with antimicrobial proteins[296]. Lastly, cfDNA may also be actively secreted with extracellular vesicles and exosomes to maintain tissue homeostasis by removing damaged DNA[297]. As the half-life of cfDNA in the circulation is estimated between 16 minutes to 2.5 hours, cfDNA is an attractive "real-time" snapshot of disease burden[121].

The predictive utility of cfDNA serial analysis is gaining traction as a novel biomarker in predicting oncological outcomes in patients with breast cancer. Panagopoulou et al. analysed circulating cfDNA in a cohort of patients with metastatic breast cancer and demonstrated that elevated cfDNA levels correlated with treatment resistance, shorter progression- free survival and poorer survival outcomes[122]. The prognostic utility of cfDNA has shown promise in several other cancer fields. Park et al demonstrated that cfDNA levels correlated with survival outcomes in patients with hepatocellular carcinoma treated with radiotherapy [123]. Similarly, quantitative high measures of cfDNA were associated with poorer oncological outcomes in patients with metastatic colorectal cancer treated with FOLFIRI treatment [124] and patients with advanced Non-Small Cell Lung Cancer (NSCLC) treated with the Epidermal Growth Factor Receptor (EGFR) tyrosine kinase inhibitor Osimertinib [125]. Furthermore, cfDNA serial monitoring has shown promise in the early detection of disease relapse. Fleming and colleagues' research centred on cell-free DNA (cfDNA) dynamics peri-operatively and discovered that cfDNA 48 hours post-surgery served as an important biomarker to identify those at risk of early recurrence in colon cancer [126]. These findings were corroborated by Chen et al who performed a seminal study to investigate the precise perioperative dynamic changes in circulating tumour DNA (ctDNA) in patients with early-stage lung cancer. Elevated ctDNA detection on the third day after R0 resection was reported as the baseline value for postoperative lung cancer surveillance[127]. CfDNA is not only used as a cancer biomarker for prognostication and disease status but is also increasingly being applied as input for downstream molecular analysis.

The adoption of cfDNA as a tool in clinical decision making means it is essential to determine the optimal pre-analytical processing and cfDNA quantification techniques. Currently, there are significant inconsistencies among biospecimen collection, processing and storage practices globally [298]. The Reference Institute for Bioanalytics (RfB) and European Molecular Genetic Quality Network (EMQN) performed the first external quality assessment (EQA) and reported a lack of harmonization in cfDNA extraction methodology across forty-two institutions in ten European countries, which resulted in an overall genotyping error

rate of 6.09%. The main inconsistencies related to plasma storage temperature and duration, volume of plasma used, extraction kit, and quantification and genotyping methods[299]. To standardise cfDNA pre-analytical processing and reduce DNA degradation, analytical artifacts and contamination introduced by varying practices, the National Cancer Institute's (NCI) Biorepositories and Biospecimen Research Branch (BBRB) developed cfDNA-specific guidelines that are based upon published evidence and have been vetted by a panel of internationally recognised experts in the field[298]. The guidelines include optimal recommendations for each procedural step, from preparations for biospecimen collection to quantification and quality assessment of cfDNA (Figure 6A). Using biospecimen evidence-based best practice we aimed to examine the peri-operative dynamics of cfDNA with clinicopathological features of patients undergoing breast cancer surgery.

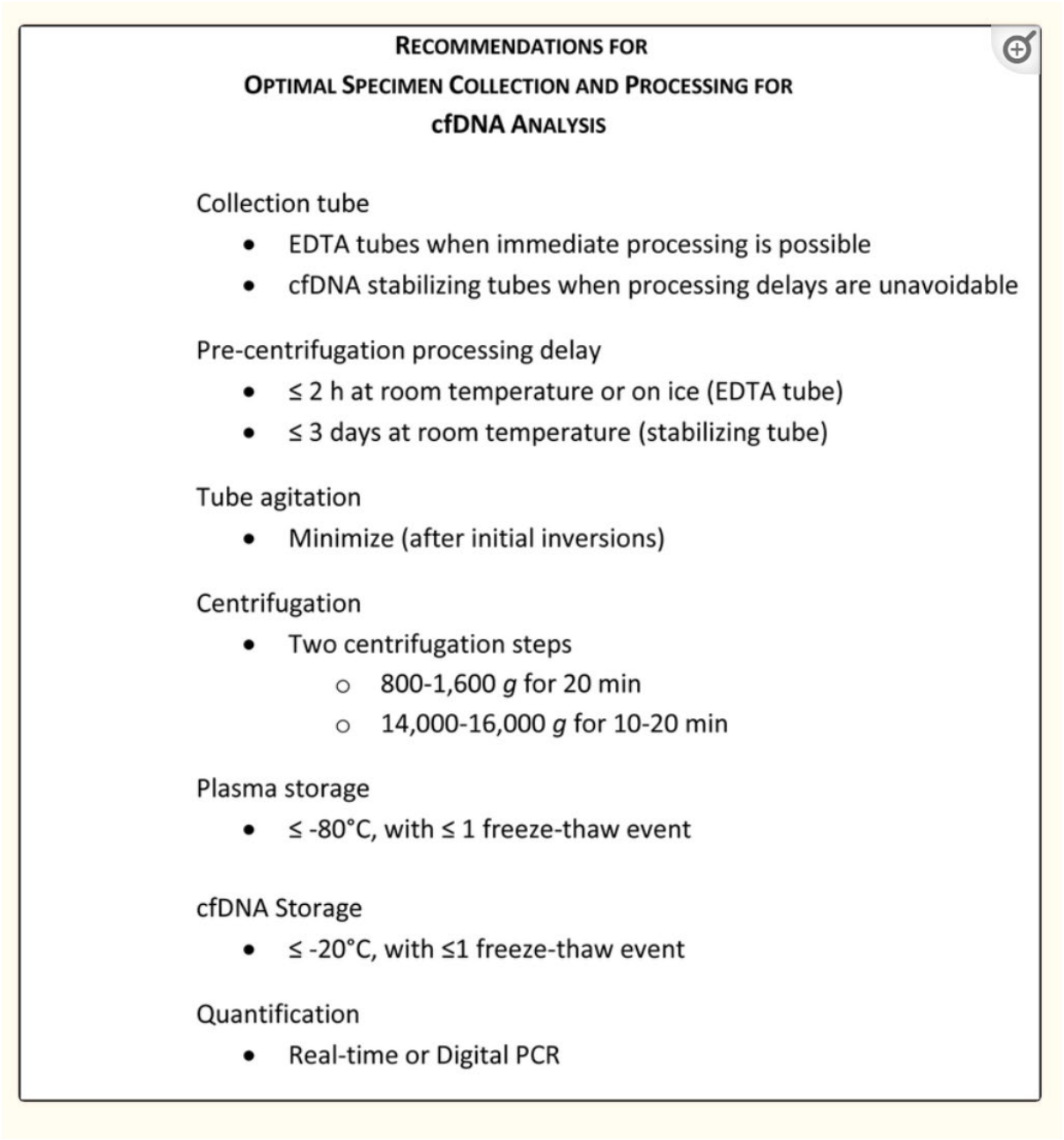


Figure 6A: Retrieved from Greytak SR, Engel KB, Parpart-Li S, Murtaza M, Bronkhorst AJ, Pertile MD, Moore HM. Harmonizing Cell-Free DNA Collection and Processing Practices through Evidence-Based Guidance. Clin Cancer Res. 2020 Jul 1;26(13):3104-3109.

6.3 Aims & Objectives

Aim: The aim of this study is to define peri-operative plasma cfDNA dynamics in patients with non-metastatic breast cancer and cfDNA association with certain tumour characteristics.

Objectives.

1. We aim to define the peri-operative dynamics of cfDNA in patients with non-metastatic breast cancer to determine whether cfDNA concentration is associated with certain tumour characteristics.
2. To determine whether cfDNA levels correlate with peri-op inflammatory mediators.
3. To determine whether peri-operative cfDNA dynamics are associated with survival outcomes.

6.4 Methodology

6.4.1 Patient Recruitment, Treatment & Follow-up

To analyse the relationship between peri-operative cfDNA and breast tumour characteristics, surgical intervention, 30-day morbidity and two-year cancer outcomes, a prospective group of patients with non-metastatic breast cancer were recruited. Patients undergoing breast cancer surgery and treatment at our institute are being actively recruited to the ethically approved ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study. This study is sponsored by Cancer Trials Ireland (NCT01840293) and open across Irish centres. This study is a sub-study of the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity, and sample collection is within the remit of the biospecimen protocol.

Patients presenting for elective breast cancer surgery, with non-metastatic disease, aged 18-85 years of age were screened for inclusion. Exclusion criteria were applied as follows: received neoadjuvant chemotherapy or hormonal therapy, active infection at the time of surgical intervention.

All patients with breast cancer were discussed at the regional Multi-Disciplinary Meeting (MDM) and managed in keeping with National Cancer Control Programme (NCCP) recommendations. In this institution all patients with breast cancer are routinely screened for axillary nodal disease using ultrasonography +/- fine needle aspiration (FNA) of indeterminate or suspicious nodes. Axillary lymph node dissection is recommended to patients with positive axillary nodal disease receiving upfront surgery. Image guided breast conserving surgery is performed using radiological placed guide wires or a Radiofrequency Identification Device (RFID) tag localisation. Tumour phenotype was recorded from final histopathological reports. Oestrogen receptor/progesterone receptor were considered positive when >10% of cells showed positive staining. HER2 status was assessed by immunohistochemistry (IHC) and Fluorescent in Situ Hybridization (FISH) confirmation in cases with 2+ by IHC. HER2 positivity was defined as either 3+ with IHC and/or amplified with FISH.

Blood samples were phlebotomised peri- operatively from patients at the following time-points– pre-operatively the morning of surgery (pre-op), 3-5 hours post-operatively (early post-op), 10-14 days post-operatively (late post-op). Patients with positive invasive margins or large volume nodal disease requiring further surgery had their 2-week post op sample deferred until 2 weeks after surgery is complete. Plasma samples were stored in K3EDTA bottles and centrifuged at 2500 rpm for 15 mins within 1 hour of phlebotomy. Alternatively, samples were stored in Streck tubes and centrifuged at 2500rpm for 15 mins within 48hours of phlebotomy. Plasma samples were aliquoted into collection tubes (500 µL and 1500 µL), labelled with trial codes and stored at -80 degree Celsius.

6.4.2 Sample Storage

Plasma and tissue samples were stored at -80°C in a dedicated biobank freezer housed in a well-ventilated room containing generator backup power. Freezers were monitored 24/7 using internal and external temperature probes, to ensure the safe preservation of samples and secure alarm system. An automatic emergency call is

made to one of three full-time laboratory staff members if a freezers temperature rises above -75°C so that freezer problems can be assessed and any problems rectified. Aliquots were stored in 500µL and 1500 µL vials and labelled with time/date and trial code. Freezer opening time was restricted to prevent samples being exposed to freeze/thaw cycles. Once extracted, cfDNA samples were stored in -20°C dedicated freezer.

6.4.3 Cell Free DNA Extraction

Cell-free DNA (cfDNA) was extracted using the QIAamp Circulating Nucleic Acid kit (Qiagen, Cat. No. / ID: 55114) via the four processes of lysis, binding, washing and elution (Chapter 3, Figure 3D).

1. Lysis: Free-circulating nucleic acids in biological fluids are usually bound to proteins or enveloped in vesicles, requiring an efficient lysis step to release nucleic acids for selective binding to the QIAamp Mini column. Hence, samples are lysed under highly denaturing conditions at elevated temperatures in the presence of proteinase K and Buffer ACL, which together ensure inactivation of DNases and RNases and complete release of nucleic acids from bound proteins, lipids, and vesicles.
2. Binding: To ensure optimal binding of the circulating nucleic acids to the silica membrane (the QIAamp Mini membrane), buffer ACB is added to the lysate. The lysate is then transferred onto a QIAamp Mini column, and circulating nucleic acids are adsorbed from a large volume onto the small silica membrane as the lysate is drawn through by vacuum pressure. Salt and pH conditions ensure that proteins and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp Mini membrane. A vacuum manifold (e.g., the QIAvac 24 Plus with the QIAvac Connecting System) and a vacuum pump capable of producing a vacuum of –800 to –900 mbar (e.g., QIAGEN Vacuum Pump) are required for

the protocol. A vacuum regulator should be used for easy monitoring of vacuum pressures and convenient vacuum release.

3. Washing: cfDNA remain bound to the membrane, while contaminants are efficiently washed away during 3 wash steps.
4. Elution: In a single step, highly pure circulating nucleic acids are eluted in Buffer AVE, equilibrated to room temperature. Elution of pure nucleic acids Elution is performed using 100µl of Buffer AVE.

The Protocol "Purification of Circulating Nucleic Acids from 5 mls of Plasma" was utilised. Samples were mixed with PBS to bring the total sample volume to 5mls if required. 500µl of proteinase K and 4mls of buffer ACL (containing 1 µg carrier RNA) were added to the samples and incubated at 60° Celsius for 30 minutes. 9mls of buffer ACB was added to the lysate and incubated on ice for 5 mins. The samples were then transferred into QIAamp Mini columns via tube extenders which allowed the lysate to pass through the QIAamp Mini Column Silica membrane via vacuum. Buffer ACW1, buffer ACW2 and ethanol were added to the QIAamp mini columns sequentially. The mini columns were centrifuged at 20,000 g for 3 minutes and then incubated at 56degree Celsius on a heat block for 10 minutes to dry the membranes completely. CfDNA was finally eluted with 100µl of AVE buffer, labelled and stored in -20degree Celsius freezer. CfDNA samples were exposed to one freeze thaw cycle for quantification purposes.

6.4.4 CfDNA Quantification

Traditionally cfDNA was quantified using a spectrophotometer. This technology is based on Beer-Lambert Law which is the concept that biomolecules absorb light at characteristic wavelengths. Measurement of light absorption, by a spectrometer, is used to detect compounds and to measure their concentration in solution. Accuracy is a major concern in spectrophotometry as dsDNA, ssDNA, RNA, free nucleotides and primers cannot be discriminated from each other in a sample**.** RNA

contamination in a DNA sample leads to overestimation of DNA concentration by UV spectrophotometry.

Fluorometry or spectrofluorometry is another type of electromagenetic spectroscopy which measures the light emitted from fluorogenic molecules called fluorophores. Fluorescence based quantitation methods take advantage of this mechanism by employing dye molecules that have been designed to preferentially bind to selected nucleic acids. This type of assay requires a dilution series of known concentration to create a standard curve. Compared to UV spectrophotometric methods, fluorescence is more specific as it is less affected by other components present in samples.

CfDNA samples were quantified using the Denovix DS 11-FX Spectrofluorometer and the DeNovix dsDNA High Sensitivity Quantification Assay. This assay creates a two point-standard curve which covers a range of 250 pg/µL to 1500 ng/ µL. CfDNA samples were brought to room temp and then vortexed briefly to minimize reagent loss on the cap. To prepare the working solution 10mls of the assay buffer was added with 100µl of the dye.

For each standard and unknown sample, 190µl of the working solution was added to a labelled tube. 10ul of standards and 10µl of unknown samples were added to the tubes and mixed well. After being incubates at room temperature for 5 mins. Standards were measured from low concentrations to high and a standard curve was created. Once the standard curve was complete unknown samples were measured in triplicate. A mean of the three values was then calculated as a final concentration (ng/µl).

6.4.5 Systemic Inflammatory Mediator Analysis

IL-6, IL-8, TNF-α, and IL1-β were measured using electrochemiluminescence with the Human Pro-Inflammatory Panel I multispot assay manufactured by MSD (Meso Scale Discovery)® (Gaithersburg, Maryland, USA) using the steps outlined in chapter 4. A panel of 5 cytokines, IL-6, IL-8, TNFα, IL-10 and IL1-β, were measured in blood plasma samples following manufactures instructions. Antibodies were incubated with

unique Linkers at room temperature for 30 min and at 4 °C overnight. U-PLEX 96-well plates were coated with the Linker-coupled Antibodies and incubated at room temperature for 1 hr while shaking at 1000 rpm. Plates were washed three times with PBS-T (PBS with 0.05% Tween-20). A volume of 25 µL of undiluted plasma samples, calibrators and dilution buffer (blank), each in duplicate, were added to individual wells on the sample plate which were pre-filled with 25 µL of dilution buffer and incubated at room temperature for 1 h while shaking at 1000 rpm. Each plate was washed three times with PBS-T and reconstituted with read buffer prior to analysing on the MSD Sector Imager. The MSD Discovery Workbench 4.0 12 software (LSR_4_0_12) produced a curve using a four-parameter logistic regression model with 1/y2 weighting to determine cytokine concentration. An inter-assay analysis was performed to ensure inter-assay plate variability was <15%.

6.4.6 Outcome Analysis

Statistical analysis was performed using SPSS, version 28 and graphs generated using GraphPad PRISM, version 8.4.2. For demographic and clinicopathological data, categorical data was presented by the numbers and percentages in each. Continuous variables were described by their median and interquartile ranges. Perioperative cfDNA levels were represented in ng/µL and mean and SEM levels graphically displayed with statistical significance categorised as: $p<0.05$.

6.4.7 CfDNA, Clinicopathological Features & Mediators of Systemic Inflammation

Descriptive statistics were used to assess clinicopathological variables. Our primary aim was to evaluate if cfDNA levels significantly changed peri-operatively and whether cfDNA levels were associated with tumour characteristics (size, grade, subtype, hormonal status, LVI). A repeated measure analysis of variance (ANOVA) was used to compare mean cfDNA values peri-operatively followed by multiple comparison analysis using Bonferroni.

Chi squared tests were used for associations between categorical variables. The Kruskal-Wallis test was performed to determine statistical differences between

continuous or ordinal and categorical variables. The mean cfDNA value was compared across clinicopathologic groupings using Mann-Whitney-Wilcoxon test. The association between cfDNA and 30-day post-operative morbidity, specifically post-operative wound infectious complications was reported using odds ratios (OR), 95% confidence interval and p-values generated with chi-square (X^2) testing. To assess the association between two continuous variables, spearman correlation was performed. For all of the above analyses, statistical significance was observed at $p < 0.5$.

6.4.8 CfDNA and Breast Cancer Disease Free Survival (DFS)

Breast cancer disease free survival (DFS) was defined as the absence of new cancer deposits at the site of tumour resection (local recurrence) or in distant organs (distant recurrence). Disease Student t-test was used to compare mean perioperative cfDNA levels between those disease free and those with disease-recurrence at two years post-operatively. Statistical significance was observed at (two-tailed) observed at $p<0.05$

Patients were stratified into “cfDNA low” and “cfDNA high” groups according to the median cfDNA value for each peri-operative time point as there is no defined cut-off value stated in the literature. The association between cfDNA and four year cancer recurrence was analysed using Mantel Cox log-rank test and Kaplan Meier curves produced. Statistical significance was observed at (two-tailed) observed at $p<0.05$

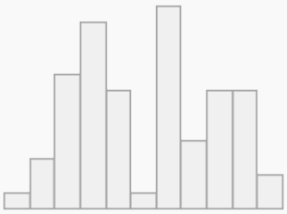
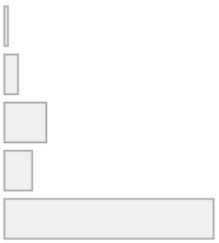
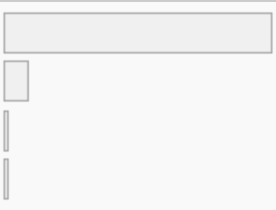
6.5 Results

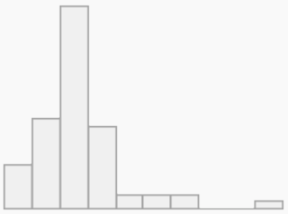
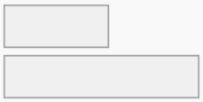
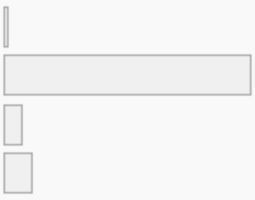
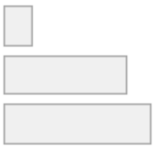
6.5.1 Clinicopathological Characteristics

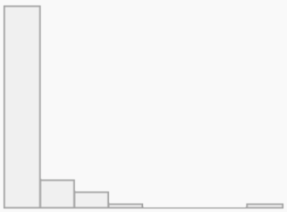
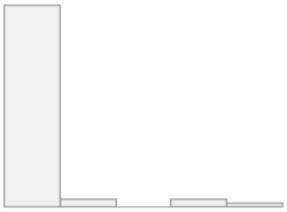
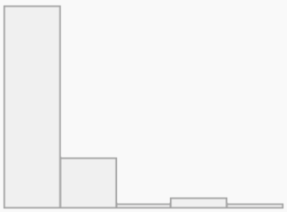
Sixty-three patients who fullfilled the inclusion criteria over an 18 month period were included in the study with a median age of 61(IQR 23.5). Sample collection was during the Covid-19 pandemic. Forty-four patients (69.8%) had a Wide Local Excision and Sentinel Lymph node Biopsy(WLE&SLNB). Nine patients (14.3%) had a Mastectomy and Sentinel Lymph Node Biopsy (Mastectomy&SLNB). A wide local excision and

Axillary Lymph Node Dissection (WLE&ALND) was performed on six patients (9.5%) and three patients had a mastectomy & ALND(4.8%). One patient opted to have a bilateral mastectomy and ALND (1.6%). Fifty-six (88.9%) patients had an Invasive Ductal Carcinoma (IDC) subtype whilst five patients had Invasive Lobular Carcinoma (7.9%). Of the remaining patients, one patient had mixed lobular/ductal subtype and one patient had a metaplastic subtype. Twenty-eight patients included in the study had lymph node positive disease (44.4%). The majority of patients had stage T2 disease (N=42, 66.7%) with a median tumour size of 25mm (IQR 12). Fifty-two patients (82.5%) had ER+/HER2- disease, six patients (9.5%) had TNBC, four patients (6.3%) had ER+/HER2+ disease and only one patients had ER-/HER2+ breast cancer. Fifty-seven patients(90.5%) had either grade 2 or grade 3 disease and the remaining six patients had grade 1 disease. Fifty-four percent (N=34) had associated LVI present in the tumour specimen (Table 1).

Table 1: Characteristics of Included Patients (N=63)

Age	Mean (sd) : 58.4 (13.4) min ≤ med ≤ max: 32 ≤ 61 ≤ 81 IQR (CV) : 23.5 (0.2)	30 distinct values		63 (100.0%)	0 (0.0%)
Operation	1.Bilateral Mastectomy&ALND 2. Mastectomy&ALND 3. Mastectomy&SLNB 4. WLE&ALND 5. WLE&SLNB	1 (1.6%) 3 (4.8%) 9 (14.3%) 6 (9.5%) 44 (69.8%)		63 (100.0%)	0 (0.0%)
Subtype	1. IDC 2. ILC 3. Metaplastic 4. Mixed	56 (88.9%) 5 (7.9%) 1 (1.6%) 1 (1.6%)		63 (100.0%)	0 (0.0%)
Lymph Node Positivity	Min : 0 Mean : 0.4 Max : 1	0 : 35 (55.6%) 1 : 28 (44.4%)		63 (100.0%)	0 (0.0%)

No. of Positive Nodes	Mean (sd) : 1.4 (4.4) min ≤ med ≤ max: 0 ≤ 0 ≤ 31 IQR (CV) : 1 (3.2)	0 : 35 (55.6%) 1 : 18 (28.6%) 2 : 4 (6.3%) 3 : 2 (3.2%) 4 : 2 (3.2%) 16 : 1 (1.6%) 31 : 1 (1.6%)		63 (100.0%)	0 (0.0%)
T Size	Mean (sd) : 1.9 (0.6) min ≤ med ≤ max: 1 ≤ 2 ≤ 3 IQR (CV) : 0 (0.3)	1 : 15 (23.8%) 2 : 42 (66.7%) 3 : 6 (9.5%)		63 (100.0%)	0 (0.0%)
Size in mm	Mean (sd) : 28.6 (15.8) min ≤ med ≤ max: 4 ≤ 25 ≤ 96 IQR (CV) : 12 (0.6)	29 distinct values		63 (100.0%)	0 (0.0%)
ER	1. neg 2. pos	7 (11.1%) 56 (88.9%)		63 (100.0%)	0 (0.0%)
PR	1. neg 2. pos	22 (34.9%) 41 (65.1%)		63 (100.0%)	0 (0.0%)
HER2	1. neg 2. pos	58 (92.1%) 5 (7.9%)		63 (100.0%)	0 (0.0%)
Hormonal Expression	1. ER-/HER2+_ 2. ER+/HER2- 3. ER+/HER2+ 4. TNBC	1 (1.6%) 52 (82.5%) 4 (6.3%) 6 (9.5%)		63 (100.0%)	0 (0.0%)
Grade	Mean (sd) : 2.4 (0.7) min ≤ med ≤ max: 1 ≤ 2 ≤ 3 IQR (CV) : 1 (0.3)	1 : 6 (9.5%) 2 : 26 (41.3%) 3 : 31 (49.2%)		63 (100.0%)	0 (0.0%)
DCIS	1. no 2. yes	11 (17.5%) 52 (82.5%)		63 (100.0%)	0 (0.0%)
LVI	1. no 2. yes	29 (46.0%) 34 (54.0%)		63 (100.0%)	0 (0.0%)

cfDNA_T1	Mean (sd) : 3.8 (5.9) min ≤ med ≤ max: 0 ≤ 1.8 ≤ 38.9 IQR (CV) : 2.8 (1.5)	61 distinct values		63 (100.0%)	0 (0.0%)
cfDNA_T2	Mean (sd) : 17.6 (44.5) min ≤ med ≤ max: 0 ≤ 3.2 ≤ 204 IQR (CV) : 6.3 (2.5)	59 distinct values		60 (95.2%)	3 (4.8%)
cfDNA_T3	Mean (sd) : 3.8 (4.6) min ≤ med ≤ max: 0 ≤ 2.1 ≤ 21.8 IQR (CV) : 3.9 (1.2)	52 distinct values		55 (87.3%)	8 (12.7%)

Axillary lymph node dissection = ALND, Sentinel lymph node biopsy = SLNB, Wide local excision = WLE, IDC = Invasive ductal carcinoma, ILC = Invasive lobular carcinoma, Oestrogen receptor = ER, Progesterone receptor = PR, Human epidermal growth factor receptor = HER2, Triple negative breast cancer = TNBC, Ductal carcinoma in situ = DCIS, Lymphovascular invasion = LVI, cfDNA = Cell free DNA, cfDNA_T1 = Cell free DNA Time point 1 (pre-op), cfDNA_T2 = Cell free DNA Time point 2 (early post-op), cfDNA_T3 = Cell free DNA Time point 3 (late post-op)

6.5.2 CfDNA Dynamics Peri-Operatively

Sixty-three patients had pre-op blood samples phlebotomised, of which 60 (96.7%) had early post-op samples and 55 (85%) had late post op samples collected. Pre-operatively the mean cfDNA concentration was 3.82ng/µl (SEM = 0.74). This increased significantly early post-op in keeping with surgical insult to 17.59 ng/µl (SEM = 5.75). Late post-op there was a significant decrease in cfDNA to near baseline with a mean value of 3.78ng/µl (SEM = 0.62) (Figure 6B). Post op cfDNA concentration did not appear to differ significantly according to surgical procedure (Figure 6C). CfDNA levels in patients who underwent a mastectomy & SLNB reduced early post-

op, from 6.04ng/µl pre-op to 1.65ng/µl early post-op however this was not statistically significant (Figure 6D).

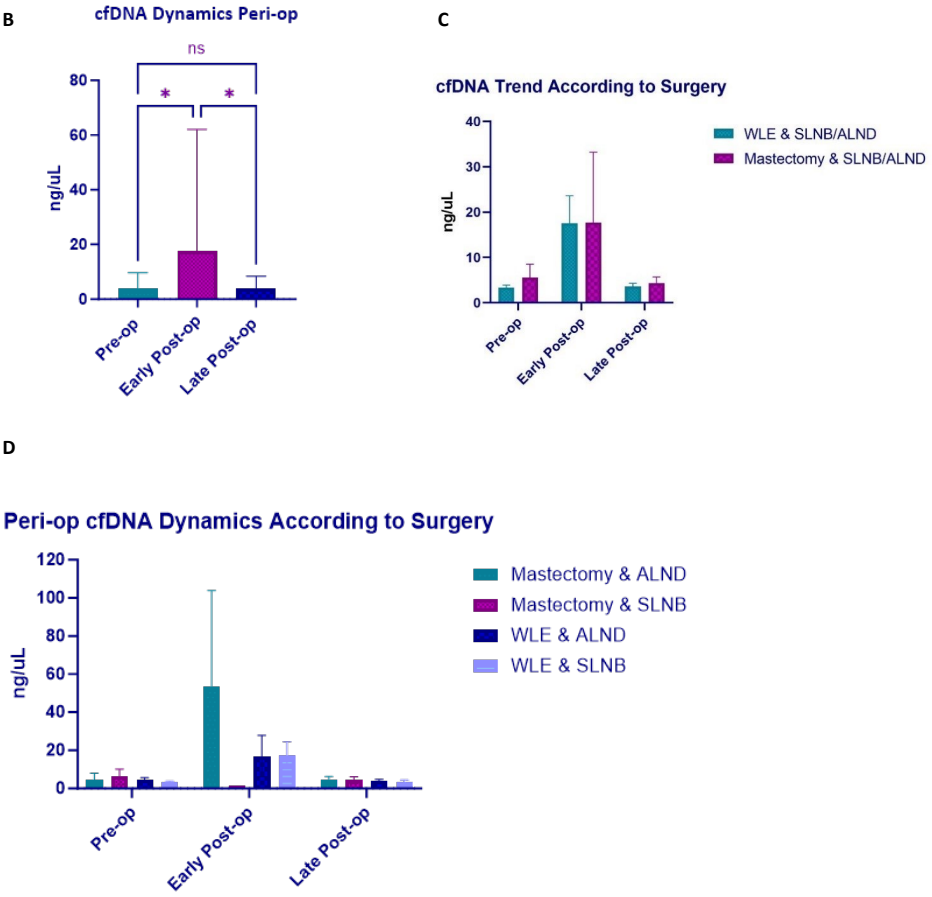


Figure 6 B-D. Peri-operative cfDNA trend **B.** cfDNA release pre, 4 hours post -op and late (two-weeks) post-op, **C.** cfDNA release peri-operatively in patients who underwent a wide local excision (WLE) and sentinel lymph node biopsy (SLNB) or axillary lymph node dissection (ALND) compared to patients who had a mastectomy and SLNB/ALND **D.** cfDNA release peri-operatively comparing the cohorts who had a mastectomy and ALND, mastectomy and SLNB, WLE and ALND and WLE and SLNB.

6.5.3 CfDNA Association with Clinicopathological Features

Pre-op, early post-op, and late post-op cfDNA concentrations were assessed for associations with categorical clinicopathological characteristics using Mann-Whitney U test. There was no significant association between peri-operative cfDNA concentration and tumour histology, lymph node status, tumour size, hormonal expression, or grade. Patients with associated LVI in their tumour had statistically higher cfDNA concentrations early post-op (Table 2).

Table 2: CfDNA Association with Clinicopathological Features

	CfDNA Pre-op	**CfDNA Early Post-op**	**CfDNA Late Post-op**
Histological subtype	0.360	0.519	0.966
Lymph node status	0.300	0.766	0.798
Number of positive nodes	0.314	0.500	0.402
Tumor size	0.223	0.964	0.984
ER pos	0.527	0.137	0.122
HER2 pos	0.206	0.721	0.383
Hormonal subgroup	0.198	0.371	0.357
Grade	0.139	0.096	0.713
LVI	0.155	0.011*	0.376

ER= oestrogen receptor, HER2 = human epidermal growth factor receptor, LVI = lymphovascular invasion

6.5.4 CfDNA Association with Inflammatory Mediators

Of the 63 patients with cfDNA samples available peri-operatively, 36 patients had peri-op IL-6, IL-8 and TNFa levels quantified, 12 patients had IL-10 levels measured and 18 had IL1b concentrations available. Linear correlation analysis of peri-op cfDNA concentrations with individual systemic mediators of inflammation are presented in Table 3. A significant linear relationship was observed between early post-op cfDNA concentration and early post-op IL-8 levels ($r = 0.430$, $p = 0.009$).

A significant inverse relationship was observed between pre-op cfDNA and early post op IL1b. Significant inverse relationships were also detected between late post-op cfDNA concentration and post op IL1b levels (both early post-op and late post-op).

Table 3: Correlation of cfDNA levels with Systemic Mediators of Inflammation at Three Peri-Operative Time Points (r coefficient (p-value))

	CfDNA Pre-op	**CfDNA Early Post-op**	**CfDNA Late Post-op**
Age	-0.35(0.787)	-0.52(0.693)	0.142(0.302)
IL6 T1	-0.139(0.420)	0.235(0.167)	-0.238(0.162)
IL6 T2	-0.156(0.362)	0.290(0.087)	-0.028(0.873)
IL6 T3	-0.184(0.282)	0.057(0.739)	-0.255(0.133)
IL8 T1	-0.243(0.153)	0.232(0.174)	-0.185(0.281)
IL8 T2	-0.235(0.168)	0.430(0.009*)	-0.155(0.365)
IL8 T3	-0.120(0.487)	0.156(0.365)	-0.198(0.248)
TNFa T1	-0.151(0.380)	0.049(0.776)	-0.132(0.444)
TNFa T2	-0.109(0.527)	0.008(0.923)	-0.193(0.259)
TNFa T3	-0.060(0.728)	-0.021(0.903)	-0.137(0.424)
IL10 T1	0.462(0.131)	-0.175(0.587)	NA
IL10 T2	0.028(0.931)	0.140(0.665)	-0.112(0.729)

IL10 T3	-0.217(0.499)	0.000(1.0)	0.308(0.331)
IL1b T1	-0.365(0.080)	0.377 (0.069)	-0.350(0.094)
IL1b T2	-0.614(0.001*)	0.177(0.408)	-0.491(0.015*)
IL1b T3	-0.401(0.052)	0.156(0.467)	-0.558(0.005*)

Spearmann correlation co-efficient: r <0.3 -> poor correlation, r = 0.3-0.5 -> fair correlation, r = 0.6-0.8 -> moderately strong correlation, r >0.8 -> very strong correlation.

6.5.5 CfDNA and Post-Operative Sequelae

Eleven patients developed post-op complications within 30 days post surgery (17.5%). Six (9.5%) of these patients developed surgical site infections as defined by the Centre for Disease Control (CDC) [300]. Three patients developed haematomas (4.8%), two of which required interventional radiological drainage. Two patients developed Covid19. Patients with high cfDNA levels pre-op, early post-op or late post-op were not at increased risk of developing complications within 30 days following surgery. Twenty-nine patients (46%) received adjuvant chemotherapy following a risk benefit analysis assesment with a Consultant Medical Oncologist. Table 4.

Table 4: Risk of Developing Post-Operative Complications with High cfDNA levels.

	Overall Complication (wound infection, haematoma, Covid19)	**Wound Infection**
High CfDNA Pre-op	OR 1.02(CI .279-3.797)	OR 4.83(CI 0.530-43.96)
High CfDNA Early Post-op	OR 0.306 (CI 0.72- 1.291)	OR 1.0(CI 0.185-5.403)
High cfDNA Late Post-op	OR 1.31(CI 0.349-4.95)	OR 6.14 (CI 0.667-56.477)

CfDNA = cell free DNA, OR = odds ratio, CI = confidence interval

Table 5: Summary Table of Patients with Breast Cancer Cancer Recurrence

Age at diagnosis	Subtype	Hormonal Expression	T stage	N status	No. of Nodes Positive	Grade	Time to Recurrence (months)	Site of Recurrence
76	IDC	ER+/ PR-/Her2-	1	Negative	NA	3	18	Axilla
46	IDC	ER+/ PR-/Her2-	2	Positive	2	3	29	Brain
61	ILC	ER+/ PR+/Her2-	2	Positive	31	3	20	Thorax
73	IDC	TNBC	2	Negative	NA	3	6	Brain

IDC = invasive ductal carcinoma, T stage = tumor stage, N stage = nodal stage, TNBC = triple negative breast cancer, ER= oestrogen receptor, HER2 = human epidermal growth factor receptor, PR= progesterone receptor.

6.5.6 CfDNA and Breast Cancer Disease Free Survival

Median follow up time was 20months (IQR 11 months). Of the 63 patients with cfDNA samples available peri-operatively, four patients experienced recurrence of their disease. Median time to recurrence was 19 months (IQR 8 months). Three had distant metastatic disease (lung, brain and pulmonary) and one patient had local recurrence of disease. There was no statistically significant difference in the mean cfDNA value between patients that had disease recurrence and those that remained disease free at any of the peri-operative time points (Pre-op p=0.6255, Early post-op p= 0.0695, Late post-op p=0.6465). Simialrly there was no difference in DFS between patients who had high cfDNA levels compared to low cfDNA levels pre-op, early post-op or late post-op (p=0.9850, p=0.261, p=0.3351). Figures 6E-G.

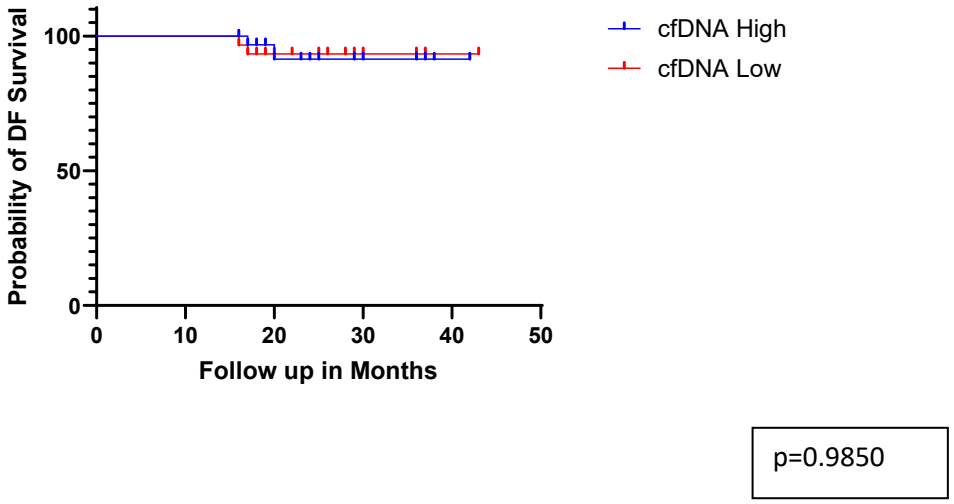


Figure 6E: Disease Free Survival Kaplan Meir Curves Stratified According to Pre-operative cfDNA Levels

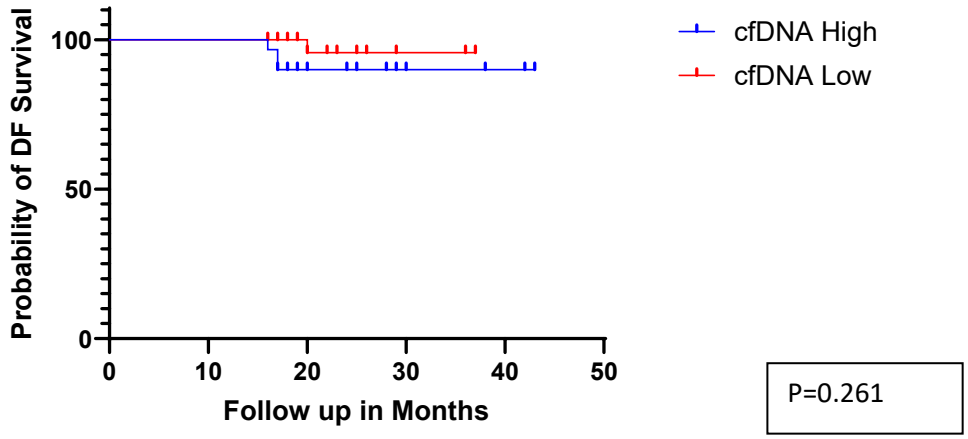


Figure 6F: Disease Free Survival Kaplan Meir Curves Stratified According to Early Post-operative cfDNA Levels

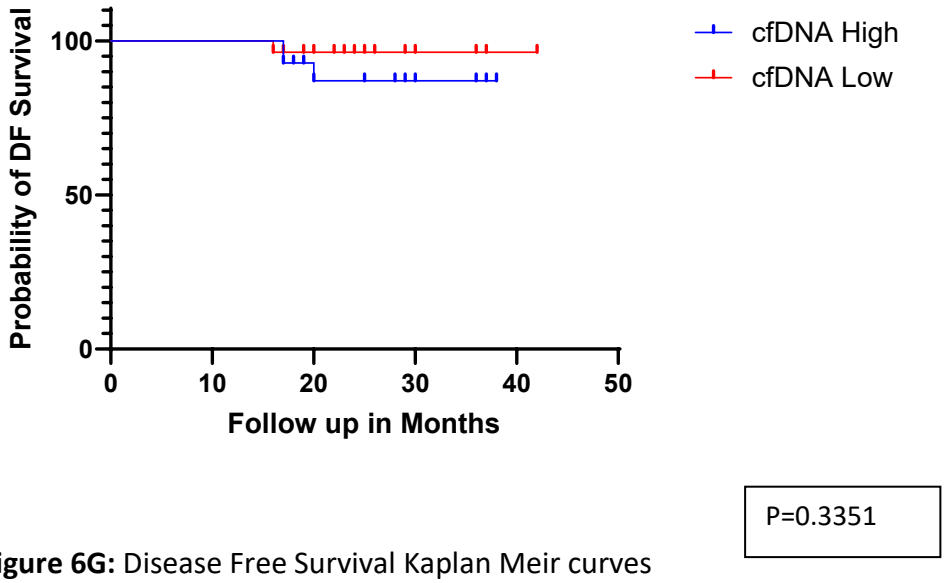


Figure 6G: Disease Free Survival Kaplan Meir curves Stratified According to Late Post-operative cfDNA Levels

6.6 Discussion

Image-based screening provides an estimated 20% reduction in breast cancer mortality [114] however mammography incurs several downsides including false positive rates, limited sensitivity and patient discomfort [115]. Reliable breast cancer blood-based biomarkers are limited in clinical practice. The most widely used blood-based tumour marker CA-15 lacks sensitivity, only clinically relevant in stage IV disease and is hindered by a long half-life in circulation reducing the potential to monitor treatment response [116]. Liquid biopsy techniques have emerged as an advantageous alternative to tumour marker analysis as it allows for non-invasive serial monitoring of disease status. This study analysed the peri-operative dynamics of cfDNA in patients undergoing breast cancer to determine whether surgery has an impact on cfDNA concentration. CfDNA concentration increased significantly early post-operatively and returned to near baseline two week post-surgery. Whilst the half-life of cfDNA is approximately 2 hours, cfDNA concentration can remain elevated

for several days to weeks after trauma, depending on the severity of trauma incurred[301]. Patients who underwent colorectal and invasive bladder cancer surgery were found to have persistently elevated cfDNA concentrations upto 4 weeks post-operatively[302]. However, as breast cancer surgeries are generally shorter operations that do not involve entrance into the visceral cavity a shorter return to baseline cfDNA concentration was evident in our cohort. Patients who underwent more extensive breast cancer and axillary surgery trended towards an increase early post-operative cfDNA concentration, though this was not statistically significant. As post-operative ctDNA detection has been proposed to guide post-treatment surveillance, it's imperative to determine the optimum timing of ctDNA analysis to eliminate surgical trauma cfDNA from confounding the results. Previous studies have shown that trauma-induced cfDNA could affect the ability to detect ctDNA and only few techniques have the sensitivity required to avoid contmaination from trauma-induced cfDNA[303]. We demonstrated that the total cfDNA level increases immediately post-surgery and elevation returns to baseline approximately 2 weeks after surgery. Two week post- breast cancer surgery should be considered as an appropriate time-point for blood collection to perform ctDNA analysis.

In this study, we aimed to determine whether peri-operative cfDNA dynamics were associated with clinicopathological features and survival outcomes. Patients with LVI in their tumour had a significantly elevated early post-operative cfDNA concentration compared to patients without LVI. Tumour LVI is a hallmark of tumour aggressiveness and a well established prognostic marker in breast cancer studies [304, 305]. One possible explanation is that LVI positive tumours have the ability to cross the basement membrane and influence neighbouring cells in the tumour microenvironment, elliciting a more pronounced surgical cfDNA release in the post-operative period. A positive correlation between early post-operative IL-8 release and early post-operative cfDNA concentration was evident in our cohort. IL-8 is an important inflammatory chemokine with autocrine and/or paracrine tumour-promoting potential. Clinical studies have shown that increased serum IL-8 levels are associated with positive lymph node status and higher-stage breast tumours [208]. Furthermore, elevated systemic IL-8 levels are a poor prognostic indicator in patients

with breast cancer[58, 59]. The pro-inflammatory cytokine IL-6 also trended to correlate positively with cfDNA concentration in the early post-operative period but this was not statistically significant. These correlates suggest that the peri-operative period is a complex process with networks of immunological and genomic crosstalk. Therapeutic attenuation with immunomodulatory agents in the peri-operative space could provide a window of opportunity to suppress tumour-promoting inflammation post breast cancer surgery.

Various studies have reported that an increase in post-treatment cfDNA blood levels may indicate the presence of residual tumor and incur worse breast cancer survival outcomes[307, 308]. Data from our cohort did not corroborate with these findings as we did not find a significant association between peri-operative cfDNA concentration and disease free survival. On visual inspection of the Kaplan Meier graphs, patients with high early and late post-operative cfDNA appeared to have a worse DFS but this was not statistically significant. These results are limited by a short median follow-up time of 20 months and a low number of cancer recurrences (N=4). In the future, we aim to re-analyse the data to determine the relationship between peri-operative cfDNA concentration and five year disease-free and overall survival rates.

6.7 Limitations

There were a number of limitations to this study. Whilst the prospective recruitment of patients with non-metastatic breast cancer strengthens the methodological design of the study, a sufficient follow up period could not be achieved within the timeframe of this MD thesis to derive any meaningful prognostic results. We look forward to obtaining five- and ten-year disease-free survival data to determine whether peri-operative cfDNA predicted those likely to experience disease recurrence. Furthermore, the correlation analysis between peri-operative cfDNA levels and inflammatory mediators was limited by the fact that only 36 out of the 63 patients had cytokine data available. In contrast, there were many strengths to the study including the prognostic design and pre-defined inclusion criteria. However, the biggest strength of the study is strict adherence to biospecimen evidence-based best

practice as each procedural step, from preparations for biospecimen collection to quantification and quality assessment of cfDNA was performed in accordance with the National Cancer Institute's (NCI) Biorepositories and Biospecimen Research Branch (BBRB) protocol.

6.8 Conclusion

Peri-operative changes in breast cancer cfDNA are dynamic with a significant rise in cfDNA concentration detected four hours post-surgery and a return to near baseline two weeks post-operatively. Early post-operative cfDNA release appeared to correlate positively with both IL-8 release and the presence of tumour LVI. These clinical correlates suggest the presence of peri-operative immunological and genomic crosstalk. Immunomodulation in the peri-operative space could provide an opportunity to suppress tumour-promoting inflammation post breast cancer surgery.

Chapter 7: Association of Circulating Tumour DNA (ctDNA) with Disease Free Survival in Breast Cancer – A systematic review and meta-analysis.

7.1 Abstract: Association of Circulating Tumour DNA (ctDNA) with Disease Free Survival in Breast Cancer–A systematic review and meta-analysis.

Background

Fragmented DNA is continuously released into the circulation following apoptosis and necrosis of both cancerous and non-cancerous cells. When it is released by cancer cells, it is specifically known as circulating tumour DNA (ctDNA). We performed a systematic review and meta-analysis to determine the clinical utility of ctDNA as a prognostic biomarker for predicting breast cancer outcomes.

Methods

A systematic review and meta-analysis was performed in keeping with PRSIMA guidelines. Primary outcome was the ability of ctDNA to identify disease free survival /relapse free survival in breast cancer. Secondary outcomes focused on subgroup analysis in the setting of early breast cancer and metastatic breast cancer. Statistical analysis was performed using Revman, version 5.

Results

Thirteen studies including 1,809 patients were suitable for inclusion. ctDNA mutation detection (both pre and post treatment) was significantly associated with worse disease-free survival (DFS) (HR 3.38,95% CI 2.36-4.84, p = <0.0001). ctDNA detection was significantly associated with a reduction in disease free survival in both the early breast cancer subgroup (HR 7.03, CI 3.4- 14.55, p=<0.0001) and metastatic/locally advanced subgroup (HR 2.18, 95% CI 1.69-2.81, p<0.0001). Pre- and post-treatment plasma sample collection was analysed in both early and metastatic groups. The post treatment group encompassed both surgical and/or oncological therapy. Pre-treatment and post-treatment plasma detection of ctDNA was significantly

associated with reduced DFS (HR 3.03, 95% CI 2.18-4.21, p=<0.00001, HR 8.17, 95% CI, 1.91-65.89, p = 0.05).

Conclusion

Circulating tumour DNA is an important prognostic biomarker of reduced breast cancer disease free survival. Detection of elevated plasma ctDNA can predict patients at high risk of relapse and therefore may provide an excellent method to stratify risk and personalise patient follow-up.

7.2 Introduction

Breast cancer is one of the most common cancers affecting women worldwide [309]. Despite significant advances in treatment regimes, screening and surveillance, the risk of disease recurrence persists for years after the initial diagnosis [310]. Local and systemic breast cancer recurrence can be difficult to detect early due to the lack of associated clinical symptoms and the limitations of radiological investigations. Mammography-based screening provides an estimated 20% reduction in breast cancer mortality rates but has significant downsides including a high false-positive rate, limited sensitivity, and patient discomfort [115, 311]. Therefore, there is considerable interest in new non-invasive methods for early disease detection.

Liquid biopsy is a non-invasive technique which can yield important diagnostic, therapeutic and prognostic information in many types of cancer [128] . A component of liquid biopsy known as circulating tumour DNA (ctDNA) has shown promise in the detection of breast cancer and predicting clinical and cancer outcomes[100, 129]. ctDNA is a sub-group of cell-free DNA (cfDNA) which is present in healthy individuals and is released from non-malignant cells [130]. ctDNA is found within the circulation of patients with cancer owing to the continuous release of fragmented DNA from necrotic and apoptotic cancer cells, and it accounts for between 0.01% to as much as 50% of total cfDNA in cancer patients[131, 132]. These fragments have a length of approximately 166, 332, or 498 base pairs and exhibit tumour-specific alterations, including point mutations, chromosomal rearrangement, copy-number variation (CNV), and DNA methylation[133, 134]. ctDNA levels are influenced by tumour size and burden therefore serial measurement of ctDNA can provide real time non-invasive monitoring of response to treatment and early detection of recurrence [135].

Personalised medicine and precision oncology emerged as an exciting concept in the late 90's with the advent of Trastuzumab for HER2+ breast tumours and Imatinib for BCR-ABL Chronic Myeloid Leaukaemia [136]. Around the same time, independent studies reported the presence of specific KRAS and NRAS point mutations in ctDNA from pancreatic adenocarcinoma [137]. The objective is to provide the most accurate and effective oncological treatment to patients based on

individuals' genetic profile [130]. ctDNA can not only be used as a blood-based biomarker of response to treatment, but it can also indicate tumour sensitivity to specific chemotherapy regimens [138]. PIK3CA and BRCA1 are common breast cancer mutations and significant research has been invested into targeting these mutations. The PARP inhibitor Olaparib is more effective in the presence of BRCA1 mutation whilst PIK3CA suggest sensitivity to the MTOR inhibitor Everolimus [101, 103].

ctDNA is advantageous as a biomarker as it has a short half-life in the bloodstream, and this characteristic makes it a very important tool in analysing real-time situation of mutation dynamics and tumour burden after surgery or systemic treatment. ctDNA is also emerging as an important adjunct in screening certain types of cancer. Detection of certain genes, including APC, BRAF, KRAS and CTNNB1 in circulating plasma is highly associated with the presence of colorectal cancer or advanced adenomas in colorectal cancer screening[139]. Monitoring ctDNA levels has the potential to gauge response to treatment and aid in early detection of disease progression or recurrence. Thus, the aim of this study is to determine the association of ctDNA with breast cancer disease-free survival (DFS) and progression-free survival in early, locally advanced, and metastatic breast cancer.

7.3 Methods

This study adhered to the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA and Meta-Analysis of Observational Studies in Epidemiology)[312]. Analysis and results were extracted from previous ethically approved studies therefore patient consent and ethical approval was not required. The study was prospectively registered with PROSPERO, registration no. 152173.

7.3.1 Search Strategy

An electronic search was conducted using the Cochrane library, Sciencedirect, PubMed and Embase. All studies included up to 31/10/2021 and all languages were included. The following search terms/MESH terms were used: (ctDNA (Mesh) OR circulating tumour DNA OR liquid biopsy) AND (breast cancer (Mesh) OR breast

carcinoma OR breast tumour) AND (prognosis (Mesh) OR survival). All titles were initially screened and appropriate abstracts were reviewed. Each of the publications' bibliographies and Google scholar were manually searched for relevant articles. The last date of search was 31/10/2021. Where data in publication was insufficient, the authors contacted authors of relevant studies seeking permission to access data to extrapolate hazard ratios.

7.3.2 Outcomes

The primary outcome was to determine the clinical utility of ctDNA to identify disease free survival /relapse free survival in breast cancer. For the purpose of data reporting, disease free survival will include relapse free survival and progression free survival. Subgroup analysis was performed to identify the clinical utility of ctDNA in the metastatic versus early disease population. Similarly, subgroup analysis was performed to determine whether pre-treatment or during-treatment (surgery or chemotherapy) sampling of ctDNA was more prognostic. The quality of the studies included was assessed using the Newcastle-Ottawa Scale (NOS). For the NOS, points were awarded for patient selection (maximum four points), outcome assessment (maximum three points) and comparability of cohort (maximum two points) each added to a maximum of nine points [313]. The risk of bias was assessed using the Risk of Bias in Non-Randomized Studies of Intervention (ROBINS-I Tool; Cochrane Bias Methods Group) [314]. Publication bias was assessed via visualisation of the forest plots.

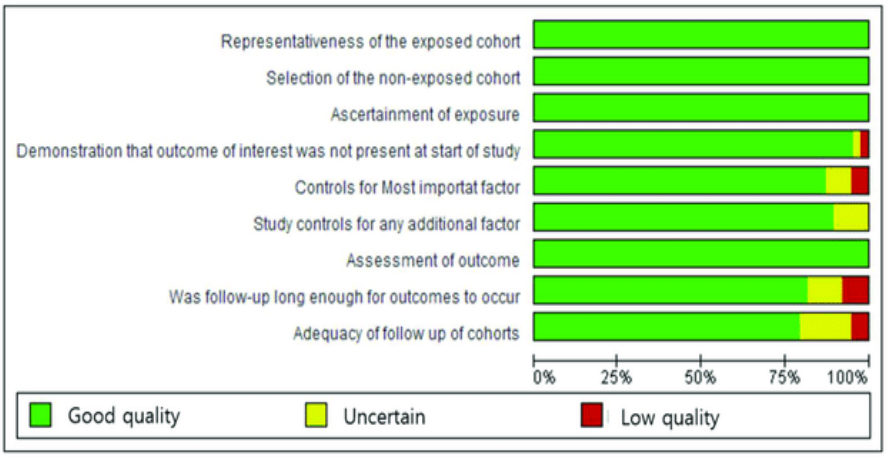


Figure 7A: Newcastle-Ottawa Scale

7.3.3 Inclusion Criteria

The studies had to meet the following prespecified inclusion criteria: (1) a ctDNA blood sample was measured; (2) DFS, progression-free survival, or relapse-free survival was reported as an HR; and (3) the patient population only had breast cancer. Studies were excluded if cell-free DNA was measured, urinary ctDNA was measured, the outcomes of interest (DFS, relapse-free survival, or overall survival) were not reported, or the HR could not be extrapolated from the available data.

7.3.4 Data Extraction

The available literature was reviewed according to the above predefined strategy and criteria. The following variables were extracted: title and study details (year, journal, design, country), study population characteristics (sample size, target mutation, cancer stage, method of quantification, time of sampling, outcome measure) and ctDNA data .

7.3.5 Statistical Analysis

Statistical analysis was performed using the Review Manager 5, *RevMan 5* (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark). Outcome data was reported as Hazard ratios (HR). Hazard ratios reported in the study were used when available, otherwise it was extrapolated using the available data. The 95% confidence interval (95% CI) were estimated using the Mantel-Haenszel method. HR of >1 favoured a worse outcome i.e. a reduced disease free/progression free survival associated with elevated ctDNA levels. 95% confidence interval could not cross unity to be considered statistically significant. Heterogeneity was assessed using I^2 statistics, with >50% considered as significant heterogeneity. A fixed-effects model was preferred to random-effects model when there was no significant heterogeneity and vice versa when there was significant heterogeneity (I^2>50%). Pooled effect estimates (hazard ratios) of differences were calculated using random effects models, accounting for potential inter study heterogeneity. p-value of <0.05 were considered significant.

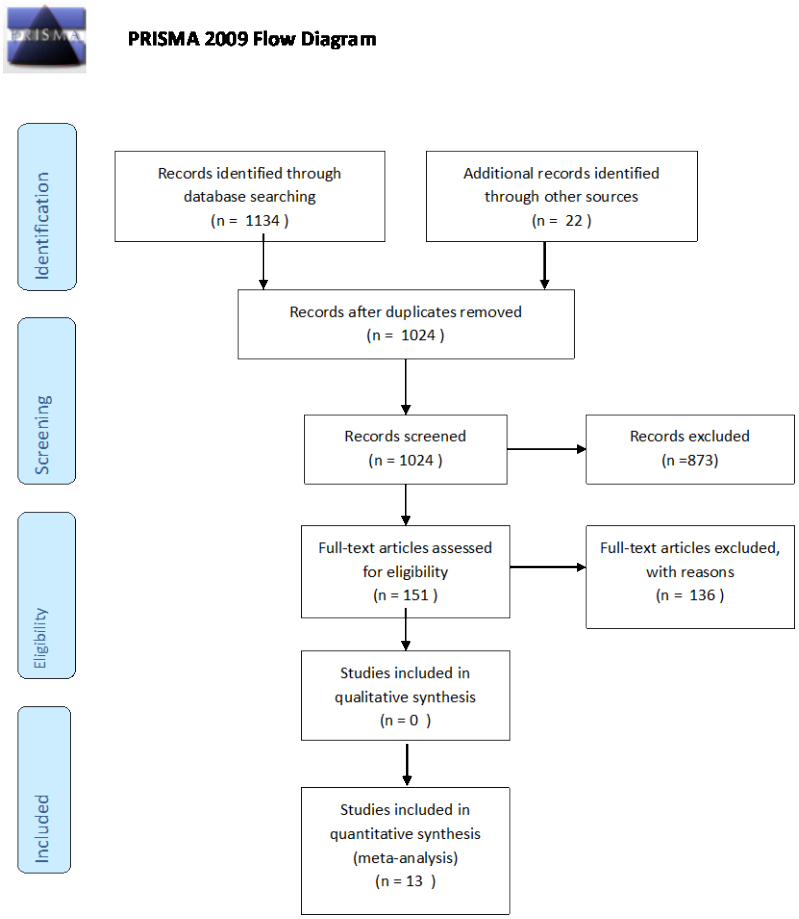


Figure 7B: PRISMA Flow Diagram

7.4 Results

7.4.1 Eligible Studies

A total of 1156 publications were found using the predefined search terms, of which, 13 studies were eligible for inclusion. Nine prospective population studies were included [190, 191, 315-320] while the remaining four studies were retrospective cohort studies [138, 321-323]. All studies were published between 2002 and 2021. Studies were comparable, reporting ctDNA levels as a prognostic indicator of breast cancer outcomes. Characteristics and Newcastle Ottawa scores are presented in Table 1.

The studies differed in their methodology. Six studies examined ctDNA levels as a marker of progression free survival amongst patients with advanced or metastatic breast cancer [138, 190, 191, 319-321]. The other seven studies reported ctDNA levels and disease-free survival for patients with early breast cancer [315-318, 322-324]. The timing of ctDNA sampling also varied between studies. Six studies analysed ctDNA samples prior to starting treatment (surgical or oncological) [138, 315, 318, 319, 321, 322] , whilst four studies recorded ctDNA after commencing treatment to predict oncological outcomes[190, 191, 317, 324]. Three studies analysed ctDNA post Neoadjuvant Chemotherapy (NACT) to predict future recurrence risk [316, 320, 323].

Quantification and measurement of ctDNA levels varied between the studies. Hu et al, Ma et al, Chandarlapaty et al and Schiavon et al [138, 190, 191, 321] analysed serial measurements of ctDNA in metastatic breast cancer patients. Hu and colleagues utilised a receiver under the operating curve (ROC) to determine the optimum ctDNA cut off value for predicting disease progression[138]. Ma et al and Chandarlapaty et al measured mutation clusters and mutation threshold respectively to ellicit the prognostic value of ctDNA variations[191, 321]. Schiavon et al investigated whether the detection of ESR1 mutation influenced oncological outcomes[190]. O'Leary et al collected serial plasma samples at baseline and during treatment as part of the PALOMA-II trial and calculated a circulating tumour fraction cut-off [320]. Kruger and colleagues measured baseline ctDNA prior to commencing Everolimus and NGS findings for each variant were expressed as mutant ctDNA molecule numbers per mL plasma and number of hotspot mutations[319]. Magbanua et al and Radovich et al both used a targeted panel to identify the presence of ctDNA and calculated a variant threshold to determine ctDNA positivity [323, 324]. Magbanua analysed ctDNA pre and post NACT whilst Radovich et al only measured ctDNA post NACT [323, 324] The remaining five studies examined early breast cancer disease and reported a delta trend in ctDNA over time [315-318, 322]. ctDNA mutation detection was reported as a poor prognostic indicator of DFS.

The target mutations differed between studies. The most common mutations that were targeted were TP53, PIK3CA and ESR1. Hu et al and Coombes et al used

sophisticated Whole Exome Sequencing technology to detect somatic variants[138, 317]. Seven studies utilised a targeted panel of prespecified mutations to detect mutant variants [315, 316, 319-321, 323, 324]. Chandarlapaty et al and Schiavon et al investigated the ESR1 mutation whilst Silva et al studies p53 mutations exclusively [190, 191, 318]. Fiegl et al measured serum RASSF1A DNA methylation one year post surgery[322]. The follow up period ranged from 12months to 3 years.

7.4.2 Patient Characteristics

A total of 1,809 patients were analysed. Patients' characteristics are reported in table 1. Hu et al, Ma et al, Garcia-Murillas et al, Coombes et al and Magbanua et al [138, 315, 317, 321, 323] examined all breast cancer subtypes whilst Chen et al[316] and Radovich et al[324] focused on triple negative breast cancers [17]. ER positive breast cancer was the subtype studied by Fiegl et al[322], Chandarlapaty et al [191], Kruger et al [319], O'Leary et al [320] and Schiavon et al [190]. Silva et al did not comment on the hormone profile of the breast cancers included in their study[318].

7.4.3 ctDNA Level and Disease-Free Survival (DFS)

All thirteen studies were included in the initial meta-analysis. This analysis included studies which performed ctDNA analysis prior to treatment, and after completion of treatment and there was a statistically significant worse disease-free survival observed in patients with detectable ctDNA (HR 3.38, 95% CI 2.36-4.84, p=<0.0001). There was significant heterogeneity amongst the studies (I^2 = 70%). Figure 7C.

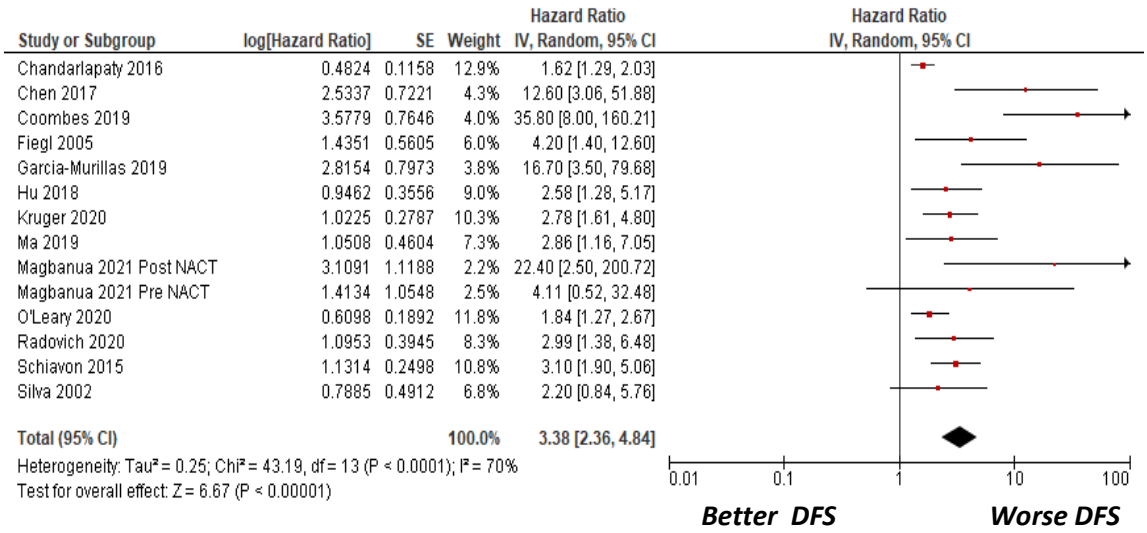


Figure 7C: ctDNA and Disease-Free Survival/Progression Free Survival

7.4.4 CtDNA Analysis in Patients with Metastatic and Early Breast Cancer

Subgroup analysis was performed on the metastatic and advanced breast cancer patient cohort and six studies were eligible for inclusion. ctDNA level was significantly associated with a lower relapse-free survival rate (HR 2.18, 95% CI 1.69-2.81, p < 0.00001, I^2 =45%). Figure 7D. With regards to the early breast cancer studies, a significantly worse disease-free survival was associated with an elevated ctDNA level (HR 7.03, 95% CI 3.4-14.55, p = <0.0001). Magbanua et al reported pre and post treatment ctDNA hazard values and both were included in this subgroup analysis[323]. Overall, there was considerable heterogeneity between the studies (I^2 = 61%, Figure 7E).

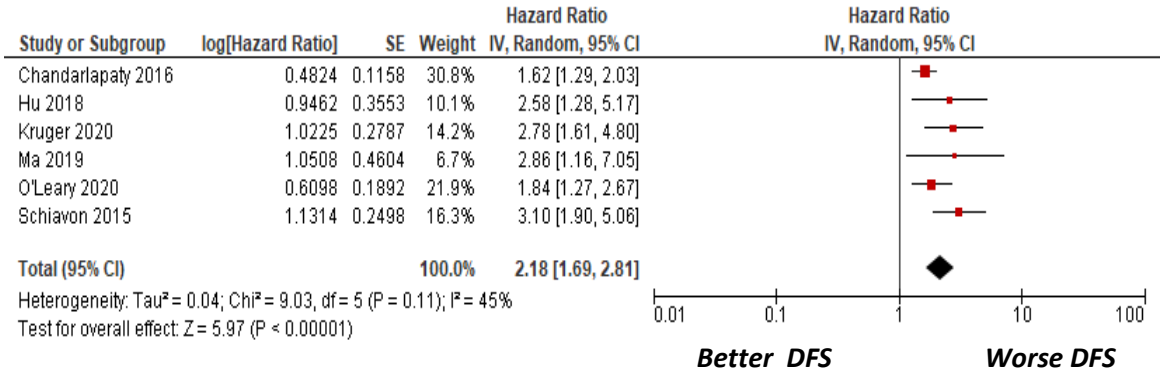


Figure 7D: Subgroup analysis; ctDNA as a predictor of PFS in metastatic breast cancer

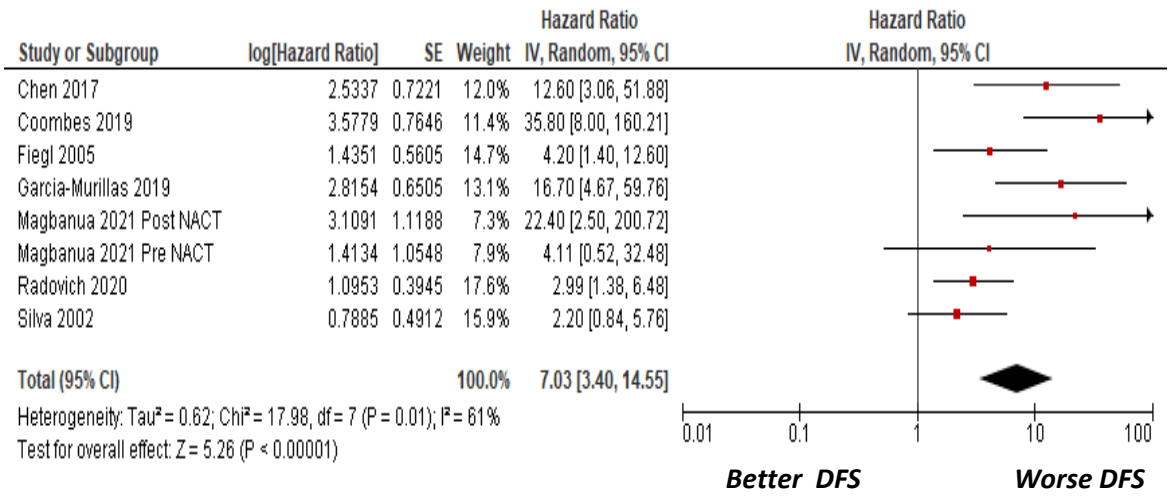


Figure 7E: Subgroup analysis; ctDNA as a predictor of DFS in early breast cancer

7.4.5 ctDNA Analysis Pre and Post Commencement of Treatment

In order to determine whether timing of ctDNA sample collection influenced the predictive value of ctDNA, subgroup analysis was performed comparing pre-treatment and during/post-treatment samples. ctDNA samples collected prior to commencement of oncological or surgical treatment showed that patients with an elevated ctDNA had a worse DFS (HR 3.03, 95% CI 2.18-4.21, p = <0.00001) with no heterogeneity observed (I^2 = 0%). Figure 7F. Samples obtained after commencing oncological treatment or after surgery showed a worse DFS with elevated ctDNA levels (HR 8.17, 95% CI, 1.91-65.89, p = 0.05). However, this was statistically insignificant within a very heterogenous study group (I^2 = 91%). Figure 7G.

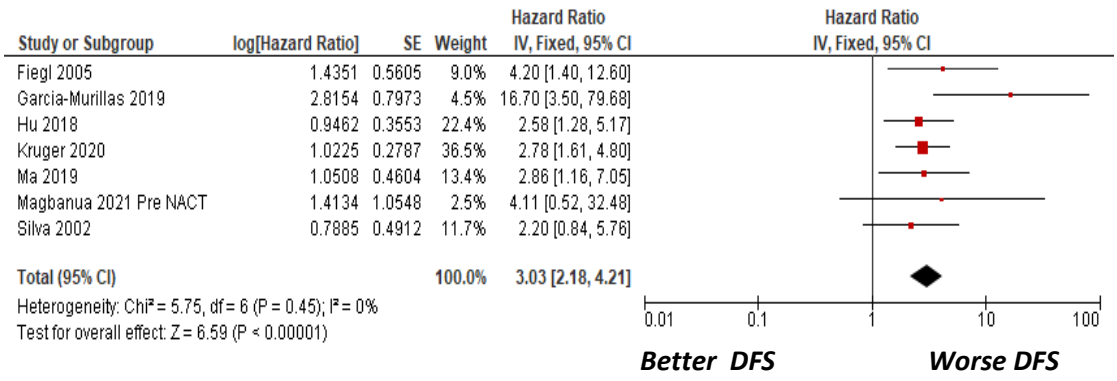


Figure 7F: Subgroup analysis; ctDNA analysis at baseline/prior to treatment

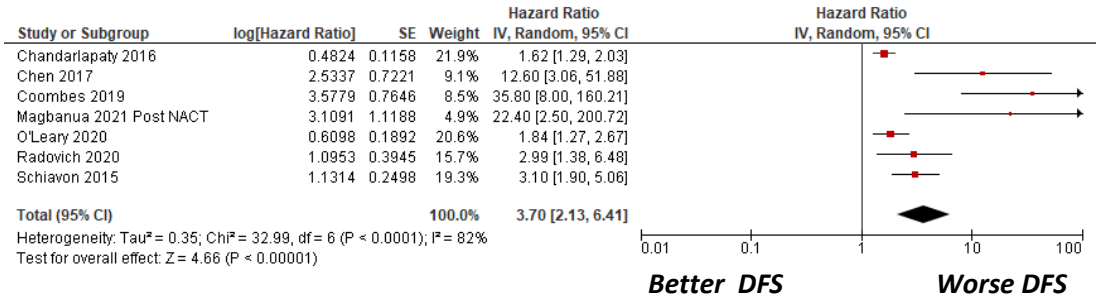


Figure 7G: Subgroup analysis; ctDNA analysis post/during treatment

7.4.6 ctDNA Analysis Post Completion of Neoadjuvant Chemotherapy (NACT)

Three studies included patients who had NACT. CtDNA analysis post completion of NACT predicted worse DFS in patients with breast cancer (HR 7.18, 95% CI 1.95-26.46, P = 0.003, I^2 = 68%). Figure 7H.

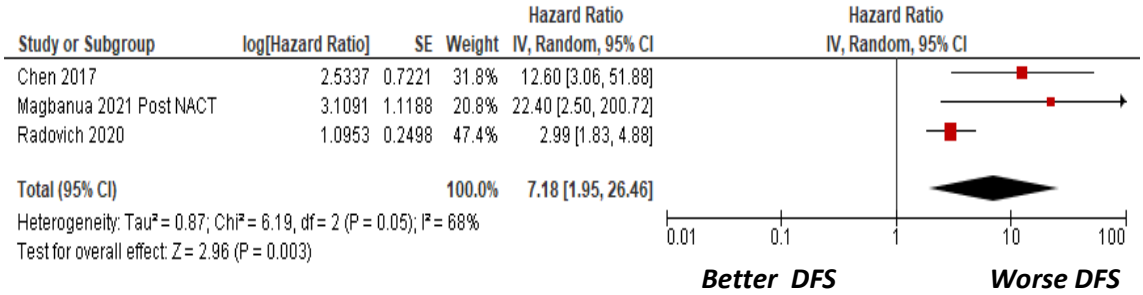


Figure 7H: Subgroup analysis; ctDNA analysis post completion of NACT

7.5 Discussion

Meta-analysis of the published data demonstrates that elevated ctDNA levels can help predict breast cancer outcomes. ctDNA mutation status is a poor prognostic indicator and this proved significant for data pertaining to both early and advanced breast cancer disease. Subgroup analysis from this study demonstrated that ctDNA mutation detection indicates unfavourable survival outcomes. To the best of our

knowledge this is the first meta-analysis solely examining the predictive value of ctDNA mutation status in breast cancer.

These findings concur with previously published meta-analysis reporting on the utility of liquid biopsy in breast cancer [315, 316]. Lee et al analysed 69 studies to determine the prognostic value of liquid biopsy in predicting lymph node metastases, recurrence and survival in breast cancer[325]. Similarly Tan et al reported that circulating cell-free DNA is a strong predictive and prognostic marker in patients with breast cancer[326]. Both of these meta-analyses included studies with cfDNA, ctDNA and circulating tumour cells.

The utility of ctDNA as a predictive biomarker is gaining much appreciation in the field of colorectal cancer, pancreatic cancer, gastric cancer amongst many others [327-330]. ctDNA was first discovered in 1948 however it's only recently come to fruition due to major advances in technological development and detection method[331]. ctDNA is composed of tiny fragments, accounting for as little as 0.01% of the total circulating DNA (cell free DNA) with a very short half-life (less than 2 hours)[332]. One of the original methods for detecting circulating tumor DNA involved DNA isolation, bisulfite modifications and methylight analysis [331, 333], this was the detection method adopted by Fiegl et al in 2005[322].

In more recent years, sophisticated polymerase chain reaction (PCR) based techniques have been adopted to detect ctDNA in various cancers. One of the practical limitations of PCR is that knowledge of the particular genetic mutation is required before it can be quantified. Tumour analysis is necessary prior to mutation alteration measurement unless the target is well established such as TP53, PIK3CA, and ERBB2 in breast cancer[144]. Seven of the studies included in this study utilised PCR techniques to measure ctDNA mutation [190, 191, 315, 317, 318, 320, 321]. ctDNA detection by next generation sequencing (NGS) based techniques is a major advancement in tumour prognostics as this technique sequences millions of DNA templates in parallel enabling rapid identification of tumour specific alterations in ctDNA[334] . Unlike PCR-based techniques, prior knowledge of the underlying mutation is not required in order to quantify genomic alterations using NGS.

Furthermore, NGS-based techniques allow detection of several mutations in multiple genes. Hu et al and Chen et al utilised NGS based techniques to collate data for their studies[138, 316]. Recent advances in PCR and NGS-based techniques has paved the way for ctDNA as a potential prognostic biomarker in breast cancer.

The clinical application of ctDNA detection in breast cancer lies in its' prognostic potential. With the advent of Oncotype DX and MammaPrint, the application of chemotherapy is becoming more individualised and overtreatment should be avoided if possible. Oncotype DX (Genomic Health Inc., Redwood City, CA) is a clinically validated twenty-one-gene genomic assay that can quantify the risk of breast cancer recurrence and the degree of benefit associated with chemotherapy[335] . This tool has revolutionised oncological management of patients with early-stage breast cancer however the score does not absolutely reflect residual disease burden. There is a dire need for circulating biomarkers that can reflect tumour burden and highlight selected patients that would benefit from adjuvant systemic treatment. This meta-analysis shows that ctDNA can reflect tumour burden and monitor response to therapy.

The clinical utility of ctDNA as a prognostic marker can be applied to both early and metastatic breast cancer disease as demonstrated by subgroup analysis in this study. Another potential benefit of ctDNA is that it can detect residual disease after neoadjuvant therapy (NAT). McDonald et al demonstrated high accuracy for assessment of molecular response and residual disease during neoadjuvant therapy using ctDNA analysis[334]. It is worth mentioning that this study was limited by the fact that one patient had high volume residual disease post NAT with undetectable ctDNA, this was potentially due to a limited number of mutations assayed for this patient. The usefulness of ctDNA as a prognostic biomarker during NAT has been validated by a number of further studies with various breast cancer subtypes[336, 337]. In the context of metastatic breast cancer and multi-line chemotherapy resistance, Hu et al proposed that drug selection should be streamlined by identifying drug-sensitive gene mutations in ctDNA[138].

Despite excellent advances in surgical and oncological treatment, breast cancer recurrence rates remain high. The hazard of recurrence is highest during the first 5 years (10.4%) with a peak during the first two years[338]. Current surveillance methods used to detect asymptomatic patients are based on clinical examination and radiological imaging. Garcia-Murillas et al performed a multicentre prospective study on a cohort of 101 female patients and demonstrated that ctDNA analysis detected disease relapse before it became radiologically/clinically apparent with a median time of 10.7 months. Interestingly brain-only metastasis was less commonly detected by ctDNA which is an important consideration should ctDNA analysis be incorporated into follow up surveillance[315].

7.6 Limitations

There are a number of limitations to this study. The main limitation is the heterogeneity of the included studies. Another drawback is that all of the relevant literature pertaining to this subject could not be included in the analysis due to lack of data available. Efforts were made to obtain relevant data from the different authors but correspondence was not reciprocated. Regarding the aforementioned methods for ctDNA analysis, the included studies differed in the techniques they adopted for quantifying ctDNA. A standardised technique needs to be established in order to introduce ctDNA analysis into routine clinical practice.

7.7 Conclusion

In conclusion this meta-analysis found that ctDNA analysis can predict disease free survival/progression free survival in breast cancer patients. It's clinical utility ranges from early breast cancer cohorts to patients with metastatic breast cancer with multi-line drug resistance. ctDNA is an exciting clinical biomarker that can be used to identify preclinical metastases in patients following breast cancer treatment. In an era of personalised medicine, ctDNA shows promise as a tool for guiding future precision medicine.

Table 1: Characteristics of Included Studies

Study	Year	Country	Sample Size	Target mutation	Quantification of ctDNA	Method of ctDNA analysis	Breast Cancer Subtype	Breast Cancer Stage	NOS	Pre/ Post Rx	Design
Kruger et al	2020	Netherlands	164	The Oncomine Breast Assay	Somatic mutation detection	NGS	ER+/ HER2-	IV	8	Pre	PTS
Magbanua et al	2021	USA	84	Targeted panel of 16 genes	Variant threshold	NGS/ PCR	All	I-III	8	Pre/ Post NACT	RTS
Radovich et al	2020	USA	196	Targeted panel of 11 genes	Variant threshold	NGS	TNBC	I-III	8	Post NACT	PTS
Coombes et al	2019	UK	49	Whole Exome Sequencing	Somatic variant detection	PCR	All	I-II	8	Post	PTS
O Leary	2021	UK	455	Targeted panel of 17 genes	Somatic mutation detection/optimized cutoff	PCR	ER+	IV	8	Pre/ Post	PTS
Ma et al	2019	China	37	Targeted panel of 368 genes	Mutation cluster (heterogeneity)	PCR	All	I-III	8	Pre	RTS
Garcia-Murillas et al	2019	UK	101	Targeted panel of 14 genes	ctDNA detection	PCR	All	I-II	8	Pre	PTS
Hu et al	2018	China	68	Whole Exome Sequencing	ROC curve for ctDNA levels	NGS	All	III/IV	8	Pre	RTS
Chen et al	2017	USA	38	Oncomine Research panel of 134 genes	Somatic mutation detection	NGS	TNBC	I-III	7	Post NACT	PTS
Chandarlapa et al	2016	USA	156	ESR1	Mutation amplitude threshold	PCR	ER +	IV	7	Post	PTS
Schiavon et al	2015	UK	171	ESR1	Somatic mutation detection	PCR	ER +	IV	6	Post	PTS
Fiegl et al	2005	Austria	148	methylated RASSF1A DNA	RASSF1A methylation detection	DNA isolation	ER +	I-II	6	Pre	RTS
Silva et al	2002	Spain	142	TP53	Loss of heterogeneity in polymorphic marker, mutation detection	PCR	Not specified	I-II	7	Pre	PTS

NOS = Nottingham Ottawa Scale, NGS = next generation sequencing, PCR = polymerase chain reaction, TNBC = triple negative breast cancer, ER = oestrogen receptor, RTS = retrospective, PTS = prospective, PFS = progression free survival, RFS = recurrence free survival, DFS = disease free survival

Chapter 8: Breast Cancer Circulating Tumour DNA Detection in the Peri-Operative Period

8.1 Abstract: Breast Cancer Circulating Tumour DNA Detection in the Peri-Operative Period

Background

In recent years, circulating tumour DNA (ctDNA) has emerged as a non-invasive method for prognostic stratification, monitoring treatment response and minimal residual disease testing. Unlike traditional tissue biopsies, ctDNA can give a molecular snapshot of the primary tumour and overcome the complexities of sampling bias and intratumour heterogeneity. The aim of this study was to analyse ctDNA mutation status from preoperative and postoperative cfDNA samples to determine whether it can serve as a biomarker to predict disease outcomes.

Methods

This prospective biomarker cohort study is a sub-study of the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity (NCT01840293) research study open across Irish centres and sample collection is within the remit of the biospecimen protocol. cfDNA was extracted using QIAamp Circulating Nucleic Acid kit, quantified using the Denovix DS 11-FX Spectrofluorometer and sequenced using the Illumina Novoaseq 6000 platform (Next Generation Sequencing performed by Novogene).

Results

Forty-five pre- and post-operative patient samples were suitable for ctDNA analysis. ctDNA was detected in 93% of samples pre-operatively and 89% of samples post-operatively. The number of pre-operative ctDNA variants detected correlated positively with pre-operative cfDNA concentration (r= 0.314, p= 0.035). ctDNA positivity was not associated with tumour grade, size or lymph node positivity. Two patients experienced disease recurrence within the follow up period (median 22 months) and ctDNA was detectable in both their pre- and post-op samples. Kaplan Meier estimates of disease-free survival stratified by the presence of ctDNA did not

illustrate any significant difference in DFS. Patients who underwent ALND had a significant increase in post-op ctDNA variant detection compared to those who underwent SLNB using Fisher Exact test (OR 21.71, p=0.0063).

Conclusion

Results from this study demonstrate that ctDNA can be detected peri-operatively using a commercially available ctDNA assay. An increase in post-operative ctDNA burden was evident in patients who went more extensive axillary surgery suggesting a bigger surgical insult can alter ctDNA dynamics. Longer term follow up is required to determine the prognostic potential of peri-operative ctDNA variant dynamics.

8.2 Introduction

Breast cancer is the most commonly diagnosed cancer amongst women globally and the fourth leading cause of cancer death[339]. Approximately 70% of breast cancers are oestrogen-receptor (ER) positive and many of these patients will be effectively cured of their disease with contemporary treatment options [186]. Despite excellent advances in neoadjuvant and adjuvant therapies which reduces the risk of recurrence and death, up to 30% of patients with non-metastatic breast cancer will experience disease recurrence after treatment [340]. Although recurrences can occur early, the majority of patients with hormone receptor-positive breast cancer occur more than 5 years after initial diagnosis, with the risk extending for decades [341].

Contemporary adjuvant systemic therapies can reduce the risk of recurrence however current tools for risk stratifying patients who will derive survival advantage from adjuvant chemotherapy are imperfect and do not reflect minimal residual disease [342, 343]. The American Society of Clinical Oncology recommends 10 years of adjuvant endocrine therapy for women presenting with stage II or III ER+ breast cancer as previous trials have reported an improvement in distant disease-free survival of 1.4-1.9% compared to 5 years of endocrine therapy [34, 94, 344]. Despite convincing data on the benefits of extended endocrine therapy in high- risk ER+ breast cancer, there are limited tools to assess in real-time which therapies are achieving their intended effects. More individualised methods to detect minimal residual disease are warranted to enable adjuvant therapies to be escalated to the subset of patients with high-risk disease and spare other patients of potentially adverse effects of unnecessary treatments[343]. Furthermore, minimal residual disease status reflects early tumour development and tumour relapse and represents a window of opportunity for assessment and intervention [345].

In recent years, circulating tumour DNA (ctDNA) sequencing has emerged as a non-invasive method for prognostic stratification, treatment response and minimal residual disease testing [346, 347]. Circulating cell free DNA (cfDNA) are DNA fragments ranging from 150 to 200 base pairs in length mainly derived from apoptotic or necrotic cells[348]. CtDNA is released from tumour cells, contains tumour-specific genetic and epigenetic abnormalities and accounts for <0.01% of

total cfDNA thus requiring high sensitivity detection techniques[349]. Unlike traditional tissue biopsies, ctDNA can give a molecular snapshot of the primary tumour and overcome the complexities of sampling bias and intratumour heterogeneity[349]. Recent advances in sequencing technology and ctDNA analysis have enabled ctDNA analysis to be incorporated into routine oncological care to identify molecular targets and monitor disease burden [350]. Next generation sequencing (NGS), including whole exome sequencing (WES) and whole genome sequencing (WGS) is a high-throughput technique that can search for previously unidentified genetic variations. The sensitivity of NGS for single nucleotide variants (SNV) detection is about 4-10% in tissue samples, however detection of rare mutations in ctDNA is more difficult as it's an extremely low percentage in cfDNA[351]. To enable NGS to cover broad areas across the genome and improve sensitivity, targeted NGS approaches that analyse specific areas of the genome have been established [351]. The first FDA approved ctDNA MRD assay was introduced in 2018 in the context of B-cell acute lymphoblastic leukaemia. Since then, two classes of ctDNA-based assays applicable to solid tumours have been developed and introduced to oncological care. The first of which is tumour-informed assays such as CAPP-Seq, Safe-Seq and Signatera, whereby mutations from the primary tumour are known to enable a limit of detection as low as 0.01% variant allele frequency [352-354]. Tumour-naïve or tumour-independent assays are the second class of ctDNA assays on the market (Guardant Health Reveal test) that incorporate broad panel-based sequencing for genotyping and reach a limit of detection limit of about 0.2% [355].

Despite excellent technological advances in NGS techniques, there are several technical and biological factors that account for the generation of false-negative and false-positive ctDNA results[351]. The volume of plasma, low DNA shedding by certain tumours and location of metastatic disease all impact the detection ability of ultradeep sequencing techniques[351, 356]. Similarly, technical errors during library preparation, bioinformatical analytical errors and clonal variations such as clonal haematopoeisis of indeterminate potential (CHIP) all contribute to false-positive ctDNA results[351, 357, 358]. A potential method of overcoming the limitation of the

test's sensitivity is to perform serial ctDNA monitoring to increase the confidence of calling low-frequency variants in cfDNA[351].

Several studies have demonstrated the clinical utility of ctDNA in early breast cancer[359-361]. Garcia-Murillas et al demonstrated that ctDNA detection during follow-up was associated with a high risk of future relapse in early-stage breast cancer. Furthermore, ctDNA detection had a median lead time of 10.7 months, enabling disease recurrence identification before it was radiologically apparent[359]. Similarly, Cavallone and colleagues reported that detection of ctDNA early during treatment and late at the end of neoadjuvant chemotherapy before surgery was strongly predictive of residual disease at surgery[362]. Furthermore, a meta-analysis published by our group showed that both pre- and post-treatment ctDNA detection was significantly associated with reduced disease-free survival in patients with breast cancer[105]. Post-operative ctDNA detection in several lung cancer studies have showed that ctDNA identified patients who were likely to experience recurrence after surgery prior to traditional radiological methods [357, 363].

There is limited data available on peri-operative ctDNA detection in breast cancer. The peri-operative period, refers to the timeframe from a few days before to several days-weeks after surgery and is a window of opportunity for decision making on adjuvant treatment and follow-up protocols because adjuvant therapies are usually commenced within two months post-operatively [364, 365]. Additionally, the peri-operative period has been proposed to have pro-metastatic potential because surgery and tissue trauma ensues a complex multifaceted inflammatory response[366]. Systemic and local activation of the innate immune response is an essential response to promote tissue healing and elimination of immunogenic pathogens however may inadvertently create an environment which promotes circulating tumour cells and growth of micrometastatic disease[82]. Nakona and colleagues demonstrated that tumour manipulation during pancreatic surgery has the ability to shift several patients from ctDNA mutant KRAS negative pre-operatively to mutant KRAS positive post-operatively. Furthermore, the change in expression to mutant KRAS after resection was an independent biomarker for poor overall survival in patients with pancreatic cancer [367]. Therefore, the aim of this study was to

analyse ctDNA mutation status from preoperative and postoperative cfDNA samples to determine whether it can serve as a biomarker to predict disease outcomes.

8.3 Methodology

8.3.1 Aims & Objectives

The aim of this study is to determine the peri-operative dynamics of circulating tumour DNA (ctDNA) in breast cancer and define its prognostic potential.

Objectives.

1. To define the mutational landscape of ER+ breast cancer in a cohort of Irish women using a commercially available comprehensive genomic profiling test for solid tumors called The Novogene Precision Medicine 2.0 (NovoPM V2.0). This NGS-based assay analyses 484 genes for clinically important alterations at the DNA level.
2. To examine the peri-operative dynamics of ctDNA in patients with early ER+ breast cancer using the NovoPM V2.0 to determine whether ctDNA is associated with certain tumour characteristics.
3. To determine whether peri-operative ctDNA dynamics are associated with survival outcomes.

8.3.2 Patient Recruitment, Treatment & Follow-up

To analyse ctDNA mutation status from pre-operative and post-operative cfDNA samples, a prospective group of patients with non-metastatic breast cancer were recruited. Patients undergoing breast cancer surgery and treatment at our institute are being actively recruited to the ethically approved ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity Study. This study is sponsored by Cancer Trials Ireland (NCT01840293) and open across Irish centres. This study is a sub-study of the ICORG 09-07 Breast Cancer Proteomics and Molecular Heterogeneity, and sample collection is within the remit of the biospecimen protocol.

Patients presenting for elective breast cancer surgery, with non-metastatic ER+/HER-2 negative disease, aged 18-85 years of age were screened for inclusion.

Exclusion criteria were applied as follows: received neoadjuvant chemotherapy or hormonal therapy, active infection at the time of surgical intervention, known germline mutation in BRCA 1/2.

All patients with breast cancer were discussed at the regional Multi-Disciplinary Meeting (MDM) and managed in keeping with National Cancer Control Programme (NCCP) recommendations. In this institution all patients with breast cancer are routinely screened for axillary nodal disease using ultrasonography +/- fine needle aspiration (FNA) of indeterminate or suspicious nodes. Axillary lymph node dissection is recommended to patients with positive axillary nodal disease receiving upfront surgery. Image guided breast conserving surgery is performed using radiological placed guide wires or a Radiofrequency Identification Device (RFID) tag localisation. Tumour phenotype was recorded from final histopathological reports. Oestrogen receptor/progesterone receptor were considered positive when >10% of cells showed positive staining. HER2 status was assessed by immunohistochemistry (IHC) and Fluorescent in Situ Hybridization (FISH) confirmation in cases with 2+ by IHC. HER2 positivity was defined as either 3+ with IHC and/or amplified with FISH.

Peripheral blood samples were collected pre-operatively (the morning of surgery) and post-operatively (10-14 days post-surgery). Patients with positive invasive margins or large volume nodal disease requiring further surgery had their post-op sample deferred until 2 weeks after their surgery was complete. Similarly, patients who had re-excision of margins for DCIS and had incidental invasive disease on histological analysis had their post-operative sample deferred until two weeks post-operative to ensure that patients did not have invasive disease at the time of sampling. Plasma samples were stored in K3EDTA bottles and centrifuged at 2500 rpm for 15 mins within 1 hour of phlebotomy. Plasma samples were aliquoted into collection tubes (500 µL and 1500 µL), labelled with trial codes and stored at -80 degree Celsius. Up to 40mls of blood was obtained as per protocol.

8.3.3 Sample Storage

Plasma and tissue samples were stored at -80°C in a dedicated biobank freezer housed in a well-ventilated room containing generator backup power. Freezers were monitored 24/7 using internal and external temperature probes, to ensure the safe preservation of samples and secure alarm system. An automatic emergency call is made to one of three full-time laboratory staff members if a freezers temperature rises above -75°C so that freezer problems can be assessed and any problems rectified. Aliquots were stored in 500µL and 1500 µL vials and labelled with time/date and trial code. Freezer opening time was restricted to prevent samples being exposed to freeze/thaw cycles. Once extracted, cfDNA samples were stored in -20°C dedicated freezer.

8.3.4 Cell Free DNA Extraction

Cell-free DNA (cfDNA) was extracted using the QIAamp Circulating Nucleic Acid kit (Qiagen, Cat. No. / ID: 55114) via the four processes of lysis, binding, washing and elution. Steps previously outlined in Chapter 3 and Chapter 6.

8.3.5 CfDNA Quantification

cfDNA was quantified using the Denovix DS 11-FX Spectrofluorometer and the DeNovix dsDNA High Sensitivity Quantification Assay as outlined in Chapter 3 and Chapter 6.

8.3.6 Next Generation Sequencing

Next Generation Sequencing (NGS) of the extracted cfDNA samples was outsourced to NovoGene for paired sequencing using the Illumina Novaseq 6000 platform, employing NGS technology with the NovoPM 2.0 targeted gene panel. This panel comprises 484 genes, including the 324 genes present in the FDA-approved FoundationOne CDx gene panel, among which are the breast cancer genes of interest (Novogene Co, 2023). Targeted NGS is a powerful and highly efficient technique in

the field of genomics. Unlike whole-genome sequencing, which involves sequencing an individual's entire genetic code, targeted NGS focuses on specific regions of interest, such as genes or genomic regions associated with cancer. By honing in on these specific targets it offers several advantages, including reduced sequencing costs, faster turnaround times, and the ability to detect genetic variants with high precision[199].

8.3.7 Library Preparation

Library preparation is a crucial step in the process of NGS, as it involves the conversion of DNA or RNA samples into a format suitable for high-throughput sequencing. The key steps involved in library preparation are outlined in Figure 6. This intricate process begins with fragmentation of the input genetic material, followed by the addition of unique DNA adapters to each fragment. These adapters serve as barcodes and sequencing primers, allowing for the identification of individual DNA fragments and their subsequent amplification. Through various enzymatic reactions, the library preparation workflow also involves end repair, A-tailing, adapter ligation, and PCR amplification, ultimately generating a pool of DNA fragments ready for sequencing. Proper library preparation is an essential, labour-intensive step in the NGS workflow to ensure accurate, reproducible, and high-quality sequencing results. The quality of the library preparation process significantly impacts the success of downstream applications, including variant calling, gene expression analysis, and genome assembly[201]. (Figures 8B-C)

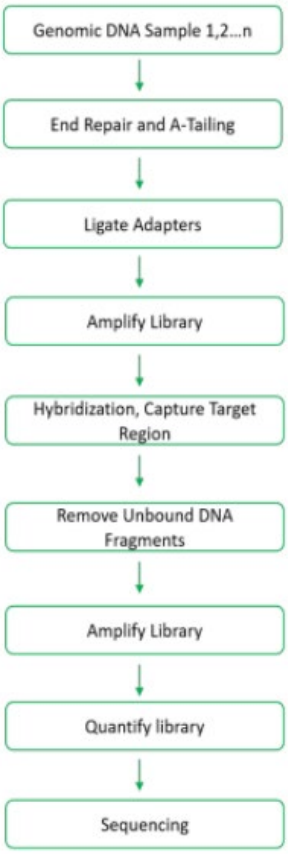


Figure 8B: Library Construction and Sequencing Workflow of NovoPM V2

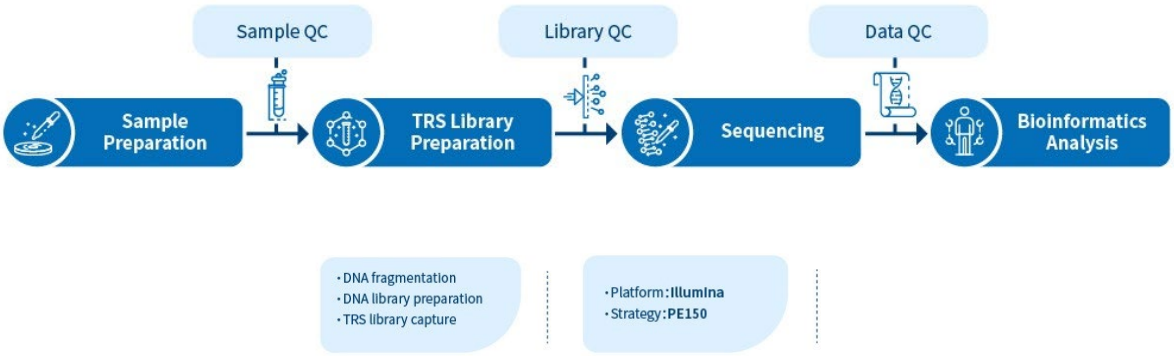


Figure 8C: Novogene Workflow for NovoPM V2

8.3.8 Bioinformatics

Bioinformatical support was provided by Sandra Martinex who analysed the data as part of her MSc in Bioinformatics and Computational Biology with UCC. The raw data files were provided by Novogene containing cfDNA sequences in FASTQ format. These files consisted of 184 raw data files, corresponding to the forward and reverse read of each patient sample at both timepoints. These raw data files served as the foundation for subsequent bioinformatic analyses, which followed a tumor-independent approach. The bioinformatic analysis pipeline involved 6 primary stages including quality control, data trimming, alignment to the Human Reference Genome GRCh38, Deduplication, Variant calling (SNP's and Indel identification) and Variant Annotation. The software required for each analytical stage is outlined in Figure 4. The variant calling step is a critical step in DNA variant discovery and this involves comparing read sequences aligned to the genome to identify variations in base pairs. The software required for this (GATK:Mutect2) specifically identifies Single Nucleotide Variants (SNV's) and small insertions and deletions (INDELs). The next important step in deciphering clinically relevant variants and evaluating their potential pathogenicity involves variant annotation using a combination of biological annotation sources including GENCODE, ClinVar and the Catalogue of Somatic Mutations in Cancer (COSMIC). When an allele frequency threshold >1% was established across the three separate population databases within the ClinVar dataset the sample was considered ctDNA positive.

8.3.9 Statistical Analysis

Statistical analysis was performed using SPSS V.28 and Graphpad Prism Version 10.1. (*GraphPad Software Inc.). Descriptive analysis was used to depict baseline characteristics. Student T test or Mann Whitney tests were used to determine differences in peri-op ctDNA detection. Chi-squared or Fisher's exact test (ranksum) were carried out to assess associations between ctDNA detection and clinical variables. Binary logistic regression analysis was performed to predict the likelihood

of ctDNA positivity based on dichotomous clinical outcomes including LVI, lymph node positivity, tumour grade 3 and tumour size >20mm. Correlation between continuous variables was analysed using Pearson correlation. Values <0.05 were considered significant. Disease free survival analysis was performed using Kaplan Meier curve and log rank analysis. It was not possible to perform binary logistic regression analysis on RUNX1, PIK3CA and CDH1 mutations due to low detection in peri-operative samples.

8.4 Results

8.4.1 Clinicopathological Features

At both timepoints, one patient sample could not be analysed due to a corrupted file that could not be opened or repaired (sample 843). Forty-five out of the sequenced 46 samples were suitable for downstream analysis. Patient demographic and clinicopathological features are outlined in table 1. Median age of the 45 included patients was 61 (IQR 25.5). The majority of patients underwent a wide local excision and sentinel lymph node biopsy (78%) and had invasive ductal carcinoma (IDC) subtype (91%). Over half of the included patients (58%) were lymph node negative and 91% of patients had a tumour size <5cm. The median follow-up time was 22 months with an interquartile range of 8.5months.

Table 1: Patient Demographic, Clinical and Tumour Characteristics N=45

Age	Median 61 (IQR 25.5)
Surgery	N (%)
Wide Local Excision & Sentinel Lymph Node Biopsy	35 (78%)
Wide Local Excision & Axillary Lymph Node Dissection	3 (7%)
Mastectomy & Sentinel Lymph Node Biopsy	5 (11)

Mastectomy & Axillary Lymph Node Dissection	2 (4%)
Subtype	
Invasive Ductal Carcinoma (IDC)	41 (91%)
Invasive Lobular Carcinoma (ILC)	3 (7%)
Mixed	1 (2%)
Lymph node status	
Positive	19 (42%)
Negative	26 (58%)
Tumour Size	
T1	12 (27%)
T2	29 (64%)
T3	4 (9%)
T4	0
Hormone expression	
ER+/HER2 -	41 (91%)
ER+/HER2+	4 (9%)
Grade	
1	3 (8%)
2	19 (50%)
3	16 (42%)
Lymphovascular Invasion (LVI)	
Present	23 (51%)
Absent	22 (49%)
Ductal Carcinoma in Situ (DCIS)	
Present	37 (82%)
Absent	8 (18%)
Follow up	22 months (IQR 8.5)

8.4.2 ctDNA Detection

Pre-operatively (T1) 42 out of 45 patients (93%) cfDNA samples were classified as ctDNA positive when at least one mutated variant was found, and 3 patients were considered ctDNA negative when there was no detectable mutation found. Similarly, post-operatively (T2), 40 of the 45 patients cfDNA samples (89%) were identified as ctDNA positive, and 5 patients were classified as ctDNA negative. The number of mutated variants between pre- and post-op samples decreased in 13 of the 45 patients, increased in 11 and remained the same in 21 of the breast cancer patients studied. There was no significant difference between pre- and post-op number of ctDNA variants detected in cfDNA samples(p=0.7963) (Figure 8D). When each of the ctDNA variants were analysed individually, there was no significant difference between pre- and post-op detection of GATA, KIT, ATM, BRCA1, BRCA2, TP53, RUNX1, PTEN, CDH1, PIK3CA and ERBB2 using Student t-test.

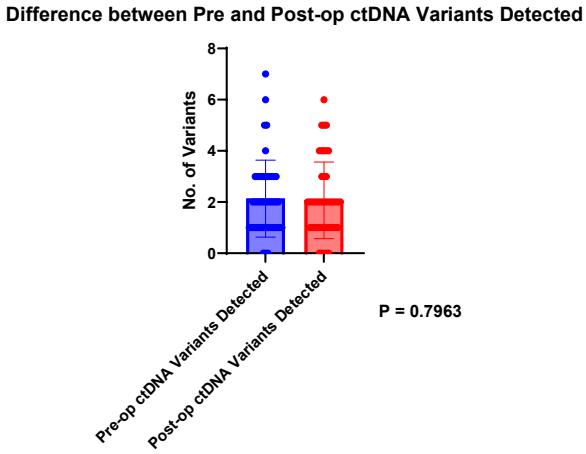


Figure 8D: Difference between Pre- and Post-op ctDNA Variants Detected

8.4.3 Peri-Operative Changes in Individual ctDNA Variants

The peri-operative changes in individual ctDNA variant detection were also analysed. Patients that gained gene mutations post-operatively relative to pre-operative detection were in the ERBB2, CDH1, GATA3 and KIT genes. Pre-operatively 18 (40%) patients exhibited somatic mutations in the ERBB2 gene, while 19 (42%) post-surgery

patient samples had ERBB2 gene mutations. There was one patient (2%) with somatic mutations detected in the CDH1 gene which increased to two patients (4%) post-operatively. The BRCA2 gene was mutated in 12 (27%) patients pre-operatively and 16 (36%) patients post-operatively. There were 15 patients (33%) with mutations in GATA3 pre-operatively and 16 (36%) patients post-operatively. There were 5 (11%) patients with a mutation in the KIT pre-operatively which increased to 7 (16%) post-operatively. Two genes were consistently mutated both pre- and post-operatively. TP53 was mutated in the cfDNA of 10 patients (22%) both pre- and post-operatively. Similarly, the RUNX1 gene exhibited identical mutations patterns with 2 (4%) patients found to have a mutation in this gene both pre- and post-operatively.

Patients lost gene mutations post-operatively relative to pre-operatively for the genes AMT, BRCA1, PTEN and PIKCA3. There were 9 (20%) patients pre-operatively and 8 (18%) patients post-operatively with somatic mutations in the ATM gene. The BRCA1 gene exhibit mutations in 9 (20%) patients pre-operatively and 7 (16%) patients post-operatively. The number of patients with somatic mutations at the gene PTEN decreased from 3 (7%) pre-operatively to 1 (2%) post-operatively. The somatic mutation in gene PIKCA3 was found in 1 (2%) patient pre-operatively and was not detected post-operatively.

8.4.4 Association Between Peri-op ctDNA & Clinicopathological Features

Binary logistic regression analysis was performed to analyse the likelihood of an association between peri-op ctDNA detection and multiple clinicopathological features (ie tumour size >20mm, lymph node positivity, LVI, DCIS, grade 3, and disease recurrence). Data was expressed as odds ratios (OR), representing the odds of ctDNA detection in the presence of different clinicopathological features. There was no significant association between pre- and post-op ctDNA detection and the various clinicopathological features (Table 2A). Similarly, when each of the ctDNA variants were analysed individually, there was no significant association between peri-operative detection of any of the ctDNA variants and clinicopathological variables (Table 2B-I, Table 3A-I).

Table 2A: Pre-op ctDNA detection Association with Clinicopathological Features

	Coefficient (SE)	OR	CI	P value
Tumour size >20mm	2.053	0.903	0.061-190.174	0.551
Lymph node status	1.719	0.374	0.013-10.86	0.551
No. of Positive nodes (>3)	16021.071	3.64	.000	0.998
LVI	1.715	0.320	0.011-9.223	0.507
DCIS	1.715	>100	.000	.998
Grade 3	7222.713	0.00	.000	0.998
Recurrence	20896.497	0.00	.000	0.999

Table 2B: Pre-op ctDNA GATA3 Positivity N =15

	Coefficient (SE)	OR	CI	P value
Tumour size >20mm	0.826	0.372	0.074-1.877	0.231
Lymph node status	0.837	0.477	0.093-2.463	0.377
No. of Positive nodes (>3)	1.436	1.068	0.064-17.8	0.963
LVI	0.747	1.338	0.244-4.841	0.697
DCIS	0.952	2.201	0.341-14.210	0.407
Grade 3	0.762	1.087	0.244-4.841	0.913
Recurrence	22000.574	0.000	0.000	0.999

Table 2C: Pre-op ctDNA KIT Positivity N = 5

	Coefficient (SE)	OR	CI	P value
Tumour size >20mm	1.467	0.170	0.010-3.014	0.227
Lymph node status	1.641	2.273	0.091-56.651	0.617
No. of Positive nodes (>3)	9318.994	>1000	0.000	0.998
LVI	1.379	0.298	0.020-4.455	0.298
DCIS	13015.771	>100	0.000	0.999
Grade 3	9318.994	0.000	0.000	0.998
Recurrence	19255.052	>100	0.000	0.999

Table 2D: Table 2Pre-op ctDNA ATM Positivity N = 9

	Coefficient (SE)	OR	CI	P value
Tumour size >20mm	0.944	0.612	0.096-3.892	0.603
Lymph node status	0.916	1.323	0.220-7.961	0.760
No. of Positive nodes (>3)	1.425	0.797	0.049-13.005	0.873
LVI	0.857	1.296	0.241-6.958	0.762
DCIS	0.973	0.669	0.099-4.506	0.679
Grade 3	0.901	0.924	0.156-5.343	0.920
Recurrence	1.440	0.490	0.029-8.231	0.620

Table 2E: Pre-op ctDNA BRCA1 Positivity N = 9

	Coefficient (SE)	OR	CI	P value
Tumour size >20mm	1.036	0.237	0.031-1.808	0.237
Lymph node status	0.999	3.279	0.463-23.235	0.235
No. of Positive nodes (>3)	17595.659	0.000	0.000	0.999
LVI	0.884	0.772	0.137-4.369	0.770
DCIS	1.332	2.534	0.186-34.470	0.485

Grade 3	0.890	1.113	0.194-6.368	0.904
Recurrence	1.674	0.405	0.015-10.768	0.589

Table 2F: Pre-op ctDNA BRCA2 Positivity N = 12

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	0.965	0.961	0.145-6.369	0.967
Lymph node status	0.954	0.808	0.125-5.239	0.823
No. of Positive nodes (>3)	1.326	4.834	0.359-65.061	0.235
LVI	0.830	0.693	0.136-3.525	0.658
DCIS	114054.236	>100	0.000	0.999
Grade 3	14054.236	>100	0.000	0.999
Recurrence	1.479	0.761	0.042-13.807	0.854

Table 2G: Pre-op ctDNA TP53 Positivity N = 10

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	0.973	0.697	0.104-4.691	0.711
Lymph node status	1.495	2.975	0.518-17.083	0.222
No. of Positive nodes (>3)	17571.283	0.000	0.000	0.999
LVI	0.882	0.446	0.079-2.515	0.360
DCIS	1.264	2.217	0.186-26.404	0.529
Grade 3	0.844	1.706	0.326-8.915	0.527
Recurrence	1.669	0.364	0.014-9.578	0.854

Table 2H: Pre-op ctDNA PTEN Positivity N = 3

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	10832.937	>100	0.000	0.999
Lymph node status	1.350	2.237	0.159-31.524	0.551
No. of Positive nodes (>3)	16790.973	0.000	0.000	0.999
LVI	1.366	1.824	0.125-26.543	0.660
DCIS	12840.326	>100	0.000	0.999
Grade 3	1.342	0.615	0.044-8.520	0.717
Recurrence	20302.738	>100	0.000	0.999

Table 2I: Pre-op ctDNA ERBB2 Positivity N = 18

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	0.777	1.255	0.274-5.751	0.770
Lymph node status	0.771	0.458	0.101-2.075	0.311
No. of Positive nodes (>3)	1.189	1.908	0.186-19.602	0.295
LVI	0.709	0.629	0.157-2.526	0.513
DCIS	0.840	1.223	0.236-6.341	0.811
Grade 3	0.725	1.158	0.280-4.798	0.840
Recurrence	1.376	1.127	0.076-16.725	0.931

Table 3A: Post-op ctDNA detection Association with Clinicopathological Features

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	4478.457	0.00	0.000	0.997
Lymph node status	1.723	0.620	0.021-18.166	0.782
No. of Positive nodes (>3)	2.181	1.27	0.018-91.243	0.913
LVI	9329.261	0.000	0.000	0.995
DCIS	6924.460	2.20	.000	0.996

Grade 3	6924.460	8.49	.000	0,996
Recurrence	7690.485	4.02	.000	0.996

Table 3B: Increase in ctDNA Variant Detection Post-op N = 11

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	1.003	1.667	0.233-11.903	0.610
Lymph node status	0.957	0.580	0.089-3.781	0.569
No. of Positive nodes (>3)	1.386	0.243	0.016-3.682	0.308
LVI	0.900	0.631	0.108-3.687	0.609
DCIS	1.305	0.455	0.035-5.874	0.546
Grade 3	1.024	2.845	0.383-21.156	0.307
Recurrence	1.571	0.158	0.007-3.424	0.239

Table 3C: Post op GATA ctDNA Detection N = 16

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	0.890	0.242	0.042-1.392	0.110
Lymph node status	0.874	0.385	0.069-2.138	0.275
No. of Positive nodes (>3)	1.638	0.719	0.029-17.817	0.840
LVI	0.797	2.161	0.453-10.307	0.334
DCIS	1.027	2.698	0.359-20.115	0.336
Grade 3	0.825	0.714	0.142-3.597	0.683
Recurrence	21021.764	0.000	0.000	0.999

Table 3D: Post op KIT ctDNA Detection N = 7

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	1.247	0.240	0.021-2.761	0.252
Lymph node status	1.485	0.413	0.022-7.583	0.551
No. of Positive nodes (>3)	8339.566	>100	0.000	0.998
LVI	1.179	2.340	0.232-23.601	0.471
DCIS	12823.284	>100	0.000	0.998
Grade 3	12152.202	0.714	0.142-3.597	0.998
Recurrence	21834.284	0.196	0.000	1.000

Table 3E: Post op ATM ctDNA Detection N = 8

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	0.988	0.523	0.075-3.627	0.512
Lymph node status	0.949	1.433	0.223-9.209	0.705
No. of Positive nodes (>3)	16213.106	0.000	0.000	0.999
LVI	0.880	1.072	0.191-6.020	0.937
DCIS	1.232	1.820	0.163-20.344	0.627
Grade 3	0.957	0.868	0.133-5.665	0.883
Recurrence	1.717	0.485	0.114-95.637	0.486

Table 3F: Post op BRCA1 ctDNA Detection N = 7

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	1.487	0.374	0.020-6.906	0.509
Lymph node status	1.366	8.666	0.596-125.968	0.114
No. of Positive nodes (>3)	1.705	0.211	0.007-5.969	0.362
LVI	1.142	0.736	0.079-6.897	0.788
DCIS	1.522	1.059	0.054-20.904	0.970

Grade 3	1.304	0.691	0.054-20.904	0.970
Recurrence	1.832	6.602	0.182-239.698	0.303

Table 3G: Post op BRCA2 ctDNA Detection N = 16

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	0.952	1.098	0.170-7.088	0.922
Lymph node status	0.934	0.371	0.060-2.313	0.288
No. of Positive nodes (>3)	1.370	1.402	0.096-20.545	0.805
LVI	0.805	2.532	0.523-12.258	0.248
DCIS	13464.562	>100	0.000	0.999
Grade 3	0.819	2.123	0.427-10.559	0.358
Recurrence	1.490	0.304	0.016-5.634	0.424

Table 3H: Post op TP53 ctDNA Detection N = 10

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	0.996	0.763	0.108-5.372	0.786
Lymph node status	0.900	2.858	0.460-16.684	0.243
No. of Positive nodes (>3)	17475.701	0.000	0.000	0.999
LVI	0.892	0.435	0.076-2.501	0.351
DCIS	1.267	2.147	0.179-25.697	0.546
Grade 3	0.904	1.478	0.251-8.698	0.666
Recurrence	1.724	3.319	0.113-97.411	0.487

Table 3I: Post op ERBB2 ctDNA Detection N = 18

	Coefficient (SE)	**OR**	**CI**	**P value**
Tumour size >20mm	0.804	1.580	0.327-7.640	0.570
Lymph node status	0.800	0.381	0.079-1.825	0.227
No. of Positive nodes (>3)	1.422	0.862	0.053-13.980	0.917
LVI	0.733	0.608	0.145-2.557	0.497
DCIS	0.872	0.952	0.172-5.262	0.955
Grade 3	0.783	0.810	0.174-3.762	0.788
Recurrence	1.468	1.940	0.109-34.491	0.652

8.4.5 Association Between Number of Pre-operative ctDNA Variants Detected & Clinicopathological Features

Pearson correlation analysis was performed between the number of pre-op ctDNA variants detected and multiple continuous outcomes including number of positive nodes, tumour size, grade, pre-op cell free DNA concentration and age (Table 4). Pre-op ctDNA concentration correlated positively with the number of pre-op ctDNA variants detected ($r = 0.314$, $p = 0.035$). A higher number of positive lymph nodes was associated with disease recurrence ($r = 0.561$, $p = <0.001$). There was a positive

correlation between increasing tumour size/T stage and lymph node positivity (r = 0.392, p = 0.008, r = 0.504, p = <0.001).

Table 4: Correlations Between Number of Pre-Operative ctDNA Variants & Clinicopathological Variables

		No. of Positive Nodes	Size in mm	Pre-op cfDNA Conc.	Grade	T Stage	Age	Lymph Node Positivity	Recurrence	No. of Pre-op ctDNA Variants
No. of Positive Nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	.345*	.561**	-.115
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.020	<.001	.454
	N	45	45	45	45	45	45	45	45	45
Size in mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	.392**	-.172	-.099
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.008	.257	.517
	N	45	45	45	45	45	45	45	45	45
Pre-op cfDNA Conc.	Pearson Correlation	.217	-.045	1	.242	-.074	.037	-.113	.090	.314*
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.459	.559	.035
	N	45	45	45	45	45	45	45	45	45
Grade	Pearson Correlation	.222	.159	.242	1	.174	.036	.229	.270	-.120
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.131	.073	.431
	N	45	45	45	45	45	45	45	45	45
T Stage	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	.504**	-.073	-.182
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	<.001	.633	.231
	N	45	45	45	45	45	45	45	45	45
Age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	-.051	.043	-.060
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.739	.780	.696
	N	45	45	45	45	45	45	45	45	45
Lymph Node Positivity	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	1	.132	-.077
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739		.386	.616
	N	45	45	45	45	45	45	45	45	45
Recurrence	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	.132	1	-.084
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.386		.583
	N	45	45	45	45	45	45	45	45	45
No. of Pre-op ctDNA Variants	Pearson Correlation	-.115	-.099	.314*	-.120	-.182	-.060	-.077	-.084	1
	Sig. (2-tailed)	.454	.517	.035	.431	.231	.696	.616	.583	
	N	45	45	45	45	45	45	45	45	45

Pearson correlation co-efficient: r < 0.3 = poor correlation, r = 0.3-0.5 = fair correlation, r = 0.6-0.8 = moderately strong correlation, r > 0.8 = very strong correlation. *correlation is significant at the 0.05 level (two tailed)

**correlation is significant at the 0.02 level (two tailed)

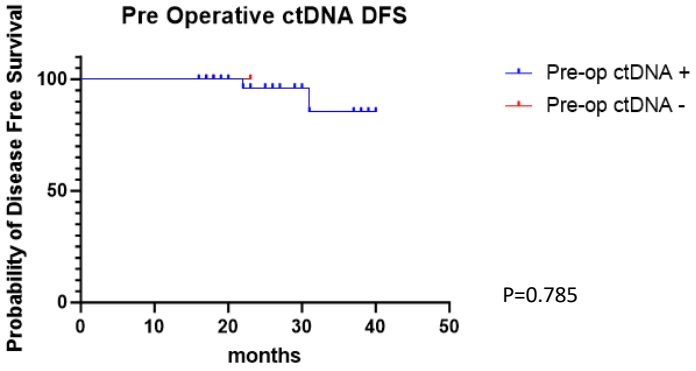
8.4.6 Peri-op ctDNA Detection & Disease-Free Survival Analysis

Only two out of the 45 included patients had disease recurrence during the follow up period of 22 months (IQR 8.5). The clinical details of the patients who experienced disease recurrence are outlined in Table 5. ctDNA was detectable in both pre- and post-op samples in patients who had disease recurrence. Kaplan Meier estimates of disease-free survival stratified by the presence of ctDNA pre- and post-operatively did not illustrate any significant difference in DFS (Figure 8E-H). There was a trend towards a worse DFS for those patients who had an increase in the ctDNA variants detectable post-op compared to pre-op but this was not statistically significant (p=0.9435) (Figure 8I).

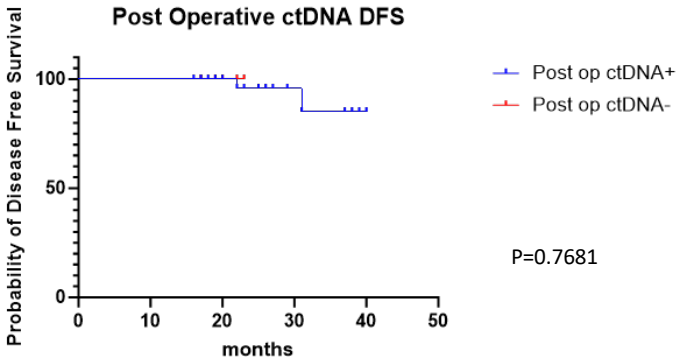
Table 5: Disease Recurrence N = 2

Age	Surgery	T Stage	Lymph Node Positivity	Grade	LVI	ctDNA Pre-Op	ctDNA Post-Op	Time of Recurrence	Site of Recurrence
76	Mastectomy & SLNB	1	Neg	3	Pos	Pos	Pos	18months	Axilla
46	WLE & SLNB	2	Pos	3	Pos	Pos	Pos	29months	Brain

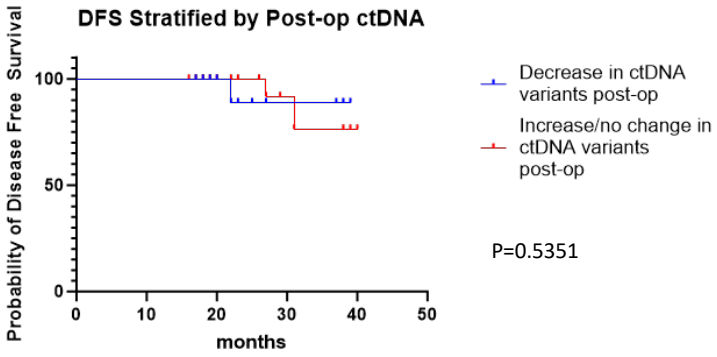
E



F



G



H

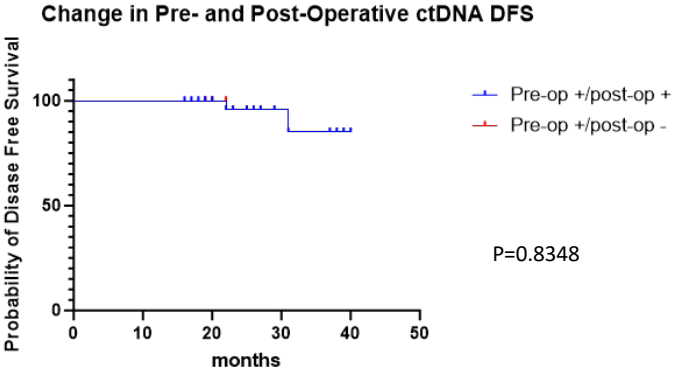


Figure 8. E. Kaplan-Meier Estimates of Disease Free Survival (DFS) stratified by pre-operative ctDNA **F.** Kaplan-Meier Estimates of Disease Free Survival (DFS) stratified by post-operative ctDNA **G.** Kaplan-Meier estimates for DFS for patients' whose ctDNA changes from detectable pre-operatively to undetectable post-operatively compared with patients whose ctDNA status remains negative both pre- and post– operatively **H.** Kaplan-Meier estimates for DFS for patients' whose ctDNA remains detectable post-operatively compared to patients' whose ctDNA converts to negative post-operatively.

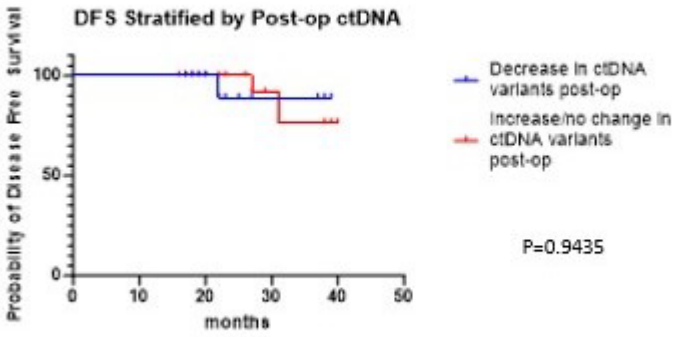


Figure 8I. Kaplan-Meier Estimates of Disease-Free Survival (DFS) stratified by ctDNA variant change post-operatively

8.4.7 Association Between Number of Post-op ctDNA Variants Detected & Clinicopathological Features

Pearson correlation analysis was performed to determine whether there was an association between the number of post-op ctDNA variants detected and different clinicopathological features. The number of post-operative ctDNA variants detected did not appear to correlate with tumour grade, size, lymph node positivity or age (Table 5).

Table 5: Correlations between No. of Post-op ctDNA Variants & Clinicopathological Variables

		No. of Positive Nodes	Size in mm	Pre-op cfDNA Conc.	Grade	T Stage	Age	Lymph Node Positivity	Recurrence	No. of Pre-op ctDNA Variants
No. of Positive Nodes	Pearson Correlation	1	.345*	.504**	.392**	.229	-.079	-.130	.252	-.051
	Sig. (2-tailed)		.020	<.001	.008	.131	.606	.396	.095	.739
	N	45	45	45	45	45	45	45	45	45
Size in mm	Pearson Correlation	.345*	1	.152	.102	.222	.204	-.209	.711**	.044
	Sig. (2-tailed)	.020		.320	.505	.142	.179	.168	<.001	.772
	N	45	45	45	45	45	45	45	45	45
	Pearson Correlation	.504**	.152	1	.778**	.174	-.098	-.197	.067	-.117

Pre-op cfDNA Conc.	Sig. (2-tailed)	<.001	.320		<.001	.253	.523	.195	.660	.443
	N	45	45	45	45	45	45	45	45	45
Grade	Pearson Correlation	.392**	.102	.778**	1	.159	-.189	-.105	-.144	-.066
	Sig. (2-tailed)	.008	.505	<.001		.296	.215	.491	.347	.668
	N	45	45	45	45	45	45	45	45	45
T Stage	Pearson Correlation	.229	.222	.174	.159	1	-.063	-.096	.218	.036
	Sig. (2-tailed)	.131	.142	.253	.296		.679	.532	.150	.812
	N	45	45	45	45	45	45	45	45	45
Age	Pearson Correlation	-.079	.204	-.098	-.189	-.063	1	-.130	.162	.063
	Sig. (2-tailed)	.606	.179	.523	.215	.679		.394	.288	.682
	N	45	45	45	45	45	45	45	45	45
Lymph Node Positivity	Pearson Correlation	-.130	-.209	-.197	-.105	-.096	-.130	1	-.010	-.142
	Sig. (2-tailed)	.396	.168	.195	.491	.532	.394		.950	.353
	N	45	45	45	45	45	45	45	45	45
Recurrence	Pearson Correlation	.252	.711**	.067	-.144	.218	.162	-.010	1	-.082
	Sig. (2-tailed)	.095	<.001	.660	.347	.150	.288	.950		.593
	N	45	45	45	45	45	45	45	45	45
No. of Pre-op ctDNA Variants	Pearson Correlation	-.051	.044	-.117	-.066	.036	.063	-.142	-.082	1
	Sig. (2-tailed)	.739	.772	.443	.668	.812	.682	.353	.593	
	N	45	45	45	45	45	45	45	45	45

Pearson correlation co-efficient: r < 0.3 = poor correlation, r = 0.3-0.5 = fair correlation, r = 0.6-0.8 = moderately strong correlation, r > 0.8 = very strong correlation. *correlation is significant at the 0.05 level (two tailed)

**correlation is significant at the 0.02 level (two tailed)

8.4.8 Post-op ctDNA Variant Detection According to Surgical Approach

Patients who underwent ALND had a significant increase in post-op ctDNA variant detection compared to those who underwent SLNB using Fisher Exact test (OR 21.71, p=0.0063). Figure 8J.

This was independent to lymph node positivity as there was no significant difference in post-op ctDNA variant dynamics in lymph node positive and lymph node negative cohorts (p=0.6361).

There was no significant difference in post-op ctDNA variant change in patients who had a mastectomy compared to WLE (p=0.4667).

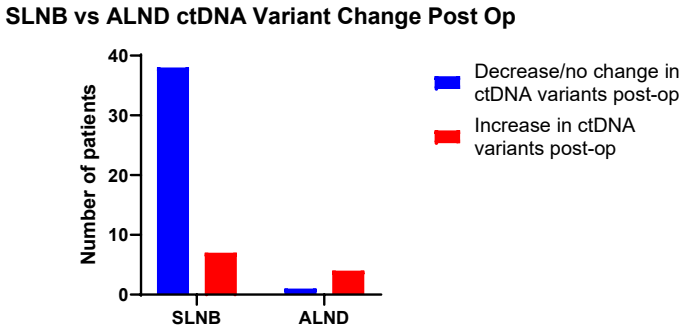


Figure 8J: Comparisons between SLNB & ALND Post-Operative ctDNA Changes

8.5 Discussion

Genomics is the foundation stone of building a precision medicine construct. Results from this study suggest that ctDNA is detectable peri-operatively in patients undergoing breast cancer surgery and there is some reduction in ctDNA variant detection post-operatively (p = 0.7963). This is the first study to examine the mutational landscape of ER+ breast cancer in a cohort of Irish women using a commercially available comprehensive genomic profiling test (NovoPM V2.0). Circulating tumour DNA was detectable in 93% of samples pre-operatively and 89% of cfDNA samples post-operatively which is significantly higher than previously reported. Radovich and colleagues used the commercially available FoundationOne Liquid assay (Foundation Medicine Inc) to examine the association between post treatment ctDNA detection and disease recurrence in triple negative breast cancer and only 65% of the patient cohort had detectable ctDNA post neoadjuvant chemotherapy (NACT)[324]. Their study concluded that patients with ctDNA positive disease post NACT had a significantly poorer disease-free survival compared to patients with ctDNA negative disease (HR 2.99, p = 0.006). Several other studies have reported the clinical significance of serial ctDNA monitoring to detect early disease recurrence in breast cancer [323, 368]. The prognostic utility of ctDNA extends beyond breast cancer studies to include colorectal cancer, pancreatic cancer,

melanoma and lung cancer[328, 369-371]. Chen et al devised a prospective cohort study to investigate perioperative dynamic changes in ctDNA in patients with lung cancer and found that ctDNA decays rapidly in the circulation[127]. They concluded that ctDNA detection on the third post-operative day after R0 resection can be used as a baseline for post-operative surveillance. Throughout the literature the estimated half-life of ctDNA in the circulation is reported to range from minutes to hours[372-374]. This makes ctDNA analysis an attractive biomarker option as it's a real-time time reflection of disease burden and can potentially aid with decision making regarding adjuvant therapies.

Only two patients in our study cohort experienced disease recurrence within the short follow up period of 22 months therefore it's not surprising that ctDNA detection was not significantly associated with DFS. Breast-cancer recurrences after 5 years of adjuvant endocrine therapy continue steadily from 5 to 20 years with risks ranging from 10 to 41% depending on tumour stage and grade[375]. ER-positive recurrences can develop throughout a patients' lifetime, with half occurring more than 5 years from diagnosis thus a longer follow up period is required to capture the true clinical utility of post-operative ctDNA detection[375]. The temporal pattern of ER positive breast cancer recurrence is unique and centres on the theory of minimal residual disease and tumour dormancy[376]. This is a complex process whereby cancer cells are able to escape the primary tumour, survive therapeutic intervention, travel to sanctuary sites where they can lie dormant as disseminated tumour cells (DTC's) before ultimately re-activating as distant metastatic disease[376, 377]. Late recurrences, that occur beyond 5 years are only modestly reduced by extending adjuvant endocrine therapy for an additional time period [378] therefore, methods to identify women who are at high risk for late recurrence are warranted to develop therapeutic strategies for this high risk cohort. Minimal residual disease (MRD), which is broadly classified as persistent evidence of cancer that is not detectable with standard clinical or radiographic assessment may provide seeds for subsequent recurrence. In breast cancer, dormant disseminated tumour cells in the bone marrow(BM) is a surrogate marker of minimal residual disease with precise prognostication however BM aspiration is an invasive, painful procedure [379].

CtDNA is a less invasive alternative that allows real-time monitoring of MRD and response to treatment [380]. Our study findings demonstrate that up to 89% of patients had persistent ctDNA present post breast cancer surgery. The high detection of ctDNA post-operatively could be attributed to the fact that all patients were ER+ and had not received adjuvant endocrine therapy. Furthermore, 19 (42%) patients were classified as lymph node positive on final histology but only 5 (11%) patients had an axillary lymph node dissection due to fulfilment of Z11 criteria[381]. Oncotype DX (Genomic Health, Inc., Redwood City, CA, USA) is a clinically validated multigene prognostic and predictive assay that can be performed on primary Fixed Formalin Paraffin Embedded tumour samples to predict likelihood of recurrence of early-stage breast cancer [110]. Whilst, OncotypeDX is the only multigene assay validated for prediction of chemotherapy benefit, as well as prognosis [110] it does not provide information on the presence of MRD. In recent years, several clinical trials have assigned patients into intervention trials aiming to eradicate MRD and prevent metastatic outgrowth according to ctDNA detection including the DARE trial, TRAK TN and the SUCCESS A trial [382, 383]. Whilst ctDNA detection appears to accurately reflect MRD and predict recurrence rates, the optimal timing of therapeutic intervention upon ctDNA detection remains challenging.

Blood samples were collected pre- and post-surgery to analyse ctDNA dynamics peri-operatively as pre-clinical research has suggested that the surgical insult can promote the growth of new metastases and facilitate seeding of micrometastases into the circulation [4, 5]. The pro-metastatic potential of the peri-operative period was first described by Paget and Halsted at the turn of the century who found that patients who underwent radical resection of their breast cancer did not survive as long as those managed conservatively [81]. Surgery and tissue trauma ensues a complex multifaceted inflammatory response. Systemic and local activation of the innate immune response is an essential response to promote tissue healing and elimination of immunogenic pathogens however may inadvertently create an environment which promotes circulating tumour cells and growth of micrometastatic disease [82]. In chapter four we found that surgical magnitude and a more extensive surgical approach appears to correlate with the level of post-operative inflammatory

mediator release. Similarly, patients who underwent a more extensive axillary lymph node dissection had a significantly higher increase in the number of post-operative ctDNA variants detected compared to patients who had SLNB, independent of lymph node status. The potential effect of the peri-operative inflammatory milieu on breast cancer ctDNA dynamics has been somewhat overlooked in the literature. Lee et al studied ctDNA dynamics as a potential marker of adjuvant chemotherapy benefit following surgery for resectable pancreatic cancer. Whilst over 50% of patients converted to KRAS negative post-operatively, 2 patients with undetectable ctDNA levels before surgery were found to have detectable ctDNA following surgery[328]. These results are in line with our own findings that whilst ctDNA detection generally decreases post-operatively with removal of the tumour bulk, there are a certain cohort of patients who will convert to ctDNA positive or increased ctDNA variant detection post-surgery. Post-operative ctDNA positivity infers a significantly poorer recurrence free survival rate compared to ctDNA negative cohorts with a HR of 5.4 ((p<0.0001)[328]. Whether a similar trend will occur with our patient cohort with longer term follow up remains to be explored.

The NovoPM V2.0 genomic profiling test was utilised to interrogate the complete coding regions of 468 genes and the introns of 43 genes. This panel comprises 484 genes, including the 324 genes present in the FDA-approved FoundationOne CDx gene panel, among which are the breast cancer genes of interest (Novogene Co, 2023). Eleven breast cancer relevant genes were analyzed individually including GATA, KIT, ATM, BRCA1, BRCA2, TP53, RUNX1, PTEN, CDH1, PIK3CA and ERBB2. These somatic variants were selected as genes of interest as they are the most frequently mutated breast cancer genes outlined in the COSMIC and TCGA databases with clinically relevant implications of their discovery. CtDNA detection not only has a role in disease surveillance and monitoring but the presence of certain somatic mutations including PIK3CA, BRCA1, BRCA2 and ERBB2 has important therapeutic opportunities as their presence can also indicate tumour sensitivity to specific chemotherapy regimens [138]. PI3K3CA aberration suggests sensitivity to the mechanistic target of rapamycin (mTOR) inhibitor Everolimus and PIK3CA inhibitor Alpesilib [101, 103]. Both of which are approved for advanced ER+ breast cancer. The

Poly Adenosine Diphosphate-Ribose Polymerase (PARP) inhibitor Olaparib is effective in the presence of germline BRCA1 mutation. More recently, a phase II trial is underway to determine whether somatic BRCA1/2 mutant metastatic breast cancer may also respond to PARP inhibition[384]. We await the outcome of this landmark trial as confirmatory results could extend the potential efficacy of PARPi beyond germline BRCA carriers.

Though no association between peri-operative and clinicopathological features was observed in this study, ctDNA has been associated with tumour size, stage and metastasis in multiple studies across various cancer types [385] [386-388]. Strijker and colleagues found that ctDNA detection was more prevalent in patients with metastatic liver disease compared to those with non-metastatic pancreatic cancer [389]. Similarly, tumour mutational burden appeared to correlate with ctDNA in patients with metastatic melanoma compared to localised disease [387]. Our study did not find an association between ctDNA size, lymph node status or grade but we did demonstrate a positive correlation between ctDNA tumour variants with total cfDNA concentration. In the setting of metastatic breast cancer, cfDNA concentration is a valuable clinical adjunct as an independent prognostic marker of treatment response[390]. Furthermore, studies have suggested that combining cfDNA concentration with other markers could enhance the prognostic power in patients with various types of cancer [390, 391]. We aim to closely analyse the cohort of patients with both elevated cfDNA and detectable ctDNA to determine whether both variables combined confer worse survival outcomes.

8.6 Limitations

There were a number of limitations to this study. Whilst the prospective, homogenous recruitment of patients with ER+, non-metastatic breast cancer strengthens the methodological design of the study, a sufficient follow up period could not be achieved within the timeframe of this MD thesis to derive any meaningful prognostic results. We look forward to obtaining five- and ten-year

disease-free survival data to determine whether peri-operative ctDNA predicted those likely to experience disease recurrence. Another significant limitation of the study is lack of availability of the buffy coat for sequencing of matching white blood cell DNA (WBC DNA) to exclude germline or clonal haematopoiesis of indeterminate potential (CHIP) variants. The frequency of detecting CHIP increases with age, previous systemic cancer treatment and smoking [392]. CHIP variants detectable in cfDNA could lead to false-positive results in CHIP-related genes such as TP53 and ATM which can be mitigated via parallel sequencing of WBC DNA. Certain gene alterations are unlikely to be CHIP false-positives such as PIK3CA and ESR1 ligand-binding domain mutations in breast cancer[393]. It is possible that variant mutations in TP53 and ATM genes detected in our patient cohort were CHIP related aberrations. However, we used multiple clinical databases to encompass only those variants estimated to occur in the same variant allele frequency range associated with breast cancer to reduce the potential of CHIP detection [393]. As more commercial ctDNA sequencing platforms come to the fore (without parallel analysis of WBC DNA), its paramount to explore the impact of clonal haematopoiesis –related variants as a source of biologic noise. These clonal variants represent important biological confounders and serve as a deterrent towards incorporating commercially available NGS services who do not correct for clonal haematopoeises into clinical practice[394].

8.7 Conclusion

Results from this study demonstrate that ctDNA can be detected peri-operatively using a commercially available ctDNA assay. An increase in post-operative ctDNA burden was evident in patients who went more extensive axillary surgery. Longer term follow up is required to determine the prognostic potential of peri-operative ctDNA variant dynamics.

Chapter 9: Thesis Conclusion

This thesis explored the peri-operative dynamics of inflammatory cytokines, cell-free DNA and circulating tumour DNA in relation to breast cancer characteristics and oncological outcomes. A significant elevation in IL-6 and IL-8 was observed in the early post-operative period and this elevation correlated positively with the magnitude of surgery. As emerging evidence suggests that patients who undergo more extensive breast cancer surgery have worse oncological outcomes, one wonders if the heightened inflammatory response could be the causative mechanism. Our study shows there is a significant inflammatory response post breast cancer surgery. As previous studies have demonstrated the negative association between IL-6 and breast cancer outcomes, the peri-operative space provides an exciting opportunity to therapeutically attenuate the hosts immunological response. Further research is required to determine whether manipulation of systemic mediators in the peri-operative period would lead to better survival outcomes.

To better understand the breast tumour microenvironment, gene expression analysis of IL-6 related genes was performed. In contrast to colon cancer, preliminary research has suggested that the normal adjacent to tumour tissue in breast cancer has a higher expression of IL-6 compared to the tumour tissue itself. I was able to validate this finding using a small sample size from our biobank. Furthermore, IL-6 related genes including Leptin, JAK1, CD38, FABP4 and Adiponectin gene expression were significantly higher in NAT compared to matched tumour samples. The morphological features evident in the NAT could be justified using Slaughters' "field cancerization" theory describing a cumulative process of carcinogenesis via acquisition of step-wise genetic alterations, resulting in the NAT tissue composed of morphologically normal but molecularly altered cells[269]. Transcriptomic and epigenetic aberrations suspend the NAT in an intermediate, pre-neoplastic state susceptible to tumorogenic transformation[268]. Potentially, certain individuals are at an increased risk of developing cancer and cancer recurrences because of gene expression in their histologically normal breast epithelium[270].

Potentially, certain individuals are at an increased risk of developing cancer and cancer recurrences because of gene expression in their histologically normal breast epithelium. Findings from this section of the thesis suggest that the NAT could potentially serve as a biomarker to predict patients at higher risk of disease recurrence.

The next section of the thesis was a prospective biomarker study aiming to define the dynamics of cfDNA and ctDNA in the peri-operative period in breast cancer. Prior to embarking on this prospective study, I conducted a systematic review and meta-analysis to determine the prognostic utility of ctDNA in predicting breast cancer outcomes. Eight studies reporting on 739 cases in total were suitable for inclusion. ctDNA mutation detection (both pre and post treatment) was significantly associated with worse disease-free survival (DFS) (HR 4.44,95% CI 2.29-8.61, p=<0.0001). ctDNA detection was significantly associated with a reduction in disease free survival in both the early breast cancer subgroup (HR 8.32, CI 3.01- 22.99, p=<0.0001) and metastatic/locally advanced subgroup (HR 1.91, 95% CI 1.35-2.71, p=0.0003). Findings from this meta-analysis provided the foundations required to explore cfDNA and ctDNA dynamics in the peri-operative period. I also sought to explore the prognostic utility of cfDNA and ctDNA and whether an association between these biomarkers and clinicopathological features existed.

One hundred and seventy-eight cfDNA samples from 63 patients were analysed with a median age of 61(IQR 23.5). Pre-operatively the mean cfDNA concentration was 3.82ng/µl (SEM = 0.74). This increased significantly early post-op in keeping with surgical insult to 17.59 ng/µl (SEM = 5.75). Late post-op there was a significant decrease in cfDNA to near baseline with a mean value of 3.78ng/µl (SEM = 0.62). Patients with associated ductal carcinoma in situ (DCIS) and lymphovascular invasion (LVI) in their tumour had statistically higher cfDNA concentrations early post-operatively. A significant linear relationship was observed between early post-op cfDNA concentration and early post-op IL-8 levels (R = 0.430, p = 0.009). Forty-five pre- and post-operative patient samples were suitable for ctDNA analysis. ctDNA was detected in 93% of samples pre-operatively and 89% of samples post-operatively. The number of pre-operative ctDNA variants detected correlated positively with pre-

operative cfDNA concentration (R = 0.314, p=0.035). ctDNA positivity was not associated with tumour grade, size or lymph node positivity. Two patients experienced disease recurrence within the follow up period (median 22 months) and ctDNA was detectable in both their pre- and post-op samples. Patients who underwent ALND had a significant increase in post-op ctDNA variant detection compared to those who underwent SLNB using Fisher Exact test (OR 21.71, p=0.0063). Kaplan Meier estimates of disease-free survival stratified by the presence of ctDNA did not illustrate any significant difference in DFS.

Whilst the prospective, homogenous recruitment of patients with non-metastatic breast cancer strengthens the methodological design of the study, a sufficient follow up period could not be achieved within the timeframe of this MD thesis to derive any meaningful prognostic results. We look forward to obtaining five- and ten-year disease-free survival data to determine whether peri-operative cfDNA and ctDNA predicted those likely to experience disease recurrence.

In summary, the peri-operative period is underpinned by a complex interaction between local and systemic inflammatory mediators, cfDNA and ctDNA. Longer term follow up is required to determine the prognostic potential of these peri-operative biomarkers.

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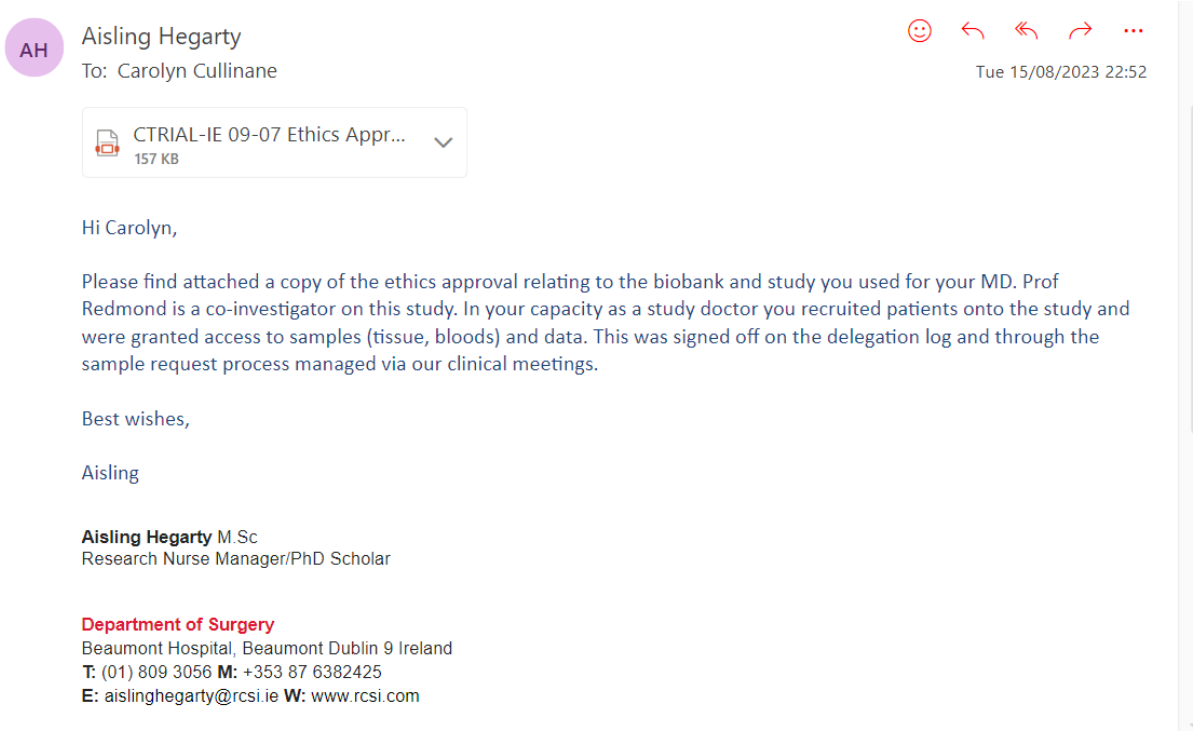
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Appendix 1: Ethical Approval Letter



AH

Aisling Hegarty

To: Carolyn Cullinane

Tue 15/08/2023 22:52

CTRIAL-IE 09-07 Ethics Appr...
157 KB

Hi Carolyn,

Please find attached a copy of the ethics approval relating to the biobank and study you used for your MD. Prof Redmond is a co-investigator on this study. In your capacity as a study doctor you recruited patients onto the study and were granted access to samples (tissue, bloods) and data. This was signed off on the delegation log and through the sample request process managed via our clinical meetings.

Best wishes,

Aisling

Aisling Hegarty M.Sc
Research Nurse Manager/PhD Scholar

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Appendix 2: Ethical Approval

Our ref: ECM 3 (a) 11/05/2021 & ECM 4 (m) 11/01/11

31st May 2021

Professor Paul Redmond

Consultant Surgeon

Cork University Hospital

Wilton

Cork

Re: 09-07 Breast cancer proteomics and molecular heterogeneity.

Dear Professor Redmond

The following documents have been approved by the committee:

Ø Cover Letter dated 16th March 2021 (revisions rec. 8th April 2021)

Ø Amendment Application Form signed 1st April 2021

Ø Amendment Summary Version 1 Protocol Version 10 dated 24th February 2021

Ø Study Protocol Version 10 dated 24th February 2021

Ø Patient Information Leaflet & Informed Consent Form Master Type A Version 11 dated 25th February 2021 Cork University Hospital Version 4 dated 3rd March 2021

Ø Patient Information Leaflet & Informed Consent Form Master Type C Version 1 dated 25th February 2021 Cork University Hospital Version 1 dated 3rd March 2021

Ø Study Questionnaire Version 2 dated February 2021.

Yours sincerely

_____

Professor David Kerins

Chairman

Clinical Research Ethics Committee

of the Cork Teaching Hospitals

CC: Magdalena Mroz, Magdalena.Mroz@cancertrials.ie

COISTE EITICE UM THAIGHDE CLINICIÚIL

Clinical Research Ethics Committee of the Cork Teaching Hospitals

University College Cork

Tel: +353-21-4901901 Lancaster Hall

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Cork

Ireland

Appendix 3: Pearson Correlation between Pre-op ctDNA Variants & Clinicopathological Variables

Correlations

		number_of_positive_nodes	size_mm	cfDNA_T1ngul	grade	t_size	age	GATA3	lymph_node_status	Recurrence_status_16.08.23
number_of_positive_nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	-.137	.345*	.561**
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.370	.020	<.001
	N	45	45	45	45	45	45	45	45	45
size_mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	-.089	.392**	-.172
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.562	.008	.257
	N	45	45	45	45	45	45	45	45	45
cfDNA_T1ngul	Pearson Correlation	.217	-.045	1	.242	-.074	.037	.204	-.113	.090
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.180	.459	.559
	N	45	45	45	45	45	45	45	45	45
grade	Pearson Correlation	.222	.159	.242	1	.174	.036	-.172	.229	.270
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.257	.131	.073
	N	45	45	45	45	45	45	45	45	45
t_size	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	-.193	.504**	-.073
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	.203	<.001	.633
	N	45	45	45	45	45	45	45	45	45
age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	.062	-.051	.043
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.685	.739	.780
	N	45	45	45	45	45	45	45	45	45
GATA3	Pearson Correlation	-.137	-.089	.204	-.172	-.193	.062	1	-.223	-.189
	Sig. (2-tailed)	.370	.562	.180	.257	.203	.685		.141	.214
	N	45	45	45	45	45	45	45	45	45
lymph_node_status	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	-.223	1	.132
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739	.141		.386
	N	45	45	45	45	45	45	45	45	45
Recurrence_status_16.08.23	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	-.189	.132	1
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.214	.386	
	N	45	45	45	45	45	45	45	45	45

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		number_of_positive_nodes	size_mm	cfDNA_T1ngul	grade	t_size	age	lymph_node_status	Recurrence_status_16.08.23	KIT
number_of_positive_nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	.345*	.561**	-.012
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.020	<.001	.938
	N	45	45	45	45	45	45	45	45	45
size_mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	.392**	-.172	.081
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.008	.257	.595
	N	45	45	45	45	45	45	45	45	45
cfDNA_T1ngul	Pearson Correlation	.217	-.045	1	.242	-.074	.037	-.113	.090	-.034
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.459	.559	.826
	N	45	45	45	45	45	45	45	45	45
grade	Pearson Correlation	.222	.159	.242	1	.174	.036	.229	.270	-.308*
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.131	.073	.040
	N	45	45	45	45	45	45	45	45	45
t_size	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	.504**	-.073	-.014
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	<.001	.633	.928
	N	45	45	45	45	45	45	45	45	45
age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	-.051	.043	.006
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.739	.780	.971
	N	45	45	45	45	45	45	45	45	45
lymph_node_status	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	1	.132	-.016
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739		.386	.917
	N	45	45	45	45	45	45	45	45	45
Recurrence_status_16.08.23	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	.132	1	-.094
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.386		.537
	N	45	45	45	45	45	45	45	45	45
KIT	Pearson Correlation	-.012	.081	-.034	-.308*	-.014	.006	-.016	-.094	1
	Sig. (2-tailed)	.938	.595	.826	.040	.928	.971	.917	.537	
	N	45	45	45	45	45	45	45	45	45

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		number_of_positive_nodes	size_mm	cfDNA_T1ngul	grade	t_size	age	lymph_node_status	Recurrence_status_16.08.23	ATM
number_of_positive_nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	.345*	.561**	-.063
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.020	<.001	.681
	N	45	45	45	45	45	45	45	45	45
size_mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	.392**	-.172	.083
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.008	.257	.587
	N	45	45	45	45	45	45	45	45	45
cfDNA_T1ngul	Pearson Correlation	.217	-.045	1	.242	-.074	.037	-.113	.090	-.144
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.459	.559	.347
	N	45	45	45	45	45	45	45	45	45
grade	Pearson Correlation	.222	.159	.242	1	.174	.036	.229	.270	-.017
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.131	.073	.910
	N	45	45	45	45	45	45	45	45	45
t_size	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	.504**	-.073	-.039
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	<.001	.633	.799
	N	45	45	45	45	45	45	45	45	45
age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	-.051	.043	-.020
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.739	.780	.896
	N	45	45	45	45	45	45	45	45	45
lymph_node_status	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	1	.132	.022
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739		.386	.883
	N	45	45	45	45	45	45	45	45	45
Recurrence_status_16.08.23	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	.132	1	.089
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.386		.561
	N	45	45	45	45	45	45	45	45	45
ATM	Pearson Correlation	-.063	.083	-.144	-.017	-.039	-.020	.022	.089	1
	Sig. (2-tailed)	.681	.587	.347	.910	.799	.896	.883	.561	
	N	45	45	45	45	45	45	45	45	45

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		number_of_positive_nodes	size_mm	cfDNA_T1ngul	grade	t_size	age	lymph_node_status	Recurrence_status_16.08.23	BRCA1
number_of_positive_nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	.345*	.561**	-.099
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.020	<.001	.517
	N	45	45	45	45	45	45	45	45	45
size_mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	.392**	-.172	-.186
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.008	.257	.221
	N	45	45	45	45	45	45	45	45	45
cfDNA_T1ngul	Pearson Correlation	.217	-.045	1	.242	-.074	.037	-.113	.090	.231
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.459	.559	.127
	N	45	45	45	45	45	45	45	45	45
grade	Pearson Correlation	.222	.159	.242	1	.174	.036	.229	.270	-.017
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.131	.073	.910
	N	45	45	45	45	45	45	45	45	45
t_size	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	.504**	-.073	-.234
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	<.001	.633	.121
	N	45	45	45	45	45	45	45	45	45
age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	-.051	.043	.184
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.739	.780	.227
	N	45	45	45	45	45	45	45	45	45
lymph_node_status	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	1	.132	.022
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739		.386	.883
	N	45	45	45	45	45	45	45	45	45
Recurrence_status_16.08.23	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	.132	1	.089
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.386		.561
	N	45	45	45	45	45	45	45	45	45
BRCA1	Pearson Correlation	-.099	-.186	.231	-.017	-.234	.184	.022	.089	1
	Sig. (2-tailed)	.517	.221	.127	.910	.121	.227	.883	.561	
	N	45	45	45	45	45	45	45	45	45

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		number_of_positive_nodes	size_mm	cfDNA_T1ngul	grade	t_size	age	lymph_node_status	Recurrence_status_16.08.23	BRCA2
number_of_positive_nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	.345*	.561**	.235
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.020	<.001	.125
	N	45	45	45	45	45	45	45	45	44
size_mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	.392**	-.172	-.059
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.008	.257	.706
	N	45	45	45	45	45	45	45	45	44
cfDNA_T1ngul	Pearson Correlation	.217	-.045	1	.242	-.074	.037	-.113	.090	-.117
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.459	.559	.450
	N	45	45	45	45	45	45	45	45	44
grade	Pearson Correlation	.222	.159	.242	1	.174	.036	.229	.270	-.053
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.131	.073	.732
	N	45	45	45	45	45	45	45	45	44
t_size	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	.504**	-.073	.016
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	<.001	.633	.917
	N	45	45	45	45	45	45	45	45	44
age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	-.051	.043	-.025
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.739	.780	.871
	N	45	45	45	45	45	45	45	45	44
lymph_node_status	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	1	.132	-.019
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739		.386	.904
	N	45	45	45	45	45	45	45	45	44
Recurrence_status_16.08.23	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	.132	1	.037
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.386		.812
	N	45	45	45	45	45	45	45	45	44
BRCA2	Pearson Correlation	.235	-.059	-.117	-.053	.016	-.025	-.019	.037	1
	Sig. (2-tailed)	.125	.706	.450	.732	.917	.871	.904	.812	
	N	44	44	44	44	44	44	44	44	44

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		number_of_positive_nodes	size_mm	cfDNA_T1ngul	grade	t_size	age	lymph_node_status	Recurrence_status_16.08.23	TP53
number_of_positive_nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	.345*	.561**	-.088
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.020	<.001	.566
	N	45	45	45	45	45	45	45	45	45
size_mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	.392**	-.172	-.015
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.008	.257	.924
	N	45	45	45	45	45	45	45	45	45
cfDNA_T1ngul	Pearson Correlation	.217	-.045	1	.242	-.074	.037	-.113	.090	.121
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.459	.559	.427
	N	45	45	45	45	45	45	45	45	45
grade	Pearson Correlation	.222	.159	.242	1	.174	.036	.229	.270	.121
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.131	.073	.428
	N	45	45	45	45	45	45	45	45	45
t_size	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	.504**	-.073	-.021
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	<.001	.633	.892
	N	45	45	45	45	45	45	45	45	45
age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	-.051	.043	-.076
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.739	.780	.619
	N	45	45	45	45	45	45	45	45	45
lymph_node_status	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	1	.132	.084
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739		.386	.583
	N	45	45	45	45	45	45	45	45	45
Recurrence_status_16.08.23	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	.132	1	.071
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.386		.641
	N	45	45	45	45	45	45	45	45	45
TP53	Pearson Correlation	-.088	-.015	.121	.121	-.021	-.076	.084	.071	1
	Sig. (2-tailed)	.566	.924	.427	.428	.892	.619	.583	.641	
	N	45	45	45	45	45	45	45	45	45

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		number_of_positive_nodes	size_mm	cfDNA_T1ngul	grade	t_size	age	lymph_node_status	Recurrence_status_16.08.23	PTEN
number_of_positive_nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	.345*	.561**	-.040
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.020	<.001	.794
	N	45	45	45	45	45	45	45	45	45
size_mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	.392**	-.172	.052
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.008	.257	.736
	N	45	45	45	45	45	45	45	45	45
cfDNA_T1ngul	Pearson Correlation	.217	-.045	1	.242	-.074	.037	-.113	.090	-.099
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.459	.559	.517
	N	45	45	45	45	45	45	45	45	45
grade	Pearson Correlation	.222	.159	.242	1	.174	.036	.229	.270	-.009
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.131	.073	.952
	N	45	45	45	45	45	45	45	45	45
t_size	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	.504**	-.073	.083
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	<.001	.633	.586
	N	45	45	45	45	45	45	45	45	45
age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	-.051	.043	-.002
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.739	.780	.989
	N	45	45	45	45	45	45	45	45	45
lymph_node_status	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	1	.132	.132
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739		.386	.386
	N	45	45	45	45	45	45	45	45	45
Recurrence_status_16.08.23	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	.132	1	-.071
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.386		.641
	N	45	45	45	45	45	45	45	45	45
PTEN	Pearson Correlation	-.040	.052	-.099	-.009	.083	-.002	.132	-.071	1
	Sig. (2-tailed)	.794	.736	.517	.952	.586	.989	.386	.641	
	N	45	45	45	45	45	45	45	45	45

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		number_of_positive_nodes	size_mm	cfDNA_T1ngul	grade	t_size	age	lymph_node_status	Recurrence_status_16.08.23	ERBB2
number_of_positive_nodes	Pearson Correlation	1	.102	.217	.222	.152	.044	.345*	.561**	-.123
	Sig. (2-tailed)		.505	.152	.142	.320	.772	.020	<.001	.423
	N	45	45	45	45	45	45	45	45	45
size_mm	Pearson Correlation	.102	1	-.045	.159	.778**	-.066	.392**	-.172	-.182
	Sig. (2-tailed)	.505		.770	.296	<.001	.668	.008	.257	.233
	N	45	45	45	45	45	45	45	45	45
cfDNA_T1ngul	Pearson Correlation	.217	-.045	1	.242	-.074	.037	-.113	.090	.063
	Sig. (2-tailed)	.152	.770		.109	.631	.808	.459	.559	.680
	N	45	45	45	45	45	45	45	45	45
grade	Pearson Correlation	.222	.159	.242	1	.174	.036	.229	.270	-.028
	Sig. (2-tailed)	.142	.296	.109		.253	.812	.131	.073	.853
	N	45	45	45	45	45	45	45	45	45
t_size	Pearson Correlation	.152	.778**	-.074	.174	1	-.117	.504**	-.073	-.064
	Sig. (2-tailed)	.320	<.001	.631	.253		.443	<.001	.633	.677
	N	45	45	45	45	45	45	45	45	45
age	Pearson Correlation	.044	-.066	.037	.036	-.117	1	-.051	.043	-.082
	Sig. (2-tailed)	.772	.668	.808	.812	.443		.739	.780	.594
	N	45	45	45	45	45	45	45	45	45
lymph_node_status	Pearson Correlation	.345*	.392**	-.113	.229	.504**	-.051	1	.132	-.147
	Sig. (2-tailed)	.020	.008	.459	.131	<.001	.739		.386	.335
	N	45	45	45	45	45	45	45	45	45
Recurrence_status_16.08.23	Pearson Correlation	.561**	-.172	.090	.270	-.073	.043	.132	1	-.036
	Sig. (2-tailed)	<.001	.257	.559	.073	.633	.780	.386		.813
	N	45	45	45	45	45	45	45	45	45
ERBB2	Pearson Correlation	-.123	-.182	.063	-.028	-.064	-.082	-.147	-.036	1
	Sig. (2-tailed)	.423	.233	.680	.853	.677	.594	.335	.813	
	N	45	45	45	45	45	45	45	45	45

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).