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Analysis of Cytokine Expression in Breast Cancer

A thesis submitted to the National University of Ireland as partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

by

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Under the supervision of
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And the direction of
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Discipline of Surgery, Clinical Science Institute
National University of Ireland, Galway
2013
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Dedication

I lovingly dedicate this work to my husband James, who is my source of strength and courage
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAFs</td>
<td>CCR4 associated factors</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
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<td>CCL2</td>
<td>chemokine (C-C motif) ligand 2</td>
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<tr>
<td>CXCL5</td>
<td>chemokine (C-X-C motif) ligand 5</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EASR</td>
<td>European Age Standardised Rate</td>
</tr>
<tr>
<td>E-cad</td>
<td>Ecadherin</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-Mesenchymal Transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERBB2</td>
<td>v-erb-b2 avian erythroblastic leukemia viral oncogene homolog</td>
</tr>
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<td>FAP</td>
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<td>FSH</td>
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<td>industrial methylated spirits</td>
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<td>KDR</td>
<td>kinase insert domain receptor</td>
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<tr>
<td>LAF</td>
<td>laminar air flow</td>
</tr>
<tr>
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<td>Luteinising Hormone</td>
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<td>MMPs</td>
<td>matrix metalloproteases</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRPL19</td>
<td>39S ribosomal protein L19, mitochondrial</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NBCRI</td>
<td>National Breast Cancer Research Institute</td>
</tr>
<tr>
<td>NCRI</td>
<td>National Cancer Registry Ireland</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NPI</td>
<td>Nottingham Prognostic Index</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatories</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl Isomerase A</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T Expressed and Secreted</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RQ</td>
<td>relative quantity</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>Real-time Quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SCP2</td>
<td>sterol carrier protein 2</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TAMs</td>
<td>tumor-associated macrophages</td>
</tr>
<tr>
<td>TAN</td>
<td>tumor-associated neutrophil</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor β1</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>transforming growth factor, beta receptor II</td>
</tr>
<tr>
<td>TIMPs</td>
<td>tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNM</td>
<td>TNM Classification of Malignant Tumours</td>
</tr>
<tr>
<td>UICC</td>
<td>Union for International Cancer Control</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
VEGFR2  vascular endothelial growth factor receptor 2
WASR  World Age Standardised Rate
WHO  World Health Organization
α-SMA  alpha smooth muscle actin
Communications arising from this work

Published papers

Relationship between CCL5 and transforming growth factor β1 (TGFβ1) in breast cancer
PMID 21658938

Influence of stromal-epithelial interactions on breast cancer in vitro and in vivo
Potter SM, Dwyer RM, Hartmann MC, Khan S, Boyle MP, Curran CE, Kerin MJ.
PMID: 21344235

Published abstracts

Effect of Monocyte Chemotactic Protein-1 (MCP-1) on breast cancer cell proliferation and gene expression
Hartmann MC, Dwyer RM, Boyle MP, Potter SM, Kerin MJ
Targeted Anticancer Therapies (TAT), March 23-25 2009, Amsterdam, The Netherlands
ANNALS OF ONCOLOGY, 20: 28-28 Suppl. 3 MAY 2009

Characterization of primary tumour stromal cells and their potential role in the breast cancer microenvironment
Hartmann MC, Dwyer RM, Potter SM, Dockery P, Kerin MJ
ECCO 13-34TH ESMO Multidisciplinary Congress September 20-24 2009
EJC SUPPLEMENTS, 7 (2): 105-105 SEP 2009
Investigation of potential role of CCL5 in breast cancer progression
Analysis of primary breast tumour stromal cells and their potential role in disease progression
Hartmann MC, Dwyer RM, Kerin MJ
San Antonio Breast Cancer Symposium (SABCS) December 9-13 2009, Texas, USA
CANCER RESEARCH, 69 (24): 761S-761S Suppl. 3 DEC 2009

**Oral presentations**

Potential role of MCP-1 in the breast cancer microenvironment
Sylvester O’Halloran Meeting, February 6-7 2009, Limerick, Ireland

Gene expression signature of primary breast tumour stromal cells
34th Sir Peter Freyer Surgical Symposium & Memorial Lecture September 4-5 2009, Galway, Ireland

Potential of circulating chemokines as serum tumour markers in breast cancer
Society of Academic & Research Surgery (SARS) January 6-7 2010, London, UK

Correlation between circulating and tumour tissue CCL5 and TGFβ1 in breast cancer patients
35th Sir Peter Freyer Surgical Symposium & Memorial Lecture September 03-04 2010 (Plenary Session)

Relationship between CCL5 and TGFβ1 in breast cancer patients at both systemic and cellular levels
1st British Breast Cancer Research Conference September 15-17 2010
Posters

Circulating and tumour tissue levels of CCL5/RANTES in breast cancer
Irish Association for Cancer Research (IACR) March 3-5 2010, Galway, Ireland

Characterization of primary tumour stromal cells and their potential role in the breast cancer microenvironment
ECCO 13-34TH ESMO Multidisciplinary Congress September 20-24 2009

Analysis of primary breast tumour stromal cells and their potential role in disease progression
San Antonio Breast Cancer Symposium (SABCS) December 9-13 2009, Texas, USA

CCL5 in Breast Cancer: Circulating systemic levels and gene expression in the primary tumour microenvironment
American Association for Cancer Research (AACR) 101st Annual Meeting April 17-21 2010, Washington, DC

Awards

2008 Research Fellowship from National Breast Cancer Research Institute (NBCRI)
2009 Breast Cancer Ireland Research Fellowship
Abstract

Introduction: It has long been recognized that cancer develops in sites of chronic inflammation. Tumours seem to seize molecular pathways seen in wound healing and the inflammatory response and as a consequence appear as “wounds that do not heal”. However, the underlying molecular mechanisms facilitating the interconnection of inflammation and cancer remain poorly understood. This body of work aims to analyse mediators of inflammatory response and carcinogenesis on a systemic, breast tissue and cellular level in breast cancer.

Methods: Circulating levels of CCL5 and TGFβ1 were measured in breast cancer patients and age matched controls using ELISA. Gene expression levels of CCL5, CCR5, TGFβ1, TGFβRII, CCL2, CCR2, MMP3 and FAP were analysed in corresponding tumour tissue, normal tissue, and isolated tumour and normal stromal cells using RQ-PCR. CCL5, TGFβ1 and VEGF and menstrual hormones (LH, FSH, Oestradiol, Progesterone) were quantified in serum samples from healthy, premenopausal volunteers.

Results: Systemic levels of TGFβ1 were significantly elevated in breast cancer patients compared to controls. A significant positive correlation between circulating CCL5 and TGFβ1 was observed, and mirrored at the gene expression level in tumour tissue from the same breast cancer patients. In primary stromal cells a negative correlation was observed between CCL5 and TGFβ1. CCL5, CCR5, TGFβ1, MMP3 and FAP expression was significantly higher in tumour compared to normal breast tissue. A significant negative correlation was observed between circulating CCL5, Oestradiol and Progesterone ($r = -0.50$, $r = -0.39$ respectively, $p<0.05$). Gene expression levels of CCL5, FAP, VEGF and MMP3 were found to be increased in tumour compared to normal primary stromal fibroblasts. The cytokine TGFβ1 was found to be decreased in tumour stromal cells.

Conclusion: CCL5 expression is elevated in the tumour microenvironment. The data support a role for hormonal control of circulating CCL5 and also highlight a potentially important relationship between CCL5 and TGFβ1 in breast cancer.
Chapter 1 Introduction


1.1 Overview of Breast Cancer

Breast cancer is the most common cancer diagnosed in women and is the one of the leading causes of cancer related death in women worldwide, second only to lung cancer [1, 2]. Globally each year breast cancer accounts for 1 in 10 reported cases [3]. According to recent epidemiological studies, post-menopausal women represent the majority of breast cancer patients and the mean age of first diagnosis is 58 years. Patient demographics reveal higher incidence of the disease in northern and western European countries [4, 5]. Women in less developed regions such as Eastern Africa, Central America and Eastern Asia have lower incidence rates of breast cancer, but higher mortality rates [6, 7].

The high incidence of breast cancer in developed countries has been attributed to multiple factors. The change in lifestyle of Western women with delayed child bearing, lower parity and use of postmenopausal hormones are contributing factors as well as obesity and physical inactivity [6]. In addition, increased incidence has been attributed to widespread use of mammographic screening and early detection of breast cancer [4, 5].

Treatment of breast cancer has evolved greatly over the last 50 years. Radical mastectomy used to be the only treatment option in the 1960’s. Radiotherapy and adjuvant chemotherapy became widely available two decades later [8]. Cancer centres improved care by providing an integrated service for breast cancer patients. Antiestrogen therapies are widely available and research and clinical trials are underway to provide individualized treatment options [9, 10]. Mortality rates have declined in developed countries due to early diagnosis of breast cancer using mammographic screening and improved therapies, including use of adjuvant antiestrogen and chemotherapy [4].
1.1.1 Incidence and Survival

In Ireland, as in most developed countries, incidence of breast cancer has been on the rise (Table 1.1). Most cases of invasive breast cancer were reported in postmenopausal women.

<table>
<thead>
<tr>
<th>Year of diagnosis</th>
<th>Number of Cases</th>
<th>Crude Rate</th>
<th>EASR</th>
<th>WASR</th>
<th>Age 50+ Number of cases</th>
<th>% of All Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>1796</td>
<td>95.38</td>
<td>101.96</td>
<td>74.59</td>
<td>1319</td>
<td>73.44</td>
</tr>
<tr>
<td>2000</td>
<td>1906</td>
<td>99.97</td>
<td>105.51</td>
<td>76.78</td>
<td>1459</td>
<td>76.39</td>
</tr>
<tr>
<td>2001</td>
<td>2034</td>
<td>105.17</td>
<td>110.78</td>
<td>80.71</td>
<td>1545</td>
<td>75.89</td>
</tr>
<tr>
<td>2002</td>
<td>2179</td>
<td>110.55</td>
<td>117.21</td>
<td>85.29</td>
<td>1683</td>
<td>77.20</td>
</tr>
<tr>
<td>2003</td>
<td>2224</td>
<td>111.04</td>
<td>116.72</td>
<td>85.66</td>
<td>1654</td>
<td>74.44</td>
</tr>
<tr>
<td>2004</td>
<td>2187</td>
<td>107.56</td>
<td>111.44</td>
<td>81.86</td>
<td>1603</td>
<td>73.53</td>
</tr>
<tr>
<td>2005</td>
<td>2209</td>
<td>106.61</td>
<td>109.97</td>
<td>80.58</td>
<td>1643</td>
<td>74.48</td>
</tr>
<tr>
<td>2006</td>
<td>2265</td>
<td>106.91</td>
<td>109.02</td>
<td>79.54</td>
<td>1695</td>
<td>74.77</td>
</tr>
<tr>
<td>2007</td>
<td>2492</td>
<td>114.95</td>
<td>119.6</td>
<td>87.64</td>
<td>1868</td>
<td>75.08</td>
</tr>
<tr>
<td>2008</td>
<td>2811</td>
<td>126.86</td>
<td>132.42</td>
<td>97.17</td>
<td>2086</td>
<td>74.77</td>
</tr>
<tr>
<td>2009</td>
<td>2766</td>
<td>123.39</td>
<td>126.29</td>
<td>92.33</td>
<td>2082</td>
<td>75.99</td>
</tr>
<tr>
<td>2010</td>
<td>2724</td>
<td>120.81</td>
<td>122.68</td>
<td>89.75</td>
<td>2062</td>
<td>75.69</td>
</tr>
</tbody>
</table>

Table 1.1 Incidence for female breast cancer in Ireland: incidence 2004-2009, Crude Rate: Number of cases per 100,000 women per year. EASR: European Age Standardised Rate. WASR: World Age Standardised Rate [3]
Chapter 1 Introduction

In the past 10 years in Ireland incidence of breast cancer has increased, while mortality has decreased (Table 1.2).

| Five-year relative survival (with 95% confidence interval) by diagnosis year | EUROCARE-4 |
|---|---|---|---|---|---|
| **all 15-99, age-standardized (95% Confidence Interval)** | 69.8% (68.5-71.0%) | 76.6% (75.6-77.5%) | 82.7% (81.5-83.7%) | 82.1% (81.1-82.9%) | 76.2% (74.3-78.2%) |
| **Ireland** | | | | | 79.0% (78.1-80.0%) |
| **Europe average** | | | | | |
| **15-44 (95% Confidence Interval)** | 75.7% (72.7-78.3%) | 82.0% (79.7-84%) | 84.9% (82.2-87.1%) | 84.8% (82.7-86.5%) | |
| **45-54 (95% Confidence Interval)** | 78.6% (76.3-80.6%) | 84.8% (83.1-86.2%) | 88.4% (86.5-90.0%) | 88.6% (87.1-89.8%) | |
| **55-64 (95% Confidence Interval)** | 72.9% (70.3-75.3%) | 80.9% (79.1-82.5%) | 87.5% (85.6-89.2%) | 86.9% (85.3-88.2%) | |
| **65-74 (95% Confidence Interval)** | 69.9% (66.7-72.8%) | 75.0% (72.4-77.4%) | 81.6% (78.5-84.2%) | 80.3% (77.9-82.4%) | |
| **75-99 (95% Confidence Interval)** | 63.9% (59.4-68.2%) | 64.8% (61.2-68.2%) | 70.2% (65.5-74.7%) | 68.8% (65.3-72.2%) | |

**Table 1.2 Five-year relative survival for female breast cancer by diagnosis year and age at diagnosis**

Most recently, the National Cancer Registry Ireland (NCRI) reported a total of 2724 cases of breast cancer in Ireland in 2010. As shown in Table 1.2, breast cancer incidence rates are among the highest in Europe with survival rates below the European average. The mortality rate for breast cancer has decreased in recent years due to increased patient awareness, earlier diagnosis and treatment. In the period between 2005 and 2009 breast cancer accounted for 679 female deaths per year [3].
Chapter 1 Introduction

1.1.2 Subtypes of breast cancer

Breast cancer is not a single entity but a heterogeneous disease, consisting of several biologically distinct subtypes [11-14]. The World Health Organization (WHO) recognizes 18 different breast cancer types [15]. The disease has been classified histologically into lobular and ductal carcinoma, reflecting the origin of cancer cells from the epithelial lining of either milk producing lobules or milk ducts. The classification based on morphology has limitations and newer methods have been developed including immunohistochemistry and gene expression profiling (Figure 1.1). Several distinct breast cancer subtypes were described through analysis of gene expression patterns of 496 genes using DNA microarrays [11].
Figure 1.1 Molecular profiling of breast cancer.

(A) The tumour specimens were divided into five (or six) subtypes based on differences in gene expression. The cluster dendrogram showing the five (six) subtypes of tumours are coloured as: luminal subtype A, dark blue; luminal subtype B, yellow; luminal subtype C, light blue; normal breast-like, green; basal-like, red; and ERBB2+, pink. (B) The full cluster diagram scaled down. The coloured bars on the right represent the inserts presented in C–G. (C) ERBB2 amplicon cluster. (D) Novel unknown cluster. (E) Basal epithelial cell-enriched cluster. (F) Normal breast-like cluster. (G) Luminal epithelial gene cluster containing ER [13].
Molecular profiling identified five main categories of breast cancer subtypes: luminal A, luminal B, human epidermal growth factor receptor-2 positive, basal-like and normal like subtypes [11, 13]. These main molecular breast cancer categories have been found to correlate reliably with biomarker subtypes (Table 1.3) [16].

<table>
<thead>
<tr>
<th>Molecular category</th>
<th>Biomarker subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>Estrogen receptor (ER) positive (+) and/or progesterone receptor (PR) positive/HER2-</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER positive and/or PR+/HER2+</td>
</tr>
<tr>
<td>HER2 overexpressing</td>
<td>ER-/PR-/HER2+</td>
</tr>
<tr>
<td>Basal-like</td>
<td>ER-/PR-/HER2-</td>
</tr>
</tbody>
</table>

*Table 1.3 Molecular breast cancer categories correlate with biomarker subtypes*

It has been recognized in recent years, that breast cancer subtypes differ in prevalence, clinical outcome, therapeutic options, metastasis, and survival, age and race distribution [14, 17, 18]. Knowledge of breast cancer subtypes and hormone receptor status has implications for systemic treatment in breast cancer patients as targeted treatment options are now available [19, 20]. Treatment for breast cancer includes often surgery, radiation therapy, and chemotherapy depending on disease stage. In addition, therapies targeted at tumour expressed proteins such as HER2 and ER have become available for breast cancer patients. Trastuzumab (Herceptin®) is successfully used systemically in patients with HER2 positive breast cancer [21]. It is a monoclonal antibody that binds to the HER2 receptor, thus blocking its pro-tumourigenic pathways [22, 23]. Tamoxifen is used specifically for patients with estrogen receptor positive breast cancer. Its active metabolite, hydroxytamoxifen, acts as an antagonist of the estrogen receptor in breast tissue [19]. Estrogen signalling induces breast cancer cell growth. Hydroxytamoxifen binds to the estrogen receptor without activating it and
therefore blocks growth signals to breast cancer cells [24]. Aromatase inhibitors block the action of the enzyme aromatase which converts androgens to estrogen. Postmenopausal patients with estrogen receptor positive breast cancer are treated with aromatase inhibitors as the main source of systemic estrogen are peripheral tissues in this group [20, 25].

The most prevalent breast cancer subtype has been reported to be luminal A, followed by luminal B, basal-like subtype and the least prevalent subtype is HER2 overexpressing [14, 18]. Investigation of clinicopathological subtypes revealed that patients with luminal A subtype tended to be older and presented with smaller tumours [18]. In addition, patients with luminal A subtype were less likely to be lymph node positive. Studies have shown significant differences in length of overall survival between breast cancer subtypes. Shortest overall survival and disease free survival was observed in basal-like subtype (ER-/PR-/HER2-) [14, 17, 18]. Intrinsic breast cancer subtype distribution varies among ethnic groups, it has been shown that African American women are diagnosed significantly more often with basal-like breast cancer than their Caucasian counterparts. In addition, younger women are significantly more likely to suffer from basal-like breast cancer subtype, which carries the worst prognosis [14]. Breast cancer diagnosis and treatment remains a challenge as the disease is multifaceted and individual patients’ clinical response to available treatment and outcome vary broadly [12]. For optimal results, an individualized treatment plan should be available for all breast cancer patients. Preclinical experiments and clinical trials are currently underway to investigate targeted therapies and elucidate the best use of these drugs in combination with each other and standard therapies.

### 1.1.3 Breast cancer classification and clinicopathological parameters

The TNM classification system is widely used to classify and diagnose breast cancer and it describes tumour size (T), number and location of lymph node metastases (N) and metastatic spread to distant parts of the body (M) [26]. The International Union Against Cancer (UICC) employs the TNM system to
define breast and other solid tumour progression. The UICC stages 0-IV represent groups of TNM status (Table 1.4)[26].
<table>
<thead>
<tr>
<th>UICC stage</th>
<th>TNM</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis</td>
<td>N0</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Stage I</td>
<td>T1</td>
<td>N0</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T0</td>
<td>N1</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>T1</td>
<td>N1</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>T2</td>
<td>N0</td>
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<tr>
<td>Stage IIB</td>
<td>T2</td>
<td>N1</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>T3</td>
<td>N0</td>
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<tr>
<td>Stage IIIA</td>
<td>T0</td>
<td>N2</td>
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<td>T1</td>
<td>N2</td>
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<td>N2</td>
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<td></td>
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<tr>
<td>Stage IIIB</td>
<td>T4</td>
<td>N0, N1, N2</td>
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<td>Stage IIIC</td>
<td>T0-4</td>
<td>N3</td>
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<td>Stage IV</td>
<td>T0-4</td>
<td>N0-3</td>
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</tbody>
</table>

*Table 1.4 UICC and TNM staging for breast cancer [26]*
The UICC and TNM staging gives important prognostic information and is also crucial in determining the optimal treatment option for the breast cancer patient. Survival rates drop with each increment in stage (Table 1.5).

<table>
<thead>
<tr>
<th></th>
<th></th>
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<tr>
<td>OBSERVED SURVIVAL</td>
<td></td>
</tr>
<tr>
<td>Period</td>
<td>1-year (%)</td>
</tr>
<tr>
<td>Stage I</td>
<td></td>
</tr>
<tr>
<td>1994-98</td>
<td>98.0%</td>
</tr>
<tr>
<td>1999-03</td>
<td>98.5%</td>
</tr>
<tr>
<td>2004-08</td>
<td>99.0%</td>
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<td>Stage II</td>
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<td>96.9%</td>
</tr>
<tr>
<td>2004-08</td>
<td>97.8%</td>
</tr>
<tr>
<td>Stage III</td>
<td></td>
</tr>
<tr>
<td>1994-98</td>
<td>85.3%</td>
</tr>
<tr>
<td>1999-03</td>
<td>89.1%</td>
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<td>59.8%</td>
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<td>2004-08</td>
<td>66.3%</td>
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</tbody>
</table>

| RELATIVE SURVIVAL                           |                                                      |
| Period                                      | 1-year (%)                                           | 95% CI                | 5-year (%) | 95% CI                  |
| Stage I                                     |                                                      |
| 1994-98                                     | 99.6%                                                | [92.3, 100%]           | 93.6%      | [89.5, 96.2%]           |
| 1999-03                                     | 99.8%                                                | [92.6, 100%]           | 96.2%      | [92.5, 98.1%]           |
| 2004-08                                     | 100.0%                                               | [-]                    | 96.1%      | [91.2, 98.3%]           |
| Stage II                                    |                                                      |
| 1994-98                                     | 96.2%                                                | [95.0, 97.1%]          | 77.6%      | [75.3, 79.8%]           |
| 1999-03                                     | 97.6%                                                | [96.5, 98.3%]          | 85.0%      | [82.9, 86.8%]           |
| 2004-08                                     | 98.7%                                                | [97.7, 99.2%]          | 89.5%      | [86.7, 91.7%]           |
| Stage III                                   |                                                      |
| 1994-98                                     | 84.6%                                                | [81.8, 87.0%]          | 51.2%      | [47.2, 55.1%]           |
| 1999-03                                     | 90.2%                                                | [87.9, 92.2%]          | 58.7%      | [54.8, 62.4%]           |
| 2004-08                                     | 91.4%                                                | [89.3, 93.2%]          | 66.4%      | [61.4, 71.0%]           |
| Stage IV                                    |                                                      |
| 1994-98                                     | 54.2%                                                | [49.9, 58.3%]          | 19.4%      | [15.9, 23.2%]           |
| 1999-03                                     | 58.6%                                                | [54.4, 62.4%]          | 22.0%      | [18.5, 25.7%]           |
| 2004-08                                     | 65.7%                                                | [62.2, 68.9%]          | 28.1%      | [23.7, 32.8%]           |

Table 1.5 Relationship of UICC stage and survival in Ireland [2]

In the most recent diagnostic period, 2004-2008, a patient diagnosed with stage I breast cancer has a prognosis of relative 1-year survival that approaches 100% (Table 1.5) [2]. However, if diagnosed with stage IV disease in the same time period, the 1-year relative survival rate drops to 66% (Table 1.5).

In Ireland, there was an improvement in observed and relative survival during the diagnostic periods between 1994-1998 and 2004-2008 respectively across all stages of breast cancer (Table 1.5) [2].
The Bloom-Richardson grade is another classification tool for breast cancer and it is based on invasive breast cancer morphology [27]:

1) The degree of tumour tubule formation (percentage of breast cancer composed of tubular structures)
2) The mitotic activity of the tumour (rate of breast cancer cell division)
3) The nuclear pleomorphism of tumour cells (nuclear grade, change in cell size and uniformity)

The Bloom-Richardson grade is calculated by scoring each of the three above named features from 1 to 3. These values are added up to comprise the final score ranging from 3 to 9 and are subdivided in the following manner:

Grade 1 tumour (well-differentiated): score 3-5
Grade 2 tumour (moderately-differentiated): score 6-7
Grade 3 tumour (poorly-differentiated): score 7-9

The grade 3 poorly differentiated tumours carry the worst prognosis.

The Nottingham Prognostic Index (NPI) is a prognostic tool used to evaluate survival following surgery for breast cancer [28, 29]. The formula for calculation of NPI includes three factors that were found to be significantly associated with survival: Tumour size, tumour grade and number of lymph nodes with metastatic spread.

\[ \text{NPI} = [0.2 \times S] + N + G \]

- S is the size of the index lesion in centimetres
- N is the number of lymph nodes involved: 0 LN = 1; 1-3 LN = 2; >3 LN = 3
- G is the grade of tumour: Grade 1 = 1, Grade 2 = 2, Grade 3 = 3
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The NPI score calculates five year survival rates for breast cancer patients (Table 1.6).

<table>
<thead>
<tr>
<th>NPI Score</th>
<th>Prognosis/5-year survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;/=2.0 to &lt;=2.4</td>
<td>Excellent prognosis/93% 5yr survival</td>
</tr>
<tr>
<td>&gt;2.4 to &lt;=3.4</td>
<td>Good prognosis/85% 5yr survival</td>
</tr>
<tr>
<td>&gt;3.4 to &lt;=5.4</td>
<td>Moderate prognosis/70% 5yr survival</td>
</tr>
<tr>
<td>&gt;5.4</td>
<td>Poor prognosis/50% 5yr survival</td>
</tr>
</tbody>
</table>

*Table 1.6 Nottingham Prognostic Index*
Chapter 1 Introduction

1.2 Inflammation and cancer

1.2.1 The hallmarks of cancer

Carcinogenesis is understood to be a multistep process during which cells progressively acquire a number of neoplastic characteristics or “hallmarks” [30]. Initially, this concept included six hallmarks of cancer and described the key mutations in cell physiology that underlie malignant progression: tumour cell proliferation; escape from growth suppressors; invasion and metastasis; replicative immortality, angiogenesis and resisting cell death (Figure 1.2) [30].

![Figure 1.2 The Hallmarks of Cancer](image)

In the last decade, understanding of carcinogenesis has evolved and two hallmarks have emerged - deregulation of cellular metabolism and evasion of cancer cells from immune recognition and destruction (Figure 1.3) [30]. Inflammation, the body’s physiological response to tissue injury and infection has been found to support most cancer hallmark capabilities. Genomic instability and mutation enables carcinogenesis through acquisition of hallmark functions (Figure 1.3).
The importance of tumour stroma in carcinogenesis has been investigated by many groups [32-36]. Evidence suggests that the tumour microenvironment influences all hallmark capabilities of cancer through reciprocal interactions with tumour epithelial cells [33, 37].
1.2.2 Mediators of physiological inflammation

Tumour-promoting inflammation is emerging as an “enabling characteristic”, an important step in carcinogenesis [38-41]. A connection between inflammation and cancer was reported as early as 1863 by Virchow, who observed cancer developing in sites of chronic inflammation [42]. Inflammation has important physiological functions in wound healing and defence against infection [43]. In response to tissue damage or to fight infection, the body sets in motion a cascade of events. Leucocytes (neutrophils, monocytes and eosinophils) migrate from the venous system to the site of tissue injury, activated and directed by a network of chemotactic signals [44]. These signals include chemokines such as Monocyte Chemoattractant Protein -1, -2, and -3 (MCP-1/CCL2, MCP-2/CCL8 and MCP-3/CCL7), Transforming Growth Factor beta (TGFβ) and platelet factor 4 (PF-4). Activated macrophages/monocytes at the site of tissue injury are the main source of growth factors and cytokines (insulin-like growth factor (IGF), TGFβ1, tumour necrosis factor -α (TNF-α) and interleukin (IL)-1). This signalling network has a profound effect on all cell types in the local inflammatory environment e.g. endothelial, mesenchymal, epithelial and neuroendocrine cells. Fibroblasts migrate to sites of tissue repair and deposit collagen in response to factors such as TGFβ1, β2 and β3 and IL -1. Activated macrophages stimulate production of matrix metalloproteases (MMPs) which play an important role in facilitating transmigration through the endothelium to sites of injury. Mast cells synthesize and store histamines, cytokines and proteases such as heparin, histamine, MMPs and vascular endothelial growth factor (VEGF). The activated mast cells release these factors in their local inflammatory microenvironment [44, 45].

Physiological inflammation is self-limiting and once tissue repair is complete, the inflammatory environment is restored to its former balance. In this context, TGFβ is an activator of inflammatory cells in early inflammation and involved in leucocyte recruitment and regulation of MMP secretion. Once immune cells are activated, TGFβ suppresses their ability to proliferate and differentiate. This negative feedback loop also reduces activation of immune cells in response to
other cytokines [46]. In conclusion, TGFβ is a critical factor in wound healing and plays a role in resolving inflammation.

1.2.3 Tumour promoting inflammation

The link between inflammation and cancer has been described in the literature as two pathways: an extrinsic and intrinsic pathway (Figure 1.4) [47, 48].

Figure 1.4 Pathways that connect inflammation and cancer [48]
The extrinsic pathway leads to carcinogenesis through chronic inflammatory conditions such as chronic hepatitis which increases greatly the risk of liver cancer. There are numerous examples for infectious agents and inflammatory conditions which increase the risk of developing cancer (Table 1.7).

<table>
<thead>
<tr>
<th>Chronic inflammatory conditions associated with neoplasms</th>
<th>Associated neoplasm</th>
<th>Aetiologic agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory bowel disease, Crohn’s disease, chronic ulcerative colitis</td>
<td>Colorectal carcinoma</td>
<td></td>
</tr>
<tr>
<td>Reflux oesphagitis, Barrett’s oesophagus</td>
<td>Oesophageal carcinoma</td>
<td></td>
</tr>
<tr>
<td>Asbestosis, silicosis</td>
<td>Mesothelioma, lung carcinoma</td>
<td>Asbestos fibres, silica particles</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>Lung carcinoma</td>
<td>Silica, asbestos, smoking (nitrosamines, peroxides)</td>
</tr>
<tr>
<td>Cystitis, inflammation</td>
<td>Bladder carcinoma</td>
<td>Chronic indwelling , urinary catheters</td>
</tr>
<tr>
<td>Lichen sclerosus</td>
<td>Vulvar squamous cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>Gingivitis, lichen planus</td>
<td>Oral squamous cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>Chronic pancreatitis, hereditary pancreatitis</td>
<td>Pancreatic carcinoma</td>
<td>Alcoholism, mutation in trypsinogen gene on Ch. 7</td>
</tr>
<tr>
<td>Sialadenitis</td>
<td>Salivary gland carcinoma</td>
<td></td>
</tr>
<tr>
<td>Sjögren syndrome, Hashimoto’s thyroiditis</td>
<td>MALT lymphoma</td>
<td></td>
</tr>
<tr>
<td>Skin inflammation</td>
<td>Melanoma</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>Cancers associated with infectious agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Opisthochis, Cholangitis</td>
<td>Cholangiosarcoma, colon carcinoma</td>
<td>Liver flukes (Opisthochis viverrini), bile acids</td>
</tr>
<tr>
<td>Chronic cholecystitis</td>
<td>Gall bladder cancer</td>
<td>Bacteria, gall bladder stones</td>
</tr>
<tr>
<td>Gastritis/ulcers</td>
<td>Gastric adenocarcinoma, MALT</td>
<td>Helicobacter pylori</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>Hepatocellular Carcinoma</td>
<td>Hepatitis B and/or C virus</td>
</tr>
<tr>
<td>Mononucleosis</td>
<td>B-cell non-Hodgkin’s lymphoma, Burkitt’s lymphoma</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>AIDS</td>
<td>Non-Hodgkin’s lymphoma, squamous cell carcinomas, Kaposi’s sarcoma</td>
<td>Human immunodeficiency virus, human herpes virus type 8</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>Skin carcinoma in draining sinuses</td>
<td>Bacterial infection</td>
</tr>
<tr>
<td>Pelvic inflammatory disease, chronic cervicitis</td>
<td>Ovarian carcinoma, cervical/anal carcinoma</td>
<td>Gonorrhoea, chlamydia, human papillomavirus</td>
</tr>
<tr>
<td>Chronic cystitis</td>
<td>Bladder, liver, rectal carcinoma, follicular lymphoma of the spleen</td>
<td>Schistosomiasis</td>
</tr>
<tr>
<td>MALT, mucosa-associated lymphoid tissue</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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Table 1.7 Chronic inflammatory conditions associated with neoplasms [44]

The intrinsic pathway fosters a tumour promoting microenvironment through oncogene activation in the absence of underlying chronic inflammatory disease. Breast cancer is an example of an inflammatory tumour microenvironment in the absence of chronic inflammatory conditions (Figure 1.4) [48]. It is thought that neoplastic transformed cells generate a tumour microenvironment rich in immune cells and inflammatory mediators such as chemokines, growth factors, MMPs and proteases and kinases. The two pathways, intrinsic and extrinsic, both result in activation of transcription factors such as nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α in tumour cells (HIF1α). The transcription factors modulate the production of proinflammatory factors in tumour cells, for example chemokines, cytokines and prostaglandins. Pro-inflammatory factors not only recruit immune cells, they also activate the same transcription factors (NF-κB, STAT3, HIF1α) in cells of the tumour microenvironment (stromal cells, leucocytes and tumour cells) that will result in increased pro-inflammatory signalling. In the context of breast cancer, the NF-κB pathway has been shown to promote the invasion of breast cancer cells by regulating the expression of MMP1 and MMP2 proteases [49]. In addition NF-κB pathway has been implicated in breast cancer stem cell expansion [50]. The positive feedback loop creates a chronic inflammatory tumour microenvironment that promotes tumour progression, invasion and metastasis [48] (Figure 1.4). Key factors in cancer-related inflammation include major inflammatory cytokines such as IL-1, IL-6, IL-23 and TNF-α. Chemokines (CCL2, CCL20, IL-8 and CCL5) recruit Monocytes/Macrophages into the tumour microenvironment and promote angiogenesis. In breast cancer, the cytokine CXCL12 and its receptor have been found to be involved in tumour cell homing to lymph nodes. Proteases (MMP3, MMP7, MMP9 and MMP10) play a role in tumour cell invasion and dissemination [48]. Tumour epithelial cell are involved in fostering an inflammatory microenvironment. Although inflammatory mediators exhibit pro as well as anti-tumourigenic functions, the inflammatory microenvironment greatly enables hallmark capabilities of
cancer (Figure 1.3). In this context, the use of anti-inflammatories in breast cancer is discussed in section 1.2.4.

1.2.4 Therapeutic potential of anti-inflammatory drugs

Tumours seem to seize molecular pathways seen in wound healing and as a consequence appear as “wounds that do not heal” [44]. However, the underlying molecular mechanisms facilitating the interconnection of inflammation and cancer remain poorly understood. Recent research investigated the effect of anti-inflammatory agents such as aspirin on cancer incidence and progression. In colorectal cancer, it has been shown that non-steroidal anti-inflammatory agents such as aspirin reduce incidence and mortality [51-53]. Prophylactic use of aspirin has been shown to reduce the incidence of breast cancer [54]. Breast cancer mortality has also been shown to be decreased due to aspirin use. In this context, it has been reported recently, that Cyclooxygenase 2 (COX-2) expression in breast tumours is linked to worse prognosis. This is interesting since aspirin and other non-steroidal anti-inflammatories (NSAID) inhibit COX-2. However, survival benefit of breast cancer patients on aspirin or other NSAIDS has been independent of COX-2 expression [54, 55]. Recent reports suggest that cyclooxygenase inhibitors such as celecoxib could block brain metastasis in a mouse model of breast cancer [56].

In breast cancer the inflammatory tumour microenvironment should also be a treatment target in addition to malignant epithelial cells. Anti-inflammatory agents have shown some promise, however the exact mechanisms of action are still poorly understood [54].
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1.3 The breast cancer microenvironment

1.3.1 Overview of the breast cancer microenvironment

In the past, cancerous lesions were seen as merely an agglomeration of cancer cell clones. In the past decade, cancers are increasingly recognized as complex, organised entities with an abundance of different specialized cell types [57]. Similarly, the interaction of tumours and their microenvironment and the impact on tumourigenesis, proliferation and metastasis was not recognized and investigated until recently.

Breast tumours consist of many different cell types including not just carcinoma cells, but also additional epithelial cell types, stromal cells, adipose cells, endothelial cells, pericytes and infiltrating lymphocytes (Figure 1.5) [31, 36]. Fibroblasts constitute the predominant cell type in tumour stroma of carcinomas and are referred to in the literature as “cancer-associated fibroblasts” [31, 32]. It has been recognized in the last decade, that stromal fibroblasts play a tumour promoting and even tumour inducing role in carcinogenesis [32]. Myofibroblasts, a subset of these cells, can be identified by α smooth muscle actin expression (α-SMA). Interestingly, these cells are rarely found in healthy tissues; however they appear in sites of chronic inflammation and wound healing. Inflammation is an enabling characteristic of cancer progression and the activation of fibroblasts has an important role in this context. Cancer cells as described in section 1.2.3 have the ability to induce transcription factors that are involved directly in the activation of fibroblasts or indirectly through secretion of chemokines/cytokines which are mediators of a reactive inflammatory microenvironment [32, 36, 44].
In addition to cancer cells, tumours of the breast consist of stromal cells (fibroblasts, myofibroblasts), endothelial cells, pericytes, macrophages, neutrophils and lymphocytes. Different matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are synthesized by stromal cells, cancer cells and cancer cells undergoing Epithelial-to-Mesenchymal Transition (EMT) taken from Egeblad and Werb [58].

*Figure 1.5 Expression of MMPs and TIMPs in breast tumours.*
1.3.2 Matrix metalloproteinases and cancer progression

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases and are mediators of physiological and pathological tissue restructuring processes including wound healing, inflammation and carcinogenesis [59]. Their principle mode of action is cleavage of substrates such as growth factor precursors, growth factor binding proteins, receptor tyrosine kinases, cell adhesion molecules and components of extracellular matrix. They are involved in several steps of carcinogenesis: cancer cell growth, migration, invasion, metastasis and angiogenesis [59, 60]. Interestingly, most MMP’s are produced by stromal cells in the tumour microenvironment, not cancer cells [59].

As discussed in 1.2.1, there are six fundamental hallmarks of cancer and MMPs have been found to play a significant role in these steps in cancer progression (Figure 1.6a-f).
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Figure 1.6a-c Functions of MMPs in cancer progression taken from Egeblad and Werb [58].

The matrix metalloproteinases (MMPs) have both cancer-promoting and cancer–inhibiting functions [58]. a) MMPs inhibit growth of cancer cells by liberating TGF-β from the latent TGF-β complex. b) MMPs promote survival of cancer cells by liberating IGF and by cleavage of FAS ligand (FASL), a ligand for the death receptor FAS. MMPs also promote cancer cell death by changing the ECM composition, which influences integrin signalling. c) MMPs promote angiogenesis by increasing the bioavailability of the pro-angiogenic growth factors vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), and TGF-β.
MMPs act antiangiogenically through cleavage of plasminogen and Col-XVIII, resulting in generation of the anti-angiogenic factors angiostatin and endostatin [58].

MMPs have the ability to either promote or inhibit tumour cell growth. On the one hand, MMPs activate growth stimulating factors such as insulin-growth-factor (IGF) through degradation of IGF-binding protein (IGF-BP) and release the cell membrane bound precursor of TGF-α. On the other hand, MMPs inhibit tumour cell growth by liberating TGF-β from the latent TGF-β complex (Figure 1.6a) [58, 61].

MMPs were reported to be involved in regulation of cancer cell survival and to display pro- as well as anti-apoptotic features (Figure 1.6b) [58, 62]. Elevated MMP3 in mammary epithelial cells has been shown to induce apoptosis [58, 62, 63]. MMPs were found to unfold proangiogenic signalling through release of TGF-β from its latent state (Figure 1.6c). The proteases also increased bioavailability of VEGF and fibroblast growth factor 2 (FGF-2), which lead to an increase in migration and proliferation of endothelial cells [64, 65]. The ability of MMPs to cleave and degrade components of the extracellular matrix (ECM) also promoted angiogenesis because it facilitated invasion of endothelial cells into tumour stroma [58, 66]. MMPs also cleaved substrates which act as suppressors of angiogenesis: angiostatin and endostatin (Figure 1.6c) [60, 67].
Figure 1.6d-f Functions of MMPs in cancer progression taken from Egeblad and Werb [58]

d) The MMPs regulate invasion by degrading structural ECM components. MMPs also promote invasion by cleavage of the adhesion molecules CD44 and Ecadherin (E-cad). MMPs might inhibit metastasis by cleavage of CXCL12, a chemokine of the CXC family that promotes breast cancer metastasis. e) MMPs promote EMT by cleaving the cell-adhesion molecule E-cad and by liberating TGF-β. f) Reactive inflammatory cells provide some of the key MMPs involved in cancer progression. MMPs play a role in activating TGFβ from its latent state. TGFβ has been found to be involved in homing of immune cells into the tumour microenvironment [68]. Adapted from Egeblad and Werb [58]
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It was reported in the literature, that tumour invasion was aided by MMPs through cleavage of ECM components and cell adhesion molecule CD44 and E-cadherin [69-71]. E-cadherin is a well characterized cell-adhesion molecule. Evidence in the literature suggested that MMP3 and MMP7 were cleaving E-cadherin, which induced EMT [72]. EMT has been described as the metamorphosis of a phenotypically epithelial cell to a phenotypically mesenchymal cell and is associated with increased tumour invasiveness. It has also been reported that expression of MMP-3 on its own was sufficient to induce EMT in the mammary cell line SCP2 in vitro and increase carcinogenesis in vivo [73]. When treated with recombinant MMP-3, cells belonging to the immortal mammary epithelial breast cancer cell line Scp2 lost epithelial marker cytokeratin and upregulated mesenchymal marker vimentin. In vivo experiments in mice showed, that MMP-3 expression triggered EMT in mammary epithelial cells and changed their phenotype to mesenchymal-like cells, which lead to more invasive tumours [73, 74]. MMP-3 has been shown to be expressed by the highly invasive MD-MB-231 cell line but not in less invasive breast cancer cell lines like T47D [75].

TGFβ has previously been reported to be an important factor in tumour invasion and metastasis and MMP-9 was observed to activate the growth factor from its latent stage [76]. TGFβ is known to induce EMT in breast cancer, thus promoting breast cancer invasiveness and metastasis (Figure 1.6e) [77]. Interestingly, studies have found CXCL12 to be degraded and inactivated by MMP-1, -3, -9, -13 and -14 [78]. CXCL12 is a ligand to CXCR4, which is expressed by breast cancer epithelial cells. In vivo experiments showed, that blockage of CXCL12 by antibodies resulted in reduced metastasis to lung and lymph nodes (Figure 1.6 d and e) [79].

The importance of inflammation as an enabling characteristic in cancer progression has been discussed in section 1.2.2. MMPs regulate the immune response by several mechanisms: TGFβ is activated by MMPs and suppresses the proliferation of T-lymphocytes which act as tumour suppressors [80]. MMPs cleave and inactivate or activate numerous CC and CXC chemokines which attract lymphocytes (e.g. MMP-12 cleaves CCL2, MMP-9 degrades CXCL4) [81]. Matrix metalloproteinases were implicated in cleaving CCL2 in order to activate the cytokine.
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There was also evidence of an important role for MMPs in brain metastasis of breast cancer. Protein expression of MMP-3 as well as MMP-2 and MMP-9 has been found in breast cancer brain metastases using a rat model [82]. In in vivo studies, decreased metastatic colonisation of the lungs of MMP-2 and MMP-9 deficient mice as compared to wild type mice has been found following injection with cancer cells [83]. MMP-3 genes have recently been linked to breast cancer risk and survival [84]. The hemopexin domain of the MMP-3 protein has been shown to bind with extracellular heat-shock protein 90 β (HSP90β). This interaction has been proven to be important for invasion in in vitro experiments using a three dimensional collagen-1 gel assay [85].

MMPs have multiple functions which often seem to have opposing effects in carcinogenesis and their multifaceted role in breast cancer has to be evaluated in the context of the inflammatory tumour microenvironment. MMPs take their cues from the microenvironment and can be pro- or anti-carcinogenic. Inflammatory cells synthesize, store and release several MMPs (e.g. MMP-3, -9, -12 and -14).
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1.3.3 Transforming growth factor β1 (TGFβ1)

TGFβ ligands TGFβ1, TGFβ2 and TGFβ3 are potent modulators of cell behaviour [46, 86, 87]. The cytokine TGFβ is expressed in most tissues and unlike other inflammatory cytokines (e.g. IL-10) does not require activation by immune stimuli. In the context of cancer progression, TGFβ regulates pathways important to tumour initiation, proliferation and metastasis [86, 88]. TGFβ in normal epithelial cells induces arrest of the cell cycle and this function has been implicated in cancer suppression in the early stages of oncogenesis [86, 89]. In later stages of carcinogenesis TGFβ acts as a tumour promoter by stimulating EMT and angiogenesis [58, 90]. In addition, TGFβ signalling is involved in suppression of the immune response and metastasis [91]. TGF β regulates chemotaxis in the tumour microenvironment through stimulation of expression of CXCR4 in monocytes and macrophages [92]. CXCR4 is the receptor for CXCL12, a ligand that is expressed by invasive carcinoma-associated fibroblasts [33]. It is thought that TGFβ acts as a tumour promoter in the tumour-stromal interaction by attracting immune cells into the tumour microenvironment [90].

In breast cancer patients, circulating levels of TGFβ1 and their relationship with prognosis and disease stage has been investigated by many groups [93, 94]. Elevated systemic levels of TGFβ1 were shown to drop in breast cancer patients following removal of the tumour and a significant relationship between systemic levels prior to cancer treatment and overall survival has also been reported [93-96].

Previous studies report that TGF-β1 has an important role in breast cancer cell metastasis to bone [97]. While all three isoforms of TGF-β, TGF-β1, TGF-β2 and TGF-β3 were present in bone, TGFβ1 was the most abundant. It was established in a mouse model of breast cancer that both host and tumour derived TGFβ1 were involved in metastasis to bone. TGF-β1 knockout mice showed reduced bone metastasis and a similar effect was found when TGF-β1 was neutralized in MDA-MB-231 breast cancer cells [86, 98].
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1.3.4 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is the principal modulator of endothelial cell proliferation and its functions are mediated by the high affinity tyrosine kinase receptors, KDR/Fik-1 and Fit-1. In the context of breast cancer, VEGFA and its receptor VEGFR2 have been described as the most important isotype of the cytokine [99, 100]. The important role of VEGF in angiogenesis, vasculogenesis and vascular permeability makes it an important factor in carcinogenesis [101]. VEGF is one of the most potent angiogenic cytokines and tumour-derived VEGF has been shown to play a critical role in tumour growth and angiogenesis [102]. Matrix metalloproteinase 9 (MMP9), which is a readily available factor in the inflammatory tumour microenvironment, has been found to increase the bioavailability of VEGF and induce an “angiogenic switch” which activates tumour angiogenesis from previously quiescent vessels (Figure 1.5 e) [65]. Reports of VEGF and breast cancer carcinogenesis suggest a dual role for the angiogenic cytokine. On the one hand VEGF directly stimulates endothelial cells, on the other it recruits macrophages in the tumour stroma, which are able to stimulate angiogenesis through secretion of factors including VEGF, tumour necrosis factor-alpha (TNF-alpha), and thymidine phosphorylase (TP) [103]. Tumour associated macrophages also are known to secrete monocyte chemoattractant protein1 (MCP1), which associated with breast cancer progression and decreased survival. Expression levels of VEGF and MCP1 have been found to be correlated in breast cancer [104-106]. VEGF has also been reported to contribute to breast cancer proliferation directly through VEGFR2/Flk-1 [107]. Levels of the cytokine VEGF have been shown to correlate with prognosis for patients with node-negative breast carcinoma [108-110]. Further strengthening the evidence base for a strong link between VEGF with breast cancer are findings that suggest fluctuations of systemic levels of VEGF with circulating menstrual hormones [111]. Serum VEGF was found to be significantly decreased in the luteal phase and showed a significant negative correlation with progesterone. In vitro experiments showed a decrease of VEGF expression in breast cancer cell lines when cultured with the supernatant of luteal phase serum. These results showed a potential benefit in timing breast cancer surgery
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with the menstrual cycle of premenopausal patients because low levels of the angiogenic cytokine VEGF could decrease the possibility of micrometastasis establishment [111, 112]. The important influence of VEGF on survival of breast cancer patients has been reported to vary with hormone receptor status of breast cancer subtypes [113].

1.3.5 CCL2

Chemokine (C-C motif) ligand 2 (CCL2) is also known as monocyte chemoattractant protein 1 (MCP-1) and belongs to the CC chemokine family [114, 115]. Recombinant CCL2 is a chemoattractant to T-lymphocytes, monocytes and dendritic cells and the chemokine is known to recruit those immune cells to sites of tissue injury or inflammation [116]. CCL2 has been found to be secreted by tumour epithelial, as well as stromal cells in the breast cancer microenvironment using immunohistochemistry. Several reports showed highest stromal expression of the chemokine in fibroblasts and macrophages. However, endothelial cells also expressed CCL2 [117-121]. The cytokine was significantly expressed in cancerous breast epithelial cells and its expression was seen as a characteristic gained through carcinogenesis. A relationship between expression levels of the chemokine and breast cancer progression has been investigated. CCL2 expression has been found to be significantly correlated with higher tumour grade, positive lymph node status, low levels of differentiation and poor prognosis [104, 122, 123]. Several studies investigated systemic levels of the chemokine with contradictory results [124]. While a clear correlation between breast cancer progression and CCL2 expression could be established in breast cancer tissue, more evidence of an association with systemic CCL2 levels is needed [116, 125].

The chemokine CCL2 is found in the plasma membrane of human endothelial cells, attached by its glycosaminoglycan side chain [126]. It has been reported in the literature that CCL2 promoted angiogenesis directly on the endothelial cells in mice bearing mammary tumours. Also, an indirect mechanism has been described through the recruitment of macrophages into the tumour microenvironment, which released angiogenic factors such as VEGF [104, 106, 126, 127]. The tumour-promoting, positive feedback loop between CCL2,
VEGF and TAMs in the tumour microenvironment is an example of how inflammatory mediators are involved in tumour progression (Figure 1.7) [128].

![Diagram of Pro-tumour processes influenced by CCL2 in breast cancer](image)

**Figure 1.7 Pro-tumour processes influenced by CCL2 in breast cancer**

The interaction of CCL2, TAMs and the angiogenic regulator VEGF potentially induces a positive feedback loop within the tumour microenvironment to greatly augment tumour progression as well as metastasis in other organ systems [128].

Stromal cell derived CCL2 has been found to act as the key mediator in tumour associated macrophage (TAM) recruitment into the breast tumour which promotes angiogenesis and tumour growth [103, 118, 129]. Studies reported that stromal CCL2 contributes to breast cancer progression in vivo. The cytokine was found to induce tumour associated macrophage infiltration in tumour bearing mice, which promotes tumour growth [118, 130]. Recent reports also suggested that stromal cell derived CCL2 promoted lung metastasis in an in vivo mouse model of breast cancer [131]. In addition, anti-CCL2 therapy decreased tumour growth and reduced recruitment of immune cells in mice [118]. Recent studies on breast cancer cells in vitro suggested that
the CCL2/CCR2 signalling axis was involved in breast cancer cell motility and survival through phosphorylation of Smad3 and p42/44MAPK proteins [132]. Several in vivo and in vitro studies showed a link between the CCL2/CCR2 signalling axis and breast cancer metastasis to bone and lungs. CCL2 specific antibodies have been shown to reduce TAM recruitment to the tumour, and decrease breast cancer motility and invasion [126, 133, 134]. The above mentioned studies confirmed that the chemokine CCL2 played an important role in breast cancer proliferation, invasion and metastasis. The usefulness of the CCL2/CCR2 signalling axis as a therapeutic target will need further evaluation in the context of other major players in the inflammatory tumour microenvironment.

1.3.6 CCL5

The chemokine CCL5 is also known as Regulated upon activation T-cell expressed and secreted (RANTES). The chemotactic cytokine plays an important part in inflammation through activation of T cells, monocytes, dendritic cells, natural killer cells, eosinophils and basophils [135]. An association between CCL5 and multiple cancer types has been reported, with the most striking findings reported in relation to breast cancer [116]. CCL5 binds to multiple receptors including CCR1, CCR3 and CCR4, with CCR5 recognised as its principal receptor [136].

Conflicting reports exist in relation to the role of CCL5 in breast cancer progression. On a systemic level, Niwa et al found elevated plasma levels of the chemokine using ELISA in breast cancer patients compared to healthy controls, and reported a correlation with disease stage [137]. Another group also observed significantly elevated serum CCL5 in breast cancer patients compared to healthy controls, although no significant change was detected between patients with metastatic and non-metastatic disease [138].

Using immunohistochemistry, tissue from patients with advanced breast carcinoma was reported to have elevated levels of CCL5 [122], with the protein rarely found in biopsies taken from healthy patients. Also joint CCL5 and CCL2 expression in the same breast tumour tissues has been correlated
with more advanced disease [117]. In an in vivo model of breast cancer, tumours secreting lower levels of CCL5 were shown to have reduced metastatic potential [139]. Tumour derived CCL5 has also been implicated in reduced T-cell response and shown to support in vivo growth of murine mammary carcinoma [140]. In contrast, Kurt et al reported that tumour-derived CCL5 on its own had no role in breast cancer progression [141].

CCL5 protein expression at diagnosis has been correlated to clinical outcome in stage II breast cancer patients, suggesting a potential role for the chemokine as a biomarker for the disease independent of oestrogen receptor-α (ER-α) status [142]. In combination with ER status the prognostic strength of CCL5 was greatly improved in Stage II patients [142]. It has been reported that Mesenchymal Stem Cells (MSCs) increase the rate of osteosarcoma lung metastasis via secretion of CCL5 [143]. MSCs are found within the stroma of breast tumours and are thought to promote breast cancer metastasis [37]. The chemokine CCL5 and its receptor CCR5 have been implicated in breast cancer cell proliferation in vitro through mTOR-dependent mRNA translation [144].

1.3.7 Fibroblast activation protein

Fibroblast activation protein (FAP), also known as seprase, belongs to the serine protease family [145]. It has been implicated in a number of physiological and pathological conditions that were previously associated with activation of stromal fibroblasts such as wound healing, inflammation, arthritis and fibrosis of lung and liver [146, 147]. In epithelial cancers it was found to be selectively expressed on the cell-surface of tumour associated stromal fibroblasts in the tumour microenvironment [148]. Interestingly, in vitro experiments showed that TGFβ induced upregulation of FAP expression on fibroblasts [145].

FAP has been found to cleave components of the extracellular matrix, and recent evidence suggested that other functions of the protein such as MMP-9 activation were also responsible for enhanced tumour growth [149]. Gene expression profiling studies detected high expression levels of FAP in tumour tissue and low expression levels in normal tissue [150]. In preclinical studies
using in vivo models of breast cancer, FAP has been shown to have an important role in carcinogenesis. Overexpression of FAP in fibroblasts were shown to improve xenograft tumour take and promoted tumour growth as well as support angiogenesis [151]. FAP has been investigated by several groups as a therapeutic target, because it is specifically expressed on activated fibroblast in the tumour microenvironment, but does not appear in normal tissue. FAP knockdown in breast tumour bearing mice using short hairpin RNA showed reduced tumour growth and angiogenesis [152].

1.3.8 The tumour microenvironment and response to chemotherapy

Chemotherapeutic agents are widely used in the treatment of breast cancer. The mechanisms of action of chemotherapeutic agents remain poorly understood. Chemotherapy has cytotoxic effects not only on the malignant epithelial breast cancer cells but also cancer associated fibroblasts (CAF) and endothelial and immune cells such as tumour associated macrophages (TAMs) [153, 154]. It is currently under investigation to what extent the breast cancer microenvironment is involved in cancer regression and response to chemotherapy. El Hilali et al demonstrated that DNA damaging chemotherapeutic agents reduced overall tumour mass without reducing the total number of tumour cells [155]. Van der Kuip et al recognized the importance of investigating cells of the breast cancer microenvironment in terms of response to chemotherapy. This group developed a tissue culture method combined with a novel read out system for both tissue cultivation and rapid assessment of drug efficacy together with the simultaneous identification of different cell types within non-fixed breast cancer tissues. This method has potential significance for studying tumor responses to anticancer drugs in the complex environment of a primary cancer tissue [154]. In addition, Van der Kuip described an increased response to chemotherapy in tissue slice culture compared to isolated cell culture. These findings suggested a significant contribution of the tumour microenvironment to a cancer’s response to chemotherapy. Isolated primary tumour stromal cells from breast cancer
showed a high rate of resistance to paclitaxel when compared to breast cancer cell lines [156, 157].

In summary, response to chemotherapy in breast cancer is influenced by interactions between the epithelial cell compartment and stromal cell compartment in breast cancer microenvironment [156]

1.4 Circulating menstrual hormones and their influence on cytokines

The importance of inflammatory mediators such as CCL5 and growth factors such as TGFβ1 has been recognized in the context of breast cancer progression. Fluctuation of systemic cytokines in breast cancer progression has been previously described [125]. Variations of pro-inflammatory systemic cytokines with the menstrual cycle in premenopausal breast cancer patients could have implications in the context of timing of surgical intervention. It has been hypothesized that cyclical variations of cytokines could have an effect on the establishment of micro metastasis in breast cancer patients. A previous study reported cyclical variation of VEGF, and its importance in terms of choosing the optimal time point for surgery in the menstrual cycle of premenopausal breast cancer patients [111]. Previous studies support the hypothesis that the second half of the menstrual cycle is the most favorable time for surgery [158-160].

Previous studies have shown that Estradiol replacement therapy decreased serum inflammatory cytokines e.g. CCL5 and CCL2 [161]. This inverse relationship between circulating hormones and cytokines has been reported in the context of atherosclerosis [161]. It has been observed that postmenopausal women using estrogen replacement display reduced risk of cardiovascular disease [162]. Inflammatory mechanisms and mediators are now known to play a key role in progression of atherosclerosis [163, 164]. Estrogen has been found to down regulate mediators of the inflammatory response such as inflammatory cytokines [164, 165]. The inverse relationship between estrogen and inflammatory cytokines has been described by Christodoulakos et al. [161]
who investigated the effect of estrogen and the selective estrogen-receptor modulator raloxifen on circulating inflammatory mediators CCL5 and CCL2 in postmenopausal women [161]. The authors hypothesized that estrogen depletion in postmenopausal women is associated with stimulation of mediators of inflammation such as cytokines. This leads to an increase in atherosclerosis and cardiovascular disease in postmenopausal women [161]. The group showed that treatment with estradiol and raloxifene significantly decreased circulating CCL5. However, Joffroy et al reported an induction of TGFβ1 through antiestrogen treatment of in breast cancer cells in vitro [166]. The group further hypothesized that the TGFβ pathway plays a role in treatment resistance to antiestrogens through localized suppression of the immune response [166].
1.5 Hypothesis and aims

In this work, the cytokines CCL5 and TGFβ1 were selected to be investigated on a systemic as well as tissue and cellular level in breast cancer patients. These two factors have long been known to play a role in breast cancer invasion, metastasis and angiogenesis. In this work it was hypothesised, that a potential association between prominent tumour promoting growth factor TGFβ1 and inflammatory cytokine CCL5 exists and this association was investigated on a systemic level, tissue and stromal cell gene expression level. This work aimed to analyse CCL5 in the highest number of serum samples to date and in addition, to investigate tissue samples from a subset of the same breast cancer patients from whom serum samples were studied.

This body of work aimed to correlate systemic levels of these cytokines with clinicopathological characteristics. In this study it was also hypothesized that, in healthy volunteers, levels of circulating menstrual hormones potentially influence circulating cytokines. Potential fluctuations of systemic levels of inflammatory mediators with the menstrual cycle of premenopausal breast cancer patients has potential implications for timing of surgery as well as systemic treatment options such as hormone therapeutics and potential use of neoadjuvant anti-inflammatories.

This work aimed to investigate a potential association between CCL5 and TGFβ1 through gene expression analysis of whole breast tissue samples. It was hypothesized that a potential relationship between the two factors exists on a tissue gene expression level as well as systemic level. It was also hypothesized that gene expression of chemokines, proteases and growth factors differ in tumour and normal breast tissue and that a potential association between these factors exists.

Stromal fibroblasts are the predominant factor in the breast tumour microenvironment and their tumour promoting role is still being investigated. It has been described in the literature that normal stromal cells differ from stromal cell found in the tumour microenvironment. This work focused on gene expression levels of CCL5 and TGFβ1 in particular to investigate a potential association between these factors. This work aimed to characterise tumour and normal stromal cells through gene expression analysis of chemokines, growth
Chapter 1 Introduction

factors and proteases. It was hypothesized in this study that cytokine gene expressions differ significantly in tumour, TAN and normal stromal cells. Changes in gene expression profile of stromal fibroblasts are important as they might potentially impact on outcome and therapeutic treatment options of breast cancer patients.
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2. 1 Discipline of Surgery Biobank

Breast tissue samples used in this study were retrieved from the Biobank storage system of the Discipline of Surgery at National University of Ireland Galway which was established in 1992. Ethical permission for research related sample storage was granted by the Galway University Hospitals Clinical Research Ethics Committee. Following written informed consent from each patient, the research samples and related demographic, clinical and pathological details of study participants were collected and stored. The Galway University Hospital research programme was introduced to the prospective study participants by the clinician and the specialist breast care nurse. The patients read information provided on a consent form and signed and dated same (see Appendix).

Each patient sample was assigned an alphanumerical code and was stored according to the storage location assigned by the laboratory information management system (Shire). Clinicopathological parameters were also stored using Shire. The breast cancer study cohort was identified using Shire which displayed typical distributions of clinicopathological parameters such as epithelial subtypes, stage and grade.

2.2 Analysis of circulating cytokine levels and their relationship to circulating menstrual hormones

2.2.1 Blood collection

A cohort of patients (n=102) was identified of whom preoperative blood samples were available, which was representative of a typical symptomatic group of breast cancer patients. Blood samples from age matched healthy female volunteers (n=66) with no past or present history of malignant or inflammatory conditions were collected in an outpatient facility. In order to investigate the relationship between systemic CCL5, TGFβ1, VEGF and circulating menstrual hormones, serum samples were obtained from 15 healthy premenopausal volunteers every week for four consecutive weeks (60 samples in total). All blood samples were collected with informed consent in Vacutainer
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Serum Separator Tubes II (Becton Dickinson). Samples were allowed to clot for 30 min and centrifuged at 2,000 rpm at 4°C for 10 min in a Sorvall RT6000D centrifuge. The serum was then aliquoted and stored at -80°C until required.

2.2.2 Measurement of target protein

The detection of target protein was carried out using an enzyme immunoassay technique, the sandwich ELISA. In principle, the target protein is bound in between two antibodies (Figure 2.1), the capture antibody and the detection antibody. The two layers of antibodies and the target protein in between inspired the name “sandwich ELISA”. The target protein has to have at least two antigenic sites for the capture and detection antibody to be suitable for a sandwich ELISA. The capture and detection antibodies can be monoclonal or polyclonal. Monoclonal antibodies recognise a single epitope that allows fine detection and quantification of small differences in antigen. The microplate is precoated with a monoclonal capture antibody specific to the target protein. The immobilized antibody binds target protein contained in samples and standards that are added to the wells. Following washing steps to remove any unbound substances, a horseradish peroxidase enzyme (HRP) linked polyclonal detection antibody is then added which selectively binds to the target protein. The enzyme horseradish peroxidase (HRP), found in the roots of plant horseradish, is used to increase detectability of a target molecule. HRP oxidizes a substrate using hydrogen peroxide as the oxidizing agent which results in a characteristic change that is detectable by spectrophotometric methods. Another washing step ensures removal of unbound reagents. Substrate solution is then added and reacts with enzyme linked to the detection antibody. Colour develops in the process of the reaction in proportion to the target protein quantity. Colour development is subsequently stopped and colour intensity measured based on absorbance.
Method:
Quantification of concentrations of circulating levels of CCL5 and TGFβ1 was carried out on 168 serum samples (102 breast cancer, 66 healthy controls) using Quantikine® Enzyme Linked Immunosorbent Assay (ELISA) kits (R&D Systems). Circulating levels of CCL5, TGFβ1 and VEGF were determined in healthy volunteers (n=15 weekly for four weeks) and correlated to phases of the menstrual cycle and circulating menstrual hormones. These kits are designed to detect chemokine levels using a target specific antibody on pre-coated 96 well microplates. The assay targeting CCL5, VEGF and TGFβ1 was carried out following the manufacturer’s protocol as shown in Figure 2.2. Variations of the protocol for TGFβ1 appear in brackets.

Figure 2.1 Schematic of sandwich ELISA using HRP-linked detection antibody and CCL5, TGFβ1 and VEGF specific capture antibodies
Figure 2.2 Flowchart of ELISA protocol for measurement of CCL5, VEGF and TGFβ1.
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Minor variations of protocol required for TGFβ1 ELISA are shown in brackets.

The serum samples were removed from their designated storage location in the -80°C freezer and thawed on crushed ice. In the case of TGFβ1, activation of latent TGFβ1 to immunoreactive TGFβ1 was required prior to assaying. This involved addition of 20 µL of 1N HCL to 40µL of serum sample and incubation at room temperature for 10 min followed by neutralization with 1.2N NaOH/0.5 M HEPES. The activated samples were then diluted 20-fold using Calibrator Diluent RD5-53. 10 µl activated sample was added and 190 µl Calibrator Diluent RD5-53. TGFβ1 could then be detected in the serum samples. In order to detect CCL5, serum samples were diluted using Calibrator Diluent RD 6-11 which was provided in the kit and was diluted in different concentrations depending on sample type. For the purpose of analysing serum samples, 20 ml of deionised water was added to 20 ml of Calibrator Diluent RD 6-11 to achieve a 1:1 Dilution. Serum samples were then diluted adding 165µl Calibrator Diluent RD 6-11 to 55µl of serum sample. Serial dilution of recombinant standards of the target proteins was carried out to achieve seven different concentrations as shown in Figure 2.3.

![Figure 2.3 Serial dilution of recombinant protein standards for ELISA](image)

The absorbance readings of the dilutions were used to create a standard curve and calculate results. The standard was provided as a powder and reconstituted...
with 5 ml of Calibrator Diluent RD 6-11. The reconstituted standard represented 2000 pg/ml CCL5 (or VEGF, TGFβ1). Six tubes were filled with 500 µl of Calibrator Diluent RD 6-11 and labelled according to the concentration of standard. Thereafter, 500 µl of reconstituted standard was pipetted into tube labelled 1000pg/ml. This serial dilution was carried out as shown in Figure 2.3 until a concentration of 31.2 pg/mL standard was achieved. 100 µl (or 50 µl) of RD 1 W (or RD1-21) Assay Diluent was added to each well. 100 µl (or 50 µl) of standard/sample was added per well according to their position on the plate plan. Each sample/standard was analysed in duplicate. The 96 well microplate was incubated for 2 hours at room temperature in order to bind the target to the antibody. The content of each well is aspirated and wells washed three times (or four times) using wash buffer provided in the kit. 480 ml of distilled water was added to 20 ml of provided wash buffer to create this working solution. 200 µl (or 100 µl) of target protein conjugate was added to each well. The 96 well microplate was covered with new adhesive strip and incubated for one hour (or two hours) at room temperature. Each well was aspirated and washed three times (or four times). The Substrate Solution is prepared by mixing colour reagents A and B. The substrate solution should be made up no longer than 15 minutes in advance. 200 µl (or 100 µl) of Substrate Solution was added to each well. The microplate was covered in aluminium foil to protect it from light and incubated for 20 (or 30) minutes at room temperature. Colour developed in proportion to the amount of target protein bound initially. 50 µl (or 100 µl) of Stop Solution was added to each well to halt the colour development. The optical density of each well was determined within 30 min using a microplate (Multiskan RC) reader set to 450 nm wavelength ( wavelength correction at 570 nm) and the chemokine concentration determined from the standard curve.

Data obtained by ELISA was analysed using a statistical software package (Minitab Version 15 for Windows). The assumption of normality of all data was confirmed using the Kolmogorov-Smirnov test (a significance value of more than 0.05 indicates normal distribution). Once normality of the data was established, parametric tests were carried out. Independent two sample t-test, one way ANOVA and repeated measures ANOVA was carried out in order to
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compare mean values between two independent samples as appropriate. Continuous variables of interest are summarised numerically by Mean (SEM), and graphically using boxplots. The degree of relationship between pairs of response variables was assessed using the Pearson or Spearman correlation coefficient. Scatterplot smoothers were employed to indicate the likely relationship between variables in a population. All tests were two tailed and results with a p<0.05 were considered statistically significant.
2.2.3 Quantification of circulating levels of menstrual hormones

In order to investigate a potential relationship between circulating menstrual hormones and cytokines, blood was collected and serum extracted as described in section 2.2.1 from 15 healthy, premenopausal volunteers weekly for four weeks (total n=60). Luteinising Hormone (LH), Follicular Stimulating Hormone (FSH), Oestradiol and Progesterone were measured in serum samples (n=60) by direct chemiluminescence, using a Siemens ADVIA® Centaur™ Immunoassay System. The mid-cycle phase was determined by a peak of circulating levels of LH as shown in Figure 2.4.

Figure 2.4 The human menstrual cycle [167]
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The mid-luteal phase of the menstrual cycle was established by a peak in systemic Progesterone. Other phases of the menstrual cycle were extrapolated from these time points.

2.3 Cell culture

This study involved use of both commercially available epithelial breast cancer cell lines and primary cultures of fresh breast tissue specimens. Cells were isolated from breast tumour specimens and specimens harvested from tumour associated normal areas of the breast. Tumour associated normal refers to tissue harvested at least 2 cm distal from the outer margins of the tumour mass. Cells isolated from breast tissue harvested at reduction mammoplasty served as normal controls.

2.3.1 Cell lines

Details of cell lines used in the course of this work are shown in Table 2.1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MDA-MB-231</th>
<th>T47D</th>
<th>Sk-Br-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease</td>
<td>Adenocarcinoma</td>
<td>Ductal carcinoma</td>
<td>Ductal carcinoma</td>
</tr>
<tr>
<td>Origin</td>
<td>Pleural effusion</td>
<td>Pleural effusion</td>
<td>Pleural effusion</td>
</tr>
<tr>
<td>Receptor Status</td>
<td>ER-,PR-,Her2/neu-</td>
<td>ER+,PR+,Her2/neu-</td>
<td>Her2/neu+</td>
</tr>
<tr>
<td>Media Requirements</td>
<td>Leibowitz L15</td>
<td>RPMI 1640</td>
<td>McCoys 5A</td>
</tr>
<tr>
<td>Epithelial Subtype</td>
<td>Basal/Triple Negative</td>
<td>Luminal A</td>
<td>Her2</td>
</tr>
</tbody>
</table>

Table 2.1 Description of breast cancer cell lines employed in this study
Abbreviations: ER: oestrogen receptor, PR: progesterone receptor, Her2/neu: human epidermal growth factor receptor 2
Maintenance and culture of breast cancer cells was always carried out adhering to aseptic principles. The risk of contamination was reduced through the obligatory use of laboratory coats and gloves. The Discipline of Surgery cell culture laboratory is equipped with laminar air flow (LAF) hoods, a BHA48 Faster Biohazard Cabinet for breast cancer cell lines which ensures a sterile, pathogen free environment for in vitro work. Dedicated hoods for both primary and cell line culture were available and excluded any possible cross contamination. The air flow in the hoods was cleared for 15 mins before and after use, and the hoods were also disinfected using 70% industrial methylated spirits (IMS). The surface of all equipment introduced to the hood was wiped with 70% IMS to remove contaminants. While working in the hood care was taken to separate clean from soiled equipment and waste.

Breast cancer cell lines were maintained in a Steri-Cycle CO₂ HEPA Class 100 Incubator (Thermo Electron Corporation) at 37°C and 5% CO₂ with humidified air. Separate incubators were available for cell lines and primary cells to prevent cross contamination. The cells were regularly inspected for signs of contamination including turbidity and colour change of medium. Phenol red was a constituent of all media types used, maintaining red colour at neutral pH and changing to yellow colour as pH decreases. Microscopic examination using an Olympus CK2 microscope was carried out to assess cell adherence, confluence, morphology and the presence of bacterial/fungal contaminants.

The MDA-MB-231 cell line displays the triple negative epithelial subtype. The cells were cultured in Leibowitz L15 media. The T47D cell line is characterised by expressing Oestrogen and Progesterone receptors and was grown in RPMI 1640. The SK-BR-3 cell line is Progesterone and Oestrogen receptor negative and over expresses HER/neu, these cells were cultured in McCoy’s 5a medium. 10% Fetal Bovine Serum (FBS), penicillin G (2 units/ml) and streptomycin (100µg/ml) was added to all media. Media was changed 2-3 times weekly and cells passaged every 7 days.
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2.3.2 Passaging of cell populations

Cell lines were passaged at 80-90% confluence in order to maintain the cells in the log growth phase. The flasks were sprayed and wiped with 70% IMS before introducing them into the cell line hood where the spent media was decanted aseptically. The cell monolayer was rinsed in phosphate buffered saline (PBS). This step removes traces of media and FBS that could impair the function of the trypsin, a cell dissociation reagent. Trypsin was warmed in a 37°C water bath prior to usage, which is the optimal temperature for the enzyme to exert its function. Then 5ml of the warmed trypsin was added to each flask and by gently tilting the flask from side to side the cell layer was immersed in the enzyme. After one minute, excess trypsin was decanted aseptically into the waste bottle. The flasks were then incubated for 4-5 min at 37°C to allow the enzyme to stay at its optimal reaction temperature. The detachment of cells from the plastic substrate was confirmed using the microscope and if necessary cell detachment was aided mechanically by gently tapping each flask. In case not all cells lifted easily, the flasks were returned again to the incubator and inspected at intervals. However, cells were not longer than 10 min in contact with trypsin to prevent cell death. Once adherent cells were detached, trypsin was inactivated by adding serum-containing media to each flask. The cell suspension was then transferred to a 15ml falcon tube and centrifuged at 1000 rpm for 4 min. The supernatant was removed and the cell pellet resuspended in 1-2 ml of appropriate media. This step removed any trace of trypsin and a homogenous cell suspension was achieved by gently pipetting the cells up and down.

2.3.3 Cell count

Following passaging and trypsinising of cell populations as described in 2.3.2, cell number and viability were determined using the NucleoCounter® (ChemoMetec). This automated device consists of an integrated fluorescence microscope and detects signals from the fluorescent dye, propidium iodide (PI), a DNA complexing agent which is immobilized within a cell collection cassette. Propidium iodide can be taken up into the nucleus of non-viable cells through the permeable plasma membranes as shown in Figure 2.5.
Figure 2.5 Operating principle of nucleocounter

PI cannot be taken up through the intact plasma membranes of viable cells and only complexes with DNA in non-viable cells. Therefore, lysis reagents are added to the cell suspensions to permeabilize the cell membranes and determine total cell count.

**Method:**

As shown in Figure 2.6, the total cell count (viable and non-viable cells) was determined by adding 100µl of lysis buffer Reagent A to 100µl of cell suspension in a 1.5 ml eppendorf. The tube was vortexed, 100 µl of Reagent B (stabilising buffer) added and vortexed again. The cell lysate was then loaded into a disposable NucleoCassette and the immobilised PI in the interior of the NucleoCassette stained the cellular DNA. The NucleoCassette was then placed in the automated NucleoCounter and 2 µl of the stained mixture was
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automatically transferred to the NucleoCassette measurement chamber. Green light excites the PI-DNA intercalation and the red light emitted is registered in the CCD camera for correlation into a cell count. In order to ascertain the viable cell count, total cell count and non-viable cell count was measured. The non-viable cell count was then subtracted from the total cell count as in the formula below:

\[ \text{Viable Count} = \text{Total Count} - \text{Non-Viable Count} \]
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Cell counting protocol

Total Cell Count

50µl sample of cell suspension

↓

Vortex

Add 50µl sample of Lysis Buffer

↓

Vortex

Add 50µl sample of Stabilising Buffer

↓

Vortex

Load into NucleoCassette

Non-Viable Cell Count

100 µl sample of cell suspension

↓

Vortex

Read on NucleoCounter

Figure 2.6 Flow chart of cell counting protocol
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The percentage of viability was also determined

\[ \% \text{ Viability} = \frac{(CtMt-CnvMnv)}{CtMt} \times 100 \]

\( Ct \) = total cell concentration in the original cell suspension (displayed result)
\( Mt \) = Multiplication factor used for total cell count (as the stabilised lysate is prepared using equal volumes of cell suspension, lysis buffer and stabilizing buffer, the multiplication factor is 3)
\( CnV \) = Concentration of non-viable cells in the NucleoCassette (displayed result) \( MnV \) = Multiplication factor used for the counting of non-viable cells (counting was done without dilution of the cell suspension prior to the analysis, thus the multiplication factor was 1).

\[ \text{Total Seeding Volume Required} = \frac{\text{Cells Needed Per Flask}}{\text{Viable Count}} \]

Limits for accuracy of the NucleoCounter are 3 x 10^5 cells/ml (minimum) and 2 x 10^6 cells/ml (maximum)

2.3.4 Cryopreservation and recovery of epithelial breast cancer cells

Stocks of breast cancer cell lines were maintained by freezing excess cells. Cells were trypsinised into a single cell suspension and counted as described in 2.3.2. Cells were frozen in 2ml cryovials (Nunc) maintained on ice during the freezing process. Each cryovial contained 1x10^6 cells in 1.8 ml of complete medium as well as 5% of sterile filtered Dimethylated Sulfoxide (DMSO, Sigma-Aldrich) and were inverted to mix the contents completely. Once full, the cryovials were immediately transferred to a box containing an isopropanol bath, also known as “Mr. Frosty” (Nalgene labware, Thermo Fischer scientific). The purpose of the “Mr Frosty” is to allow cells to gradually freeze at a rate of -1°C per minute. Mr Frosty was immediately transferred to the -80°C freezer for a minimum of 3 hours and then the cryovials were transferred to liquid nitrogen for long term storage.

The cryovials were retrieved from their storage location using personal protective equipment and swirled in a 37°C water bath in order to quickly thaw
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the cells. The cells need to be thawed and transferred to media quickly to prevent cell death through cytotoxicity of the added DMSO which is toxic to cells at 37°C. The vials were thawed until only a small portion of ice was left and the cells were transferred to the labelled media containing flasks. The cell culture media was changed after 24 hours of incubation to remove any trace of cyto-destructive DMSO. Cell morphology and adherence was assessed using an Olympus CK2 microscope.

2.3.5 Fresh breast tumour specimen collection

Following written informed consent, fresh specimens of human breast cancer were harvested from patients undergoing surgery. Tissue was also harvested from tumour associated normal regions of the breast which refers to tissue at least 2 cm distal to the outer margin of the tumour mass. Tissue harvested from patients undergoing reduction mammoplasty served as normal controls. Following harvest, all breast tissue samples were placed into a tube containing complete stromal medium. Stromal medium consists of Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 1% P/S and 10% heat inactivated FBS. For heat inactivation, tubes containing FBS were placed in a water bath at 55°C for 30 min.

2.3.6 Primary cell culture protocol

Freshly harvested tissues were washed twice in phosphate buffered saline (PBS), supplemented with double strength P/S. Subsequently they were minced finely using crossed scalpels. Tissues were digested with 0.1% collagenase type III at 37°C for 12-18 hrs in stromal medium. Cell fractions were separated using differential centrifugation as shown in Figure 2.7. The digested tissue was centrifuged at 400 RPM for 1 min. The pellet thus formed contained mixed cells and small fragments of partially digested tissue, termed the organoid fraction. The resulting supernatant was centrifuged at 700 RPM for 2 min. The pellet thus formed contained the epithelial fraction. The resulting supernatant was centrifuged at 1000 RPM for 4 min. The pellet thus formed contained the stromal/fibroblast fraction. All three pellets (organoid, epithelial and fibroblast fractions) were resuspended in 2ml stromal medium. The cells were maintained
at 37°C, 5% CO₂. The organoid and epithelial fractions were cultured in a 3:1 organoid medium: stromal medium solution for the first 24 hours, after which they will be transferred to complete organoid medium. Organoid medium consists of DMEM supplemented with Hydrocortisone 0.5µg/ml, 1% P/S, Bovine Serum Albumin 0.75g/ml, Epidermal Growth Factor 5ng/ml, and Cholera Toxin 5µg/ml. The stromal fraction was cultured in stromal medium which selects for fibroblast growth.
Figure 2.7 Schematic of subculture of primary breast cells (adapted from Valerie Speirs)[168]
2.4 In vitro ApoGlow cell proliferation assay

The ApoGlow® Assay Kit is manufactured by Lonza Rockland Incorporated. The principle of this assay is based on bioluminescent measurement of ATP that is present in all metabolically active cells. This measurement can be used to calculate the ADP:ATP ratio and distinguish between proliferation, apoptosis and necrosis [169]. The bioluminescent method uses luciferase, which generates light from ATP and luciferin according to the following reaction

\[
\text{Luciferase} \quad \text{ATP} + \text{Luciferin} + O_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO}_2
\]

\[
\text{Mg}^{++} \text{ LIGHT}
\]

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. The optimal reaction temperature for luciferase is at ambient temperature (18°C-22°C) and therefore the assay is carried out at room temperature. Measurement of ATP utilising bioluminescence has the advantage of being very sensitive and convenient to use. ADP is measured by its conversion to ATP, which is subsequently detected using luciferase.

The ADP: ATP ratio was calculated in order to distinguish between proliferation, apoptosis and necrosis in the breast cancer epithelial cells in the following way:

**Apoptosis:** lower level of ATP and increase in ADP, thus an increase in ADP: ATP ratios over controls

**Necrosis:** lower levels of ATP but greatly increased ADP, thus a marked increase in ADP: ATP ratios over controls

**Arrested Proliferation:** lower levels of ATP than control with little or no change in ADP: ATP ratio,

**Proliferation:** markedly elevated ATP values compared to controls with no significant increase in ADP levels
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No Effect: similar levels of ATP with little or no change in ADP

Light is emitted from ATP and luciferin, a reaction catalysed by the enzyme luciferase. The emitted light is measured using a luminometer and corresponds to the amount of ATP present. ADP is measured by its conversion to ATP which is subsequently detected, and the ADP: ATP ratio can be calculated.

Method:
Breast cancer cells (MDA-MB-231, Sk-Br-3) were seeded into white walled 96 well plates (white wall plates are designed to reduce well-to-well crosstalk during luminescent assay) at a density of $8 \times 10^3$ cells per well. Cells were incubated for 48 – 72 hours with various concentrations of CCL5 (25-100 ng/ml) and TGFβ1 (2 ng/ml). Cellular ATP was quantified using the ApoGlow Adenylate Nucleotide Ratio Assay following the assay protocol depicted in Figure 2.8.
ApoGlow® Assay Procedure

1. Set up cell culture and incubate for required time
2. Add 100 µl NRR to cells. Wait for 5 minutes
3. Add 20 µl NMR. Measure luminescence (Reading A).
4. Wait 10 min
5. Add ADP-CR
6. Measure luminescence (Reading B)
7. Wait for 5 minutes
8. Measure luminescence (Reading C)

Figure 2.8 Flowchart of ApoGlow® assay protocol
Throughout the incubation period, media was changed at 24 hour intervals. The media was removed and 100µl of nucleotide releasing reagent per well was added and incubated for at least five minutes at room temperature. The 96 well microtitre plate was placed in a Luminoskan Ascent Luminometer (Thermo Fischer Scientific). Bioluminescence was measured (Reading A) immediately after the automated addition of 20µl of nucleotide monitoring reagent per well. After 10 minutes, 20µl of ADP converting reagent was added per well and luminescence was recorded by an immediate 1 second integrated reading (Reading B). Following a 5 minute delay, a final 1 second integrated reading (Reading C) was obtained and the ADP: ATP ratios were then calculated from these readings as follows:

Reading A: ATP
Reading B: luminescence baseline for ADP
Reading C: follows conversion of all ADP into ATP
Reading C – Reading B: ADP

ADP: ATP ratio: (Reading C – Reading B)/Reading A
2.5 *In vitro cell migration*

**Background:**
Transwell Permeable Supports (Corning) were used to track migration of tumour epithelial cell lines from the upper chamber across a tissue culture treated polycarbonate porous membrane, to the lower chamber as depicted in Figure 2.9.

![Figure 2.9 Schematic of migration assay setup](image)

**Method:**
Migration of the commercially available breast cancer cell line MDA-MB-231 was assessed in response to CCL5 and TGFβ1 alone and in combination using Transwell™ Permeable Supports (Corning, Sigma Aldrich). The breast cancer cell lines are able to migrate from the upper chamber (6.5 mm diameter inserts) across a tissue culture treated polycarbonate porous membrane (8µm pore size) towards the lower chamber. The epithelial breast cancer cell lines are known to migrate towards FBS enriched media as well as towards chemokines.
The porous membranes were hydrated using basal media for approximately 1 hour prior to usage to improve cell attachment. The experiments were setup using a 24 well format. Each well contained 600µl medium (with and without chemoattractants) and each insert 100 µl of cell suspension (7.5x10⁴/ml). The wells contained different conditions in duplicate: FBS enriched medium as a positive control, medium containing bovine serum albumin as negative control, TGFβ1 (concentration 2ng/ml), CCL5 (25ng/ml), CCL5 (50ng/ml), TGFβ1 (2ng/ml) in combination with CCL5 (25ng/ml) and TGFβ1 in combination with CCL5 (50ng/ml).

The MDA-MB-231 cell suspension was prepared in nutrient free basal medium at a concentration of 7.5x10⁵ cells/ml, so that each insert with 100µl of cell suspension contained 7.5 x 10⁴ cells.

The insert was lifted and the rehydration medium removed and then the insert was placed in well containing chemoattractant. Immediately, 100µl of cell suspension was added into the insert. All conditions were carried out in duplicate. The basal medium below the insert served as a negative/baseline control. Serum rich medium served as a positive control. Since CCL5 and TGFβ1 were diluted in bovine serum albumin (BSA), it was necessary to introduce this as a control in case the diluent itself induced cell migration. The plate containing the inserts was returned to the incubator and migration allowed to proceed for 18hrs.

The membranes were then harvested to count number of migrated cells. A cotton swab was used to scrub the top of the membrane in order to remove any cells remaining on top of the membrane. Scrubbing was repeated a second time after a fresh swab was wetted in medium. The scrubbed inserts were transferred to a 24 well plate containing ice cold Methanol for 15mins at RT to fix cells. The inserts were then transferred to a 24-well plate containing Haematoxylin, for 3mins at RT. The membranes were rinsed twice in two separate beakers of dH₂O (serial washes) to remove all traces of unbound Haematoxylin. Subsequently, the inserts were inverted on tissue and left to dry. The membrane was then excised using a scalpel, and mounted on a slide on top of a drop of immersion oil. A further drop of immersion was placed on top of the membrane followed by a coverslip. The membrane was excised by holding a scalpel and rotating the membrane against it, in order to prevent membrane
Chapter 2 Materials and Methods

gathering/folding. A small amount of membrane was left attached and then removed with forceps. Migrated cells were then counted in 5 fields of view per membrane. Consistent fields of view on each membrane were used as shown in Figure 2.10 to get an accurate representation of migration.

*Figure 2.10 Schematic depicting five fields of view*
2.6 Gene expression analysis

2.6.1 RNA extraction

All RNA extraction procedures were carried out in an ESCO Biosafety cabinet. RNA can be destroyed by ribonucleases (RNases) which are stable and active enzymes. Even very small amounts of RNases can destroy RNA and care has to be taken to avoid any contamination. This is achieved by using dedicated hoods, wearing a laboratory coat and handling samples using gloves. Aseptic technique reduces the risk of RNase contamination from common sources such as the surface of the skin or dust particles. Disposable plastic ware is recommended as they are generally RNase free without the need for pretreatment. Barrier pipette tips should be used when pipetting RNA samples as this reduces risk of contamination.

Total RNA extraction was carried out on frozen normal and tumour breast tissue (approximately 100mg) which was retrieved from the Discipline of Surgery Biobank. The tissue was homogenised using a bench-top homogenizer (Kinematica AG) in 1 mL of QIAzol (Qiagen).

For extraction of RNA from cell culture populations, cells were trypsinised as described in section 2.3.2. Cell suspensions were centrifuged using an Eppendorf Centrifuge 5810 (1000 rpm for 4 mins). Following removal of the supernatant, the cell pellets were stored at -80°C in the freezer. The frozen pellets were thawed initially on ice, and 1 ml of QIAzol (Qiagen) added to induce cell lysis. Following continuous pipetting, a homogenous suspension was achieved.

Method:
The homogenates were transferred to a 1.5ml collection tube (Eppendorf), 200 µl of chloroform added and the mixture vortexed for 15 seconds. The suspensions were left for 10 mins at room temperature and centrifuged at 4°C, 14000 rpm for 15 min in an Eppendorf Centrifuge 5417R. This resulted in an upper, aqueous (clear) phase which contains RNA and was transferred to a 15ml labelled falcon tube. 3.5 x volume Ethanol (100%) was then added as
shown in Figure 2.11. The mixture was vortexed briefly and pipetted onto Qiagen® RNeasy MiniSpin Columns in aliquots of 700µl. The spin columns were centrifuged at 4ºC; 14000 rpm for 20 seconds. This step was repeated until the whole sample passed through the spin column, leaving the RNA bound to the filter portion of the spin column as shown in Figure 2.11. The following washing step with 350 µl of buffer RW1 was carried out to remove contaminants from the RNA. The RW1 buffer was pipetted onto the spin column which was centrifuged at 4ºC; 14000 rpm for 20 seconds and the eluent is discarded. 80 µl of DNase/Buffer RDD mix is then pipetted onto the membrane and incubated at room temperature for 15 min. The mix contained 70 µl Buffer RDD and 10 µl DNase. This step was employed to degrade any contaminating genomic DNA. For the second time, an aliquot of 350µl of RW1 buffer was added and the spin column centrifuged at 4ºC; 14000 rpm for 20 seconds. Subsequently, the spin columns were washed with 500 µl of RPE buffer and centrifuged for 20 seconds. This step was repeated and the second time the spin columns were centrifuged for 2 min to dry the membrane. Every trace of ethanol was removed to ensure complete elution of RNA in the next step. The spin columns that contained the membrane on which RNA was bound were placed in a new collection tube and 30 µl of chilled nuclease free water was added directly onto the membranes. The columns were centrifuged at 4ºC; 14000 rpm for 1 minute and the RNA was eluted with the nuclease free water into the collection tube. The RNA extract was transferred to a pre-labelled Sarstedt storage tube and an aliquot of 1.3 µl removed for Nanodrop analysis as described in section 2. The storage tube was placed at an assigned location in the laboratory freezer at -80ºC.
Figure 2.11 Schematic of RNA extraction protocol
2.6.2 RNA analysis (nanodrop)

Analysis of RNA integrity and purity was carried out using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). Prior to RNA purity analysis, the sample pedestal was wiped clean. The instrument was calibrated with nuclease free water and samples measured as ‘Nucleic Acid’ and sample type ‘RNA-40’. A 1.1 µl aliquot of RNA was pipetted onto the apparatus sample pedestal and the sample arm compressed the aliquot in order to form a sample column by capillary action and enable absorbance readings. Spectral measurements were made with a tightly controlled pathlength of 0.1 cm.

RNA concentration was automatically calculated using the formula:

\[
\text{RNA concentration (ng/µl) = (A260 x e)/b}
\]

A\text{260} = absorbance at 260 nm  
\(e\) = extinction coefficient (ng-cm/ml)  
\(b\) = pathlength (cm)

Purity of RNA samples was inferred by absorbance readings at 260/280 nm. A 260/A280 absorbance ratio between 1.8 and 2.2 was deemed acceptable. A 260/A280 ratio of 2 indicates 100% RNA purity.

2.6.3 Reverse transcription

Messenger RNA (mRNA) was reverse transcribed into complementary DNA strands using the reverse transcriptase enzyme SuperScript™ III reverse transcriptase (Invitrogen) in a Captair Biosafety Cabinet. This involved conversion of single stranded RNA into single stranded cDNA. This conversion was necessary for amplification using polymerase chain reaction
Chapter 2 Materials and Methods

(PCR). Each sample contained a total of 1 µg RNA in a volume of 11.67 µl. The samples were brought to 11.67 µl using nuclease free water.

Volumes equivalent to 1µg of RNA were calculated based on nanodrop values (RNA quantity) with the following formula:

\[
\text{Volume required for 1 µg RNA} = \frac{1}{\text{RNA Concentration (ng/µl)}} \times 1000
\]

The non-template controls (NTC) consisted of 11.67 µl of nuclease free water. The purpose of the NTC was to control for contamination. The NTC contains all reagents except the sample and no amplification should be detected. Each sample was combined with 1.33 µl of Master Mix I (MMI), consisting of 0.33µl random primers and 1 µl of dNTP mix (10 mM). The samples were transferred to the Gene Amp PCR 9700 system and the denaturation program selected (4 min at 65°). A separate cDNA synthesis mix (Master Mix II) was prepared containing 4µl of 5x RT buffer, 1 µl of DTT (0.1M), 1µl of RNaseOUT™ (40U/µl, Invitrogen) and 1µl of SuperScript™ III RT (200U/µl, Invitrogen) for each cDNA reaction. 7µl of MMII was added to the samples giving a final volume of 20 µl. The mixture was incubated at 25°C for 5 minutes, 50°C for 60 minutes and finally 70°C for 15 minutes to denature the RT enzyme.

2.6.4 Real time quantitative PCR

PCR is a method to detect a target sequence through amplification of starting template DNA or cDNA. The PCR method employs thermal cycling, which are cycles of repeated heating and cooling. The thermal cycling steps separate the DNA double helix into two DNA strands at a high temperature, this procedure is called DNA melting. Each single strand DNA serves as a template in DNA synthesis which is catalysed by the DNA polymerase at low temperature. The target DNA sequence is selectively amplified using primers (short DNA fragments) which are complementary to the target region and DNA polymerase, an enzyme that catalyses DNA replication. The heat-stable DNA
polymerase used is called Taq polymerase and is an enzyme originally found in the bacterium Thermus aquaticus. This Taq polymerase uses single stranded DNA as a template and synthesises a new DNA strand from nucleotides (DNA building blocks). The result is a DNA polymerase driven chain reaction, each synthesised DNA template serves as a template itself in an exponentially increasing amplification procedure. 

During the exponential phase of the reaction the amplified sequences double during each cycle of denaturation, primer annealing and template extension. As reagents are being used up, the reaction slows down and enters the plateau phase. Quantification of PCR products is not possible using endpoint analysis as the plateau phase of the reactions is already reached at this stage as shown in Figure 2.12.

Figure 2.12 Reaction kinetics in PCR ("Critical Factors for Successful Real-Time PCR", Qiagen handbook)
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The plateau phase of the reaction process is a result of depletion of PCR components and the high concentration of amplification products which hinder primer annealing. The same amount of starting template can result in different yields using endpoint PCR analysis. The limitations of the technique, however, were overcome with real-time PCR. This method differs from endpoint PCR because it measures the amount of amplification product present at each cycle. The quantification of PCR product is carried out using fluorescently labelled DNA based probes as shown in Figure 2.13.

*Figure 2.13 Schematic of function of fluorescently labelled DNA probes in RT-PCR*

These probes carry a fluorescent reporter at one end and opposite from it a quencher of fluorescence, which inhibits detection of fluorescence initially as shown in Figure 2.13. At the start of PCR, the probe and the primer both anneal to a DNA target and polymerisation of a new DNA strand is initiated as depicted in Figure 2.13. Once the polymerase reaches the probe, its 5’-3’-
Chapter 2 Materials and Methods

exonuclease degrades the probe. As a result, the fluorescent reporter is separated from the quencher, resulting in an increase in fluorescence. The intensity of fluorescence is proportional to the amount of amplification product present if the reaction is in the exponential phase as shown in Figure 2.13 and if all reactions proceed with similar efficiency.

**Method:**

RQ-PCR reactions were carried out in final volumes of 10µl using an ABI Prism-7900 Fast Sequence Detection System. Reactions consisted of the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Fast Master Mix (2X)</td>
<td>5µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>3.5µl</td>
</tr>
<tr>
<td>cDNA</td>
<td>1µl</td>
</tr>
<tr>
<td>Pre-developed Assay Reagent (PDAR)</td>
<td>0.5µl</td>
</tr>
</tbody>
</table>

Standard thermal cycling conditions were applied consisting of 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds.

Quantitative real-time PCR gene expression results were normalised using endogenous control genes. These reference genes should be expressed at a constant level across all sample groups in a study, and should not be influenced by study treatments or conditions.

Endogenous reference genes correct for variations in reverse transcription efficiency and amount of input cDNA. MRPL19 and PPIA have been recommended as endogenous control genes for RQ-PCR expression and validated as suitable for analysis of breast tissue within the Discipline of Surgery [170]. The gene targets are shown in Table 2.2.
<table>
<thead>
<tr>
<th>Designation</th>
<th>Synonym</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL5</td>
<td>Regulated upon activation normal T-cell expressed and secreted (RANTES)</td>
<td>Immunomodulatory function, metastasis, invasiveness</td>
</tr>
<tr>
<td>CCR5</td>
<td>CCL5 Receptor</td>
<td>Immunomodulatory function, metastasis, invasiveness</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor beta one</td>
<td>Angiogenesis, vasculogenesis, EMT induction</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>Transforming growth factor beta receptor two</td>
<td>Angiogenesis, vasculogenesis, EMT induction</td>
</tr>
<tr>
<td>MMP3</td>
<td>Matrix Metalloproteinase 3</td>
<td>Invasiveness</td>
</tr>
<tr>
<td>FAP</td>
<td>Fibroblast activation protein</td>
<td>Fibroblast growth, modulation of stromal-epithelial interactions</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl Isomerase A (cyclophilin A)</td>
<td>Endogenous control</td>
</tr>
<tr>
<td>MRPL19</td>
<td>Mitochondrial Ribosomal Protein L19</td>
<td>Endogenous control</td>
</tr>
</tbody>
</table>

*Table 2.2 Gene targets analysed in breast tissue, epithelial breast cancer cell lines and primary stromal cells*

### 2.6.5 Amplification efficiencies

For PCR results to be valid and comparable, it is essential that PCR reactions are equally efficient as explained in section 2.7.4 Therefore, primer efficiency was determined prior to sample analysis. Serially diluted cDNA samples were prepared to create a standard curve. The cDNA was diluted using nuclease free
Chapter 2 Materials and Methods

water and the following concentrations prepared: cDNA neat, 1:10, 1:100, 1:1000, 1:10000. PCR was carried out as described in section 2.7.4 with the exception that PCR reactions were run as Absolute Quantification (Standard Curve) Assays. Standard deviation between replicate samples of 0.3 or less was deemed acceptable.
Primer efficiency in a PCR reaction should approach 100 ± 10% and was calculated using the formula below

\[
\text{Efficiency} = \left(10^{\frac{1}{\text{Slope}}}-1\right) \times 100
\]

(Slope refers to the dilution curve)

2.7 Statistical analysis of data

2.7.1 Analysis of systemic cytokine and hormone levels

All data was tested for normal distribution using the Kolmogorov-Smirnov test and normality was confirmed by a p-value greater than 0.5. Differences between groups were analyzed using Analysis of Variance (ANOVA) test. Any statistically significant difference between two groups was confirmed using the two sample t-test. Correlation between two variables was determined using Pearson correlation method following confirmation of normality.

Power calculations to determine the minimal sample size for each experiment were not carried out. There was a limited amount of primary stromal cell populations available, that could be cultured in the time frame of this thesis and all available populations were analyzed. It lies in the nature of the experiments carried out that sample sizes were not primarily determined by a statistical test with statistical accuracy in mind. The factors that determined the sample sizes of the cell work were time, access to whole tissue specimen and success in primary cell culture.
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2.7.2 Analysis of RQ-PCR results

Gene expression levels were calculated in this study using the relative quantification method. The ratio was determined between the amount of target gene in the samples and the amount of endogenous control gene, a housekeeping gene present in all samples. This ratio is then used to compare gene expression levels in different samples. The endogenous control gene was expressed at similar levels in all samples.

All samples and non-template controls were analysed in triplicate and inter-assay controls included to correct for variations when linking several 96-well plates. Standard deviations between triplicates were required to be <0.3. The following formula was applied to generate relative quantities [171]:

\[
\Delta Ct = \text{Average Ct Target gene} - \text{Average Ct Endogenous Reference Gene} \\
\text{(Average of PPIA and MRPL19)}
\]

\[
\Delta\Delta Ct = \Delta Ct \text{ Target} - \Delta Ct \text{ Baseline Target (e.g. untreated control)}
\]

Relative Quantity = \(2^{-\Delta\Delta Ct}\)

RQ-PCR data were log transformed and analysed using a statistical software package (Minitab Version 15 for Windows). The assumption of normality of all data was confirmed using the Kolmogorov-Smirnov test (a significance value greater than 0.05 indicates normal distribution). The normal distribution of RQ-PCR data was illustrated using scatterplots. Once normality of the data was established, parametric tests were carried out. Independent two sample t-test, one way ANOVA and repeated measures ANOVA was carried out in order to compare mean values between two independent samples as appropriate. Continuous variables of interest were summarised numerically by Mean (SEM), and graphically using boxplots and scatterplots. The degree of relationship between pairs of response variables was assessed using the Pearson or Spearman correlation coefficient as appropriate and paired t-tests were used analysing related samples. Scatterplot smoothers were employed to
indicate the likely relationship between variables in a population. All tests were two tailed and results with a p<0.05 were considered statistically significant.
Chapter 3 Systemic CCL5 and TGFβ1 levels in breast cancer patients
3.1 Introduction

The importance of inflammatory mediators such as CCL5 and growth factors such as TGFβ1 is well recognized in the context of breast cancer progression. A recent study described fluctuations of circulating chemokines such as CCL2, CXCL12 and CXCL8 in breast cancer patients and found a significant correlation with disease progression and prognosis [125].

The inflammatory chemokine CCL5 is also known as Regulated upon activation T-cell expressed and secreted (RANTES) and plays an important part in inflammation through activation and chemotaxis of immune cells [135]. Previous studies have investigated systemic CCL5 in breast cancer to evaluate its role in breast cancer progression and as a potential biomarker of the disease [137, 138]. Niwa et al. found elevated plasma levels of the chemokine and reported a correlation with disease stage [137]. Another group also observed significantly elevated serum CCL5 in breast cancer patients compared to healthy controls, although no significant change was detected between patients with metastatic and non-metastatic disease [138]. Previous studies could not conclusively prove an association between circulating CCL5 levels and prognosis in breast cancer patients and it was the aim of the current study to correlate systemic CCL5 levels in breast cancer patients with clinicopathological characteristics.

The cytokine TGFβ1 is a member of the TGFβ superfamily which is involved in regulating pathways important to tumour initiation, proliferation and metastasis [86, 88]. TGFβ in normal epithelial cells induces arrest of the cell cycle and this function has been implicated in cancer suppression in the early stages of oncogenesis [86, 89]. In later stages of carcinogenesis TGFβ acts as a tumour promoter [90]. The effects of TGFβ are mediated by three ligands, TGFβ1, TGFβ2 and TGFβ3, through TGFβ type 1 and TGFβ type 2 receptors [172, 173]. In previous studies it was reported that TGFβ1 is the predominant isoform found in blood of breast cancer patients [90, 174]. Systemic levels of TGFβ1 in cancer patients and a potential relationship with prognosis was investigated by a number of groups [93, 96, 175]. It was reported that elevated
systemic levels of TGFβ1 drop in breast cancer patients following removal of the tumour [95]. It has also been found that circulating TGFβ1 levels prior to cancer treatment correlate with overall survival [95, 96]. Further investigation is needed to identify sensitive and specific biomarkers for breast cancer that are also minimally invasive. In this context, the potential of systemic chemokines and growth factors as markers of breast cancer progression warrants further research.

3.2 Aims

1. Measurement of circulating levels of CCL5 in breast cancer patients compared to age-matched healthy controls and investigation of a potential relationship with clinicopathological details.

2. Measurement of circulating levels of TGFβ1 in breast cancer patients compared to age-matched healthy controls and investigation of a potential relationship with clinicopathological details.

3. Investigate potential relationship between circulating CCL5 and TGFβ1

3.3 Materials and Methods

Preoperative blood samples were collected with informed consent from 102 breast cancer patients and 66 controls. Blood samples from age matched healthy female volunteers (n=66) with no past or present history of malignant or inflammatory conditions were collected in an outpatient facility. Serum samples were isolated from whole blood as described in section 2.2.1. Systemic levels of CCL5 and TGFβ1 were measured in serum samples from breast cancer patients using Quantikine® Enzyme Linked Immunosorbent Assay (ELISA) as described in section 2.2.2.

3.4 Results

3.4.1 Details of study group

CCL5 and TGFβ1 levels were measured in serum samples obtained from 102 breast cancer patients and 66 healthy controls. Clinicopathological details of
this group are shown in Table 3.1. In this breast cancer cohort, 40 patients were premenopausal (range 31–49 years, mean age 43 years) and 62 postmenopausal (range 49–79 years, mean age 61 years). The breast cancer and control groups contained a similar proportion of pre- and postmenopausal women. Postmenopausal status was defined as amenorrhea for more than 12 months. Systemic levels of CCL5 and TGFβ1 were also measured in 66 age-matched controls. In the control group, 26 participants were premenopausal (range 21–49, mean age 40 years) and 40 postmenopausal (range 49–81 years, mean age 62 years). At the time of recruitment, 52 patients had axillary node negative disease. The study cohort displayed characteristics of a typical breast cancer cohort as shown by the distribution of epithelial subtypes and histological types (Table 3.1). Luminal A was the predominant tumour epithelial subtype found in the study cohort and the majority of tumours displayed ductal histology (Table 3.1).
<table>
<thead>
<tr>
<th>Tumour Characteristics</th>
<th>Breast cancer</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Patients</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>66</td>
</tr>
<tr>
<td>premenopausal</td>
<td>40 (39.2)</td>
<td>26 (39.4)</td>
</tr>
<tr>
<td>postmenopausal</td>
<td>62 (60.8)</td>
<td>40 (60.6)</td>
</tr>
<tr>
<td>Tumour Characteristics</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>66 (64.7)</td>
<td></td>
</tr>
<tr>
<td>Lobular</td>
<td>15 (14.7)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>10 (9.8)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>11 (10.8)</td>
<td></td>
</tr>
<tr>
<td>Epithelial subtype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>68 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>9 (8.8)</td>
<td></td>
</tr>
<tr>
<td>Her-2/neu</td>
<td>6 (5.9)</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>8 (7.8)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>11 (10.8)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10 (9.8)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>36 (35.3)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37 (36.3)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>19 (18.6)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 Characteristics of study cohort of breast cancer patients (n=102) and normal controls (n=66) from whom circulating serum levels of CCL5 and TGFβ1 were measured using ELISA.
Chapter 3 Systemic CCL5 and TGFβ1 levels in breast cancer patients

3.4.2 Circulating CCL5 levels in breast cancer patients and healthy controls

Systemic CCL5 serum levels were measured in samples from 102 breast cancer patients (preoperative) and 66 controls with an equivalent proportion of pre- and post-menopausal subjects in each group. The data are presented as boxplots, which depict groups of data graphically through a box shaped graph with whiskers. The bottom and top of the box represent the 25th and 75th percentile (lower and upper quartiles, respectively), and the band near the middle of the box is always the 50th percentile (median). The whiskers represent the minimum and the maximum of all the numerical data. Outliers in the data are not included in the box, but represented by an asterisk (*).

There was no significant difference detected in serum CCL5 levels of breast cancer patients (Mean (SEM) 46.1 ng/ml (4.0) ng/ml) compared to healthy controls (45.4 (3.4) ng/ml, Figure 3.1). The breast cancer cohort was further subdivided based on the epithelial subtype of the primary tumour as depicted in Figure 3.1. While the lowest systemic levels of CCL5 were observed in the Luminal A subtype and the highest in the invasive basal subtype, there was no significant difference between circulating levels of CCL5 in each subgroup.
Serum CCL5 levels in 102 breast cancer patients and 66 age-matched controls. There was no significant difference (p>0.081) detected in serum CCL5 levels of breast cancer patients (Mean (SEM) 46.1 ng/ml (4.0 ) ng/ml) compared to healthy controls (45.4 (3.4) ng/ml).
Breast cancer patients were also subdivided based on lymph node status at time of blood collection. Circulating CCL5 levels were observed to decrease in the transition from node negative (59.6 (3.7) ng/ml) to node positive disease (≤2 lymph nodes positive 40.5 (6.3) ng/ml) and increase again as the number of positive lymph nodes increased (≥3 lymph nodes positive 50.95 (9.8) ng/ml, Figure 3.2). Statistical analysis using one way ANOVA (analysis of variance test), however, did not reveal a correlation with lymph node status.

**Figure 3.2 Circulating serum CCL5 levels and lymph node status**

Clinicopathological details of each patient were further investigated to identify any potential relationship with systemic CCL5 levels. However, no significant relationships were found between systemic CCL5 levels and UICC stage (Figure 3.3) or menopausal status (Figure 3.4).
Figure 3.3 Circulating serum CCL5 and UICC stage

Figure 3.4 Circulating serum CCL5 and menopausal status
Circulating CCL5 levels were grouped based on tumour grade. A trend towards elevated levels of CCL5 were observed in patients with lower grade tumours (median 62 ng/ml) with lower levels observed in patients with higher grade tumours (median 40 ng/ml) (Figure 3.5).

Figure 3.5 Circulating serum CCL5 and tumour grade
3.4.3 Circulating TGFβ1 levels in breast cancer patients and healthy controls

Systemic TGFβ1 serum levels were also measured in 102 breast cancer patients and 66 age-matched controls. TGFβ1 levels were found to be significantly higher in the breast cancer cohort (Mean (SEM) 27.4 (1.5) ng/ml) compared to healthy controls (14.9 (0.9) ng/ml, p<0.0001 Figure 3.6). The range of TGFβ1 levels observed in breast cancer patients (range 0.26-68.1 ng/ml) was wider compared to levels in controls (range 3.2-24.1 ng/ml). The breast cancer cohort was further subdivided based on the epithelial subtype of the primary tumour as depicted in Figure 3.6. No significant difference in circulating TGFβ1 was found between different epithelial subtypes.
Circulating TGFβ1 levels and epithelial subtype

Figure 3.6 Circulating TGFβ1 levels.

TGFβ1 levels were found to be significantly higher in the breast cancer cohort (Mean (SEM) 27.4 (1.5) ng/ml) compared to healthy controls (14.9 (0.9) ng/ml, p<0.0001). No significant difference was found when TGFβ1 levels were stratified based on epithelial subtype (p>0.987).
When grouped on the basis of nodal status, TGFβ1 levels, similar to the pattern observed in CCL5, were found to decrease from node negative (27.3 (2.2) ng/ml) to node positive disease (≤ 2 lymph nodes positive 22.0 (2.7) ng/ml), and then increase again as the number of nodes positive increased (≥3 lymph nodes positive 30.7 (3.5) ng/ml, Figure 3.7).

Figure 3.7 Circulating TGFβ1 levels and lymph node status
The breast cancer patient cohort was further subdivided based on UICC stage and tumour grade. No significant relationships were found between TGFβ1 levels and UICC stage (Figure 3.8) or tumour grade (Figure 3.9).

\[ One \text{ way ANOVA } p=0.404 \]

*outliers

\[ TGFβ1 \text{ (ng/ml)} \]

\[ 0 (n=10) \hspace{2cm} I (n=20) \hspace{2cm} II (n=38) \hspace{2cm} III (n=22) \]

\[ *\text{outliers} \]

*Figure 3.8 Circulating TGFβ1 and UICC stage*
Chapter 3 Systemic CCL5 and TGFβ1 levels in breast cancer patients

Figure 3.9 Circulating TGFβ1 and tumour grade

One way ANOVA
p=0.199

*outliers
When divided on the basis of menopausal status, a trend towards higher TGFβ1 levels in premenopausal breast cancer patients was revealed. TGFβ1 levels were also found to have a wider range in the premenopausal group (range 0.26-68.072 ng/ml, n=40) compared to postmenopausal group (range 4.077-53.06 ng/ml, n=62), despite having smaller number of patients (Figure 3.10).

![Box plot showing circulating TGFβ1 and menopausal status](image)

**Figure 3.10 Circulating TGFβ1 and menopausal status**

### 3.4.4 Investigation of relationship between systemic CCL5 and TGFβ1

Circulating CCL5 and TGFβ1 levels grouped based on lymph node status (Figure 3.2 and 3.7, respectively) were compared and a similar pattern emerged. Both cytokines displayed a decrease in systemic levels in the transition from node negative to node positive disease. Levels increased again as number of positive lymph nodes increased. A potential relationship between circulating levels of both factors in breast cancer patients and controls was then investigated. This revealed a significant positive correlation between systemic CCL5 and TGFβ1 across all serum samples examined (r=0.42, p<0.0001, n=121, Figure 3.11).
Figure 3.11 Scatterplot showing positive correlation between serum CCL5 and TGFβ1 in breast cancer patients and controls.
3.5 Discussion

Circulating chemokines, growth factors and angiogenic factors have been implicated in breast cancer progression [176, 177]. Further understanding the role of these factors in breast cancer may support their use as systemic indicators of disease prognosis. Circulating levels of these factors could potentially be used for screening and monitoring of breast cancer. Previous studies have investigated serum CCL5 as a potential biomarker for breast cancer with varying results reported [137, 138]. This raised the question as to whether circulating levels of CCL5 could represent a potential biomarker of the disease. The role of this chemokine in breast cancer progression also warrants further investigation.

Eissa et al reported a significant increase in circulating CCL5 levels in serum from breast cancer patients compared to healthy controls [138]. The group also investigated a potential correlation of the chemokine with breast cancer prognostic factors, and found significantly higher levels of serum CCL5 in patients with metastatic compared to those with non-metastatic disease. At initial stages of the current study, CCL5 levels were also found to be elevated in breast cancer patients, however when the number of samples was increased, this pattern was lost and no significant increase was found between circulating CCL5 levels in breast cancer patients compared to controls. The breast cancer cohort investigated by Eissa et al was significantly smaller (n=60) than the breast cancer group in this study (n=102). The control group of Eissa et al was also smaller (n=30) compared to healthy controls used in this study (n=66). Furthermore, the control group in the current study contained an equal proportion of pre- and post-menopausal subjects as the breast cancer group. This was important as the impact of potential confounding factors such as differences in circulating hormones were reduced.

A further study in this field was conducted by Niwa et al who reported a significant increase of CCL5 in breast cancer patients compared to healthy controls and found a correlation of CCL5 with disease stage [137]. CCL5 levels were found to increase with disease stage. It is important to note that Niwa et al analysed small numbers of patients (Stage I (n=5), Stage II (n=11),
Chapter 3 Systemic CCL5 and TGFβ1 levels in breast cancer patients

Stage III (n=12) and Stage IV (n=15). The current study also investigated CCL5 levels and their relationship with clinicopathological characteristics. In contrast to previous findings, no significant differences in CCL5 levels across tumour grade, stage or menopausal status was observed in the current study. The differing findings may have a number of explanations. The current study contains the largest cohort of breast cancer patients (n=102) and healthy controls (n=66) to date. Niwa et al used plasma samples in their study, which can be a confounding factor considering platelet bound CCL5. The inflammatory chemokine CCL5 has been discussed as a potential tumour marker for breast cancer. It is important to note, however, that CCL5 is also known to play a role in allergic inflammatory conditions such as asthma and allergic rhinitis [178, 179]. Also, elevated plasma levels of CCL5 in patients with atopic dermatitis have been found [180]. Therefore, circulating CCL5 levels can be elevated in patients as a result of variety of illnesses. In conclusion, no significant relationship between CCL5 and breast cancer was detected in the current study.

There has been a wealth of information published regarding a potential role for TGFβ1 in breast cancer progression. Studies have shown a significant elevation of serum TGFβ1 in breast cancer patients [93, 181, 182]. Sheen Chen et al found a correlation between TGFβ1 levels and TNM staging [93]. The data presented here also show a significant increase in serum TGFβ1 levels in breast cancer patients (n=102) compared to normal healthy controls (n=66). This correlates with the findings of many other studies including Chod et al [183] where plasma levels of this chemokine were found to be significantly higher among breast cancer patients (n=36) than controls (n=27). Todorovic-Rakovic et al carried out a study on breast cancer patients and TGFβ1 plasma levels [182]. The authors concluded that in early stage breast cancer, TGFβ1 might potentially act as a tumour suppressor. In those with higher grade breast cancer, the study found that elevated levels of TGFβ1 was associated with cancer progression. This study indicated the need to find the “switch in TGFβ1 action” to fully understand its potential crossover from tumour inhibitor to tumour promoter. Furthermore a study by Ivanovic et al detected increased levels of TGFβ1 in stage III/IV breast cancer patients (n=44) compared to
healthy controls (n=36) [184]. Ivanovic et al also found that elevated plasma TGFβ1 levels correlated with decreased survival of metastatic breast cancer patients [175]. In the current study, no significant difference was found when TGFβ1 levels were grouped based on breast cancer stage. However, when grouped based on lymph node status, TGFβ1 levels dropped initially and increased again as number of positive lymph nodes increased. This early decline in TGFβ1 levels and further increase again supports the evidence that in early stages TGFβ1 acts as a tumour suppressor, and at an unknown stage a change takes place causing it to allow tumour progression to take place [89, 182, 185]. The importance of TGFβ1 as a potential biomarker in breast cancer was further demonstrated by Kong et al who reported elevated plasma levels of TGFβ1 in breast cancer patients preoperatively [95], with levels decreasing following removal of the tumour [95].

The current study did not find any correlation between TGFβ1 levels and menopausal status. The previous study by Ivanovic et al [175, 184] also did not find a correlation between TGFβ1 levels and epithelial subtype, ER status or PR status.

Both TGFβ1 and CCL5 levels displayed a similar pattern when grouped based on lymph node status with circulating levels of both cytokines initially decreasing and rising again as the number of positive lymph nodes increased.

Further investigation revealed a significant positive correlation between circulating CCL5 and TGFβ1 in breast cancer. The association between the inflammatory chemokine CCL5 and TGFβ1 has not been described in the literature previously. Further investigation should focus on the molecular mechanisms that potentially link the two factors. Inflammatory chemokines that might potentially be linked directly or indirectly to the important tumour promoter TGFβ1 should be investigated as possible treatment targets.
Chapter 4 Relationship between circulating menstrual hormones and cytokines
Chapter 4 Relationship between circulating menstrual hormones and cytokines

4.1 Introduction

It has been reported previously that levels of circulating cytokines fluctuate with the menstrual cycle in premenopausal breast cancer patients [125]. This finding is of interest as it could have implications in the context of timing of surgical intervention. It has been hypothesized that cyclical variations of cytokines could have an effect on the establishment of micro metastasis in breast cancer patients. In addition two classic pathways of cancer promotion, hormones and inflammation are linked in both liver and prostate cancer [186, 187]. Potentially, fluctuating inflammatory cytokine levels with the menstrual cycle could influence the breast cancer microenvironment and have implications for cancer progression.

In the context of atherosclerosis it has been established in previous studies that an inverse relationship between circulating hormones and cytokines exists. Christodoulakos et al. showed that estradiol replacement therapy decreased serum inflammatory cytokines e.g. CCL5 and CCL2 [161]. It has been observed that postmenopausal women using estrogen replacement display reduced risk of cardiovascular disease [162]. Inflammatory mechanisms and mediators are now known to play a key role in progression of atherosclerosis [163, 164]. Estrogen has been found to down regulate mediators of the inflammatory response such as inflammatory cytokines [164, 165]. The authors hypothesized that estrogen depletion in postmenopausal women is associated with stimulation of mediators of inflammation such as cytokines. This leads to an increase in atherosclerosis and cardiovascular disease in postmenopausal women [161]. The group showed that treatment with estradiol and raloxifene significantly decreased circulating CCL5. However, Joffroy et al reported an induction of TGFβ1 through antiestrogen treatment of in breast cancer cells in vitro [166]. The group further hypothesized that the TGFβ pathway plays a role in treatment resistance to antiestrogens through localized suppression of the immune response [166].

Systemic levels of VEGF have been found to fluctuate with circulating menstrual hormones [111]. Serum VEGF was found to be significantly decreased in the luteal phase and showed a significant negative correlation with
Chapter 4 Relationship between circulating menstrual hormones and cytokines

progesterone. In vitro experiments showed a decrease of VEGF expression in breast cancer cell lines when cultured with the supernatant of luteal phase serum. These results showed a potential benefit in timing breast cancer surgery with the menstrual cycle of premenopausal patients because low levels of the angiogenic cytokine VEGF could decrease the possibility of micrometastasis establishment [111, 112]. The important influence of VEGF on survival of breast cancer patients has been reported to vary with hormone receptor status of breast cancer subtypes [113].

The influence of circulating menstrual hormones on levels of chemokines and growth factors needs to be further investigated as it has potential implications for timing of breast surgery. Optimal hormone levels in a breast cancer patient could potentially prevent micrometastasis through suppression of tumour promoting cytokines. Future study designs might also take into account the potential influence of circulating menstrual hormones on the inflammatory response and systemic cytokines.

4.2 Aims

1. Measurement of circulating CCL5, TGFβ1 and VEGF in healthy premenopausal volunteers.

2. Measurement of FSH, LH, Oestradiol and Progesterone across the menstrual cycle of healthy premenopausal volunteers

3. Investigation of a potential relationship between systemic chemokine levels and circulating menstrual hormones.

4.3 Materials and Methods

4.3.1 Measurement of circulating menstrual hormones and cytokines

The study cohort consisted of 15 healthy premenopausal volunteers which displayed normal ovulatory cycles. Serum samples were collected every week for four consecutive weeks (n=60 samples in total). Demographic data was also collected from the participants. Luteinising Hormone (LH), Follicular Stimulating Hormone (FSH), Oestradiol and Progesterone were measured in
serum samples (n=60) by direct chemiluminescence as described in section 2.2.1.

Quantification of circulating levels of CCL5, TGFβ1 and VEGF in serum samples from healthy volunteers was carried out using ELISA as described in section 2.2.2. Circulating levels of cytokines were then stratified based on phase of menstrual cycle. The mid-cycle phase was determined by an LH peak, and the mid-luteal phase established by a peak in Progesterone. Other phases of the menstrual cycle were extrapolated from these time points.

4.4 Results

4.4.1 Study group characteristics

Data collected from the participants is shown in Table 4.1.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum samples</td>
<td>60</td>
</tr>
<tr>
<td>Volunteers (healthy, premenopausal women)</td>
<td>15</td>
</tr>
<tr>
<td>Females using oral contraceptive pill</td>
<td>4</td>
</tr>
<tr>
<td>Females with ovulatory cycles</td>
<td>15</td>
</tr>
<tr>
<td>Mean age</td>
<td>25 years</td>
</tr>
<tr>
<td>Age range</td>
<td>21-42 years</td>
</tr>
</tbody>
</table>

*Table 4.1 Characteristics of study group used for investigation of relationship between chemokines and circulating menstrual hormones*
Normal ovulatory cycles which displayed a mid-cycle peak of LH were found in all study participants as shown in Table 4.2.

<table>
<thead>
<tr>
<th></th>
<th>FSH IU/L</th>
<th>LH IU/L</th>
<th>Oestrogen pmol/L</th>
<th>Progesterone (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular</td>
<td>2.5-10.2</td>
<td>1.9-12.5</td>
<td>69-905</td>
<td>0.5-4.5</td>
</tr>
<tr>
<td>Midcycle</td>
<td>3.4-33.4</td>
<td>8.7-76.3</td>
<td>130-2094</td>
<td>10.6-81.2</td>
</tr>
<tr>
<td>Luteal</td>
<td>1.5-9.1</td>
<td>0.5-16.9</td>
<td>82-939</td>
<td>14.1-89</td>
</tr>
</tbody>
</table>

Table 4.2 Ranges of circulating menstrual hormones in each phase of the menstrual cycle. FSH: Follicle Stimulating Hormone, LH: Luteinising Hormone

4.4.2 Relationship of circulating hormones with systemic CCL5

A potential correlation between circulating hormones and CCL5 was examined in serum samples from healthy premenopausal volunteers (n=15 x 4 weekly samples, total n=60). CCL5 levels were stratified based on phase of the menstrual cycle (Figure 4.1). A significant drop in CCL5 levels in the transition from late luteal/early follicular to mid follicular phase of the menstrual cycle was observed (p<0.05). This corresponds with an increase in Oestradiol levels in the first two phases of the menstrual cycle.
Chapter 4
Relationship between circulating menstrual hormones and cytokines

Figure 4.1 CCL5 levels across phases of menstrual cycle
Serum CCL5 levels were then correlated to circulating Oestradiol. A significant negative correlation between Oestradiol and CCL5 (n=60, $r_s = -0.502$, $p<0.0001$, Figure 4.2) was detected.

![Figure 4.2 Correlation of circulating CCL5 with systemic Oestradiol](image)

Serum CCL5 levels were also correlated to circulating Progesterone. A significant negative correlation between Progesterone and CCL5 (n=60, $r_s = -0.385$, $p<0.05$, Figure 4.3) was detected. No relationship between circulating LH or FSH and CCL5 was detected.
4.4.3 Relationship of circulating hormones with systemic TGFβ1

A potential relationship between circulating TGFβ1 and circulating menstrual hormones was also investigated. There was no significant variation found in TGFβ1 levels across phases of the menstrual cycle (Figure 4.4). VEGF levels were also measured in this study group, with no variation found across phases of the menstrual cycle (Figure 4.5).
Figure 4.4 TGFβ1 levels across phases of menstrual cycle.
Chapter 4 Relationship between circulating menstrual hormones and cytokines

Figure 4.5 Variation of VEGF across phases of menstrual cycle
Chapter 4 Relationship between circulating menstrual hormones and cytokines

4.5 Discussion

A potential relationship between the inflammatory chemokine CCL5 and circulating menstrual hormones has been investigated in the current study. This is of interest because cyclical variations of pro-inflammatory chemokines could have indications in the context of timing of surgical intervention in premenopausal breast cancer patients. Cyclical variations of cytokines could have an effect on the establishment of micro metastasis in breast cancer patients and previous studies have investigated this potentially important effect [111]. Timing of surgical intervention in premenopausal breast cancer patients has been reported in a number of previous studies, all of them supporting the hypothesis that the second half of the menstrual cycle is the most favorable.

The current study investigated the effect of cyclical variation of menstrual hormones in healthy premenopausal volunteers on circulating CCL5. It was observed that serum CCL5 was inversely correlated with oestradiol and progesterone. This observation provides direct evidence of a cyclical variation of the chemokine.

A direct link between circulating hormones and TGFβ1 has not been described in the literature. However, Joffroy et al reported an induction of TGFβ1 through antiestrogen treatment of breast cancer cells in vitro [166]. The group further hypothesized that the TGFβ pathway plays a role in treatment resistance to antiestrogens through localized suppression of the immune response [166]. The current study did not find a correlation of serum TGFβ1 with circulating hormones, and no correlation with phases of the menstrual cycle.

A previous study reported cyclical variation of vascular endothelial growth factor (VEGF), and its importance in terms of choosing the optimal time point for surgery in the menstrual cycle of premenopausal breast cancer patients [111]. In the current study, no difference was found in VEGF levels across phases of the menstrual cycle. The contradicting findings may be a result of the differences in sample collection. Heer et al obtained blood samples every four days starting at the first day of the menstrual cycle from 14 healthy
Chapter 4 Relationship between circulating menstrual hormones and cytokines

premenopausal volunteers. They analyzed VEGF in 8-11 samples from each participant, whereas the current study took one sample weekly for four weeks.

Data presented in the current study supports a link between CCL5 and circulating menstrual hormones. The findings are important for the design of future studies since changes in CCL5 levels could be a result of menstrual status rather than being disease related. Previous studies investigating circulating CCL5 in women have to be reevaluated in the light of the findings presented in this study. Also it is important to consider the effect of age, use of antiestrogens and time in menstrual cycle that could all impact on CCL5 results when comparing breast cancer patients and healthy controls.
Chapter 5 Gene expression analysis of whole breast tissue
5.1 Introduction

Inflammation has emerged in recent years as an enabling characteristic of cancer initiation, progression and metastasis. The tumour promoting effect of inflammatory cytokines and growth factors in the breast cancer microenvironment is increasingly recognized. CCL5 is a chemotactic cytokine and plays an important part in inflammation through activation of T cells, monocytes, dendritic cells, natural killer cells, eosinophils and basophils [135]. An association between CCL5 and multiple cancer types has been reported, with the most striking findings reported in relation to breast cancer [116]. CCL5 binds to multiple receptors including CCR1, CCR3 and CCR4, with CCR5 recognised as its principal receptor [136]. Conflicting reports exist in relation to the role of CCL5 in breast cancer progression. Using immunohistochemistry, tissue from patients with advanced breast carcinoma was reported to have elevated levels of CCL5 [122], with the protein rarely found in biopsies taken from healthy patients. Also joint CCL5 and CCL2 expression in the same breast tumour tissues has been correlated with more advanced disease [117]. In an in vivo model of breast cancer, tumours secreting lower levels of CCL5 were shown to have reduced metastatic potential [139]. Tumour derived CCL5 has also been implicated in reduced T-cell response and shown to support in vivo growth of murine mammary carcinoma [140]. In contrast, Kurt et al reported that tumour-derived CCL5 on its own had no role in breast cancer progression [141]. CCL5 protein expression at diagnosis has been correlated to clinical outcome in stage II breast cancer patients, suggesting a potential role for the chemokine as a biomarker for the disease independent of oestrogen receptor-α (ER-α) status [142]. The cytokine TGFβ regulates pathways important to tumour initiation, proliferation and metastasis [86, 88]. TGFβ in normal epithelial cells induces arrest of the cell cycle and this function has been implicated in cancer suppression in the early stages of oncogenesis [86, 89]. In later stages of carcinogenesis TGFβ acts as a tumour promoter by stimulating EMT and angiogenesis [58, 90]. In addition, TGFβ signalling is involved in suppression of the immune response and metastasis [91]. TGF β regulates chemotaxis in the tumour microenvironment through stimulation of expression of CXCR4 in
monocytes and macrophages [92]. CXCR4 is the receptor for CXCL12, a ligand that is expressed by invasive carcinoma-associated fibroblasts [33]. It is thought that TGFβ acts as a tumour promoter in the tumour-stromal interaction by attracting immune cells into the tumour microenvironment [90].

Chemokine (C-C motif) ligand 2 (CCL2) is also known as monocyte chemoattractant protein 1 (MCP-1) and belongs to the CC chemokine family [114, 115]. Recombinant CCL2 is a chemoattractant to T-lymphocytes, monocytes and dendritic cells and the chemokine is known to recruit those immune cells to sites of tissue injury or inflammation. CCL2 has been found to be secreted by tumour epithelial, as well as stromal cells in the breast cancer microenvironment. The cytokine was significantly elevated in cancerous breast epithelial cells and its expression was seen as a characteristic gained through carcinogenesis. CCL2 expression has been found to be significantly correlated with higher tumour grade, positive lymph node status, low levels of differentiation and poor prognosis [104, 122, 123]. The chemokine CCL2 has been found to promote angiogenesis in breast cancer. The chemotactic cytokine recruited macrophages into the tumour microenvironment, which released angiogenic factors such as VEGF [104, 106, 126, 127]. The tumour-promoting, positive feedback loop between CCL2, VEGF and TAMs in the tumour microenvironment is an example of how inflammatory mediators are involved in tumour progression. [128]. VEGF is the principal modulator of endothelial cell proliferation. The important role of VEGF in angiogenesis, vasculogenesis and vascular permeability makes it an important factor in carcinogenesis [101]. Reports of VEGF and breast carcinogenesis suggest a dual role for the angiogenic cytokine. On the one hand VEGF directly stimulates endothelial cells, on the other it recruits macrophages in the tumour stroma, which are able to stimulate angiogenesis through secretion of factors including VEGF, tumour necrosis factor-alpha (TNF-alpha), and thymidine phosphorylase (TP) [103]. Tumour associated macrophages also are known to secrete Monocyte Chemoattractant Protein-1 (MCP-1), which is associated with breast cancer progression and decreased survival. Expression levels of VEGF and MCP-1 have been found to be correlated in breast cancer [104-106]. Levels of the cytokine VEGF have been shown to correlate with prognosis for patients with node-negative breast carcinoma [108-110]. MMP-3, also called stromelysin-1
(Str1) cleaves collagens, laminins, elastin, fibronectin and other ECM substrates. Previous studies provided evidence that MMP-3 cleaved E-cadherin and thus was implicated in EMT in breast cancer [72]. MMP-3 has been found to promote epithelial to mesenchymal transitions in cell culture [73]. MMP-3 has been shown to be expressed by the highly invasive MD-MB-231 breast cancer cell line but not in less invasive cell lines like T47D [75]. MMP-3 genes have recently been linked to breast cancer risk and survival [84]. Fibroblast activation protein (FAP), also known as seprase, belongs to the serine protease family [145]. It has been implicated in a number of physiological and pathological conditions that were previously associated with activation of stromal fibroblasts such as wound healing, inflammation, arthritis and fibrosis of lung and liver [146, 147]. In epithelial cancers it was found to be selectively expressed on the cell-surface of tumour associated stromal fibroblasts in the tumour microenvironment [148]. Interestingly, in vitro experiments showed that TGFβ induced upregulation of FAP expression on fibroblasts [145]. Gene expression profiling studies detected high expression levels of FAP in tumour tissue and low expression levels in normal tissue [150]. In preclinical studies using in vivo models of breast cancer, FAP has been shown to have an important role in carcinogenesis. Overexpression of FAP in fibroblasts were shown to improve xenograft tumour take and promoted tumour growth as well as support angiogenesis [151]. FAP has been investigated by several groups as a therapeutic target, because it is specifically expressed on activated fibroblast in the tumour microenvironment, but does not appear in normal tissue. FAP knockdown in breast tumour bearing mice using short hairpin RNA showed reduced tumour growth and angiogenesis [152]. In order to discover potential new treatment targets in the breast cancer microenvironment, further research is still necessary to evaluate the role of cytokines and growth factors in the breast cancer microenvironment.

5.2 Aims

Gene expression profiling of tissue specimens from breast cancer patients (n=43) and healthy controls (n=16) and investigation of a potential relationship with clinicopathological data targeting:
1. The ligand CCL5 and its principle receptor CCR5

2. The ligand TGFβ1 and its receptor TGFβRII

3. Investigation of potential relationship between gene expression levels of CCL5 and TGFβ1.

4. The ligand VEGF and its receptor VEGFR2.

5. The ligand CCL2 and its receptor CCR2.

6. Fibroblast activation protein (FAP) and matrix metalloproteinase-3 (MMP3)

**5.3 Materials and Methods**

**5.3.1 Gene expression analysis of whole breast tissue**

Corresponding tissue specimens (n=43) were available on a subset of the breast cancer patients from whom serum samples had been obtained and analysed in Chapter 3. Breast tissue obtained from breast reduction mammoplasty (n=16) served as normal controls. Tissue was homogenized and RNA extracted as described in section 2.7.1, and real-time quantitative PCR (RQ-PCR) carried out targeting CCL5, CCR5, TGFβ1, TGFβRII, VEGF, VEGFR2, CCL2, CCR2, FAP and MMP3 as described in 2.7.4. Results were normalized to endogenous control genes MRPL19 and PPIA and expression levels of the respective genes in tumour tissues compared to normal tissues.

**5.3.2 Statistical Analysis**

All data was log transformed and normal distribution confirmed using the Kolmogorov-Smirnov test (a significance value greater than 0.05 indicates normal distribution). Differences between groups were analyzed using Analysis of Variance (ANOVA) test. Any statistically significant difference between two groups was confirmed using the two sample t-test. Correlation between two variables was analyzed using the Pearson correlation method following confirmation of normality. Continuous variables of interest are summarised numerically by Mean (SEM), and graphically using boxplots and scatterplots.
Chapter 5 Gene expression analysis of whole breast tissue

5.4 Results

5.4.1 Study group characteristics

Corresponding tissue specimens (n=43) were available on a subset of the breast cancer patients from whom serum samples had been obtained. Clinicopathological details are shown in Table 5.1. Distribution of epithelial subtype, grade and stage was typical for a breast cancer cohort, with the majority of patients postmenopausal with Luminal A subtype tumours.

<table>
<thead>
<tr>
<th>Breast cancer patients</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Patients</strong></td>
<td>n (%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>43</td>
</tr>
<tr>
<td>premenopausal</td>
<td>16 (37.2%)</td>
</tr>
<tr>
<td>postmenopausal</td>
<td>27 (62.8%)</td>
</tr>
<tr>
<td><strong>Tumour Characteristics</strong></td>
<td>n (%)</td>
</tr>
<tr>
<td><strong>UICC stage</strong></td>
<td>n (%)</td>
</tr>
<tr>
<td>Stage I</td>
<td>8 (18.6%)</td>
</tr>
<tr>
<td>Stage II</td>
<td>14 (32.6%)</td>
</tr>
<tr>
<td>Stage III</td>
<td>13 (30.2%)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>3 (6.9%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>5 (11.62%)</td>
</tr>
<tr>
<td><strong>Epithelial subtype</strong></td>
<td>n (%)</td>
</tr>
<tr>
<td>Luminal A</td>
<td>29 (67.4%)</td>
</tr>
<tr>
<td>Luminal B</td>
<td>9 (20.9%)</td>
</tr>
<tr>
<td>Her-2/neu</td>
<td>2 (4.7%)</td>
</tr>
<tr>
<td>Basal</td>
<td>2 (4.7%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (2.3%)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td>n (%)</td>
</tr>
<tr>
<td>1</td>
<td>10 (13.9%)</td>
</tr>
<tr>
<td>2</td>
<td>15 (34.9%)</td>
</tr>
<tr>
<td>3</td>
<td>16 (37.2 %)</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (13.9%)</td>
</tr>
</tbody>
</table>

Table 5.1 Characteristics of breast cancer patients (n=43) from whom tissue specimen were obtained for gene expression analysis
5.4.2 Expression of CCL5 and CCR5 in breast tissue

RQ-PCR was performed targeting CCL5, and its principle receptor, CCR5. Expression of CCL5 and CCR5 was significantly elevated in tissue from breast cancer patients compared to controls (p<0.001, Figure 5.1). The range of values of CCL5 in tumour tissue (range 0.1-2.08 log_{10} relative quantity) was observed to be wider compared to normal tissue (range 0.1-0.8 log_{10} relative quantity (RQ)). Similarly the range of CCR5 expression in tumour tissue (range 0.1-3.01 log_{10} RQ) was greater than normal tissue (range 0.2-1.4 log_{10} RQ). This difference was especially pronounced in the case of CCR5 expression. CCR5 expression was not detected in two tumour samples and two normal samples.

![Figure 5.1 CCL5 and CCR5 gene expression in tumour and normal breast tissue](image)

**Figure 5.1 CCL5 and CCR5 gene expression in tumour and normal breast tissue**

Potential associations between expression of CCL5 in breast tumour tissue and clinicopathological details were also investigated and no significant relationships were found as shown in Figure 5.2 a-d and Table 5.2.
Figure 5.2 a) Expression of CCL5 in breast tumour tissue grouped based on epithelial subtype b) Expression of CCL5 in breast tumour tissue grouped based on grade
Figure 5.2 c) Expression of CCL5 in tumour breast tissue grouped based on UICC stage d) Expression of CCL5 in tumour breast tissue grouped based on menopausal status
Table 5.2 Analysis of CCL5 expression in tumour breast tissue grouped based on epithelial subtype, grade, UICC stage and menopausal status
Potential associations between expression of CCR5 in breast tumour tissue and clinicopathological details were also investigated. While no significant relationships were found when CCR5 was grouped based on epithelial subtype, UICC stage and menopausal status (Figure 5.4 a-c and Table 5.3), significantly elevated expression of CCR5 was found in grade 1 (1.9 log_{10} RQ) compared to grade 2 (1.27 log_{10} RQ) and grade 3 disease (0.99 log_{10} RQ, p<0.001) as shown in Figure 5.3.

Figure 5.3 Gene expression of CCR5 based on tumour grade
Figure 5.4  a) Expression of CCR5 in breast tumour tissue grouped based on epithelial subtype b) Expression of CCR5 in breast tumour tissue grouped based on UICC stage
Figure 5.4 c) Expression of CCR5 in breast tumour tissue based on menopausal status
Table 5.3 Analysis of CCR5 expression in tumour breast tissue grouped based on epithelial subtype, UICC stage and menopausal status
Chapter 5 Gene expression analysis of whole breast tissue

Further investigation revealed a significant positive correlation between CCL5 and its receptor CCR5 (n=56, r=0.665, p<0.0001, Figure 5.5).

![Figure 5.5 Correlation of the ligand CCL5 and its principle receptor CCR5 gene expression](image)

5.4.3 Expression of TGFβ1 and TGFβRII in breast tissue

RQ-PCR was carried out targeting the ligand TGFβ1 and its principle receptor TGFβRII. TGFβ1 expression was significantly increased in tumour (1.4 log_{10} RQ) compared to normal breast tissue (0.31 log_{10} RQ, p<0.0001) while expression of TGFβRII remained unchanged as shown in Figure 5.6.
Figure 5.6 TGFβ1 and TGFβRII gene expression in tumour and normal breast tissue

TGFβ1 expression was also grouped based on epithelial subtype, grade, stage and menopausal status and analyzed using ANOVA as shown in Figure 5.7 a-d and Table 5.4. No significant associations between expression of the ligand and these parameters were observed.

Figure 5.6 TGFβ1 and TGFβRII gene expression in tumour and normal breast tissue
Chapter 5 Gene expression analysis of whole breast tissue

Figure 5.7 a) Expression of TGFβ1 in breast tumour tissue grouped based on epithelial subtype b) Expression of TGFβ1 in breast tumour tissue grouped based on grade
Figure 5.7  

c) Expression of TGFβ1 in breast tumour tissue based on UICC stage 

<table>
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<tr>
<th>Stage</th>
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<td>III</td>
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d) Expression of TGFβ1 in breast tumour tissue based on menopausal status 

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Table 5.4 Analysis of expression of TGFβ1 in tumour breast tissue grouped based on epithelial subtype, grade, UICC stage and menopausal status
Chapter 5 Gene expression analysis of whole breast tissue

TGFβRII expression was also grouped based on epithelial subtype, grade, UICC stage and menopausal status. No significant relationships between gene expression and these parameters were established as shown in Figure 5.8 a-d and Table 5.5.
Chapter 5 Gene expression analysis of whole breast tissue

Figure 5.8 a) Expression of TGFβRII in breast tumour tissue grouped based on epithelial subtype b) Expression of TGFβRII in breast tumour tissue grouped based on grade
Figure 5.8 c) Expression of TGFβRII in breast tumour tissue grouped based on UICC stage d) Expression of TGFβRII in breast tumour tissue grouped based on menopausal status
## Chapter 5 Gene expression analysis of whole breast tissue

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<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
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<td>Luminal B</td>
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*Table 5.5 Analysis of expression of TGFβRII in tumour breast tissue grouped based on epithelial subtype, grade, UICC stage and menopausal status*
Chapter 5 Gene expression analysis of whole breast tissue

The ligand TGFβ1 and its receptor TGFβRII were found to strongly correlate (n=56, r=0.532, p<0.0001, Figure 5.9).

*Figure 5.9 Correlation between gene expression of TGFβ1 and TGFβRII*
Chapter 5 Gene expression analysis of whole breast tissue

5.4.4 Correlation between CCL5 and TGFβ1 in whole breast tissue

Both CCL5 and TGFβ1 were found to be significantly elevated in breast cancer patients compared to healthy controls. Further investigation revealed a significant positive correlation between CCL5 and TGFβ1 gene expression across all tissue samples (n=59, r=0.435, p<0.001, Figure 5.10).

![Figure 5.10 Correlation between gene expression of CCL5 and TGFβ1](image_url)
5.4.5 Expression of VEGF and VEGFR2 in breast tissue

The ligand VEGF, the most important mediator of angiogenesis, and its receptor VEGFR2 were also investigated. No significant difference was found between gene expression levels of VEGF in tumour compared to normal breast tissue. Expression of its receptor VEGFR2, however, was significantly decreased in tumour compared to normal tissue (p<0.005, Figure 5.11).

![Figure 5.11 VEGF and VEGFR2 gene expression in tumour and normal breast tissue](image)

VEGF expression was also grouped based on epithelial subtype, grade, UICC stage and menopausal status as shown in Figure 5.12 a-c, Figure 5.13 and Table 5.6.
Figure 5.12 a) Expression of VEGF in breast tumour tissue grouped based on grade b) Expression of VEGF in breast tumour tissue grouped based on UICC stage
Figure 5.12 c) Expression of VEGF in breast tumour tissue based on menopausal status
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<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
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<td>0.4629</td>
<td>(0.7841, 1.1232)</td>
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</table>

Table 5.6 Analysis of VEGF expression in tumour breast tissue grouped based on grade, UICC stage and menopausal status

Expression of VEGF was also grouped based on epithelial subtype as shown in Figure 5.13 and found to be significantly higher in the Luminal A (0.97 \( \log_{10} \) RQ) and Luminal B (1.13 \( \log_{10} \) RQ) subtype compared to Her-2 (0.52 \( \log_{10} \) RQ) and Basal (0.03 \( \log_{10} \) RQ, \( p<0.0001 \), Figure 5.13) epithelial subtype.
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Significantly higher expression of VEGF was observed in postmenopausal (1.02 log_{10} RQ) compared to premenopausal patients (0.67 log_{10} RQ, p<0.05, Figure 5.14), while expression of its receptor VEGFR2 remained unchanged (Figure 5.15).

Figure 5.13 Expression of VEGF based on epithelial subtype

![Figure 5.13](image)

Figure 5.14 Expression of VEGF grouped based on menopausal status

![Figure 5.14](image)
Chapter 5 Gene expression analysis of whole breast tissue

VEGFR2 expression was also grouped based on epithelial subtype, grade, UICC stage and menopausal status as shown in Figure 5.15 a-d and Table 5.7.

Figure 5.15 a) Expression of VEGFR2 in breast tumour tissue grouped based on epithelial subtype b) Expression of VEGFR2 in breast tumour tissue grouped based on grade
Figure 5.15 c) Expression of VEGFR2 in breast tumour tissue grouped based on UICC stage d) Expression of VEGFR2 in breast tumour tissue grouped based on menopausal status
Table 5.7 Analysis of expression of VEGFR2 in tumour breast tissue grouped based on epithelial subtype, grade, UICC stage and menopausal status

VEGFR2 expression was significantly decreased in grade 3 compared to grade 1 and grade 2 disease (p<0.05, p<0.01, Figure 5.15 b, Table 5.7)
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5.4.6 Expression of CCL2 and its receptor CCR2 in breast tissue

Expression of the ligand CCL2 and its receptor CCR2, known to be associated with the inflammatory response, were also investigated. There was no significant difference found in CCL2 gene expression levels between tumour and normal breast tissue. Its receptor CCR2, however, was significantly elevated in tumour compared to normal breast tissue (p<0.05, Figure 5.16).

**Figure 5.16 CCL2 and CCR2 gene expression in tumour and normal breast tissue**

Expression of CCL2 and CCR2 was stratified based on epithelial subtype, grade, UICC stage and menopausal status as shown in Figure 5.17 a-d, Figure 5.18 a-d, Table 5.8 and Table 5.9.
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Figure 5.17  

a) Expression of CCL2 in breast tumour tissue grouped based on epithelial subtype  
b) Expression of CCL2 in breast tumour tissue grouped based on grade

Figure 5.17  

a) Expression of CCL2 in breast tumour tissue grouped based on epithelial subtype  
b) Expression of CCL2 in breast tumour tissue grouped based on grade
Figure 5.17 c) Expression of CCL2 in breast tumour tissue grouped based on UICC stage d) Expression of CCL2 in breast tumour tissue grouped based on menopausal status
### Table 5.8 Analysis of expression of CCL2 in tumour breast tissue grouped based on epithelial subtype, grade, UICC stage and menopausal status

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<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
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Figure 5.18 a) Expression of CCR2 in breast tumour tissue grouped based on epithelial subtype b) Expression of CCR2 in breast tumour tissue grouped based on grade
Figure 5.18 c) Expression of CCR2 in breast tumour tissue grouped based on UICC stage d) Expression of CCR2 in breast tumour tissue grouped based on menopausal status
Table 5.9 Analysis of expression of CCR2 in tumour breast tissue grouped based on epithelial subtype, grade, UICC stage and menopausal status

<table>
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<th>StDev</th>
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<td>CCR2</td>
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<td>1.968</td>
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<td>(1.729, 2.208)</td>
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</table>
No significant differences were found in CCL2 and CCR2 expression when stratified based on epithelial subtype, grade and menopausal status (Figure 17 a-d, Figure 18 a-d, Table 5.8 and Table 5.9). Expression of CCL2 and CCR2 was found to be significantly increased in patients with Stage 2 compared to Stage 1 disease (p<0.05, Figure 5.19 and Figure 5.20, respectively). Following the initial increase, expression of both ligand and receptor significantly decreased again in patients with UICC stage (p<0.05, Figure 5.19 and Figure 5.20, respectively). Although not significant, there was a trend towards decreased expression of CCL2 and CCR2 in UICC Stage IV disease.
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Figure 5.19 Expression of CCL2 grouped based on UICC stage

Figure 5.20 Expression of CCR2 grouped based on UICC stage
Further investigation revealed a significant positive correlation between CCL2 and CCR2 expression ($r=0.632$, $n=56$, $p<0.0001$, Figure 5.21).

![Figure 5.21 Correlation between expression of CCL2 and CCR5 in whole tissue samples](image)

As previously mentioned, a significant positive correlation was found between CCL5 and TGFβ1 ($r=0.44$, $n=56$, $p<0.001$, Table 5.10, Figure 5.10). In addition, a significant positive correlation was found between expression of CCL5 and CCL2 (Table 5.10). Expression of their principle receptors, CCR5 and CCR2, also correlated ($r=0.29$, $n=56$, $p<0.05$). It was also observed that VEGFR2 expression correlated with CCL2 and CCR2 expression ($r=0.64$, $p<0.0001$ and $r=0.38$, $p<0.01$ respectively, Table 5.10)
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<table>
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<th>Chemokine</th>
<th>r</th>
<th>n</th>
<th>P</th>
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<td>56</td>
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<td>0.38</td>
<td>56</td>
<td>0.01</td>
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*Table 5.10 Correlations between expressions of chemokines and receptors in whole breast tissue samples*
5.4.7 Expression of MMP3 in breast tissue

Expression of MMP3, an enzyme known to be involved in tissue remodeling, wound healing and tumour initiation was also investigated. Expression of MMP3 was found to be significantly increased in tumour (2.45 log₁₀ RQ) compared to normal breast tissue (1.4 log₁₀ RQ, p<0.0001, Figure 5.22).

*Figure 5.22 MMP3 gene expression in tumour and normal breast tissue*
Chapter 5 Gene expression analysis of whole breast tissue

Expression of MMP3 was also grouped based on menopausal status. MMP3 expression was significantly lower in postmenopausal patients (2.31 log$_{10}$ RQ) compared to premenopausal patients (2.63 log$_{10}$ RQ, p<0.05, Figure 5.23).

*Figure 5.23 Expression of MMP3 grouped based on menopausal status*

Expression of MMP3 was grouped based epithelial subtype, grade and stage. No significant relationship was found between MMP3 and these parameters (Figure 24 a-c, Table 5.11).
Figure 5.24 a) Expression of MMP3 in breast tumour tissue grouped based on epithelial subtype b) Expression of MMP3 in breast tumour tissue grouped based on grade
Figure 5.24 c) Expression of MMP3 in breast tumour tissue grouped based on UICC stage.
### Chapter 5 Gene expression analysis of whole breast tissue

<table>
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<tr>
<th>Target</th>
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<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
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<td>29</td>
<td>2.282</td>
<td>0.699</td>
<td>(1.984, 2.579)</td>
<td>0.791</td>
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<td></td>
<td>Luminal B</td>
<td>9</td>
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<td>0.905</td>
<td>(1.715, 2.783)</td>
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</tr>
<tr>
<td></td>
<td>Her 2</td>
<td>2</td>
<td>1.86</td>
<td>1.78</td>
<td>(0.72, 2.99)</td>
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</tr>
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<td>Basal</td>
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<td>0.594</td>
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</tr>
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<td>0.981</td>
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<td>Grade 2</td>
<td>13</td>
<td>2.572</td>
<td>0.756</td>
<td>(2.146, 2.999)</td>
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</tr>
<tr>
<td></td>
<td>Grade 3</td>
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<td>2.209</td>
<td>0.58</td>
<td>(1.798, 2.620)</td>
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<tr>
<td></td>
<td>UICC stage I</td>
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<td>2.015</td>
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<tr>
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<td>UICC stage III</td>
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</tr>
<tr>
<td></td>
<td>UICC stage IV</td>
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<td>1.904</td>
<td>0.5</td>
<td>(1.120, 2.687)</td>
<td></td>
</tr>
</tbody>
</table>

*Table 5.11 Analysis of expression of MMP3 in tumour breast tissue grouped based on epithelial subtype, grade and UICC stage*

#### 5.4.8 Expression of FAP in breast tissue

Fibroblast activation protein (FAP) is known to be expressed in the reactive stroma of epithelial cancers such as breast cancer and plays a role in wound healing. Expression of FAP was significantly elevated in tumour (1.08 log\textsubscript{10} RQ) compared to normal breast tissue (0.43 log\textsubscript{10} RQ, p<0.0001, Figure 5.25).
Gene expression analysis of whole breast tissue

Figure 5.25 Fibroblast activation protein (FAP) gene expression in tumour and normal breast tissue

Gene expression of FAP in whole tumour tissue was grouped based on epithelial subtype, grade, UICC stage and premenopausal status and no significant relationships were observed (Figure 26 a-d, Table 5.12).
Figure 5.26 a) Expression of FAP in breast tumour tissue grouped based on epithelial subtype b) Expression of FAP in breast tumour tissue grouped based on grade
Figure 5.26 c) Expression of FAP in breast tumour tissue grouped based on UICC stage d) Expression of FAP in breast tumour tissue grouped based on menopausal status
Chapter 5 Gene expression analysis of whole breast tissue

<table>
<thead>
<tr>
<th>Target</th>
<th>Clinico-pathological parameter</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
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<td>FAP</td>
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<td>1.0857</td>
<td>0.3842</td>
<td>(0.9278, 1.2435)</td>
<td>0.662</td>
</tr>
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<td>Luminal B</td>
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<td>1.095</td>
<td>0.34</td>
<td>(0.812, 1.379)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Her 2</td>
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<td>0.946</td>
<td>1.173</td>
<td>(0.345, 1.548)</td>
<td></td>
</tr>
<tr>
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<td>0.663</td>
<td>0.518</td>
<td>(0.062, 1.264)</td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
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<td>0.541</td>
<td>(0.909, 1.395)</td>
<td>0.560</td>
</tr>
<tr>
<td>Grade 2</td>
<td></td>
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<td>1.0978</td>
<td>0.2224</td>
<td>(0.8642, 1.3315)</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
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<td>14</td>
<td>0.929</td>
<td>0.397</td>
<td>(0.703, 1.154)</td>
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</tr>
<tr>
<td>UICC stage I</td>
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<td>0.9735</td>
<td>0.2226</td>
<td>(0.7110, 1.2359)</td>
<td>0.530</td>
</tr>
<tr>
<td>UICC stage II</td>
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<td>1.172</td>
<td>0.439</td>
<td>(0.966, 1.378)</td>
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<tr>
<td>UICC stage III</td>
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<td>UICC stage IV</td>
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<td>3</td>
<td>0.898</td>
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<td>(0.469, 1.326)</td>
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</tr>
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<td>premenopausal</td>
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<td>1.137</td>
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<td>(0.902, 1.371)</td>
<td>0.593</td>
</tr>
<tr>
<td>postmenopausal</td>
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<td>29</td>
<td>1.0625</td>
<td>0.3255</td>
<td>(0.9116, 1.2134)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.12 Analysis of expression of FAP in tumour breast tissue grouped based on epithelial subtype, grade, UICC stage and menopausal status
5.5 Discussion

Gene expression analysis targeting the ligand CCL5 and its receptor CCR5 in breast tissue revealed a significantly higher expression of CCL5 in breast cancer patients (n=43) compared to healthy controls (n=16). These results support previous reports carried out using immunohistochemistry [117]. Also, a significant positive correlation between CCL5 and its principle receptor was observed. This finding could potentially indicate a strong affinity of the ligand to the CCR5 receptor. Ligands can bind to a multitude of receptors and a strong association of ligand and receptor could be interpreted as a strong affinity. Previous studies investigated the affinity between CCL5 and its receptor in the context of HIV [188]. Duma et al found that CCL5 is bound by extracellular parts of the chemokine receptor CCR5 and provided a rationale for the micromolar affinity exclusively to the monomeric form of CCL5 [188].

In accordance with previous reports, expression of TGFβ1 was increased in tumour tissue compared to normal controls [94, 189]. Previous reports have linked loss of TGFβRII with tumourigenesis and lower expression of TGFβRII has been found using immunohistochemistry in tumour tissue from patients with later stage disease [190]. The current study did not find any significant difference between TGFβRII expression in tumour compared to normal breast tissue. When grouped based on tumour grade, a trend towards lower expression of TGFβRII in patients with grade 3 tumours was found. Previous studies employed immunohistochemistry, in vivo mouse models or in vitro cell culture to investigate the role of TGFβRII in breast cancer [191, 192]. The current study investigated gene expression of TGFβRII using RQ-PCR, which may explain differing findings considering gene expression does not always correlate with protein expression.

A strong positive correlation was observed between the ligand TGFβ1 and its receptor TGFβRII indicating a strong affinity between ligand and receptor as described previously in the literature [100, 190].

Further investigation of expression of CCL5 and TGFβ1 in tumour tissue revealed a significant positive correlation between the two factors.
Chapter 5 Gene expression analysis of whole breast tissue

Previous studies found expression levels of VEGF and VEGFR2 significantly elevated in breast cancer and increased expression of the factors correlated with disease stage [100, 107, 110]. Gosh et al investigated VEGFA and VEGFR2 expression using 642 paraffin embedded blocks of primary breast cancer specimens and found that increased expression of the ligand and its receptor were associated with worse outcome for breast cancer patients [110]. Nakopoulou et al determined VEGF and VEGFR2 expression in 141 paraffin embedded blocks of breast cancer specimens and found increased expression of VEGFR2 correlated with proliferation markers [107]. The group did not find any correlation of VEGFR2 with stage, histological grade and patient survival. In the current study VEGF expression was not found to be significantly higher in tumour (n=42) compared to normal (n=14) tissue. VEGFR2 expression was grouped based on grade and was found to be significantly decreased in patients with grade 3 tumours compared to patients with grade 1 and grade 2 tumours. In agreement with findings of Nakopoulo et al [107], no correlation of VEGFR2 with stage was found. The current study found significantly higher expression of VEGF in postmenopausal compared to premenopausal breast cancer patients. Greb et al investigated the relationship between VEGF expression and menopausal status in 19 breast cancer and normal tissue specimen using RQ-PCR and found no significant relationship between the parameters [193]. The current study employed a higher number of breast tissue specimens (n=43) which could have contributed to the differing findings.

Previous studies reported increased expression of CCL2 protein in breast cancer tissue using immunohistochemistry [117]. Ueno et al investigated CCL2 protein expression in 151 breast tissue samples using ELISA and found CCL2 expression correlated with prognosis [104]. Soria et al found CCL2 expression in breast cancer tissue samples (n=54), detected using immunohistochemistry, correlated with disease stage [117]. Furthermore this group found that CCL2 was not expressed in normal epithelial duct cells [117]. The current study investigated CCL2 gene expression in primary breast tissue samples using RQ-PCR. CCL2 expression was not found to be significantly different in 42 breast cancer samples compared to 14 normal controls. However, the current study found a significant elevation of CCR2 in breast cancer compared to normal
controls and a significant positive correlation between expression of CCL2 and its receptor CCR2 across all breast tissue samples.

Soria et al investigated CCL2 and CCL5 expression by detecting expression of the proteins using immunohistochemistry [117]. They found a significant positive correlation between expression of CCL2 and CCL5. The current study investigated expression of CCL2 and CCL5 using RQ-PCR and also found a significant positive correlation between expression of the two chemokines. Expression of the principle receptors of the two inflammatory chemokines were also found to be significantly correlated in the current study.

Matrix metalloproteinases (MMPs) are important in cancer progression because of their ability to process or degrade all ECM components, which represents an important step in cancer metastasis [194, 195]. Each ECM element is cleaved by a specific MMP or MMP group [196]. Previous reports in the literature found high levels of a number of MMPs to correlate with poor prognosis in cancers [197-200]. The current study found expression of MMP3 to be significantly elevated in tumour compared to normal breast tissue. Nakopoulou et al investigated MMP3 gene expression in 77 infiltrative breast carcinomas using situ hybridisation. This group found MMP3 expression exclusively in stromal cells in 72 out of 77 breast cancers and no correlation with clinicopathological characteristics was observed [201]. Other studies investigating a correlation between MMP3 and clinicopathological characteristics such as tumour size, lymph node status and grade also found no significant correlation between the parameters [202, 203]. In the current study, a potential correlation of MMP3 with clinicopathological parameters was also investigated. While no significant correlation was found between MMP3 expression and disease stage, grade and epithelial subtype, MMP3 expression was found to be significantly elevated in premenopausal compared to postmenopausal breast cancer patients. Decock et al previously found elevated expression of MMP2 in premenopausal compared to postmenopausal breast cancer patients [204]. MMPs are factors involved in cancer invasion, metastasis and epithelial-to-mesenchymal transition [195, 205]. These results could indicate a potential hormonal control of MMPs in premenopausal patients. Further investigation is necessary to determine if these findings in
premenopausal patients are a result of fluctuations of menstrual hormones and if there is a correlation with outcome in breast cancer patients.

Fibroblast-Activation Protein-α (FAP) is a membrane-bound serine protease that is expressed on the surface of reactive stromal fibroblasts [148]. FAP is present within the majority of human epithelial tumours, including breast cancer, but is not expressed in normal tissues [148, 206]. FAP has also been investigated as a potential tumour target for the development of novel treatments [206, 207]. The current study has investigated FAP gene expression and found FAP to be significantly higher in tumour compared to normal breast tissue. A potential correlation between gene expression of FAP and clinicopathological characteristics was also investigated, however, no significant correlation was identified. Huang et al reported previously that FAP promotes matrix remodelling through secretion of factors such as MMP-9 [149]. Interestingly, the current study found a significant positive correlation between FAP and MMP-3 in breast tissue, which supports the findings of previous work.

In summary the current study found a significant increase of the inflammatory chemokine CCL5 and its receptor and TGFβ1 and its receptor in tumour tissue. In addition, a significant positive correlation between the two cytokines was observed and this warrants further study to determine a potential functional relationship between the two factors in breast cancer. VEGF was found to be decreased in the more invasive Basal and the Her-2 subtype, however, the numbers in the cohort were small and this interesting finding requires further validation. An association between VEGF expression and survival in breast cancer has been described in the literature. This association has been reported to vary with intrinsic breast cancer subtypes [113]. In addition, it was found that breast cancer subtypes express distinct receptor repertoires for tumor-associated macrophage derived cytokines, such as VEGF [208].
Chapter 6 Characterization of primary breast stromal cells
Chapter 6 Characterization of primary breast stromal cells

6.1 Introduction

Tumours are increasingly recognized as complex, organised entities with an abundance of different specialized cell types [57]. Breast tumours consist of many different cell types including not just carcinoma cells, but also additional epithelial cell types, stromal cells, adipose cells, endothelial cells, pericytes and infiltrating lymphocytes [31, 36]. Fibroblasts constitute the predominant cell type in tumour stroma of carcinomas and are referred to in the literature as “cancer-associated fibroblasts” [31, 32]. Myofibroblasts, a subset of these cells, can be identified by α smooth muscle actin expression (α-SMA). Interestingly, these cells are rarely found in healthy tissues; however they appear in sites of chronic inflammation and wound healing. It has recently been recognized that CAFs play a tumour promoting and even tumour inducing role in carcinogenesis [32]. It has been reported in the literature that CAFs secreted more growth factors such as VEGF, FGF, TGFβ as well as MMPs than normal stromal fibroblasts [32, 209]. Orimo et al described that CAFs promote breast cancer growth and neoangiogenesis through the CXCL12/CCR4 signalling pathway [33]. Recent reports in the literature investigated gene expression of cancer associated fibroblasts and found significant higher expression of extracellular matrix, matrix metalloproteinases and cell cycle related genes [210, 211]. The tumour stromal cell gene expression signature has been found to be specific to histological breast cancer subtype and grade [212]. This confirms the hypothesis that stromal cells undergo extensive gene expression changes during cancer progression, similar to what has been observed in the malignant epithelium [210]. CAFs are known to mediate inflammation and angiogenesis by secreting proinflammatory cytokines, such as CXCL1, CXCL2 and CXCL5, which can recruit macrophages [213]. Erez et al showed that this effect can be reversed by inhibiting NF-kB pathway in fibroblasts [213]. CAFs maintain their activated phenotype over several passages in vitro [209]. Several factors have been described in the literature (α-SMA, vimentin and FAP) that differentiate tumour from normal stromal fibroblasts [214, 215]. It is thought that growth factors such as TGFβ and FGF play a role in activation and recruitment of fibroblast from local tissue to tumour sites [216]. EMT has also been described as a mechanism through which CAFs are recruited [217].
Chapter 6 Characterization of primary breast stromal cells

Epithelial cells can adopt a mesenchymal phenotype through signalling pathways such as TGFβ [90]. Evidence published in the literature suggests that another source of CAFs are bone marrow derived stem cells including hematopoetic stem cells and mesenchymal stem cells [218, 219]. There is increasing interest in investigating potential therapeutic targets in the tumour microenvironment, especially stromal cell targets. These new treatment avenues could potentially overcome resistance of advance stage cancers to current treatment strategies. In order to achieve this goal, further characterisation and isolation of this cell type is necessary.

6.2 Aims

1) Isolation and culture of primary breast stromal cells from tumour, tumour associated normal and normal breast tissue specimens

2) Analysis of gene expression in primary stromal cells and correlation with clinicopathological characteristics targeting

   a) The ligand CCL5 and its receptor CCR5

   b) The ligand TGFβ1 and its receptor TGFβRII

   c) The ligand VEGF and its receptor VEGFR2

   d) Fibroblast activation protein (FAP) and matrix metalloproteinase-3 (MMP3)

6.3 Materials and Methods

6.3.1 Primary stromal cell culture and gene expression analysis

Established breast cancer cell lines which are commercially available are used widely in experimental models for breast cancer. They are also referred to as immortalized cell lines because they are capable of unlimited numbers of population doubling. Breast cancer cell lines are useful for in vitro work, because yields are reliable and experiments can easily be replicated. Some of these cell lines have been used for over 20 years and they were often derived from malignant pleural effusions rather than the primary tumour. Cells from
pleural effusions could be grown without stromal contamination, which was seen as an advantage at the time. However, it has been questioned whether in vitro work utilising breast cancer cell lines can truly be a representative model of breast cancer. In recent years, it has also been questioned whether breast cancer cell lines used in isolation are an appropriate model as the stromal-epithelial interactions within the microenvironment have been shown to play a critical role in tumourigenesis [37].

Cells that are cultured directly from fresh tissue specimen are known as primary cells and recent studies suggest that primary epithelial breast cells display a more clinically relevant molecular profile of breast carcinomas [13, 168]. However, primary cells have slow doubling times and yields are low, which makes experimental design more challenging. In addition, primary cells have a limited lifespan and can only undergo few passages before they stop dividing. Fresh breast tissue specimens for primary culture were obtained from patients undergoing surgery with prior informed consent (n=27). Tissue was also harvested from tumour associated normal regions (n=18) of the breast which refers to tissue at least 2 cm distal to the outer margin of the tumour mass. Tissue derived from patients undergoing reduction mammoplasty served as normal controls (n=8). Primary stromal cells were isolated from all tissues according to the protocol described in section 2.3.6. RNA was extracted from individual cell populations, reverse transcribed and real-time quantitative PCR (RQ-PCR) performed as described in section 2.7.4. The gene targets were CCL5, CCR5, TGFβ1, TGFβRII, VEGF, VEGFR2, MMP3 and FAP. Results were normalized to endogenous control genes MRPL19 and PPIA, and expression levels of the respective genes in tumour tissues compared to normal tissues.

6.3.2 Breast cancer cell proliferation and migration assays

The effect of the cytokines CCL5 and TGFβ1 on breast cancer cell proliferation and migration was determined. Proliferation was measured using the ApoGlow proliferation assay as described in section 2.5. The principle of this assay is based on bioluminescent measurement of ATP that is present in all metabolically active cells. This measurement was used to calculate the ADP:
Chapter 6 Characterization of primary breast stromal cells

ATP ratio present in cells and distinguish between proliferation, apoptosis and necrosis. Breast cancer cells (MDA-MB-231, Sk-Br-3) were incubated for 48 – 72 hours in the presence of various concentrations of CCL5 (25-100 ng/ml) and TGFβ1 (2 ng/ml) as described in section 2.5. Migration of MDA-MB-231 cells through a porous membrane in response to chemoattractants including FBS, CCL5 and TGFβ1 in the well below was determined as described in section 2.6. The experiment was carried out in triplicate.

6.3.3 Statistical Analysis

All data was log transformed and normal distribution confirmed using the Kolmogorov-Smirnov test. Differences between groups were analyzed using Analysis of Variance (ANOVA) test. Any statistically significant difference between two groups was confirmed using the two sample t-test. Correlation between two variables was analyzed using Pearson correlation method following confirmation of normality. As appropriate for nonparametric data, differences between two groups were analyzed using the Mann-Whitney test.

The proliferation assay was carried out in triplicate and all results were averaged and depicted using a boxplot. The different groups were compared using the Mann-Whitney test.

Migration of MDA-MB-231 cells was quantified by counting cells on harvested membranes in 5 fields of view as described in section 2.6. The counts of triplicate experiments were averaged and depicted in a bar chart.
6.4 Results

6.4.1 Morphology of primary stromal cells

Fibroblasts display typical bipolar spindle morphology when cultured in media on plastic substratum as shown in Figure 6.1.

Figure 6.1 Morphology of stromal cells isolated from whole breast tissue specimens in 2D culture on a plastic substratum
Chapter 6 Characterization of primary breast stromal cells

6.4.2 Clinicopathological characteristics of study group

Gene expression analysis was carried out on primary stromal cell populations isolated from fresh breast tumour (n=27), tumour-associated normal (n=17) and normal tissue (n=8). Clinicopathological details of the breast cancer cohort is shown in Table 6.1

<table>
<thead>
<tr>
<th>Breast cancer patients</th>
</tr>
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<tbody>
<tr>
<td><strong>Number of Patients</strong></td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>premenopausal</td>
</tr>
<tr>
<td>postmenopausal</td>
</tr>
<tr>
<td><strong>Tumour Characteristics</strong></td>
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<tr>
<td><strong>UICC stage</strong></td>
</tr>
<tr>
<td>Stage 1</td>
</tr>
<tr>
<td>Stage 2</td>
</tr>
<tr>
<td>Stage 3</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td><strong>Epithelial subtype</strong></td>
</tr>
<tr>
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</tr>
<tr>
<td>Luminal B</td>
</tr>
<tr>
<td>Her-2/neu</td>
</tr>
<tr>
<td>Basal</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 6.1 Clinicopathological details of breast cancer patients (n=27) from whom primary tumour stromal cell populations were derived
6.4.3 Expression of CCL5 and its receptor CCR5 in primary stromal cells

Analysis of gene expression levels of the ligand CCL5 and its principle receptor CCR5 was carried out on tumour stromal cell populations (n=27), tumour-associated normal (18) and normal stromal cells (n=8). CCL5 expression was found to be significantly elevated in tumour stromal cells compared to normal stromal cells (p<0.05, Figure 6.2, Table 6.2). CCR5 expression was not detected in any of the stromal populations analyzed.
Chapter 6 Characterization of primary breast stromal cells

![Box plot of CCL5 expression](image)

**Figure 6.2 Expression of CCL5 in tumour, tumour-associated normal and normal stromal cells**

<table>
<thead>
<tr>
<th>Target</th>
<th>Stromal cell type</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
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<tr>
<td>CCL5</td>
<td>normal</td>
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<td>1.621</td>
<td>1.184</td>
<td>(0.875, 2.368)</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>TAN</td>
<td>18</td>
<td>2.08</td>
<td>0.867</td>
<td>(1.582, 2.577)</td>
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</tr>
<tr>
<td></td>
<td>tumour</td>
<td>27</td>
<td>2.691</td>
<td>1.12</td>
<td>(2.285, 3.097)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6.2 Analysis of CCL5 expression in tumour, tumour associated normal and normal stromal cells**

CCL5 expression was also grouped based on epithelial subtype, grade, stage and menopausal status and analyzed using ANOVA as shown in Figure 6.3 a-d and Table 6.3. No significant associations between expression of the ligand and these parameters were observed.
Chapter 6 Characterization of primary breast stromal cells

Figure 6.3 a) Expression of CCL5 in tumour stromal cells grouped based on epithelial subtype b) Expression of CCL5 in tumour stromal cells grouped based on grade
Figure 6.3  c) Expression of CCL5 in tumour stromal cells grouped based on UICC stage  
d) Expression of CCL5 in tumour stromal cells grouped based on premenopausal status
### Table 6.3 Analysis of CCL5 expression in tumour stromal cells grouped based on epithelial subtype, grade, UICC stage and menopausal status

<table>
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<th>Target</th>
<th>Clinico-pathological parameter</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
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<td>CCL5</td>
<td>Luminal A</td>
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<td>1.98</td>
<td>1.77</td>
<td>(0.67,3.30)</td>
<td>0.191</td>
</tr>
<tr>
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<td>Luminal B</td>
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<td>1.075</td>
<td>(-0.249,2.966)</td>
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</tr>
<tr>
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<td>Her 2</td>
<td>3</td>
<td>2.909</td>
<td>0.918</td>
<td>(2.374,3.445)</td>
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</tr>
<tr>
<td></td>
<td>Basal</td>
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<td>2.333</td>
<td>1.418</td>
<td>(1.197,3.470)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 1</td>
<td>4</td>
<td>3.233</td>
<td>1.22</td>
<td>(2.063,4.402)</td>
<td>0.278</td>
</tr>
<tr>
<td></td>
<td>Grade 2</td>
<td>10</td>
<td>2.16</td>
<td>1.004</td>
<td>(1.420,2.900)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 3</td>
<td>9</td>
<td>2.629</td>
<td>1.241</td>
<td>(1.849,3.409)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage I</td>
<td>7</td>
<td>2.574</td>
<td>1.372</td>
<td>(1.656,3.493)</td>
<td>0.692</td>
</tr>
<tr>
<td></td>
<td>UICC stage II</td>
<td>10</td>
<td>2.192</td>
<td>1.016</td>
<td>(1.423,2.960)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage III</td>
<td>8</td>
<td>2.861</td>
<td>1.136</td>
<td>(2.001,3.720)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage IV</td>
<td>2</td>
<td>2.59</td>
<td>1.46</td>
<td>(0.87,4.31)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>premenopausal</td>
<td>8</td>
<td>2.462</td>
<td>1.33</td>
<td>(1.612,3.313)</td>
<td>0.724</td>
</tr>
<tr>
<td></td>
<td>postmenopausal</td>
<td>19</td>
<td>2.286</td>
<td>1.099</td>
<td>(1.734,2.838)</td>
<td></td>
</tr>
</tbody>
</table>
6.4.4 Expression of TGFβ1 and its receptor TGFβRII in primary stromal cells

Analysis of expression levels of TGFβ1 and its principle receptor TGFβRII was also carried out on tumour stromal cell populations (n=27), tumour-associated normal (n=18) and normal cell population (n=8). Significantly higher expression of TGFβ1 was found in normal stromal cell populations compared to tumour and tumour-associated normal cell populations as shown in Figure 6.4 (p<0.05) and Table 6.4.

![Figure 6.4 Expression of TGFβ1 in tumour, tumour associated normal and normal stromal cells](image-url)
Chapter 6 Characterization of primary breast stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>stromal cell type</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1</td>
<td>normal</td>
<td>8</td>
<td>1.063</td>
<td>0.428</td>
<td>(0.620, 1.505)</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>TAN</td>
<td>18</td>
<td>0.391</td>
<td>0.88</td>
<td>(0.115, 0.667)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tumour</td>
<td>27</td>
<td>0.6598</td>
<td>0.3019</td>
<td>(0.4345, 0.8852)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.4 Analysis of TGFβ1 expression in tumour, tumour associated normal and normal stromal cells

Tumour stromal cell expression of TGFβ1 was grouped based on menopausal status, epithelial subtype, UICC stage and histological grade, the results are shown in Figure 6.5 a-d and Table 6.5.
Figure 6.5  

a) Expression of TGFβ1 in tumour stromal cells grouped based on epithelial subtype. 

b) Expression of TGFβ1 in tumour stromal cells grouped based on grade.

Luminal A (n=18)  Luminal B (n=4)  Her2 (n=3)  Basal (n=2) 

Grade 1 (n=4)  Grade 2 (n=10)  Grade 3 (n=9)
Figure 6.5  c) Expression of TGFβ1 in tumour stromal cells grouped based on UICC stage  d) Expression of TGFβ1 in tumour stromal cells grouped based on menopausal status
Table 6.5 Analysis of TGFβ1 expression in tumour stromal cells grouped based on epithelial subtype, grade, UICC stage and menopausal status

<table>
<thead>
<tr>
<th>Target</th>
<th>Clinico-pathological parameter</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1</td>
<td>Luminal A</td>
<td>18</td>
<td>0.498</td>
<td>0.226</td>
<td>(0.178, 0.818)</td>
<td>0.635</td>
</tr>
<tr>
<td></td>
<td>Luminal B</td>
<td>4</td>
<td>0.829</td>
<td>0.232</td>
<td>(0.377, 1.282)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Her 2</td>
<td>3</td>
<td>0.6763</td>
<td>0.2116</td>
<td>(0.5210, 0.8315)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basal</td>
<td>2</td>
<td>0.667</td>
<td>0.653</td>
<td>(0.347, 0.987)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 1</td>
<td>4</td>
<td>0.599</td>
<td>0.258</td>
<td>(0.311, 0.887)</td>
<td>0.828</td>
</tr>
<tr>
<td></td>
<td>Grade 2</td>
<td>10</td>
<td>0.6456</td>
<td>0.2562</td>
<td>(0.4422, 0.8490)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 3</td>
<td>9</td>
<td>0.697</td>
<td>0.366</td>
<td>(0.511, 0.883)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage I</td>
<td>7</td>
<td>0.6756</td>
<td>0.1712</td>
<td>(0.4709, 0.8802)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>UICC stage II</td>
<td>10</td>
<td>0.4906</td>
<td>0.2349</td>
<td>(0.3193, 0.6618)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage III</td>
<td>7</td>
<td>0.898</td>
<td>0.321</td>
<td>(0.706, 1.089)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage IV</td>
<td>2</td>
<td>0.499</td>
<td>0.428</td>
<td>(0.116, 0.882)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>premenopausal</td>
<td>8</td>
<td>0.532</td>
<td>0.296</td>
<td>(0.317, 0.747)</td>
<td>0.235</td>
</tr>
<tr>
<td></td>
<td>postmenopausal</td>
<td>19</td>
<td>0.7135</td>
<td>0.2954</td>
<td>(0.5739, 0.8532)</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 6 Characterization of primary breast stromal cells

Expression of the receptor TGFβRII was also investigated and although not significant, a similar trend as that observed in TGFβ1 expression was seen, with levels elevated in normal compared to tumour associated normal and tumour stromal cells (Figure 6.6, Table 6.6).

Figure 6.6 Expression of TGFβRII in tumour, tumour-associated normal and normal stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>stromal cell type</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβRII</td>
<td>normal</td>
<td>7</td>
<td>1.066</td>
<td>1.605</td>
<td>(0.556, 1.577)</td>
<td>0.427</td>
</tr>
<tr>
<td></td>
<td>TAN</td>
<td>18</td>
<td>0.6403</td>
<td>0.3814</td>
<td>(0.3222, 0.9584)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tumour</td>
<td>27</td>
<td>0.6781</td>
<td>0.4005</td>
<td>(0.4184, 0.9378)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.6 Analysis of TGFβRII expression in normal, tumour associated normal and tumour stromal cells
A potential relationship between TGFβRII and epithelial subtype, grade, UICC stage, menopausal status was also investigated, with no significant relationships detected. The results are shown in Figure 6.7 a-d and Table 6.7.

**Figure 6.7 a) Expression of TGFβRII in tumour stromal cells grouped based on epithelial subtype**

**Figure 6.7 b) Expression of TGFβRII in tumour stromal cells grouped based on grade**
Figure 6.7 c) Expression of TGFβRII in tumour stromal cells grouped based on UICC stage d) Expression of TGFβRII in tumour stromal cells grouped based on menopausal status
Table 6.7 Analysis of TGFβRII expression in tumour stromal cells grouped based on epithelial subtype, grade, UICC stage and menopausal status
Further investigation revealed a significant negative correlation between expression of CCL5 and TGFβ1 in primary stromal cells (n=53, r= - 0.38, p<0.05, Figure 6.8)

Figure 6.8 Correlation between CCL5 and TGFβ1 in primary stromal cells
6.4.5 Expression of VEGF in primary stromal cells

Expression of VEGF and its principle receptor VEGFR2 was also investigated in normal, tumour associated normal and tumour stromal cell populations. VEGF expression was found to be significantly lower in normal stromal cells compared to tumour associated normal and tumour stromal cells (p<0.05, Figure 6.9, Table 6.8).

Figure 6.9 Expression of VEGF in tumour, tumour associated normal and normal stromal cells
Chapter 6 Characterization of primary breast stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Stromal cell type</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>normal</td>
<td>7</td>
<td>0.66</td>
<td>0.514</td>
<td>(0.317, 1.003)</td>
<td>0.0001</td>
</tr>
<tr>
<td>TAN</td>
<td>18</td>
<td>1.5009</td>
<td>0.3944</td>
<td>(1.3026, 1.6992)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumour</td>
<td>27</td>
<td>1.7972</td>
<td>0.4132</td>
<td>(1.6353, 1.9591)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 6.8 Analysis of VEGF expression in normal, tumour associated normal and tumour stromal cells*

A potential relationship between expression of VEGF and stage, grade, menopausal status and epithelial subtype was also investigated. VEGF expression was found to be significantly lower in Stage 1 and 2 compared to Stage 3 disease (p<0.05, Figure 6.10, Table 6.9).

*Figure 6.10 Expression of VEGF in primary tumour stromal cells grouped based on stage*
Chapter 6 Characterization of primary breast stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Clinico-pathological parameter</th>
<th>UICC stage I</th>
<th>7</th>
<th>1.731</th>
<th>0.345</th>
<th>(1.458, 2.004)</th>
<th>0.013</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td></td>
<td>UICC stage II</td>
<td>10</td>
<td>1.56</td>
<td>0.352</td>
<td>(1.331, 1.788)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>UICC stage III</td>
<td>8</td>
<td>2.158</td>
<td>0.337</td>
<td>(1.902, 2.413)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>UICC stage IV</td>
<td>2</td>
<td>1.776</td>
<td>0.423</td>
<td>(1.265, 2.287)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.9 Analysis of VEGF expression in tumour stromal cells grouped based on UICC stage

No significant relationship between stromal VEGF expression and tumour grade, epithelial subtype or patient menopausal status was observed (Figure 6.11 a-c and Table 6.10).
Chapter 6 Characterization of primary breast stromal cells

Figure 6.11 a) Expression of VEGF in tumour stromal cells grouped based on epithelial subtype b) Expression of VEGF in tumour stromal cells grouped based on grade
Figure 6.11 c) Expression of VEGF in tumour stromal cells grouped based on menopausal status
Table 6.10 Analysis of VEGF expression in tumour stromal cells grouped based on epithelial subtype, grade, menopausal status

Expression of VEGFR2, a principle receptor for VEGF was also investigated in the primary stromal cell populations. Although not significant, a trend towards lower VEGFR2 expression in normal stromal cells compared to tumour associated normal and tumour stromal cells was observed (Figure 6.12, Table 6.11).
Chapter 6 Characterization of primary breast stromal cells

Figure 6.12 Expression of VEGFR2 in normal, tumour associated normal and tumour stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Clinico-pathological parameter</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR2</td>
<td>normal</td>
<td>7</td>
<td>0.661</td>
<td>0.417</td>
<td>(0.320, 1.002)</td>
<td>0.097</td>
</tr>
<tr>
<td></td>
<td>TAN</td>
<td>18</td>
<td>1.096</td>
<td>0.449</td>
<td>(0.884, 1.309)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tumour</td>
<td>27</td>
<td>1.0248</td>
<td>0.4569</td>
<td>(0.8510, 1.1986)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.11 Analysis of VEGFR2 expression in normal, tumour associated normal and tumour stromal cells

Investigation of a potential relationship between VEGFR2 expression and epithelial subtype, grade, UICC stage and menopausal status was also carried out, with no significant relationship observed (Figure 6.13 a-d, Table 6.12).
Figure 6.13 a) Expression of VEGFR2 in tumour stromal cells grouped based on epithelial subtype b) Expression of VEGFR2 in tumour stromal cells grouped based on grade
Figure 6.13 c) Expression of VEGFR2 in tumour stromal cells grouped based on UICC stage d) Expression of VEGFR2 in tumour stromal cells grouped based on menopausal status
## Chapter 6 Characterization of primary breast stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Clinico-pathological parameter</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGFR2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>I. Vascular endothelial growth factor receptor 2</td>
<td>17</td>
<td>1.175</td>
<td>0.355</td>
<td>(0.9728, 1.3772)</td>
<td>0.32</td>
</tr>
<tr>
<td>Luminal B</td>
<td></td>
<td>4</td>
<td>0.832</td>
<td>0.453</td>
<td>(0.416, 1.249)</td>
<td></td>
</tr>
<tr>
<td>Her 2</td>
<td></td>
<td>2</td>
<td>1.186</td>
<td>0.434</td>
<td>(0.597, 1.776)</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td></td>
<td>4</td>
<td>0.498</td>
<td>0.551</td>
<td>(0.081, 0.915)</td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td></td>
<td>5</td>
<td>1.147</td>
<td>0.341</td>
<td>(0.719, 1.575)</td>
<td>0.550</td>
</tr>
<tr>
<td>Grade 2</td>
<td></td>
<td>10</td>
<td>1.094</td>
<td>0.441</td>
<td>(0.791, 1.397)</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td></td>
<td>12</td>
<td>0.916</td>
<td>0.518</td>
<td>(0.640, 1.193)</td>
<td></td>
</tr>
<tr>
<td>UICC stage I</td>
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<td>7</td>
<td>1.067</td>
<td>0.278</td>
<td>(0.696, 1.437)</td>
<td>0.758</td>
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<tr>
<td>UICC stage II</td>
<td></td>
<td>10</td>
<td>0.93</td>
<td>0.625</td>
<td>(0.620, 1.240)</td>
<td></td>
</tr>
<tr>
<td>UICC stage III</td>
<td></td>
<td>8</td>
<td>1.145</td>
<td>0.379</td>
<td>(0.799, 1.492)</td>
<td></td>
</tr>
<tr>
<td>UICC stage IV</td>
<td></td>
<td>2</td>
<td>0.871</td>
<td>0.424</td>
<td>(0.178, 1.565)</td>
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</tr>
<tr>
<td>Premenopausal</td>
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<td>8</td>
<td>0.967</td>
<td>0.594</td>
<td>(0.629, 1.305)</td>
<td>0.679</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td></td>
<td>19</td>
<td>1.049</td>
<td>0.402</td>
<td>(0.8297, 1.2685)</td>
<td></td>
</tr>
</tbody>
</table>

*Table 6.12 Analysis of VEGFR2 expression in tumour stromal cells grouped based on epithelial subtype, grade, UICC stage and menopausal status*
Chapter 6 Characterization of primary breast stromal cells

Correlations between expression of target genes in stromal cells were also investigated as shown in Table 6.13. There was a significant positive correlation detected between expression of the inflammatory chemokine CCL5 and VEGF, which is involved in angiogenesis, across all stromal cell populations. CCL5 expression correlated negatively with TGFβ1, which is strongly associated with tumourigenesis. VEGF expression was strongly associated with TGFβRII expression in primary stromal cells. Expression of the ligand TGFβ1 was also strongly correlated with expression of its principle receptor TGFβRII.

<table>
<thead>
<tr>
<th>Target gene correlation in primary stromal cells</th>
<th>r</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL5 &amp; TGFβ1</td>
<td>-0.38</td>
<td>53</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VEGF &amp; CCL5</td>
<td>0.34</td>
<td>53</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VEGF &amp; TGFβRII</td>
<td>-0.35</td>
<td>53</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TGFβ1 &amp; TGFβRII</td>
<td>0.28</td>
<td>53</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 6.13 Correlations between gene expressions in primary breast stromal cells
**6.4.6 Expression of MMP3 in primary stromal cells**

Expression of MMP3, which plays an important role in tissue remodeling, was also investigated in tumour, tumour-associated normal and normal stromal cells. There was significantly lower expression of MMP3 in tumour-associated normal stromal cells compared to tumour stromal cells \((p<0.05\), Figure 6.14, Table 6.14\). There was also a trend towards lower expression of MMP3 in normal stromal cells compared to tumour stromal cell populations as shown in Figure 6.14.

*Figure 6.14 Expression of MMP3 in tumour, tumour-associated normal and normal stromal cells*
Chapter 6 Characterization of primary breast stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Stromal cell type</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP3</td>
<td>normal</td>
<td>8</td>
<td>1.82</td>
<td>1.184</td>
<td>(1.082, 2.558)</td>
<td>0.003</td>
</tr>
<tr>
<td>TAN</td>
<td></td>
<td>17</td>
<td>1.548</td>
<td>0.843</td>
<td>(1.042, 2.054)</td>
<td></td>
</tr>
<tr>
<td>tumour</td>
<td></td>
<td>27</td>
<td>2.657</td>
<td>1.104</td>
<td>(2.256, 3.059)</td>
<td></td>
</tr>
</tbody>
</table>

*Table 6.14 Analysis of MMP3 expression normal, tumour associated normal and tumour stromal cells*

A potential relationship between MMP3 and epithelial subtype, grade, UICC stage and menopausal status was also investigated, however no significant relationships were detected as shown in Figure 6.15 a-d and Table 6.15.
Figure 6.15  a) Expression of MMP3 in tumour stromal cells grouped based on epithelial subtype b) Expression of MMP3 in tumour stromal cells grouped based on grade
Figure 6.15 c) Expression of MMP3 in tumour stromal cells grouped based on UICC stage d) Expression of MMP3 in tumour stromal cells grouped based on menopausal status
Chapter 6 Characterization of primary breast stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Clinico-pathological parameter</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP3</td>
<td>Luminal A</td>
<td>17</td>
<td>2.729</td>
<td>0.648</td>
<td>(2.179, 3.280)</td>
<td>0.365</td>
</tr>
<tr>
<td></td>
<td>Luminal B</td>
<td>4</td>
<td>2.674</td>
<td>1.792</td>
<td>(1.539, 3.808)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Her 2</td>
<td>4</td>
<td>1.892</td>
<td>1.939</td>
<td>(0.757, 3.026)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basal</td>
<td>2</td>
<td>3.545</td>
<td>0.212</td>
<td>(1.940, 5.149)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 1</td>
<td>5</td>
<td>2.641</td>
<td>0.786</td>
<td>(1.595, 3.688)</td>
<td>0.730</td>
</tr>
<tr>
<td></td>
<td>Grade 2</td>
<td>10</td>
<td>2.872</td>
<td>1.103</td>
<td>(2.132, 3.612)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grade 3</td>
<td>12</td>
<td>2.485</td>
<td>1.26</td>
<td>(1.809, 3.161)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage I</td>
<td>7</td>
<td>2.905</td>
<td>0.726</td>
<td>(1.999, 3.811)</td>
<td>0.893</td>
</tr>
<tr>
<td></td>
<td>UICC stage II</td>
<td>10</td>
<td>2.664</td>
<td>1.412</td>
<td>(1.906, 3.422)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage III</td>
<td>8</td>
<td>2.437</td>
<td>1.17</td>
<td>(1.589, 3.284)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UICC stage IV</td>
<td>2</td>
<td>2.641</td>
<td>0.437</td>
<td>(0.947, 4.336)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>premenopausal</td>
<td>8</td>
<td>2.511</td>
<td>1.557</td>
<td>(1.694, 3.327)</td>
<td>0.663</td>
</tr>
<tr>
<td></td>
<td>postmenopausal</td>
<td>19</td>
<td>2.719</td>
<td>0.897</td>
<td>(2.189, 3.249)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.15 Analysis of MMP3 expression in tumour stromal cells grouped based on epithelial subtype, grade, UICC stage and menopausal status

6.4.7 Expression of FAP in primary stromal cells

Quantification of expression of FAP, a marker for reactive stromal fibroblasts, was also investigated in primary stromal cells. There was a significant increase observed in expression of FAP in tumour compared to normal stromal cells (p<0.0001, Figure 6.16, Table 6.16) and tumour associated normal stromal cells compared to normal stromal cells and tumour stromal cells (p<0.01, Figure 6.16). There was, however, no significant difference observed between
gene expression of tumour compared to tumour associated normal stromal cells.

Figure 6.16 Expression of FAP in tumour, tumour-associated normal and normal stromal cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Stromal cell type</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAP</td>
<td>normal</td>
<td>7</td>
<td>0.351</td>
<td>0.302</td>
<td>(0.073, 0.629)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>TAN</td>
<td>18</td>
<td>0.7958</td>
<td>0.3163</td>
<td>(0.6225, 0.9691)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tumour</td>
<td>27</td>
<td>1.0677</td>
<td>0.4072</td>
<td>(0.9262, 1.2092)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.16 Analysis of FAP expression normal, tumour associated normal and tumour stromal cells
Expression of FAP was also investigated grouped based on epithelial subtype, grade, UICC stage and menopausal status. No significant correlation was found between expression of FAP and any clinicopathological parameters as shown in Figure 6.17 a-d and Table 6.17.

Figure 6.17 a) Expression of FAP in tumour stromal cells grouped based on epithelial subtype b) Expression of FAP in tumour stromal cells grouped based on grade
Chapter 6 Characterization of primary breast stromal cells

Figure 6.17 c) Expression of FAP in tumour stromal cells grouped based on UICC stage d) Expression of FAP in tumour stromal cells grouped based on menopausal status
Table 6.17 Analysis of FAP expression in tumour stromal cells grouped based on epithelial subtype, grade, UICC stage and menopausal status

Further investigation revealed a significant positive correlation between expression of MMP3 and FAP as well as TGFβRII and FAP in primary stromal cells as shown in Table 6.18.
Chapter 6 Characterization of primary breast stromal cells

<table>
<thead>
<tr>
<th>Target gene correlation in primary stromal cells</th>
<th>r</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP3 &amp; FAP</td>
<td>0.32</td>
<td>53</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>FAP &amp; TGFβRII</td>
<td>0.28</td>
<td>53</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 6.18 Correlations between target gene expressions in primary stromal cells

6.4.8 Investigation of proliferation of MDA-MB-231 cells in response to CCL5 and TGFβ1

ATP readings obtained from MDA-MB-231 cells using the ApoGlow proliferation assay are depicted in Figure 6.18. Although no significant difference could be determined, there was a trend towards higher ATP levels in cells incubated with both CCL5 (25 or 50 ng/ml) and TGFβ1 (2ng/ml) compared to controls (cells incubated in media and BSA). Factors were diluted in solution containing 0.1% BSA, and so the control medium also contained 0.1% BSA.
Chapter 6 Characterization of primary breast stromal cells

**Figure 6.18 Boxplot depicting ATP levels detected in MDA-MB-231**

ADP : ATP ratio readings obtained from MDA-MB-231 cells using the ApoGlow proliferation assay are shown in Figure 6.19. There was a significant decrease in ADP : ATP ratio observed in both CCL5 (25 or 50 ng/ml) and TGFβ1 (2ng/ml) compared to controls (cells incubated in media and BSA) indicating cell proliferation.

**Figure 6.19 ADP:ATP ratio detected in MDA-MB-231**
Chapter 6 Characterization of primary breast stromal cells

6.4.9 Investigation of migration of MDA-MB-231 cells in response to CCL5 and TGFβ1

Increased migration was observed in MDA-MB-231 cells in response to CCL5 (25 or 50 ng/ml) and TGFβ1 (2ng/ml) compared to controls. However, no additive effect of the two chemokines was observed as shown in Figure 6.20.

![Figure 6.20 Migration of MDA-MB-231 cells in response to cytokines](image_url)
6.5 Discussion

The important role of the local tumour microenvironment on breast cancer progression has been increasingly recognized in recent years [36, 220]. The breast cancer microenvironment consists of different cell types such as endothelial cells, fibroblasts and immunocytes [221]. Fibroblasts represent the most common cell type in the breast tumour stroma [220]. Previous studies investigating fibroblasts derived from the reactive breast stroma found distinct changes in stromal gene expression [222]. Also, the tumour stromal gene expression pattern has been shown to correlate with outcome [223]. It has also been hypothesized that circulating stromal cell derived factors could serve as potential biomarkers and be used together with cancer cell derived markers to increase sensitivity and specificity [224, 225]. There has been suggestions that stromal cell gene expression signatures detected in diagnostic biopsies might improve personalized patient management [226].

The current study found expression of CCL5 significantly elevated in tumour, compared to normal stromal cells. Previously it was found that increased CCL5 secretion from adjacent stromal cells stimulates metastasis of breast cancer cells [37]. In a study incorporating an admix of Mesenchymal Stem Cells and epithelial breast cancer cells, MSC-secreted CCL5 was shown to exert its action in a paracrine manner and stimulate increased breast cancer metastasis in vivo [37]. It is worth noting that CCR5 expression was not detected in any of the tumour stromal cells examined in the current study, supporting paracrine action of the chemokine in the tumour microenvironment. This may also have impacted a previous report showing that CCL5 on its own has little or no effect in breast cancer growth and metastasis [141]. The investigation employed a mouse model of breast cancer established using epithelial cells alone. The current study, along with previous reports, suggests that in vivo models using mixed stromal-epithelial xenografts may be more appropriate to elucidate the true role of CCL5 in breast cancer.

The current study has found that TGFβ1 expression was significantly decreased in tumour and tumour associated normal (TAN) stromal cells compared to normal stromal cells. Although not significant, a similar pattern
was found in expression of TGFβRII, the principle receptor of TGFβ1. Ligand and receptor were found to correlate significantly in primary stromal cells. This finding provides evidence of a strong affinity between the factors.

The similar expression of TGFβ1 and TGFβRII in tumour and TAN stromal cells provides further evidence that changes in stromal gene expression occur in tissue harvested as much as 2 cm from the primary tumour site.

Interestingly, previous studies have shown that reduced TGFβ signalling in tumour stroma supports breast cancer metastasis [227]. Cheng et al reported that TGFβRII deletion in an in vivo model led to blockage of the stromal autocrine TGFβ loop and increased proliferation in fibroblasts [227]. This group also found that TGFβRII deletion in stromal cells altered the paracrine stromal-epithelial crosstalk and led to increased proliferation of the mammary epithelium [227].

Further investigation revealed a significant negative correlation between CCL5 and TGFβ1 expression across all primary stromal cells examined.

This group previously reported increased gene expression levels of CCL2 in tumour compared to normal stromal cells. It was also found that the principle CCL2 receptor CCR2 was not expressed in any of the stromal cell populations [228].

The current study also investigated expression of VEGF and its receptor in primary breast tumour stromal cells. VEGF is widely recognized as a stimulator of angiogenesis [101, 102]. This is of interest considering tumour stromal cells could potentially support tumour progression through stimulation of angiogenesis. The current study found a significant increase of VEGF expression in tumour and TAN stromal cells compared to normal stromal cells and a similar pattern, although not significant was observed in its receptor VEGR2. When VEGF expression was grouped based on stage, a significant increase in expression was found in patients with stage 3 disease compared to patients with stage 1 and stage 2 disease. This supports previous reports of a correlation of expression of VEGF with poor prognosis in the context of breast cancer [108, 109, 229]. The findings of the current study provide further
evidence of the important role of VEGF in the reactive stroma of the breast cancer microenvironment.

Nakopoulou et al investigated MMP3 gene expression in 77 infiltrative breast carcinomas using in situ hybridisation. This group found MMP3 expression exclusively in stromal cells in 72 out of 77 breast cancers and no correlation with clinicopathological characteristics was observed [201]. In the current study, a significant increase was found in expression of MMP3 in tumour stromal cells and TAN stromal cells. Although not significant, a trend towards lower expression of MMP3 in normal stromal cells was observed. This finding is interesting considering the significant role MMPs have in tumour progression through their ability to process or degrade ECM components [205, 230].

Fibroblast activation protein (FAP) expression is known to be increased in human breast cancer, but absent in normal tissue [148, 231]. FAP expression has previously been associated with extracellular matrix (ECM) degradation, invasion and tumour growth [149]. The current study found increased expression of FAP in tumour stromal and TAN stromal cells compared to normal stromal cells. This is an important finding considering that FAP expression was found to be elevated in tissue that was harvested in excess of 2 cm from the primary tissue. Huang et al reported previously that FAP promotes matrix remodelling through secretion of factors such as MMP-9 [149]. Interestingly, the current study found a significant positive correlation between FAP and MMP-3 in primary stromal cells, which supports the findings of previous work.

The findings presented in the current study provide further characterisation of tumour stromal gene expression. The results of this study also support recent evidence of the important role of stromal cells in tumour progression, potentially through increased secretion of factors such as MMP3 and VEGF.

The current study also investigated the effect of CCL5 and TGFβ1 on cell proliferation of the invasive MDA-MB-231 cell line and found a significant increase in proliferation in response to combination of the two cytokines using the ApoGlow proliferation assay. Increased proliferation in response to CCL5
was previously reported in MCF-7 cells by Zhang et al. [232]. The group assessed cell proliferation using a MTT assay [232]. Another study by Murooka et al. reported that the chemokine CCL5 exerts its proliferation promoting effect on MCF-7 through mTOR dependent mRNA translation [144]. It has been reported that TGFβ1 acts as a growth inhibitor of breast cancer cell lines through the well characterized Smad dependent pathway [233, 234]. The current study found that proliferation of MDA-MB-231 cells was increased when CCL5 and TGFβ1 were combined. This proliferation stimulating effect was not observed when either CCL5 or TGFβ1 were used in isolation.

The effect of CCL5 and TGFβ1 on migration of MDA-MB-231 cells was also investigated in the current study. Cell migration was found to increase in response to a combination of the two cytokines, however no additive effect was observed. The current study supports findings from previous studies that reported TGFβ1 signalling to be associated with increased breast cancer cell migration [192, 235, 236]. Youngs et al. previously reported the promigratory effect of the chemokine CCL5 on MCF-7 cells [133].

The findings presented in the current study provide further characterisation of tumour stromal gene expression. The results of this study also support recent evidence of the important role of stromal cells in tumour progression, potentially through increased secretion of factors such as CCL5, MMP3 and VEGF and loss of TGFβ1 signalling.
Chapter 7 Discussion
Chapter 7 Discussion

In order to understand tumourigenesis and metastasis of breast cancer, molecular pathways in the breast tumour microenvironment are currently investigated with great interest by many groups [36, 237, 238]. The inflammatory response plays an important role in cancer development and progression in the context of the breast cancer microenvironment. Chemokines and growth factors are mediators of the inflammatory response and a body of work in the literature links these factors to breast cancer progression [44, 79, 133, 238-241]. The breast cancer microenvironment contains not only malignant epithelial cells, but also fibroblasts, endothelial cells and immune cells. Stromal fibroblasts represent the predominant cell type in the breast tumour microenvironment. It is important to consider that tumour stromal cells have been shown to fundamentally differ from normal stromal cells [33]. There have been suggestions that stromal cell gene expression signatures detected in diagnostic breast cancer biopsies might improve personalized patient management [226]. In this work primary cell culture was employed as a more accurate model of the in vivo breast cancer microenvironment to study stromal cells. The inflammatory chemokine CCL5 is also known as Regulated upon activation T-cell expressed and secreted (RANTES) and plays an important part in inflammation through activation and chemotaxis of immune cells. Previous studies have investigated systemic CCL5 in breast cancer to evaluate its role in breast cancer progression and as a potential biomarker of the disease [137, 138]. Niwa et al found elevated plasma levels of the chemokine and reported a correlation with disease stage [137]. Another group also observed significantly elevated serum CCL5 in breast cancer patients compared to healthy controls, although no significant change was detected between patients with metastatic and non-metastatic disease [138]. In the current study the chemokine CCL5 was analysed in serum samples, whole breast tissue samples and in isolated primary stromal fibroblasts. Circulating protein levels of CCL5 were measured in serum samples from breast cancer patients and compared to healthy controls. Systemic protein levels of the chemokine were also analysed in healthy premenopausal volunteers and correlated with circulating menstrual hormones. Gene expression of the chemokine was studied in whole breast tissue from a subset of the same breast cancer patients and compared to normal controls. Stromal fibroblasts were isolated from breast cancer tissue specimens
and gene expression analysis of CCL5 carried out. In contrast to previous studies, which reported elevated systemic protein levels of CCL5 in breast cancer, the current study did not find a significant difference in circulating levels of the chemokine compared to healthy controls. In addition, no correlation was found between circulating CCL5 levels with clinicopathological characteristics of breast cancer patients. The current study contains the largest cohort of breast cancer patients to date, which might have contributed to the discrepancy. A significant negative correlation of the chemokine CCL5 with systemic Oestradiol and Progesterone was found in healthy premenopausal volunteers. This observation provides direct evidence of a cyclical variation of the chemokine. The findings are important for the design of future studies since changes in CCL5 levels could be a result of menstrual status rather than being disease related. Previous studies investigating circulating CCL5 in women have to be reevaluated in the light of the findings presented in this study. Also it is important to consider the effect of age, use of antiestrogens and time in menstrual cycle that could all impact on CCL5 results when comparing breast cancer patients and healthy controls. Although no significant differences in circulating CCL5 could be detected, gene expression of the chemokine and its principle receptor CCR5 was found to be significantly increased in tumour compared to normal breast tissue. This finding is important, because no correlation was observed between circulating protein levels of CCL5 in serum of breast cancer patients and its gene expression in breast tumour tissue. On a cellular level, the current study also found increased gene expression of CCL5 in primary tumour stromal fibroblasts. This finding is interesting considering that increased CCL5 secretion from adjacent stromal cells has been shown to stimulate metastasis of breast cancer cells [37]. It is worth noting that none of the primary stromal cell populations expressed the principle CCL5 receptor CCR5, suggesting a paracrine role of the inflammatory chemokine in the primary breast cancer microenvironment. Possibly, tumour stromal cells secrete CCL5 in order to attract tumour associated macrophages and cells of the immune response which support tumourigenesis. This finding underlines the importance of stromal-epithelial interactions in the breast cancer microenvironment and models of breast cancer should strive to recreate this microenvironment.
Chapter 7 Discussion

The current study found circulating levels of TGFβ1 to be significantly elevated in breast cancer patients compared to healthy controls. When measured in blood of healthy volunteers, no significant fluctuation in systemic levels of the growth factor across the menstrual cycle was detected. As previously described in the literature, gene expression of the growth factor was increased in breast cancer tissue in this study [94, 189]. Expression of its receptor TGFβRII remained unchanged. Interestingly, on a cellular level in primary stromal fibroblasts, the current study reported decreased gene expression of TGFβ1 and its receptor TGFβRII in tumour and tumour associated normal cells compared to normal stromal cells. The exact role of TGFβ1 at the cellular level as a mediator of stromal-epithelial interactions still has to be evaluated.

In this work, circulating levels of CCL5 and TGFβ1 were studied in samples from breast cancer patients and age matched healthy controls. Both systemic TGFβ1 and CCL5 levels displayed a similar pattern when grouped based on lymph node status with circulating levels of both cytokines initially decreasing and rising again as the number of positive lymph nodes increased. Further investigation revealed a significant positive correlation between circulating CCL5 and TGFβ1 which has not been described previously in the literature. Analysis of gene expression of CCL5 and TGFβ1 in tumour tissue revealed a significant positive correlation between the two factors mirroring the positive correlation of the two cytokines observed in serum. The mechanisms through which these cytokines are potentially linked remain unknown and warrant further investigation. Interestingly, this finding was reversed in isolated primary breast stromal cells with a negative correlation observed between gene expression of the two factors.

Previous work from this laboratory showed elevated systemic levels of CCL2 in postmenopausal patients [124]. In addition, this group also found previously, that circulating protein levels of CCL2 have not been found to fluctuate with hormones of the menstrual cycle [242]. The current body of work investigated the chemotactic cytokine CCL2 in breast tissue and isolated primary stromal fibroblast. Gene expression of the chemokine did not differ in breast cancer
tissue compared to normal controls, while expression of its receptor CCR2 was significantly elevated in breast cancer tissue. Previous work from this laboratory showed an increase in CCL2 expression in tumour compared to normal stromal cells [228]. Its principle receptor CCR2 was not expressed in the stromal cells. In vitro co-culture experiments showed that interaction of breast cancer epithelial cells with primary tumour, but not normal stromal cells, stimulated increased expression of CCL2. In addition, stromal cell secreted CCL2 stimulated a significant increase in epithelial cell migration, with no effect on cell proliferation in vitro observed [228]. In summary, CCL2 expression levels in whole breast tissue did not reflect findings in stromal and epithelial cells as described by Potter et al [228]. This underlines that factors that are important in epithelial-stromal interactions might not be obvious in whole tissue gene expression.

Fibroblast activation protein (FAP) expression is known to be increased in human breast cancer, but absent in normal tissue [148, 231]. FAP expression has previously been associated with extracellular matrix (ECM) degradation, invasion and tumour growth [149]. In the current study increased gene expression of FAP was found in breast cancer tissue. On a stromal cell level, increased expression of FAP in tumour stromal and TAN stromal cells compared to normal stromal cells was observed. This is an important finding considering that FAP expression was found to be elevated in stromal cells derived from tissue that was harvested in excess of 2 cm from the primary tumour. In conclusion, TAN stromal cells can exhibit gene expression changes similar to tumour stromal cells and further investigation is needed to address the question if these findings impact on outcome and treatment options.

MMPs are important factors in breast cancer progression, because of their ability to cleave components of the extracellular matrix. In the current study, MMP3 was found to be significantly increased in breast tumour compared to normal breast tissue. In primary stromal fibroblasts, MMP3 gene expression was increased in tumour compared to TAN and normal stromal cells. Huang et al reported previously that FAP promotes matrix remodelling through secretion of factors such as MMP-9 [149]. Interestingly, the current study found a significant positive correlation between FAP and MMP-3 gene expression in
breast tissue and on a cellular level in primary stromal fibroblasts, which supports the findings of previous work.

This body of work also analysed gene expression of VEGF, the most important mediator of angiogenesis, and found no significant difference between tumour compared to normal tissue. Its receptor VEGFR2 was found to be significantly decreased in tumour compared to normal breast tissue, so the ratio between ligand and receptor had changed. No correlation was found between the angiogenic factor and clinicopathological parameters. These findings differ from previous studies in the literature that reported a correlation of VEGF protein expression with poor prognosis in breast cancer patients [110]. The current study analysed fewer specimen than previous studies, which might have contributed to the differing findings. On a cellular level, the current body of work found a significant increase of VEGF expression in tumour and TAN stromal cells compared to normal stromal cells. When VEGF expression in primary tumour stromal cells was grouped based on stage, a significant increase in expression was found in patients with stage 3 disease compared to patients with stage 1 and stage 2 disease. This supports previous reports of a correlation of expression of VEGF with poor prognosis in the context of breast cancer [108, 109, 229]. The findings of the current study provide further evidence of the important role of VEGF in the reactive stroma of the breast cancer microenvironment.
Conclusion

The results of the current study show that some factors are important on the systemic level e.g. as biomarkers of disease, while others have important functions in the breast cancer microenvironment. The complex interactions in the breast tumour microenvironment are not completely understood and mediators of these interactions need further investigation. The potential link between the inflammatory chemokine CCL5 and the growth factor TGFβ1, which was demonstrated in this work in serum samples, breast tissue samples and primary stromal cells could mean a new mechanism that links inflammation to carcinogenesis. CCL5 is known to attract immune cells into the breast cancer microenvironment and this contributes to a positive feedback loop that sustains inflammatory response in the local tissues which is known to support cancer metastasis. The recent reports that support the role of anti-inflammatory drugs in cancer management prove the importance of the inflammatory response in cancer progression. The potential link between CCL5 and TGFβ1 therefore warrants further investigation to achieve a clearer understanding of the inflammatory breast cancer microenvironment. Further knowledge is the key to potentially discover novel treatment targets to block inflammatory pathways in the tumour microenvironment.


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Appendix 1 Biobank specimen collection form

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

Patient Information

Introduction
We would like to invite you to participate in a clinical research initiative at Galway University Hospitals to establish a BioBank. The purpose of the BioBank is to set up a resource that can support a diverse range of research programmes intended to improve the prevention, diagnosis and treatment of cancer. You are under no obligation to take part and if, having read the information below, you would prefer not to participate, we will accept your decision without question.

Although major advances have been made in the management of cancer, many aspects of the disease are not fully understood. It is hoped that our understanding of the disease will be improved through research. Galway University Hospitals are actively involved in research that aims to identify markers that will predict how a cancer develops, progresses and responds to a variety of treatments. This type of work requires the use of tissue and blood samples. It is hoped that it will eventually lead to improvements in the diagnosis, treatment and outcome for those who have cancer. Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future.

Your Involvement
If you volunteer to participate in our BioBank, there will be no additional risks to you outside those of your standard investigation and treatment. Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the study group. All research is covered by standard institutional indemnity insurance and is approved by a Research Ethics Committee that ensures the ethical nature of the research. Nothing in this document restricts or curtails your rights. You may withdraw your consent at any time. If you decide not to participate, or if you withdraw your consent, your standard of treatment will not be affected in any way.

Procedure
We invite all patients who are undergoing treatment and/or investigation to participate. All samples for research will be taken at the time you are attending the hospital for routine diagnostic tests.

(i) Tissue Samples
By participating, you give us consent to retain small pieces of your tissue obtained at the time of surgery. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUGH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland. This will not affect your diagnosis in any way.

(ii) Blood Samples
By participating, you give us consent to take an extra blood sample (equivalent of 4 teaspoonfuls) at the same time that your blood is being taken for routine tests. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUGH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland.

(iii) Clinical Information
By participating, you give us consent to store information relating to your diagnosis and treatment on a database. This information is only accessed by personnel directly involved in research within the Surgical Research Unit.

Further Information
If you would like further information about our BioBank, your participation and your rights, please contact the Surgical Research Unit (Tel: 091 524390).

If you would like further information about research projects that may be conducted, please contact your Consultant.

Thank you in anticipation of your assistance. Please read and sign the Consent section. I have read the attached information sheet on the above project, dated

Please Initial Box
GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

PARTICIPANT DECLARATION

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor. I understand that I may withdraw from the study at any time.

(Name of sponsor): ........................................................................

PARTICIPANT'S NAME: ...................................................................

CONTACT DETAILS: ........................................................................

PARTICIPANT'S SIGNATURE: ............................................................

DATE: ..............................................................................................

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained:

NAME OF CONSENDER, PARENT, OR GUARDIAN: ..............................................................

SIGNATURE: ......................................................................................

RELATION TO PARTICIPANT: ................................................................

DECLARATION OF INVESTIGATOR'S RESPONSIBILITY

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

NAME OF RESEARCH NURSE OR INVESTIGATOR: ..............................................................

SIGNATURE: ......................................................................................

DATE: ..............................................................................................

CONSULTANT: .....................................................................................

Keep the original of this form in the investigators file, give one copy to the participant, and send one copy to the sponsor (if there is a sponsor).
Appendix 2 Communication arising from this work

Relationship between CCL5 and transforming growth factor-β1 (TGFβ1) in breast cancer

M.C. Hartmann a, R.M. Dwyer a,b*, M. Costello a, S.M. Potter c, C. Curran a, E. Hennessy d, J. Newell c, D.G. Griffin c, M.J. Kerin a

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d Department of Clinical Biochemistry, University Hospital Galway, Ireland

1. Introduction

A connection between inflammation and cancer was reported as early as 1865 by Virchow, who observed cancer developing in sites of chronic inflammation. Tumour cells seem to utilize molecular pathways seen in wound healing and as a consequence appear as “wounds that do not heal.” However, the underlying molecular mechanisms facilitating the interconnection of inflammation and cancer remain poorly understood.

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COLL5(RANTES) is a chemokine that plays an important part in inflammation through activation of T cells, monocytes, dendritic cells, natural killer cells, eosinophils and basophils. An association between COL5 and multiple cancer types has been reported, with the most striking findings reported in relation to breast cancer. COL5 binds to multiple receptors including GPCR, CXCR3 and CXCR1, with CXCR3 recognised as its principal receptor.2

Conflicting reports exist in relation to the role of COL5 in breast cancer progression. On a systemic level, levels of COL5 were found elevated plasma levels of the chemokine using ELISA in breast cancer patients compared to healthy controls, and a correlation with disease stage.4 Another group also observed significantly elevated serum COL5 in breast cancer patients compared to healthy controls, although no significant change was detected between patients with metastatic and non-metastatic disease.5

Using immunohistochemistry, tissue from patients with advanced breast cancer was reported to have elevated levels of COL5, with the protein rarely found in biopsies taken from healthy patients. Also, joint COL5 and COL2 expression in the same breast tumor tissues has been correlated with more advanced disease.6 In an in vivo model of breast cancer, tumors secreting lower levels of COL5 were shown to have reduced metastatic potential.7 Tumor-derived COL5 has also been implicated in reduced T-cell response and shown to support in vivo growth of murine mammary carcinomas.8 In contrast, Kuroi et al. reported that tumor-derived COL5 on its own had no role in breast cancer progression.9

COL5 protein expression at diagnosis has been correlated to clinical outcomes in stage II patients, suggesting a potential role for this chemokine as a biomarker for the disease independent of estrogen receptor (ER) status.10 In combination with ER status, the prognostic strength of COL5 was greatly improved in stage II patients.11 Another study also reported an interesting link between estrogen and COL5 in the context of abscopal effect,12 where a significant decrease in circulating COL5 was found after three months of estrogen replacement and radionuclide therapy.13 This is interesting considering radon has been shown to reduce risk of invasive breast cancer.14,15 Given the pivotal role of Estrogen in disease progression, a potential link between circulating hormone levels and COL5 warrants further investigation.

Transforming growth factor- β (TGFβ) is a well-established factor in tumorigenesis.16 TGFβ signaling is involved in epithelial-to-mesenchymal transition, angiogenesis, suppression of the immune response and metastasis.17 It is thought to act as a tumor suppressor in early stage disease and to switch to tumor promotion in later stage breast cancer.18 Elevated systemic levels of TGFβ were shown to drop in breast cancer patients following removal of the tumor19 and a significant relationship between systemic levels prior to cancer treatment and overall survival has also been reported.20

The aim of this study was to further investigate a potential relationship between COL5 and breast cancer, both at the systemic and tumor tissue level. Serum COL5 levels were observed to decrease in the switch from node negative to node positive disease, and increase again as the number of positive lymph nodes increased. This pattern has previously been described for TGFβ,21 and led to further investigations into a potential relationship between the two factors. Investigation of a relationship between COL5 and circulating menstrual hormones was also performed.

2. Materials and methods

2.1. Study cohort

The study was approved by the institutional ethics committee. Prospective blood samples were obtained with informed consent from 102 breast cancer patients (Table 1). Blood samples from healthy female volunteers (n = 65) with no past or present history of malignancy or inflammatory conditions were collected in an outpatient facility.

Serum samples were also obtained from 15 healthy premenopausal volunteers every week for four consecutive weeks (n = 62) to investigate the relationship between COL5 and circulating menstrual hormones. All blood samples were collected in Vacutainer Serum Separation Tubes II (Becton Dickinson), allowed to clot for 30 min and centrifuged at 2000 rpm at 4°C for 10 min. Serum was then stored at -80°C until required.

2.2. Chemokine detection

Circulating levels of COL5 and TGFβ were measured in 118 samples (102 breast cancer, 16 healthy controls) using Quantikine® enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems).

<table>
<thead>
<tr>
<th>Table 1 - Breast cancer patient details.</th>
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<td>Number of patients</td>
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<td>Total</td>
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Please cite this article as: Hummer et al., Relationship between COL5 and transforming growth factor-β (TGFβ) in breast cancer.
2.3. Analysis of circulating menstrual hormones

Luteinizing hormone (LH), follicle-stimulating hormone (FSH), Oestradiol and Progesterone were measured in serum samples (n = 60) by direct chemiluminescence using Siemens ADVIA® Centaur® Immunoassay System. The mid-cycle phase was determined by an LH peak, and the mid-luteal phase established by a peak in Progesterone.

2.4. Analysis of gene expression

Corresponding tissue specimens (n = 42) were available on a subset of the breast cancer patients from whom serum samples had been obtained. Breast tissue obtained from breast reduction mammoplasty (n = 10) served as normal controls. At the time of harvest breast tissue was snap frozen in liquid nitrogen and homogenized in 1 ml Q-REZ lysis buffer (Qiagen Ltd.). Total RNA was then isolated using RNeasy® tissue mini kit (Qiagen Ltd.) according to manufacturer’s instructions, including an on-column DNase treatment step.

RNA was reverse transcribed using SuperScript II reverse transcriptase enzyme (Hichrom). Real-time quantitative PCR (qPCR-qPCR) was carried out using an ABI Prism 7000 (Applied Biosystems) targeting CCL5, CCR5, TGFα, and TGFβ1. Results were normalized to endogenous control genes MHP15 and PGK1 and expression levels of the respective genes in tumor tissues compared to normal tissues.

2.5. Culture of primary normal controls

To investigate gene expression on a cellular level, fresh breast tissue specimens were harvested from patients undergoing surgery with prior informed consent. Breast tissues obtained from reduction mammoplasty served as normal controls. All tissue specimens were washed twice in phosphate buffered saline (PBS) supplemented with 1000 units/ml penicillin/200 μg/ml streptomycin. They were minced finely using a metal scalpel and digested for 12–18 h in 0.1% Collagenase Type IB (Roche Diagnostics) at 37°C in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% heat inactivated FBS. Following differential centrifugation of digested tissue, the stromal fraction was cultured as stromal medium selective for fibroblast growth as described previously.26 Cells were confirmed to be positive for the stromal marker CD 90 and negative for epithelial pan-cytokeratin (MNF116, Dako).

2.6. Statistical analysis

Continuous variables of interest were summarised numerically by mean(SD), and graphically using boxplots and scatterplots. Two sample t-test, one way ANOVA and repeated measures ANOVA were used to compare mean responses as appropriate. The degree of relationship between pairs of response variables was assessed using the Pearson or Spearman correlation coefficient as appropriate. Scatterplot smoothness was employed to indicate the likely relationship between variables in a population. All analyses were performed using Minitab 15.

3. Results

3.1. Systemic CCL5 and TGFβ1 levels

Patient demographics and clinicopathological details are shown in Table 1. There was no significant difference detected in CCL5 serum levels between breast cancer patients (mean(SD) 46.24±0.11 ng/ml) compared to healthy controls (46.21±0.48 ng/ml, Fig. 2A). While divided on the basis of lymph node status CCL5 levels were found to decrease in the transition from node negative (39.60±0.7 ng/ml) to node positive disease (32 lymph nodes positive 40.56±3.9 ng/ml) and increase again as the number of positive lymph nodes increased (33 lymph nodes positive 50.95±0.8 ng/ml, Fig. 1B). While correlated with other clinicopathological characteristics including menopausal status, tumour epithelial subtype, tumour stage or tumour grade, no significant relationships were identified.

Serum TGFβ1 levels were found to be significantly higher in the breast cancer cohort (27.4±0.5 ng/ml) compared to healthy controls (14.8±0.4 ng/ml, Fig. 1C). When grouped on the basis of nodal status, TGFβ1 levels similar to the pattern observed in CCL5, were found to decrease from node negative (27.3±2.2 ng/ml) to node positive disease (23.8±2.7 ng/ml), and then increase again as the number of nodes positive increased (33 lymph nodes positive 20.7±3.1 ng/ml, Fig. 1D).

Further investigation revealed a significant positive correlation between systemic CCL5 and TGFβ1 across all serum samples examined (r = 0.42, p = 0.0001, Fig. 2).

3.2. CCL5 and circulating menstrual hormones

A potential relationship between circulating hormones and CCL5 was examined on serum samples from healthy premenopausal volunteers (n = 15) and weekly samples, total n = 40. CCL5 levels were attrasted based on phase of the menstrual cycle (Fig. 2A). A significant drop in CCL5 levels in the transition from late luteal/early follicular to mid follicular phase of the menstrual cycle was observed (p < 0.05). This corresponded with an increase in Oestradiol (n = 10) (r = 0.83, p = 0.0001). No relationship between circulating LH or FSH and CCL5 was detected.

3.3. Analysis of tissue gene expression

Gene expression analysis was carried out on corresponding tissue from breast cancer patients (n = 43), on whom circulating levels had been measured, and compared to normal tissue obtained at reduction mammoplasty (n = 10). Results were normalized to endogenous control genes and expressed as relative Quantity (RQ)27. Expression of CCL5 and CCR5 were significantly elevated in tissue from breast cancer patients compared to controls (p < 0.001, Fig. 4A). A significant positive correlation between expression of the CCL5 ligand and its principle receptor CCR5 was detected (n = 43, r = 0.52, p < 0.0001). TGFβ1 expression was significantly
Fig. 1 - (a) Circulating levels of CCL5 in breast cancer patients (n = 100) and age matched controls (n = 66). Data are presented as boxplots showing the range of circulating CCL5 in ng/ml. The interquartile range box represents CCL5 levels from the 25th percentile to the 75th percentile, with the line representing the median. There was no significant difference detected in serum levels of CCL5 in breast cancer patients compared to controls (* represents outliers). (b) Circulating serum CCL5 levels grouped based on lymph node status. Graph depicts drop in CCL5 serum levels from node negative (n = 38) (56.6 (3.1) ng/ml) to node positive disease (n = 3 lymph nodes positive 40.5 (5.5) ng/ml). CCL5 serum levels increased again as number of positive lymph nodes increased (≥3 positive lymph nodes 58.95 (6.2) ng/ml). Data are presented as boxplots. (c) Circulating serum levels of TGFβ1 in breast cancer patients (n = 100) and age matched controls (n = 66). Significantly higher levels of TGFβ1 were detected in breast cancer patients compared to normal controls (p < 0.0001). (d) TGFβ1 serum levels stratified based on lymph node status.

increased in tumour compared to normal breast tissue (p < 0.0005) whilst expression of TGFβ1 remained unchanged (Fig. 1B). A significant positive correlation between CCL5 and TGFβ1 gene expression was observed across all tissue samples (r = 0.59, p < 0.0001, Fig. 1G).

3.4. Analysis of primary breast tumour stromal cells

Gene expression analysis was carried out on primary tumour stromal cells isolated from tumour (n = 23) and normal breast tissue (n = 3) harvested at resection. Mammosphere assays were normalised using the endogenous control gene TFF3 and expressed relative to normal stromal cell gene expression. CCL5 expression was increased [Mean (SD) 0.82 (0.2)] Log2 Relative Quantity(RQ)], and TGFβ1 expression decreased [−0.120 (0.2) Log2 RQ] in tumour stromal cells compared to normal stromal cells (Fig. 1). CORT was not detected in any...
contains the largest cohort of breast cancer patients (n = 102) to date. Niwa et al. compared plasma samples from breast cancer patients (n = 68) to healthy controls (n = 10), whilst Rinaldo et al. detected elevated levels of CCL5 in serum samples from 60 breast cancer patients and 30 healthy controls. At initial stages of the current study, CCL5 levels were also found to be elevated in breast cancer patients. However, when the number of samples was increased, this pattern was lost. The control group in the current study also contained an equal proportion of pre- and post-menopausal subjects as the breast cancer group, which may impact the outcomes, particularly considering the observed relationship between CCL5 and circulating hormones.

Previous studies have shown that testosterone replacement therapy decreases serum CCL5.26 The significant negative correlation between serum CCL5, Oestradiol, and Progesterone observed in this study in healthy premenopausal volunteers provides direct evidence of a cyclical variation of this chemokine. A previous study reported cyclical variation of vascular endothelial growth factor (VEGF), and its importance in terms of choosing the optimal time point of surgery in the menstrual cycle of premenopausal breast cancer patients.27 The results presented here may warrant consideration in determining the optimal time point in the menstrual cycle for surgical intervention in premenopausal breast cancer patients.

Previous evidence suggests that circulating CCL5 is elevated in late stage breast cancer.28 29 In this study, a trend towards increased CCL5 levels was observed as the number of positive lymph nodes increased. This pattern has previously been associated with TGFβ,30 and further investigations revealed a significant positive correlation between circulating CCL5 and TGFβ1. Further investigation of expression of CCL5 and TGFβ1 in tumour tissue from a subset of the same patients also revealed a correlation between the two factors, adding further significance to this finding.

Although no difference in circulating CCL5 levels was observed, as apparent with previous reports based on immunohistochemistry,5 a significant increase in CCL5 expression in tumour tissue compared to normal tissue was detected. Also, expression of CCL5 and its principle receptor CCR5 displayed a significant positive correlation, indicating a strong affinity between the ligand and receptor. TGFβ1 gene expression was also significantly higher in tumour tissue compared to normal tissue, whilst expression of TGFβ1 remained unchanged. Whilst a positive correlation between CCL5 and TGFβ1 was observed at the systemic level, and also in whole tumour tissues, upon isolation of primary tumour stromal cells, an increase relationship between the two factors was observed.

CCL5 was found to be significantly elevated in tumour, com pared to normal stromal cells, while TGFβ1 was decreased. Importantly, previous studies have shown that reduced TGFβ signalling in tumour stromal supports breast cancer metastasis, whereas increased CCL5 secretion from adjacent stromal cells has been shown to stimulate metastasis of breast cancer cells.28 Chiang et al.28 reported that TGFβ1 deletion in an in vivo model led to blocking of the stromal autocrine TGFβ loop and increased proliferation in fibroblasts. This group also found that TGFβ1 deletion in stromal cells altered the perivascular stromal epithelial crosstalk and led to increased proliferation of the mammary epithelium.28 Furthermore,

Fig. 3. - (a) Variation of systemic CCL5 across phases of menstrual cycle (n = 12 samples over four consecutive weeks, total n = 48). The levels of CCL5 were significantly decreased in the transition from late luteal/early follicular to mid follicular phase (p < 0.05). (b) Scatter plot depicting significant negative correlation between CCL5 and Oestradiol (n = 40, r = −0.602, p < 0.001). (c) Scatter plot showing significant negative correlation between CCL5 and Progesterone (n = 60, r = −0.383, p < 0.05).
Fig. 4 - (a) Gene expression analysis targeting CCL5 and CCR5 in breast cancer tissue (n = 45) compared to normal tissue (n = 16). Significantly higher expression of CCL5 and CCR5 was found in tumour compared to normal breast tissue (p < 0.0001). The data are presented as box plots showing the range of Log2 Relative Quantity gene expression of CCL5 and CCR5. (b) Gene expression analysis targeting TGFβ1 and TGFβRII in tumour and normal breast tissue. A significant increase in TGFβ1 expression was found in breast cancer tissue compared to normal controls (p < 0.0001) whereas no significant difference in gene expression of its receptor TGFβRII was found. (c) Pearson correlation between CCL5 and TGFβ1 gene expression in all breast tissue samples examined (n = 59, r = 0.64, p < 0.001).

Fig. 5 - Box plots depicting CCL5, TGFβ1 and TGFβRII expression in primary tumour stromal compared to normal stromal cells harvested at reduction mammaplasty (n = 3). Normal stromal cell expression is represented by the reference line.

et al. also showed in an in vivo model of breast tumorigenesis, that loss of TGFβRII in tumour cells lead to increased pluri- monary metastases and decreased time to tumour formation.

In a study incorporating an admix of Mesenchymal Stem Cells and epithelial breast cancer cells, MSC-secreted CCL5 was shown to exert its action in a paracrine manner and stimulate increased breast cancer metastasis in vivo. Perilla et al. found that co-culture with tissue resident stem cells stimulated increased invasion of breast cancer cell lines, an effect which was blocked in the presence of an antibody to CCL5. The adipose-derived stromal cells were identified as the source of CCL5 secretion, which was found to be induced through the influence of tumour derived humoral factors. It is worth noting that CCL5 expression was not detected in any of the tumour stromal cells examined in the current study, supporting paracrine action of the chemokine in the tumour microenvironment. This may also have impacted a previous report showing that CCL5 on its own has little or no effect on breast cancer growth and metastasis. The investigation employed a mouse model of breast cancer established using epithelial cells alone. The current study, along with previous reports, suggests that in vivo models using mixed stromal-epithelial xenografts may be more appropriate to elucidate the true role of CCL5 in breast cancer. Although unchanged at a systemic level, CCL5 expression in the tumour microenvironment is significantly increased...
compared to healthy tissue, with the ectosomal compartment partly responsible for this. The cyclin-associated in CYCS detected in breast cancer has been shown to enhance the survival of cancer cells. It appears that CCL5 may play an important role in the primary tumor microenvironment, most likely through paracrine effects on tumor epithelial cells. This study has also identified a potentially important relationship between CCL5 and TGFp in breast cancer which warrants further investigation.

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Conflict of interest statement
None declared.

REFERENCES
Influence of stromal–epithelial interactions on breast cancer in vitro and in vivo

Shirley M. Potter · Rezin M. Dwyer · Marion C. Hartmann · Sonja Khan · Marie P. Boyle · Catherine R. Curran · Michael J. Kerin

Abstract Stromal cell-secreted chemokines including CCL2 have been implicated in the primary tumor microenvironment, as mediators of tumor cell migration, proliferation, and angiogenesis. Expression of CCL2 and its principal receptor CCR2 was analyzed by RT-PCR in primary tumor cells and breast cancer cell lines. Breast cancer cell lines (MDA-MB-231, T47D) were co-cultured directly on a monolayer of primary breast tumor and normal stromal cells, retrieved using EpCAM+ magnetic beads, and changes in expression of CCL2, CCR2, MMP11, ELK1, VIL2, and KIT detected by RT-PCR. Epithelial cell migration and proliferation in response to stromal cell-secreted factors was also analyzed. In vivo, tumor xenografts were formed by co-injecting T47D cells with primary tumor stromal cells. Following establishment, tumors were harvested and digested, epithelial cells retrieved and analyzed by RT-PCR. Whole tumor tissue was also analyzed by immunohistochemistry for CD31 and the VIL2 encoded protein Erzin. Tumor stromal cells expressed significantly higher levels of CCL2 than normal cells, with no CCR2 expression detected. Primary epithelial cells and breast cancer cell lines expressed elevated CCL2, with relative expression of CCR2 found to be higher than the ligand. Interaction of breast cancer epithelial cells with primary tumor, but not normal stromal cells, stimulated increased expression of CCL2 (6-fold), ELK1 (6-fold), VIL2 (6-fold), and MMP11 (17-fold). Factors secreted by stromal cells, including CCL2, stimulated a significant increase in epithelial cell migration, with no effect on cell proliferation in vitro observed. In vivo, the presence of stromal cells resulted in tumors of increased volume, mediated at least in part through neovascularisation demonstrated by immunohistochemistry (CD31). Admitted tumor xenografts exhibited increased expression of KIT, MMP11, VIL2, and ELK1. Elevated Erzin protein was also detected, with increased cytoplasmic localization. The results presented highlight mechanisms through which breast cancer epithelial cells can harness stromal cell biology to support tumor progression.

Keywords Breast cancer · Stromal · Epithelial · Xeno-grafts · Angiogenesis · Proliferation · Metastasis

Introduction

The primary breast tumor microenvironment plays a pivotal role in cancer initiation and progression [1]. Stromal cells are the predominant cell type in this microenvironment and evidence of their active participation in tumor progression is growing rapidly [2]. Tumor stromal cells are fundamentally different from the stroma of corresponding normal breast tissue [3], and have gene expression signatures that correlate with tumor grade and poor prognosis [4–6]. These properties appear to be retained following separation from malignant epithelial cells [7], suggesting tumor stroma is comprised of an independent fibroblastic subpopulation which supports malignant behavior [6, 8]. In invasive breast cancer, stromal cells are found in much higher proportion than in in situ carcinomas, and predominantly at the invasive front [9]. Allione et al. [10] showed that breast tumor stromal cells undergo extensive gene expression changes in progression from normal breast tissue to ductal carcinoma in situ (DCIS) to invasive ductal carcinoma.
In view of these central roles in the biology of breast cancer, understanding the mechanisms by which stromal cells mediate such effects is essential. Within the tumor microenvironment, stromal cells are the most active stromal cells [2, 11], and various paracrine mediators of their growth-promoting signals have been proposed, including cytokines, growth factors, and proteases [7, 12]. Studies implicate altered chemokine expression levels as an indicator of progression to tumorigenicity and metastatic capacity [13]. Oneto et al. [7] reported that tumor stromal cell-derived CXCL12 promotes tumor growth and angiogenesis via its cognate receptor (CXCR4) expressed by carcinoma cells.

Indeed previous work by this group showed that whole breast tumor explants secreted high levels of CCL2 (MCP-1, monocyte chemotactant protein-1) and that stromal cells were responsible for the bulk of its secretion [14]. CCL2 is a 76-amino acid protein with a primary role in the immune context, regulating recruitment of monocytes/macrophages and other inflammatory cells to damaged or infected sites [15, 16]. CCL2 is minimally expressed by normal breast epithelial ducts [16]. In contrast, extensive CCL2 protein expression has been noted in breast tumor tissue [21, 17]. CCL2 functions through its main receptor CCR2, of which there are two isoforms, CCR2A and CCR2B [19, 20]. Recent research has implicated CCL2 as an active participant in the tumor microenvironment, influencing factors such as tumor-associated macrophages, growth, angiogenesis, and metastasis [17, 21, 22]. Expression of CCL2 protein in primary breast tumors was shown to have a significant prognostic value for relapse-free survival, and correlated with high tumor grade [18, 23].

Although the mechanisms by which stromal cells promote tumorigenesis are not yet fully understood, their potential as novel therapeutic targets in breast cancer is apparent [24, 25]. However, in order for stromal-epithelial interactions, or stromal cells themselves, to emerge as appropriate targets for novel breast cancer therapies, further characterization of the molecular cross-talk between these two cell populations is required.

The results presented show that isolation of breast cancer epithelial cells following interaction with primary tumor stromal cells in vitro and in vivo, stimulates increased expression of genes associated with invasion, angiogenesis, and tumor progression. While stromal cells secreted high levels of CCL2, they were devoid of the CCR2 receptor expressed by epithelial cells, suggesting paracrine action of the chemokine, potentially mediating cell migration. Another novel finding of this study was that in vivo interaction with primary tumor stromal cells induced increased expression of VEGF/Erbb, a protein that plays a key regulatory role in breast cancer metastasis. The results presented provide valuable insight into intercellular stromal-epithelial interactions between stromal and epithelial cells in the breast tumor microenvironment, highlighting how epithelial cells can harness stromal cell biology to facilitate their invasion and progression.

Methods

Primary culture

Following ethical committee approval and written informed consent, fresh specimens of human breast cancer were harvested at surgery and primary cell cultures prepared, as described previously [14]. Normal controls were obtained from reduction mammoplasty. The digested cell suspension was separated into organized, epithelial, and stromal fractions by differential centrifugation and cultured in selective media, as described [26]. Primary stromal cells were characterized by flow cytometry using the Guava EasyCyte™ HT and analyzed using FlowJo software. Cell type-specific antibodies were used including Tact/CD90, CD105, CD73, v-SMA, CD31, MUC1/CD221, CD34, and CyclinD1 (BD Pharmingen™). Appropriate isotype control antibodies were used and the results reflect the percent of positive cells compared with isotype controls. The level of expression of fibronectin activation protein (FAP) in tumor compared to normal stromal cells was also determined by real-time quantitative PCR (RQ-PCR) as described below.

Primary tumor epithelial cells (n = 6) were isolated from excised and epithelial cell fractions. Epithelial cell adhesion molecule (EpCAM) positive cell-enriched populations were retrieved from these single cell suspensions by positive selection using EasySep magnetic beads (StemCell Technologies Inc.), according to manufacturer’s protocol.

Culture of cell lines

The following breast cancer cell lines were included in this study: T47-D, ZR-75-1, MCF-7, BT-474 (oestrogen and progesterone receptor positive, ER+, PR+); MDA-MB-231, SK-BR-3 (ER−, PR−), and MCF-10A (non-tumorigenic). These were purchased from the ATCC and cultured in Leibovitz-15 (MDA-MB-231), RPMI-1640 medium (T47-D, ZR-75-1, BT-474), McCoy 5a (SK-BR-3), and Eagle’s minimum essential medium (MCF-7) media, each supplemented with 10% FBS, and 100 IU/ml penicillin G/100 mg/ml streptomycin sulfate. MCF-10A cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM/F12) medium supplemented with 5% horse serum, 20 mg/ml epidermal growth factor, 100 mg/ml cholera toxin, 0.01 mg/ml insulin, and 500 mg/ml hydrocortisone.
Collection of conditioned medium and CCL2 quantification

Conditioned media (CM), containing factors secreted by cells, was obtained from cultures. All cell populations were seeded in 6-well plates at a density of 2 x 10^5 cells per well in 2 ml DMEM containing 2% FBS (required for chronokine stability). DMEM was used to prepare media for all cell types to ensure that differences observed were not as a result of culture conditions. CM from coculture experiments was harvested after 72 h, centrifuged (1,000 g, 4°C), and stored at -20°C until required. CM from individual stromal cell populations was harvested after 24 h in culture and used neat/undiluted, as a chemoattractant in migration assays or as a growth medium in proliferation assays. CM from stromal cells was analyzed for CCL2 content using QuantiLite® Enzyme Linked Immunosorbent Assays (R&D Systems), following manufacturers’ protocol.

In vitro cell migration assay

Transwell® Permeable Support (Corning Inc., Sigma-Aldrich) were used to track migration of MDA-MB-231 cells in response to factors secreted by stromal cells, as described [14]. Briefly, MDA-MB-231 cells were inoculated into the insert, and their migration in response to stromal cell CM, serum-free basal medium (negative control), or basal medium with 1% FBS (positive control) in the well below was quantified. MDA-MB-231 migration in response to stromal cells in the presence of a CCL2 monoclonal antibody (40 ng/mL) was also quantified. Migration cells were counted in five fields of view per membrane using an Olympus BX50 microscope and image analysis software. Each experiment was repeated in triplicate, with results expressed as mean ± SEM.

In vitro cell proliferation assay

Cell proliferation was assessed using the ViaLight™ Plus Kit (Cambrex), and based on bioluminescent measurement of ATP. Breast cancer epithelial cell lines (MDA-MB-231 and T47D) were seeded into 96-well plates (6 x 10^3 cells per well) and allowed to adhere overnight. Medium was removed and substrates added. Test substrates included tumor or normal stromal cell CM, and increasing concentrations of sCCL2 (50–300 pg/mL) standards. Blank and complete medium were used as negative and positive controls, respectively. Plates were incubated for 48 h and ATP levels measured on a Luminoskan Ascent Lumino-meter (Thermo). Data represent the mean reading of 8 wells and are expressed as mean ± SEM.

Real-time quantitative PCR

Cells were homogenized in 1 ml of Qiazol Lysis reagent and total RNA isolated using the RNeasy® Mini Kit (Qiagen Ltd) as per manufacturer’s instructions. cDNA was generated using SuperScript III reverse transcriptase enzyme (Invitrogen) and amplified by real-time quantitative PCR (RQ-PCR) using the ABI Prism 7000 (Applied Biosystems). Taqman® Universal Master Mix and Gene Expression Assays (Applied Biosystems) designed for the target genes (FAP, CCL2, CRCA, CRAR, MRP11, Rk67, VII, 2, ELK 2), and control genes Mitochondrial Ribosomal Protein L12 (MRP12), and Pepsidlysephyl Isomerase A (PPIA) were used.

Due to the low yields from primary epithelial cell cultures, the TaqMan® PreAmp Cells-to-Ct™ Kit (Applied Biosystems) was used for gene expression analysis on these cells. Briefly, this protocol involves an intermediate amplification step between reverse transcription and RQ-PCR in which the cDNA is enriched for target genes. The comparative Ct method for relative quantification was used, allowing determination of the quantity of the target gene in each sample population normalized to endogenous control genes (MRP12 and PPIA) and compared to a calibrator, and was expressed in a linear form using the formula 2^ΔΔCt [27]. Gene expression levels in primary tumor epithelial and stromal cells were determined relative to the levels in epithelial and stromal cells from normal breast tissue harvested at resection mammoplasty. In the case of epithelial cell lines, levels were expressed relative to those of non-tumorigenic MCF-10A cells. In co-culture experiments, expression levels in the epithelial cell population post co-culture were expressed relative to expression levels in these cells cultured alone.

Co-culture of tumor stromal and epithelial cells

Primary tumor stromal cells (n = 6 individual donors A–F; luminal A n = 4, luminal B n = 2), or normal stromal cells (n = 4 individual donors) suspended in stromal medium were plated into T75 cm² flasks (3 x 10^6 cells per flask) and allowed to adhere overnight. Breast cancer epithelial cell lines (MDA-MB-231 or T47D) were seeded onto the monolayers of stromal cells at the same density. The individual cell populations were also cultured alone in parallel. Co-cultures were all established in stromal medium, so any changes observed could not be attributed to differences in culture conditions. Following direct co-culture adherent cells were washed twice with PBS and trypsinized into a single cell suspension and the epithelial fraction removed using EasySep® MACS™– magnetic beads (Stem Cell Technologies Inc.). RNA was extracted from...
retrieved epithelial cells and changes in gene expression resulting from their interactions with tumor and normal stromal cells identified by RQ-PCR, as described.

Growth of breast cancer xenografts in mice

Animal studies were carried out in accordance with experimental guidelines set out by the institutional ethics committee. Female athymic nude mice (Harlan Laboratories UK Ltd.) were implanted with 17β-estradiol 50-day slow-release pellets (Innovative Research of America), to support growth of estrogen receptor positive T47D cells. Mice were divided into three groups and given a subcutaneous injection of T47D cells alone (5.6 x 10⁵ cells), or T47D cells admixed with tumor stromal cells (1 x 10⁶) derived from two individual donors (n = 6 in each group). Tumors were measured weekly using callipers and volume estimated (volume (mm³) = length x width x height x 0.52). Following 10 weeks of tumor growth, animals were killed by CO₂ inhalation and tumors harvested. Tissue for immunostaining was immediately immersed in 4% paraformaldehyde for 24 h, transferred to 30% sucrose for 24 h, snap frozen in an isopentane/liquid nitrogen bath and stored at −80°C until required for cryosectioning. Tumor tissue harvested for retrieval of epithelial cells was immediately immersed in ice-cold culture media, minced using crossed blade scalpels and digested overnight using collagenase as described [14]. Epithelial cells were then retrieved from the mixed population using EpCAM⁺ magnetic beads. Cells were pelleted and stored at −80°C until required for RNA isolation.

Immunohistochemistry

Frozen tissue samples from xenografts were cryosectionned (3 μm sections) and allowed to dry at RT followed by rehydration in PBS-0.05% Tween-20. Following blocking of endogenous peroxidases, antigen retrieval was performed using citrate buffer. Sections were then analyzed using the Ventana Discovery™ machine with antibodies specific to CD31 (AbCAM), and Erzin (AbCAM). Once staining was complete sections were washed in warm soapy water, dehydrated in serial alcohol immersions, mounted using DPX mounting medium and examined using a Leica DFC 300 FX light microscope, with Leica Software, V 2.3.4.

Statistical analysis

Data were analyzed using the software package SPSS 15.0 and are presented as mean ± SEM of triplicate experiments. Results with a P < 0.05 were considered statistically significant. All tests were two-tailed. Levene’s test confirmed equal variance of observations in each group and permitted parametric data to be compared using a student’s unpaired t test. Normality was confirmed using the Kolmogorov–Smirnov test.

Results

Epithelial and stromal cell isolation and characterization

Primary breast stromal cells displayed a typical fibroblastic morphology and when characterized by flow cytometry (Fig. 1a) were shown to be positive for CD90 (≥95% positive, Fig. 1a), CD105 (≥95% positive, Fig. 1b), CD73 (≥45% positive, Fig. 1c), and alpha-smooth muscle actin (α-SMA), and negative for CD31 (≤2% positive, Fig. 1d), CD45 (≤5% positive), MT1/MMP (≤5% positive), and cytokeratin (≤2% positive, Fig. 1e). Tumor stromal cells were found to have higher expression of α-SMA (range 69–87% positive), a marker of activated fibroblasts (myofibroblasts), than normal stromal cells (range 2–68% positive).

In agreement with previous reports [28, 59], RQ-PCR analysis also revealed significantly higher expression of fibroblast activation protein (FAP) in tumor compared to normal stromal cells (Fig. 1f, P < 0.001). Epithelial cells were selected using magnetic beads, based on EpCAM positivity and were confirmed to be cytokeratin positive (≥95% positive). Levels of expression of CCL2 and its receptor CCR2, were determined in each cell population relative to the geometric mean expression of endogenous control genes, and values expressed relative to normal counterparts (2−ΔΔCt, Fig. 2). In the case of stromal cells, expression of CCL2 was significantly higher in each population of tumor stromal cells (n = 6, A–F) compared to mean CCL2 expression from n = 4 normal stromal cells isolated from bovine mammary tissues, (n = 4, 1.05 log fold increase P < 0.05, Fig. 2a). In contrast both tumor and normal stromal cells failed to express either isoform of the CCL2 receptor, CCR2A or CCR2B. Expression of CCL2 and CCR2 was also analyzed in primary breast cancer epithelial cells (n = 6, Fig. 2b). Relative expression of CCR2 was higher than the ligand (CCL2, mean 1.45 log RQ, CCL2; mean 0.78 log RQ) (Fig. 2b). CCL2/CCR2 expression analysis was also performed on five breast cancer epithelial cell lines and mean values expressed relative to endogenous MCF-10A cells (Fig. 2c).

Effect of direct co-culture on CCL2 secretion and expression

In vitro secretion of CCL2 was quantified when the cells were cultured individually and in direct co-cultures (Fig. 3a). The baseline of the graph represents the sum of each
Fig. 1 Primary stromal cell characteristics. Example of mammary stromal cell characterization by flow cytometry (a–c). Flow cytometry control set shown in white, with analytical samples shown in grey. Stromal cells were shown to be positive for CD90 (a), CD105 (b), CD73 (c), and negative for CD31 (d), and vimentin (e). RT-PCR analysis also revealed significantly higher expression of the chemokine activation protein (TAP) in tumor (n = 52) compared to normal (n = 2) stromal cells (F, P < 0.001).

cell population secreted when cultured individually, with each bar representing the amount by which the co-culture population differed from this. Following 72-h co-culture of breast cancer cell lines on a monolayer of stromal cells, CCL2 levels were significantly higher than that seen from the individual populations (mean increase for T47D+ tumor stromal cells: 4001 ± 1953 pg/ml, MDA-MB-231 + tumor stromal cells: 5055 ± 1284 pg/ml, P < 0.05). This effect was significantly higher than that seen when these cells were co-cultured with normal stromal cells (P < 0.05).

Following in vitro co-culture with primary tumor/normal stromal cells, epithelial tumor cells were removed using EpCAM magnetic beads, and expression of CCL2 analyzed. Levels of gene expression are expressed relative to levels detected in breast cancer cells cultured alone (C). CCL2 gene expression levels reflected protein secretion trends, with expression increased in both breast cancer populations following co-culture with stromal cells (Fig. 3b). This upregulation was significantly higher when co-cultured with tumor (mean fold increase T47D 9.07 ± 2.85, MDA-MB-231 8.41 ± 4.56 compared to normal stromal cells (T47D 4.03 ± 2.37 MDA-MB-231 2.41 ± 0.77, P < 0.05). In contrast to CCL2 expression, CCR2 expression levels were decreased following co-culture compared to the cells cultured alone.

Cell migration and proliferation

MDA-MB-231 showed significantly greater chemotaxis in response to tumor compared to normal stromal cells (Fig. 4a). This effect was subsequently blocked by the addition of a monoclonal antibody to CCL2 (range 27–64% blockade). Breast cancer cells also displayed a dose-dependent increase in chemotaxis towards commercial standards of CCL2, with similar results observed in SK-BR-3 and MCF-7 cells (results not shown).

There was no significant change in proliferation of breast cancer epithelial cells in response to factors secreted by tumor or normal stromal cells (Fig. 4b). Recombinant standards of CCL2 were also found to have no effect on cell proliferation (results not shown).

Expression of invasion/proliferation associated genes

In both breast cancer cell lines, expression levels of the invasion associated gene MMP11, was significantly
increased following co-culture with tumor compared to normal stromal cells (Fig. 5a, b; 0.05). While expression levels of the proliferative marker, KRT5, increased following co-culture with normal stromal cells, it decreased when the co-cultures involved tumor stromal cells. Expression levels of the invasion associated gene VGL2, and the oncogene, ERF1, were significantly upregulated in T47D cells following co-culture in a tumor specific fashion (Fig. 5a, b; 0.005).

In vivo co-culture

T47D cells were injected alone or co-injected with tumor stromal cells subcutaneously into nude mice. These tumor stromal cells were derived from two separate human invasive ductal breast carcinomas, Tum A Stro (Luminal A, Grade 3, T1, N0) and Tum B Stro (Luminal A, Grade 2, T1, N0). T47D cells co-mixed with tumor stromal cells exhibited a faster rate of growth and generated tumors of greater volume at the endpoint of 10 weeks than T47D injected alone (mean tumor volume ± SEM, T47D 23 ± 5 mm^3, T47D + Tum A Stro 118 ± 15 mm^3, T47D + Tum B Stro 65 ± 9 mm^3, Fig. 6a). Xenografts that developed in the presence of tumor stromal cells also displayed neovascularisation, confirmed by positive staining for the endothelial marker, CD31 (Fig. 6b, c, 20×), d (40×), while tumors formed from T47D cells alone were negative for CD31 (Fig. 6b, a (40×)). Mixed stromal-xenografts displayed greater intensity of Enza staining throughout the tumors and increased cytoplasmic localization of the protein (Fig. 6c, b, arrows) compared to epithelial xenografts which predominantly displayed membranous staining (Fig. 6c, a, arrows).

Upon tumor harvesting at necropsy, mixed tumor xenografts were digested and the T47D cells retrieved with epithelial specific beads. Subsequent analysis allowed identification of gene expression changes in these isolated epithelial cells resulting from their in vivo interaction with...
Fig. 3 Interaction of tumor stromal and epithelial cells results in increased CCL2 protein secretion and gene expression. a CCL2 secretion: the baseline of the graph represents the sum of what each cell population secreted when cultured individually, with each bar representing the amount by which the co-culture population different from this. Data presented represent mean ± SEM of triplicate experiments, using six individual tumor normal populations and four individual normal stromal populations. b Following in vitro co-culture of primary stromal cells with breast cancer cell lines, epithelial cells were retrieved using EpCAM+ magnetic beads, and expression of CCL2 analyzed. Data presented represent mean fold change values ± SEM of replicate experiments, and are expressed relative to levels detected in the breast cancer cells cultured alone (2-ΔΔCt).

Fig. 4 Interaction of tumor stromal and epithelial cells in vitro effect on migration and proliferation. a Tumor secreted by primary tumor stromal cells induce increased migration of breast cancer epithelial cells. This effect was subsequently blocked by the addition of a monoclonal antibody to CCL2. b A VidasCA assay was used to assess relative proliferation of breast cancer cells (MDA-MB-231 and T47D), in response to stromal cell CM (tumor stromal cells and normal stromal cells). Proliferation induced by complete medium was taken as 100%. Results are displayed as mean ± SEM of three cell lines ±SEM from triplicate experiments.

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tumor stromal cells (Fig. 6d). The epithelial cells displayed increased expression of the invasion and migration associated genes MMP11, VIL2, and ELK1 following in vivo coculture with tumor stromal cells (range 0.2–3.6 log fold increase, Fig. 6d). Interestingly, these cells also displayed increased expression of the proliferative marker K67, which was downregulated in the in vitro coculture set-up.

Discussion

The current study highlights potential mechanisms through which malignant epithelial cells can harness stromal cell biology to facilitate their invasion into the tumor microenvironment. There is accumulating evidence pointing to a pivotal role for chemokines in controlling migration, growth, and differentiation of tumor cells [30]. The current study shows that isolated primary tumor epithelial cells displayed elevated expression of CCL2, as well as its principal receptor, CCR2. In terms of breast cancer, the potential tumorigenic role of CCL2 and CCR2 is poorly defined to date. In the current study, primary tumor stromal cells had significantly higher expression of the CCL2 gene than normal stromal cells. CCL2 exerts its effects through a principal receptor, CCR2 [31], of which two isoforms (A and B) have been identified. CCR2 receptor expression has previously been shown in two cell lines, MDA-MB-231 and Tu7D [32]. The current study reports that along with these and other breast cancer cell lines, breast cancer epithelial cells isolated from fresh breast tumors express CCR2A, while both isoforms were undetectable in tumor stromal cells, suggesting a paracrine role for the chemokine. CCL2 was detected in the CM of all tumor stromal cells examined and in significantly higher quantities than normal stromal cells. Stromal cell invasion is an integral part of cancer cell invasion during metastasis, involving changes in cell cytoskeleton and adhesion [33]. In the current study factors secreted by tumor stromal cells induced migration of breast cancer epithelial cells, shown to be mediated at least in part by CCL2. Furthermore, direct contact between tumor stromal and epithelial cells induced a synergistic increase in CCL2 secretion and gene expression. The disparity between protein secretion and gene expression levels may be accounted for by post-translational modifications, which would not be detected at the gene expression level.

It is worth noting that in vitro and in vivo interaction with tumor stromal cells induced increased expression of VIL2 in epithelial cells. Erzza, the encoded product of the VIL2 gene, is a membrane-anchored leucine-rich repeat known to regulate cell adhesion and motility, as well as overall metastatic potential [34]. Moreover, mixed tumor xenografts not only achieved increased tumor volume, but also displayed increased cytoplasmic localization of Erzza protein, compared to tumors formed from T47D cells alone. The switch from apical to cytoplasmic Erzza localization has previously been shown to correlate with high tumor grade, high K67 expression (also shown in this study) and metastasis [35].

The presence of stromal cells in vivo resulted in positive staining for CD51, indicating active neovascularization within tumor xenografts [36], a known effect of CCL2 [17, 21]. Emphasis must be placed on the fact that in this study baseline gene expression levels were analyzed in primary breast cancer epithelial cells isolated from fresh tumors; however, to support replicates of all experiments performed, breast cancer cell lines were employed in functional experiments.

The results presented also show that interaction with tumor stromal cells induced MMP11 upregulation in breast cancer epithelial cells, with a similar degree of upregulation in vivo and in vitro. MMP11 has been shown to promote tumor progression [37], and it upregulated in invasive

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Fig. 6 Interaction of tumor stromal and epithelial cells in vivo. T47D cells were injected alone or co-injected with tumor stromal cells subcutaneously into nude mice. A Mice stromal epithelial xenografts exhibited increased tumor volumes. Data represent mean ± SEM for n = 6 in each group. B Xenografts that developed in the presence of tumor stromal cells (a<0.05), (a<0.025), (a<0.001) stained positive for TGFα, indicating neovascularization, while those comprised of epithelial cells alone did not (a>0.4), a Mice stromal epithelial xenografts displayed greater invasive. Enriched and increased cytoplasmic localization of the protein (b), compared to those comprised of epithelial cells alone. Whole mount immunohistochemistry (c) staining was visible. d Upon harvesting, tumors were digested and T47D cells were retrieved from mixed xenografts with epithelial specific filters. Data are expressed as the ratio of T47D:stromal cells isolated from tumors formed from T47D cells alone. Each bar represents mean ± SEM for both groups (Tom A: Steh and Tom B: Steh, n = 6 in each group).

Relative to in vivo ductal carcinoma [4]. Furthermore tumor stromal cells also induced increased expression of ELK1 in T47D cells in vitro and in vivo. ELK1, a member of the Ets oncogene family, promotes tumor progression, and is critical to regulation of cell proliferation and apoptosis [38]. While ELK1 expression was increased in vitro (six-fold increase), the level of increase was markedly higher in vivo (54-fold).

A challenge in breast cancer research is the availability of models that faithfully reflect the complexity of the disease. The majority of in vivo xenograft models are homologous, involving the introduction of breast cancer cell lines alone into the mouse microenvironment [39]. In this and other recent studies [7, 40], breast cancer epithelial cells were admixed with tumor stromal cells isolated from human breast tumors and introduced into mice, forming heterologous 3-dimensional xenografts which more accurately simulate stromal–epithelial interactions in the tumor microenvironment. This is the first study to retrieve and analyze epithelial cells following in vivo co-culture. While the majority of targets showed similar trends in both in vitro and in vivo models, the disparity in some results can be explained by the fact that cultures grown on a non-physiological 2-dimensional substratum lack exposure to components of the extracellular matrix that are present in the 3-dimensional in vivo microenvironment [40]. Given the heterogeneity of breast cancer, no individual model reflects all aspects of the disease, however, every attempt
should be made to reflect in vivo events and models that focus on epithelial cells alone fall short on this.

Understanding the dynamic and reciprocal crosstalk between stromal and epithelial cells will deepen our knowledge of the tumorigenic process, and may also facilitate exploitation of stromal-epithelial interactions as valid targets for novel therapies.

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