The clinical and prognostic use of circulating tumour cells in breast cancer

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By

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Abstract

Adjuvant therapies such as endocrine or cytotoxic chemotherapy have been demonstrated to improve overall survival in early breast cancer patients. A blood test to monitor patients at risk of relapse is needed to identify those patients who would benefit from these treatments and those for whom it is not necessary. This is in favour of detecting disseminated tumour cells (DTCs) from painful bone marrow aspirates, currently the gold-standard method for detecting minimal residual disease (MRD). The use of circulating tumour cells (CTCs) enriched from the blood was investigated for this purpose along with their characterisation in the metastatic setting to enable individualised therapy.

Sixty-four primary breast cancer patients were followed up for up to 12 years post surgery for any MRD present. This analysis looked at measurements of DTCs in the bone marrow, CTCs in the blood and circulating-free DNA (cfDNA) in the plasma over the follow up period. Patients who had involved lymph nodes at surgery, were significantly more likely to have CTCs present than low risk patients with no nodes positive, (70% compared to 39% respectively, $p = 0.042$). Our analysis also looked at the relationship of cfDNA to DTCs and CTCs. An inverse relationship of cell death in the blood (manifesting as blood cfDNA) to bone marrow DTCs by qRT-PCR was apparent. This may be due to tumour dormancy mechanisms - cycles of tumour cell proliferation and cell death occurring in the bone marrow, evidence not shown before in patient samples. Combined use of these markers could therefore be used as a monitoring system for impending metastatic disease and a rationale for further treatment.

We also participated in a multi–centre study to assess the effects of lapatinib; a targeted therapy against two members of the human epidermal growth factor receptor family (EGFR and HER2). This was in advanced breast cancer patients and used CTCs as a surrogate marker. Our study selected patients on the basis of EGFR positivity in CTCs that were present in the blood. Four out of 12 patients (33%) demonstrated an initial decrease in the number of EGFR positive CTCs in response to Lapatinib, however this was limited and all patients were taken off study with progressive disease. We also explored a novel method in development to detect viable CTCs. This used an *in situ* hybridisation method to amplify signals from mRNA transcripts of tumour markers in CTCs. The use of CTCs is a very useful and promising tool for studying both the biology of breast cancer, and also as a non-invasive analytical tool in the clinical setting to gain predictive and prognostic information.

Statement of originality

Unless otherwise stated in the text, this thesis is the result of my own work.

Patient samples

Patient blood and bone marrow samples for the minimal residual disease study were collected by our research nurse at Charing Cross Hospital, Becky Ward. Patient samples for the clinical trial of lapatinib monotherapy were collected by Miranda Avery and Laura Woodley at Charing Cross Hospital. Professor Justin Stebbing also coordinated the collection of research samples. Other blood samples for the CTCscope method optimisation were collected by Dr Jon Krell and Dr Adrian Zebrowski.

Chapter 3

The first two blood samples from each patient for CTC assessment was performed by Sabine Riethdorf at University Medical Center Hamburg-Eppendorf as part of the initial pilot CTC study. The remaining blood and bone marrow samples were all processed and analysed at Hammersmith hospital.

Chapter 4

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Chapter 5

The RNAscope assay was developed and optimised by Advanced Cell Diagnostics, Inc, US as part of a collaboration to use this method for detecting CTCs (CTCscope). The cell preparation method was performed as part of this collaboration.

Chapter 6

The CTCscope assay and RNAscope assay were performed by Advanced Cell Diagnostics, Inc, US. The lapatinib trial coordination and blood collection was performed by Miranda Avery and Laura Woodley.

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Abbreviations

ABL – Abelson oncogene
ACIS – Automated cellular imaging system
ALDH1 - aldehyde dehydrogenase 1
APC – Allophycocyanin
ASCO – American Society of Clinical Oncology
BBD – Benign breast Disease
CEA – Carcinembryonic antigen
cfDNA - circulating-free DNA
CK – Cytokeratin
CR – Complete response
CT - Computerised tomography
CTC - Circulating tumour cell
DAB - 3,3' – diaminobenzidine
DAPI - 4',6-diamidino-2-phenylindole
DCIS – Ductal carcinoma *in situ*
DFS – Disease-free survival
DMEM - Dulbecco's Modified Eagles Medium
DMSO - Dimethyl sulphoxide
DNA - Deoxyribonucleic acid
DTC - Disseminated tumour cell
ECOG – Eastern Cooperative Oncology Group Status
EGF - Epidermal growth factor
EGFR - Epidermal growth factor receptor or HER1
ELISA – Enzyme-linked immunosorbent assay
EMA – Epithelial membrane antigen
EMT – Epithelial to mesenchymal transition
EpCAM - Epithelial cell adhesion molecule
ER - Oestrogen receptor
FCS - Foetal Calf Serum
FDA – Food and drug administration
FFPE – Formalin fixed paraffin embedded
FISH – Fluorescence *in situ* hybridisation
FITC - Fluorescein isothiocyanate
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GTC - Guandine thiocyionate
hEGF - Human Epidermal growth factor
HER2 - also known as ErbB-2, a member of the EGF family of receptors
IC_{50} - half maximal inhibitory concentration
ICC – Immunocytochemistry
IDC – Invasive ductal carcinoma
IF - Immunofluorescence
IHC - Immunohistochemistry

ILC – Invasive lobular carcinoma
LN - Lymph node
MAPK - Mitogen activated protein kinase
MET – Mesenchymal to epithelial transition
miRNA – micro-RNA
MMLV - Monkey Murine Leukaemic Virus
MUC-1 – Mucin 1 protein
MRD – Minimal residual disease
mRNA – Messenger ribonucleic acid
NHBRP - National Health Service breast screening programme
OS – Overall survival
PBMC – Peripheral blood mononucleocytes
PD – Progressive disease
PE – Phycoerythrin
pEGFR - Phosphorylated epidermal growth factor receptor
PET - Positron emission tomography
PFS – Progression-free survival
PI3 - Phophatidylinositol 3-pathway
PR – Partial response
PR - Progesterone receptor
PTEN - Phosphatase and tensin homolog
qRT-PCR - quantitative reverse transcriptase-polymerase chain reaction
RECIST - Response Evaluation Criteria In Solid Tumours
RNA – Ribonucleic acid
RT-PCR - Reverse transcriptase-polymerase chain reaction
SD – Stable disease
SERMs - Selective estrogen receptor modulators
TGFα - Transforming growth factor
TNM staging system – Tumour, Nodes, Metastasis
UbC – Ubiquitin C
VEGF - Vascular endothelial growth factor

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1.0 Introduction

1.1 Breast cancer – background

Breast cancer is now the most common cancer in the UK with an average of 125 women being diagnosed every day and around 45,000 new cases every year (1). Despite the use of mammographic screening to detect the disease at an earlier stage and the use of a range of adjuvant systemic therapies to improve prognosis, it remains a significant public health issue with more than 12,000 deaths in the UK in 2008. The National Health Service breast screening programme (NHBRP) was set up in 1998 and is estimated to save 1400 lives a year by picking up breast cancer at a very early stage using mammography (low dose x-rays) (2). This is in addition to the huge advances in treatments over the last few decades that have increased 5 year survival rates in England to 83% (3). The incidence of breast cancer is increasing however, and in the last 10 years it has risen by 5% with approximately 1 in 9 women likely to suffer in their lifetime.

The principle causes and risk factors for breast cancer are increasing age and a strong family history. It is more common in women over 50 years of age, with around 80% of cases diagnosed in this age group with rates generally increasing with age until the menopause (1). The presence of a mutation in the breast cancer susceptibility genes BRCA1 and BRCA2 increases risk significantly with 84% and 74% of women developing breast cancer respectively by the age of 80 years (4). Lifestyle factors that are known to have an affect are numerous and include the moderate intake of alcohol, nulliparity or having children later in life and the use of exogenous hormones for extended periods, for example the use of hormone replacement therapy or using the contraceptive pill (5-7).

1.2 Diagnosis and staging

A diagnosis of breast cancer follows the histopathological assessment of a needle biopsy from the breast lump. The breast lump is either picked up by mammography, ultrasound and/or by a palpable lump present. Pre-operative staging is then performed if an early invasive tumour is present. This requires ultrasound of the axilla to identify if morphologically abnormal lymph nodes are present followed by an ultrasound-guided needle biopsy. The sentinel lymph node

biopsy is used increasingly to avoid unnecessary invasive surgery to remove many lymph nodes (8). This involves removal of the lymph node nearest to the tumour to indicate if the cancer has spread locally to the lymph nodes. This local spread is one of the most important prognostic factors in breast cancer as it indicates the cancer has the capacity to metastasise and gives a worse prognosis for the patient (9). The TNM staging system is used to classify cancer spread and is based on three factors: the size of the tumour (T), the extent of lymph node metastases (N) and the presence of cancer that has spread to sites other than the breast or the lymph nodes (M) (10). Other post-operative assessments include the hormone receptor status, for example the oestrogen receptor (ER) and the progesterone receptor (PR) as well as the histological grading. The status of the human epidermal growth factor receptor 2 (HER2, discussed later) is also routinely measured. Adjuvant treatment options are then considered based on these prognostic and predictive factors.

1.3 Breast cancer types

The most common type of very early, non-invasive breast cancer is ductal carcinoma *in situ* (DCIS). This is when a tumour mass is formed in the milk ducts of the breast but has not spread through the duct walls. The other early type of early disease is lobular carcinoma in situ (LCIS). Although less common it forms in the lobules of the breast and is thought to be a marker of increased risk of breast cancer rather than a definitive precursor of malignancy (11). Invasive ductal carcinoma (IDC) is the commonest form of breast cancer accounting for around 80% of cases. It occurs when cancer has spread from the ducts and gained the capacity to invade the surrounding tissue. Similarly, invasive lobular carcinoma is also disease that has spread or infiltrated from the lobules, it is less common however, and accounts for approximately 10% of cases (1). Most invasive breast cancers diagnosed are adenocarcinomas derived from glandular tissue. The remaining cases are made up of many rare breast cancers including inflammatory, medullary, tubular, papillary and basal cancers.

1.4 Treatment

Treatment for breast cancer is a multidisciplinary activity involving surgery, radiotherapy, chemotherapy, hormone therapy and other targeted biological treatments. Generally, the earlier the diagnosis, the more effective the treatment will be. The treatment regimen will be very specific to the individual and will depend on many factors such as menopausal status, type of breast cancer, size of the tumour, stage, grade and hormone and growth factor receptor status. Treatment of breast cancer is moving in the direction of personalised, tailored therapy that also uses molecular information from tumours to indicate the best therapies. The increased number of trials of sequential or novel combination treatments such as targeted and cytotoxic therapies will also ensure the most effective treatment and also seeks to combat resistance to single agents (12). The development and use of biomarkers to predict the response to therapy is of great importance to enable the tailored management of disease.

Surgery is the primary treatment for localised breast cancer and usually requires a wide local excision of the tumour (lumpectomy), a larger part of the breast removed (quadrantectomy) or a mastectomy. Breast-conserving surgery in combination with radiation is an effective alternative to a mastectomy in many cases (13). Radiation often follows surgery as an adjuvant treatment to kill any cells left at the tumour site and to reduce chances of a local recurrence.

Chemotherapy is given in the neo-adjuvant setting to shrink tumours prior to surgery, in the adjuvant setting after surgery to kill any remaining tumour cells and in the palliative setting for patients with progressed metastatic disease to limit or control the disease. Several types of chemotherapy are often given together for maximum effect and are cytotoxic to cells in a variety of ways. For example one of the most common regimens for early invasive breast cancer is adriamycin (doxorubicin) and cyclophosphamide (AC). Doxorubicin is an anthracycline therapy; this type of cytotoxic therapy forms the central component of many adjuvant combination therapies for breast cancer (14). It inhibits the enzyme topoisomerase II stopping replication of DNA (15). Cyclophosphamide is an alkylating agent that forms a metabolite that cross-links with DNA to kill tumour cells. There are many other regimens that are given to patients in an individualised basis; this depends on a number of factors including the clinical characteristics of

the tumour, the lymph node involvement or metastatic spread and the general health and age of the patient.

1.5 Endocrine therapy

Breast cancer is predominantly hormone dependent and is the reason why anti-endocrine therapies are such critical treatments for slowing the growth of tumours in the breast. The importance of oestrogen for fuelling growth was shown more than 100 years ago when Beatson demonstrated that certain patients achieved a remission from metastatic cancer following removal of their ovaries (16). This prevented the production of oestrogen and the fuel for cancer growth. The therapies to prevent this process are numerous and involve either blocking the oestrogen receptor or lowering the amount of oestrogen produced in the body. Tamoxifen is one of the most successful therapies against breast cancer and has been used for more than 30 years. It acts by binding to the oestrogen receptor, blocking oestrogen from binding and is still recommended for pre-menopausal women with early breast cancer (17). It is usually given for 5 years to prevent any remaining tumour cells from proliferating and causing metastatic disease. Post-menopausal women who no longer have oestrogen production from the ovaries benefit from a newer type of endocrine therapy; aromatase inhibitors block oestrogen synthesis and clinical trials have shown they may be more effective compared to Tamoxifen (18). Clinical trials have investigated the use of combination therapies for example an aromatase inhibitor with targeted biological therapies for further efficacy with low toxicity (19). Other anti-endocrine treatments include ovarian ablation or suppression.

1.6 Targeted biological treatments

Biological or targeted therapies in breast cancer involve targeting specific cancer cell growth or division pathways and are becoming more common as new information about the molecular signatures of cancer becomes apparent. This is important for the discovery of efficacious cancer drugs that are more likely to be successful and that have minimal levels of toxicity that are associated with chemotherapy. The increase in the armamentarium of treatments against breast cancer is also beneficial so that alternatives are available if a therapy is not working. This is particularly significant when resistance to chemotherapy and endocrine therapy occurs. The

types of targeted therapies include anti-angiogenesis therapies such as bevacizumab, anti-growth factor receptor therapies (discussed below) and anti-cancer molecules against specific down-stream components of these pathways along with novel combinations of these drugs with standard therapies (20).

1.7 The epidermal growth factor receptor family

The ErbB family of receptor tyrosine kinases is part of a potent growth factor pathway and one of the most studied biological targets for anti-cancer therapies. They consist of four distinct receptors; the epidermal growth factor receptor (EGFR), ErbB-2 (HER2-neu), ErbB-3 (HER3) and ErbB-4 (HER4). These have an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and an intracellular tyrosine kinase domain (21). Growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF-α) and amphiregulin all bind to members of the ErbB family of receptors. This causes complex interactions including the formation of homo and heterodimers of receptors. After dimerisation, autophosphorylation of tyrosine residues occurs with subsequent triggering of signalling cascades, including the PI3 kinase/Akt and the MAPK kinase pathways (Figure 1.1). These promote tumour survival effects such as proliferation, angiogenesis, migration and the inhibition of programmed cell death (22).

1.8 The role of HER2 in breast cancer

HER2 is one of the most important targets in breast cancer alongside the oestrogen receptor. It plays an important role in the signalling of the epidermal growth factor receptor family even though it has no known ligand; it is thought to modulate signals after ligand binding to other HER-family members (23). For example, EGFR preferentially dimerises with HER2 when activated (24). The *HER2* gene is amplified in around 30% of breast tumours causing overexpression of the protein on the cell surface (25). This causes potent growth and survival effects and is one of the reasons HER2 has been shown to be a significant marker of prognosis in breast cancer and is associated with a worse prognosis (26). A monoclonal antibody called trastuzumab (Herceptin, Genentech, Inc, CA) was developed and humanized against the HER2 protein to inhibit these potent growth signals in tumour cells (27). This was found to be generally well tolerated as a single agent and was active in women with metastatic breast cancer with HER2 amplified

tumours (28). More recently, it was found to increase the clinical benefit to patients when combined with chemotherapy (29).

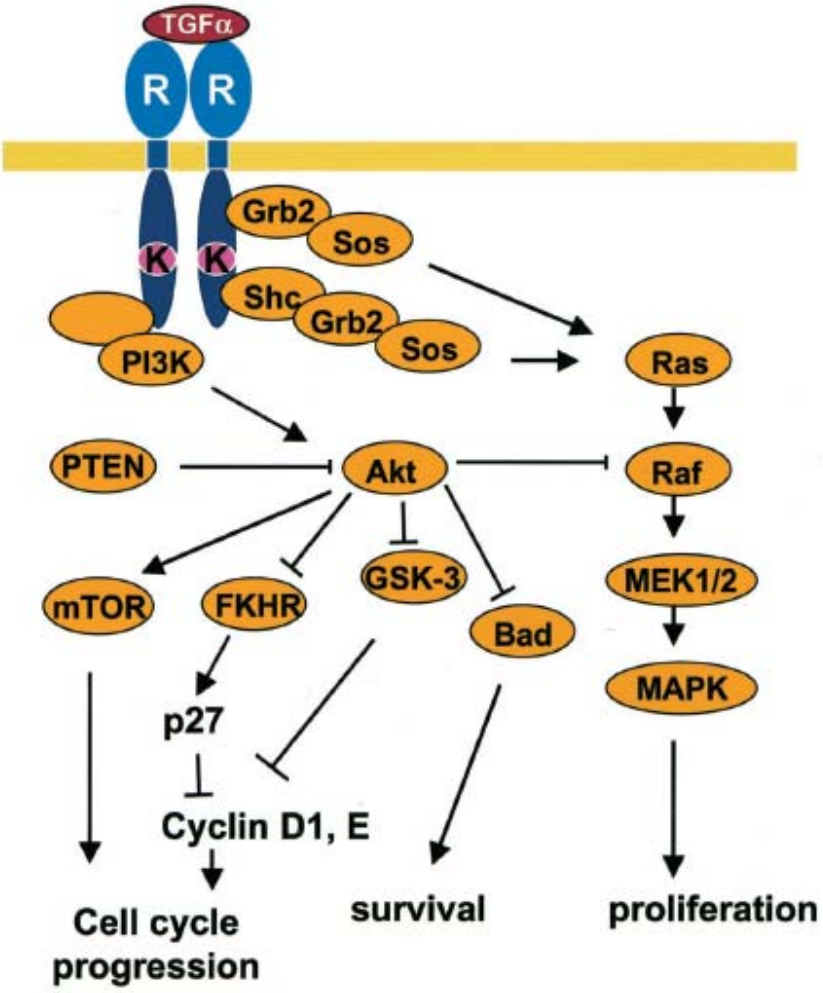


Figure 1.1: The signalling pathways involved in the human epidermal growth factor receptor family. Mitogenic signalling causes subsequent tumour promotion through activation of cell cycle progression, survival and proliferation. Adapted from (30).

1.9 EGFR in breast cancer

The huge array of drugs in development against targets in the EGF pathway is an indicator of the pivotal role it plays in tumourigenesis. The prognostic implications of the detection of EGFR in breast tumours are however, not as clear as that of HER2. A recent large scale study of 2567 breast tumours found EGFR to be over-expressed in 18% of breast tumours using a ligand binding

assay (31). A review of previous studies has found wide-ranging values of between 14 – 91% due to differing methodologies. Many of these studies also only found a weak association with progression free or overall survival (32). Some clinical trials of EGFR inhibitor therapies did not recruit patients on the basis of EGFR expression due to this lack of consensus regarding its measurement (33, 34). The over-expression of EGFR is evident in many other types of solid tumour and has been used to select patients for therapy with some success. A monoclonal antibody against EGFR (Cetuximab) was found to have activity against EGFR-expressing colorectal cancers (35). Furthermore, patients with KRAS wild type tumours benefited the most from treatment (36). Benefit from anti-EGFR therapy is also demonstrated in small cell lung cancer patients but mostly to those who have been selected based on the presence of somatic EGFR mutations (37). No such mutations have been found to be common in breast cancer which may partly explain the limited efficacy of anti-EGFR inhibitors used as single agents.

One of the most advanced of the EGFR inhibitors in clinical development is the small-molecule tyrosine kinase inhibitor Lapatinib (GW572016/Tyverb; GlaxoSmithKline, Research Triangle Park, NC) that targets both EGFR and HER2. Clinical trials have shown promising results, a study using Lapatinib as first line therapy in HER2 positive breast cancer patients showed 31% of 138 patients derived clinical benefit (38). Combination therapy trials have shown improved efficacy, Lapatinib with capecitabine markedly decreased the risk of disease progression in advanced HER2-positive patients as compared with capecitabine alone (39). Lapatinib also had a synergistic effect when given with trastuzumab to HER2 positive patients (40). In contrast, studies of Lapatinib monotherapy given to chemotherapy-refractory patients who had four or more lines of prior therapy only had a modest effect in HER2 positive patients and no effect in HER2 negative disease (41). Studies looking at the effects of Lapatinib treatment on cells have shown that inhibition of growth occurs in a number of EGFR and HER2-overexpressing cell lines from a variety of tumour types (42). When the relative HER2 and EGFR expression of 61 human normal and tumour cells lines were compared with the level of Lapatinib inhibition, the simultaneous consideration of EGFR and HER2 expression as well as tissue type yielded the best determinants for Lapatinib response (43). These and other studies, however, found Lapatinib to be more

potent in cell lines overexpressing HER2 (40). The contribution of EGFR to response to Lapatinib is poorly understood and is one of the areas investigated in this thesis.

1.10 Breast cancer subtypes and genomic signatures

The conventional prognostic indicators described above do not provide much information about the complex biology of breast cancer or the molecular and cellular heterogeneity present. One study identified several breast cancer subclasses that further defined the biology and type of breast tumours that occur. These included a basal–epithelial group or triple negative type for ER, PR and HER2, a HER2–overexpressing group, a normal–like group and a hormone receptor positive luminal A and luminal B subtype (44). This study also showed that patients with a basal subtype had a poor prognosis. Further to this, other studies have attempted to use a gene signature to categorise breast cancers and assess the likelihood of recurrence based on tumour biopsies. MammaPrint (Agendia) used a 70 gene signature to predict the prognosis of patients by looking at many genes including those involved in cell cycle, invasion, metastasis and angiogenesis (45). It is thought this signature may be able to distinguish those patients who do not require adjuvant chemotherapy and the corresponding toxicities from those who do (46). Another signature of interest is the Oncotype DX (Genomic Health) that uses a panel of 21 genes to predict the risk of recurrence (high, intermediate or low risk) to patients with ER positive tumours with no involved lymph nodes, treated with Tamoxifen (47). These assays provide valuable guidance for clinicians to select appropriate therapy, however, they do not provide 'real time' information about cancer progression in individual patients and they cannot always predict the prognosis of a patient.

1.11 The biology of metastasis

To understand the steps of cancer progression and to study this process closely it is important to find out the mechanisms involved and to study metastatic lesions. Breast cancer begins as a local disease but can spread via the lymph nodes to distant organs. This is very challenging to treat and is often the main reason for death. It is not always clear which patients will go on to develop metastatic disease even with the current prognostic indicators available. Clinically it is important to be able to identify those patients who are at risk of further disease and to give them

therapeutic intervention early enough to prevent spread. A number of processes must occur for successful dissemination and seeding of tumour cells, including invasion when cells lose cell to cell adhesion, migration into the local tissue and blood or lymph vessels, survival in the circulation followed by extravasation through blood vessel walls and colonisation of new sites for a secondary tumour to grow (Figure 1.2). The most common theory for cancer spread is that of a late event which is acquired as the tumour grows and undergoes more mutations. This means the tumour has to have been present for a sufficient period of time to grow and to gain these mutations. However, recent evidence suggests this process occurs at an early stage of tumour growth being an intrinsic component of the cancer cell (45, 48, 49).

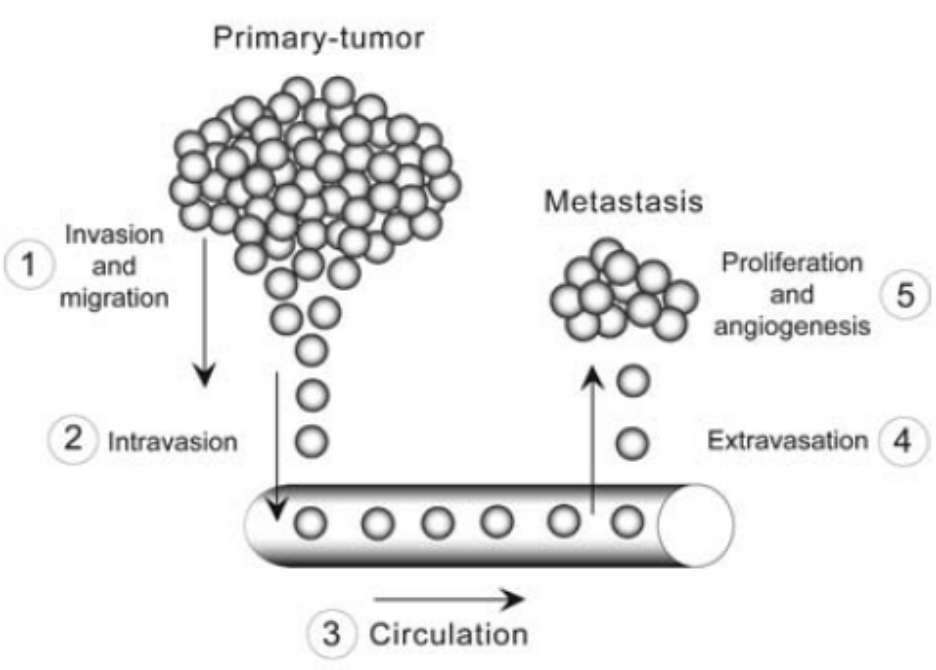


Figure 1.2: The five steps of the metastatic cascade (adapted from reference (50).

Evidence of the intrinsic capability of tumour cells to metastasise was found when untransformed mammary cells introduced into mice developed pulmonary metastatic lesions after being switched to a tumourigenic phenotype (48). In addition, pre-malignant cells transplanted into mice are able to disseminate forming micrometastases in bone marrow and lungs (51). Since the number of disseminated tumour cells was similar in small and large tumours in both patients and mouse models, a longer period of time for a tumour to grow and mutate is not necessary for

metastasis to occur. Additional evidence also indicates that the gene expression signature of the primary tumour predicts the clinical outcome in breast cancer patients (45). Genes involved in processes such as invasion, metastasis, angiogenesis and signal transduction are significantly upregulated in the primary tumour of the poor prognosis group (45). Twist, a transcription factor involved in embryonic development, has been found to be essential for the metastatic process as it induces epithelial to mesenchymal transition (EMT) (52), (53). These early developmental signals are thought to trigger the transcription of genes promoting the multiple steps of the metastatic process after early dissemination of tumour cells. Although both 'early' and 'late' hypotheses for the development of metastasis seem to be mutually exclusive, it has been recently proposed that this may not be the case as processes acting both at early and late stages of cancer progression could contribute to the process of metastasis. Metastasis may not only involve late progression of disease in terms of mutations and heterogeneity but also be an intrinsic trait of tumour cells. These processes together would ensure the multi-step process of metastasis can occur (54).

Another re-emerging area of research is to look at the microenvironment of the site of metastasis and the tropisms necessary for tumour cells to seed at a different location to the primary site. This goes back to Paget's seed and soil hypothesis in 1889 that proposed metastasis to specific organs is due to a cross-talk between the cancer cells (the seeds) and the organ in question (the soil) (55). It is known that malignant cells interact with other cells to be able to orchestrate the metastatic process of invasion and intravasation as well as other complex phenomena such as the recruitment of bone marrow cells to direct tumour dissemination (54). The study of these metastatic processes in mouse models is critical to further our understanding because *in vitro* models do not recapitulate the complex tumour/microenvironment interaction. The main strategies are firstly, the use of genetically engineered models of cancer and secondly, transplantable, xenograft models in immunocompromised mice. This enables the step-wise process of metastasis to be manipulated and explored *in vivo*. Although there are problems with the applicability of mouse models due to different genetic backgrounds to humans as well as having an incomplete immune system, recent models have sought to overcome this. One example used a breast tissue-derived orthotopic and metastatic (BOM) mouse model of breast

cancer. Normal human tissue was implanted into an immunocompromised mouse first to attempt to recreate the human mammary microenvironment. Human breast cancer cells were subsequently inoculated into the implants. This imitated tumour progression and metastasis because the breast cancer cells were able to grow in the normal tissue environment and metastasize to distant human tissues implanted in the mouse (56). In the future it is hoped that additional sophisticated animal models will be able to explore many unanswered questions such as that of metastatic dormancy and the role of cancer stem cells.

Cancer stem cells can play a role not only in the intrinsic capability of the tumour cell to metastasise but also to enter a state of dormancy. These dormant cells may then gain further aggressive properties once in the appropriate metastatic niche and achieve the heterogeneity and metastatic potential at these sites (57). As previously mentioned, both EMT and MET (mesenchymal to epithelial transition) play important roles in embryogenesis and development by forming different tissue types. The process has also been linked to cancer and cancer stem cells. One study used immortalised human mammary epithelial cells with inducible EMT which resulted in the acquisition of mesenchymal traits and the expression of stem cell markers. This showed that EMT also created stem cell properties and may be a factor in the metastasis of tumour cells with a self renewal or dormancy capability (58). Other factors at work in the process of metastasis include; transformation, dysregulation of apoptosis, tissue invasion, motility and angiogenesis. This process remains poorly characterised and is one reason why metastatic disease is so difficult to treat. One essential, commonly accepted view about cancer progression however, is that the earlier it is detected the more curable it is.

1.12 Micrometastases and their detection

Signs of the early spread of disease often go undetected by current high-resolution imaging technologies such as PET (positron emission tomography) scanning. This is due to micrometastases or single disseminated tumour cells that are too small to visualise. It is known that 30% of women have these disseminated tumour cells (DTCs) in their bone marrow at presentation of breast cancer, confirming an early metastatic event (59). The first record of single circulating tumour cells (CTCs) found in a distant site away from the primary tumour site was by

Ashworth in 1869 (60). The detection of these occult cells was then hampered for many decades by the lack of sensitive and specific methodologies and markers to distinguish epithelial-derived tumour cell in amongst several million nucleated blood or bone marrow cells. This in itself is a complex problem and is the reason why it has taken so long for any useful technique to be employed and the reason why it is still being researched so intensively now. Recent evidence suggests that even the most current sophisticated and advanced methods lack sensitivity and specificity to identify all occult tumour cells in a given blood or bone marrow sample (61).

It was around 30 years ago that sensitive immunocytochemical (ICC) techniques were developed to detect micrometastases or single tumour cells in the blood and bone marrow. One of these studies investigated the detection of an epithelial tumour marker called epithelial membrane antigen (EMA) in histological sections of liver, lymph nodes and bone marrow. The bone marrow had single tumour cells present (62). Molecular based assays followed later with the introduction of the polymerase chain reaction in the 1980s. Recent methods still use these two techniques as the basis of detection with increasingly sophisticated ways to enrich the cells beforehand. It is important to enrich and isolate the cells from the blood as much as possible to be able to phenotype them and to make sure they are of epithelial, tumour origin. Most methods use an enrichment process before detection. Two methods are mostly employed for this, the first is density-gradient centrifugation using a Ficoll–based separation liquid and the other is immunomagnetic technology. There are also numerous other novel technologies to achieve this, which are summarised in Table 1.1.

Density-gradient centrifugation was used in the early days of minimal residual disease detection and remains one of the most commonly used methods. The advantages are that it is a relatively easy and inexpensive technique to perform. The disadvantages include the loss of rare tumour cells and also the low purity of the sample (63). Recent commercial products have used tubes with extra features like a frit to separate the blood from the Ficoll to prevent the loss of cells, for example the ACCUSPIN™ System Histopaque® tube and the Vacutainer® CPT™ tube with a gel barrier layer in it for the same purpose. These methods have sought to make enrichment of rare cells easier and more routinely performed. The use of immunomagnetic or antibody-affinity

technology has been utilised in a number of ways for the further improvement of CTC detection. One approach discussed in detail later involves the conjugation of antibodies to epithelial markers to magnetic beads or particles to draw the cells out of the blood (64, 65). A recent, highly advanced approach uses a microchip device with anti-EPCAM antibody-coated microposts or coated surfaces to capture CTCs. This enables the direct enrichment of CTCs from blood as it flows through tiny channels for CTCs and in addition, allows further characterisation (66, 67).

Many other approaches exist to detect CTCs, one method uses cell filtration which is based on the larger size of tumour cells compared to blood constituents. A method called ISET (Isolation by size of epithelial tumour cell) uses a polycarbonate membrane with 8 μm pores to trap CTCs. This enables the further cytomorphological assessment and characterisation of CTCs and is a promising method (68, 69). Other novel methods to enrich CTCs include the use of intravital flow cytometry to label and visualise tumour cells in the vasculature of mice (70). Optimisation of this method is required for human use however. Alternative approaches to detect viable cells have included using an attenuated adenovirus to label CTCs by targeting only telomerase-specific, viable CTCs. Once infected, the virus is able to replicate and express green fluorescent protein for visualisation on a fluorescent scanning microscope (71). This is an interesting method that requires further study. Another approach relying on the viability of cells uses a modified version of an ELISA, using patches or spots to detect proteins from single cells. The detection of proteins associated with metastasis is used for this purpose and is a unique detection tool (72). These assays demonstrate the innovative ways CTCs can be detected. Some of the recently developed methods to detect micrometastases are summarised in Table 1.1.

Table 1.1: Recent methods for the detection of disseminated and circulating tumour cells from the bone marrow and blood

Method	Enrichment method	Immunocytochemical or molecular based	References
CellSearch™ System	Immunomagnetic using anti-EPCAM coated iron particles	Immunocytochemical	Allard *et al*., 2004 (73)
CTC chip/HB chip	Immunomagnetic using anti-EPCAM coated posts	Immunocytochemical	Nagrath *et al*., 2007 (66) Stott et al., 2010 (67)
Scanning fluorescent microscope	Ficoll gradient or filtration	Immunocytochemical	Ntouroupi *et al*., 2008 (74)
Multiphoton intravital flow cytometry	None	Immunocytochemical labelling of CTCs in *vivo*	He *et al*., 2007 (70)
MACS®	Immunomagnetic using anti-EPCAM or CD326 coated beads for negative or positive selection	Both	Wong *et al*., 1995 (64)
Filter-based microdevice	Size based filtration	Immunocytochemistry	Lin *et al*., 2010 (75)
ELISPOT	Immunomagnetic using negative depletion of CD45 positive cells	Immunocytochemical/immuno-assays	Alix-Panabières *et al*., 2005 (72)
Adnatest	Immunomagnetic	Molecular based – multiplex RT-PCR	Zieglschmid *et al*., 2005 (76)
Telomescan	RBC lysis and centrifugation	Immunocytochemical	Kojima *et al*., 2009 (71)
Cell filtration by size/ISET method	Size based filtration	Both	De Giorgi *et al*., 2010 (69) Vona *et al*., 2000 (68)
Nucleic acid-based methods for multiple markers	Ficoll gradient	Molecular based	Chen *et al*., 2010 (77)

1.13 Markers of micrometastases

There is a paucity of general tumour-specific based markers to detect micrometastases which means detection is usually based on the premise that the blood and bone marrow do not contain epithelial cells. Epithelial markers such as the cytokeratin (CK) family of structural proteins are used which are either not expressed in the blood or are expressed at low levels (78). Cytokeratins are intermediate filaments and consist of 20 proteins each belonging to a different subtype. These are either basic to neutral or acidic and are expressed in pairs in different epithelial tissues. The epithelial type is thought to express a distinct cytokeratin profile, for example, luminal (glandular) cells express CK 8 and 18 while basal (myoepithelial) cells express CK5, 6 and 17 (79). The most effective antibodies against the most common cytokeratins (CK8, 18 and 19) have been

widely investigated (80). Following this, the antibodies that targeted the widest range of cytokeratins were found to be A45-B/B3; targeting pan–CK or CK8, 18 and 19 (Micromet) and the AE1/AE3 (Sanbio) antibodies. The studies from our laboratory have used the A45-B/B3 antibody along with the detection of CK19 mRNA by quantitative reverse transcription PCR (qRT-PCR), (81-83). CK19 is the most commonly used cytokeratin marker in qRT-PCR analysis and is associated with poor prognosis in breast cancer patients (84).

Other markers include cell surface glycoproteins such as epithelial cell adhesion molecule (EpCAM, CD326). This transmembrane protein has a range of roles including adhesion, migration, tissue maintenance and cell signalling. EpCAM is known to be over-expressed in many carcinomas and has been shown to be associated with a worse prognosis in breast cancer (85). This marker is now routinely used in conjunction with CK markers to detect CTCs (discussed later). Mammoglobin is another glycoprotein discovered in 1996 to be tissue specific to the breast and overexpressed in breast cancers in 23% of cases (86). Recent studies have tended to use it as part of multimarker panels along with carcinoembryonic antigen (CEA), epithelial glycoproteins (MUC-1) and newly discovered EMT, invasion and metastatic markers (87, 88). The discovery of new markers is growing at the same pace as the discovery of cancer processes and the investigations of these in micrometastases help to confirm their function. For example, stem cell and EMT markers were expressed in CTCs from metastatic breast cancer patients adding evidence for these processes taking place in metastatic disease (89). An early marker of metastasis, TWIST was expressed in disseminated tumour cells of breast cancer patients and could be used as a marker of early disease (90).

1.14 DNA, RNA and protein biomarkers in the plasma or serum

One of the most widely used and useful serum protein biomarkers for breast cancer in the clinic is CA 15-3, which is a member of the mucin glycoprotein family (91). It is often used for monitoring metastatic breast cancer patients who are receiving treatment, in combination with other diagnostic tools such as imaging, history and physical examination. It is not recommended however, for early breast cancer screening, diagnosis or staging due to the low sensitivity in this setting and the sometimes spurious rise in levels, following the first 4 - 6 weeks of treatment (92).

This is also the case for CEA (carcinoembryonic antigen) although it has been shown to be even less sensitive than CA 15-3 (93). These markers therefore have some prognostic use in breast cancer but it is limited.

The first report of raised circulating-free DNA (cfDNA) in the serum of cancer patients compared to healthy individuals came in 1977 (94). This raised hopes for a suitable non-invasive test for breast cancer that could detect tumour-derived DNA in the blood both sensitively and specifically. This initial study found 13 ng/ml of free DNA in the serum of healthy people compared to 180 ng/ml in the serum of cancer patients. This has been confirmed in a number of studies since, both in terms of the raised quantity of cfDNA in cancer patients but also to verify that the cfDNA is tumour derived with tumour aberrations (95, 96). This is important since it is known that cfDNA is present in the serum or plasma of patients with some inflammatory conditions so a test to distinguish tumour-derived cfDNA is crucial (97, 98). It is also known that only a proportion of the cfDNA is tumour derived as it may also be from tissues surrounding the tumour or micrometastases, this varies widely from 3 – 93% of total cfDNA between cancer patients (99).

It is postulated that cfDNA derived from tumours and released into the blood stream originates from the necrosis, lysis and apoptosis of cells. Furthermore, necrosis releases larger fragments of DNA due to the relatively inefficient mechanisms of cell death that normally produce DNA fragments of 185 – 200bp by apoptosis; these are present at higher concentrations in cancer patients than healthy individuals (99). Another study looked at an integrity index of the two measurements and found the increased integrity of cfDNA was also associated with cancer (100). This provides evidence that cfDNA in breast cancer patients is tumour derived but also larger in size than in healthy controls. This has potential to be a very specific and sensitive biomarker particularly if it is combined with other markers of minimal residual disease (MRD) such as CTCs and DTCs.

The recent interest in microRNAs has prompted investigations into its use as a biomarker for breast cancer as well as providing mechanisms of tumour promotion. MicroRNAs are small noncoding RNA oligonucleotides of around 22 bp long and regulate the expression of genes post-

transcriptionally and have been strongly implicated in cancer (101). Tumour cells not only release cell-free DNA and RNA into the circulation but also microRNAs which have been found to be surprisingly stable in the blood (102, 103). One study found raised levels of the miR155 microRNA in primary breast cancer patients compared to healthy controls as well as three other microRNAs that had raised levels in metastatic patients compared to healthy controls (104). This suggests a promising future role of microRNAs as a biomarker but requires further validation and long term follow-up of patients to find their prognostic value.

1.15 Immunocytochemical and molecular PCR approaches to detect micrometastases

The two fundamental methods to detect micrometastases have used immunocytochemistry (ICC) and RT-PCR. Both use epithelial and/or tumour based markers. The advantages of using ICC approaches mean that cells can be identified not only by a marker but also by their morphology and size. The nuclear to cytoplasmic ratio can also be assessed as this is often larger in tumour cells. This and other criteria were used by a number of groups across Europe to standardise and optimise the detection of tumour cells in the bone marrow (105). As previously mentioned, one of the most common antibodies used to detect tumour cells by ICC is the A45-B/B3 antibody. The development of scanning microscopes to enable easy visualisation and identification of stained pan-CK tumour cells has also been very important for the development of this field. The laborious search for CTCs in amongst millions of white blood cells on a slide can be automated by a system called the Automated cellular imaging system (ACIS, formally developed by Chromavision Medical Systems, CA, USA) to identify specific staining of tumour cells (Figure 1.3), (106).

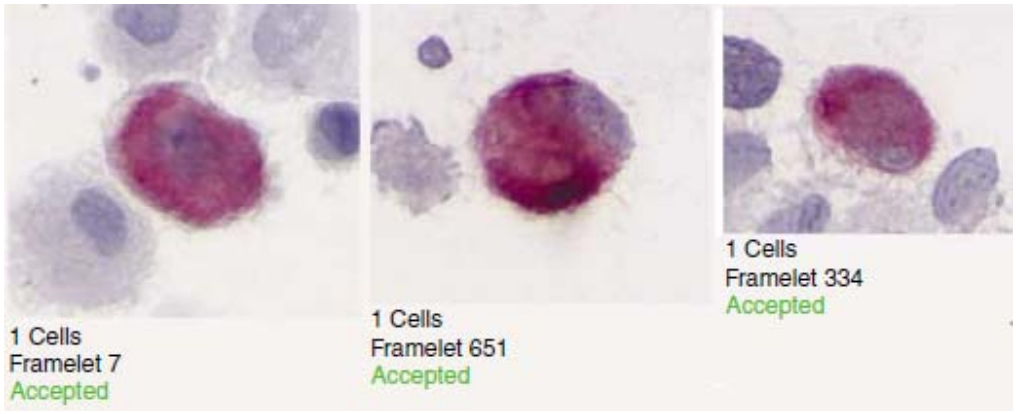


Figure 1.3: Examples of CK positive disseminated tumour cells in the bone marrow of breast cancer patients, visualised using an automated microscope, adapted from reference (107).

The most common molecular method uses quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to detect mRNA transcripts of epithelial and other tumour-based markers. The main drawback of this method is the inability to quantify tumour cells accurately as well as the problems already described with the selection of suitable tumour markers. The most commonly used marker is cytokeratin 19 although it is also expressed by blood cells, a common problem with many other markers such as CEA, Mucin 1 and gastrointestinal tumour-associated antigen-733.2 (GA733.2) (108). The presence of a *CK19* pseudogene may also cause false positivity in blood and bone marrow samples (109). A novel method to overcome this problem was developed by our laboratory which allowed for a background level of illegitimate transcripts of CK19 (83). The number of *CK19* transcripts was expressed as a percentage ratio to that of the Abelson oncogene (*ABL*) and a result of 0.1% or more (1 *CK19* transcript or more to 1000 *ABL* transcripts) was deemed positive for the presence of tumour cells. However, this method still has the drawback of not allowing single cell analysis or the characterisation of these cells. This method must also be done within a 4 hour time period due to RNA degradation. This is why the studies from our laboratory used both methods, combining the sensitivity of quantitative RT-PCR and the specificity of ICC (Figure 1.4).

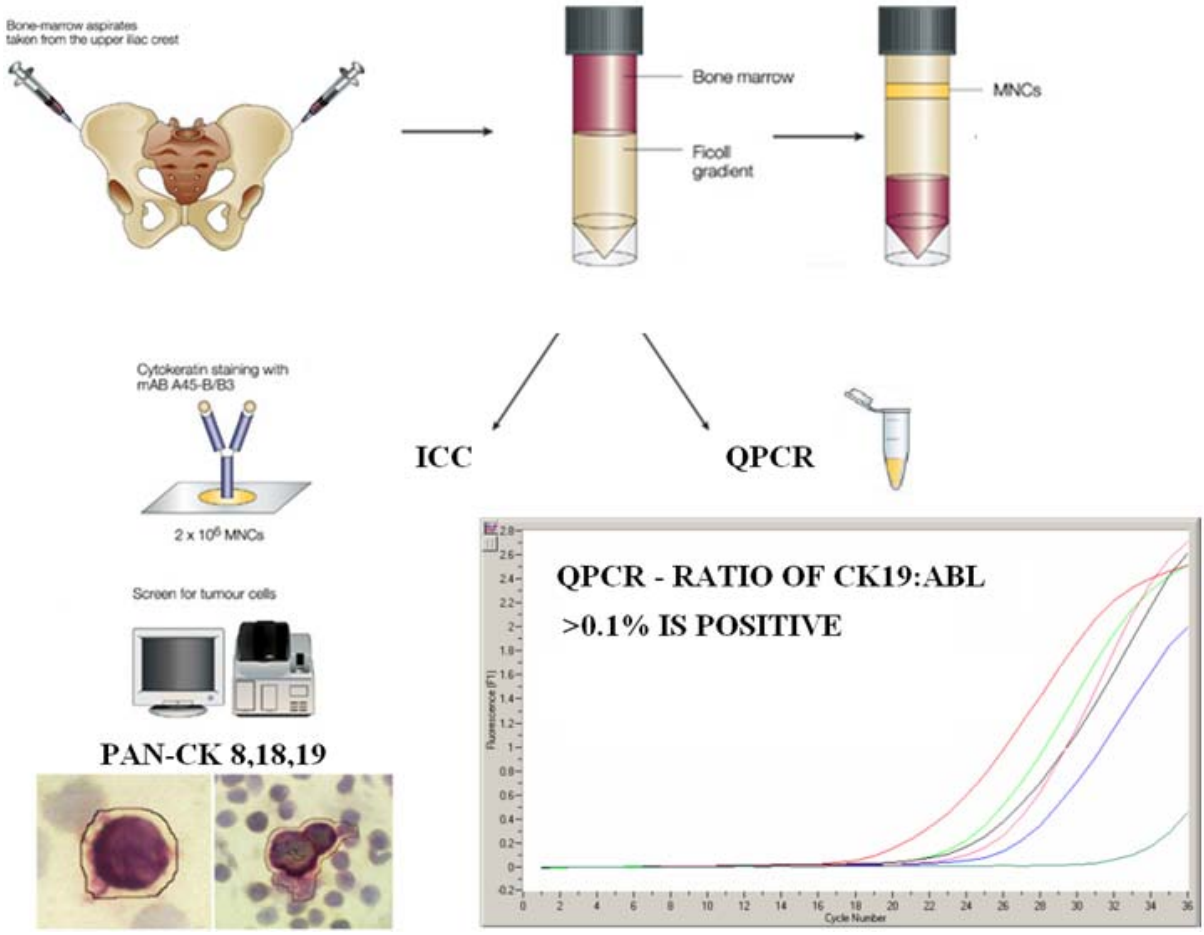


Figure 1.4: Schematic flow diagram indicating the two methods of disseminated tumour cell detection in the bone marrow (immunocytochemistry and quantitative RT-PCR), (82). Figure modified from reference (110).

Future methods of detection need to use a combination of immunocytochemistry and molecular *in situ* detection to enhance the specificity and sensitivity of detection and also to offer potential for further characterisation. This single cell detection and analysis is becoming increasingly important with the finding that CTCs are a very heterogeneous population (111). Also, the emerging evidence of the existence of a cancer stem cell sub-population means it is important to study a large enough representational proportion of CTCs in a given patient with breast cancer to identify and study those CTCs that may have metastatic potential (112). Single cell detection and characterisation also helps to prevent the detection of false positives due to many tumour markers being analysed at the same time. This is technically very challenging, particularly when

multiplexing antibodies with different assay requirements and is the reason why there are so few studies in this area. Emerging sophisticated technologies have the potential to enrich cells to enable further study, for example microchip technology allows the capture of live cells for analysis. An important study by Nagrath *et al*. in 2007 used tiny microposts coated in anti–EpCAM antibodies to capture cells allowing further molecular analysis including the identification of mutations in the EGFR gene in CTCs from non-small-cell lung cancer patients (66, 113). Additional technologies to allow single cell proteomics, genomics and transcriptomics are also being developed but are still at an early stage (114).

The detection of disseminated tumour cells in 30% of patients with primary breast cancer is well established and confers a worse prognosis to the patient (59). The main obstacle is to convincingly use this in clinical practice for monitoring purposes. The bone marrow aspirate procedure is painful and invasive and will only be tolerated for monitoring purposes once or twice a year (82, 83). This, along with the issues of sensitive and specific detection means there are too many caveats in this system for its widespread use. For example, studies from our laboratory show that many patients who have DTCs at surgery do not relapse even after 12 years of follow up. These are some of the reasons the American Society of Clinical Oncology (ASCO) still does not recommend the use of DTCs as a monitoring tool in the clinic (115). This has meant the emphasis has shifted to the more amenable, less invasive method of detecting tumour cells from the blood, despite the lower occurrence of cells (this will be discussed in-depth later). Phlebotomy is much more acceptable to patients and can be performed more routinely and frequently than bone marrow biopsies or tumour biopsies. In addition, blood can also be taken at a local hospital or general practice to be sent to a lab for testing and so is practical and less expensive. Bone marrow aspiration requires a specially trained medical professional and takes more time, for example a medical history must be taken beforehand and vital signs taken both before and after the procedure. It is therefore of paramount importance to develop a regular monitoring system using blood samples for the clinical management of breast cancer patients. It is also of great importance for novel and innovative methods to be developed or existing ones optimised to enable the more effective and specific detection and characterisation of MRD. Recent advances have enabled the more reliable, standardised detection of CTCs from blood.

1.16 The CellSearch system by Veridex LLC

The CellSearch system is a semi–automated, immuno-magnetic based method to detect circulating tumour cells from the blood and represents the only US Food and Drug Administration (FDA) approved method. This system uses very small iron particles (ferrofluid) conjugated to antibodies against epithelial cell adhesion molecule (EPCAM), to attach to and enrich the cells from 7.5 ml blood using magnets. Once these cells have been enriched, they are stained for pan-cytokeratin (CK8, 18 and 19), DAPI (for nuclear staining) and CD45 to distinguish the leukocytes from the epithelial tumour cells. These are all conjugated to fluorescent markers for measurement using an automated microscope (CellTracks Analyzer) and are displayed in a gallery of images (Figure 1.5). Additional tumour markers conjugated to a fluorescein (FITC) marker are HER2 and EGFR and can also be measured in CTCs using this system. As mentioned previously, the two most important criteria for the detection of rare cells in the blood or bone marrow are that of sensitivity and specificity. Initial studies were performed to assess and validate the system by testing blood samples from healthy controls, benign disease patients and patients with a range of metastatic cancers. Of 145 healthy women tested, 5.5% had 1 CTC per 7.5 ml of blood and none had more than 1 CTC. Similarly 199 women with benign breast disease had very few CTCs with 7.5% having 1 CTC and 1 patient having 3 CTCs per 7.5 ml of blood (73). Several studies have further demonstrated the reproducibility and concordance of multi-centre results as well as the stability of samples up to 72 hours (116).

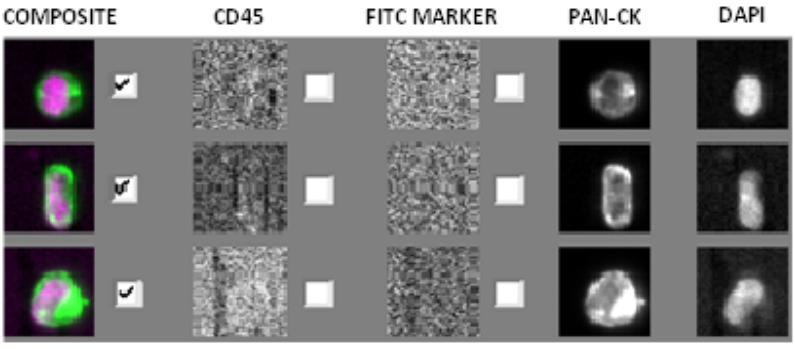


Figure 1.5: An example of a gallery of CTC images from the CellSearch system. Three CTCs are shown that are EpCAM, PAN-CK and DAPI positive and CD45 negative.

A key study for establishing the prognostic use of CTCs detected by the CellSearch system was by Cristofanilli's group in 2004 (117). Breast cancer patients (n = 177) with metastatic disease were tested before and after a new line of treatment to establish the significance of finding CTCs in 7.5 ml of blood. The presence of 5 or more CTCs in 7.5 ml of blood at the time of diagnosis of metastatic disease or before initiation of first-line therapy was associated with shorter progression free survival and overall survival. Those patients with 5 or more CTCs had a median progression free survival at baseline of 4.9 versus 9.5 months ($p = 0.0014$) from patients with less than 5 CTCs. This showed the presence of CTCs was an independent predictive factor and prognostic indicator. This was the first such study with multi-centre validation and confirmation of the important prognostic use of CTCs using a standardised method.

1.17 Circulating tumour cell measurement in early stage breast cancer

The study of disseminated tumour cells in the bone marrow has been extensively investigated in early breast cancer; however as previously mentioned the detection of circulating tumour cells in blood would be more acceptable to patients. This has been hampered due to the finding that fewer CTCs are found in the blood than in the bone marrow (82, 118, 119). Their prognostic relevance is still under scrutiny and requires large scale, long term studies with sophisticated methods of detection. The development of technologies like the CellSearch system has meant that this area of research has gained momentum. Several of these studies have been initiated such as the SUCCESS trial in Germany, a trial of adjuvant chemotherapy and is currently involved in the long-term follow-up analysis of 1489 high breast cancer patients who had involved lymph nodes or who had other high risk prognostic factors. This study has so far shown that the presence of two or more CTCs before therapy was significantly associated with poor disease-free survival (DFS) (120). Importantly, the study used 23 ml of blood instead of the standard 7.5 ml to increase the chances of detecting CTCs. The use of CTC detection as a test was therefore recommended to find out if extra secondary adjuvant treatment is necessary. Interestingly, the presence of two or more CTCs after therapy was only indicative and not significant of reduced DFS. Follow-up is ongoing to establish the prognostic use of CTCs in these patients and if a drop in CTC number after therapy will mean better survival rates.

Another large scale study is the REMAGUS trial; a phase II neoadjuvant chemotherapy trial, where CTCs were tested in 115 patients before and after therapy, this showed similar findings to the SUCCESS trial. Only the CTC measurement before chemotherapy was significantly associated with metastasis-free survival and overall survival (121). Furthermore this was true of samples even when only one CTC was detected unlike the SUCCESS trial that used a cut off of 2 or more CTCs. This shows there is promise of CTC detection used as a prognostic indicator in the early breast cancer setting, even finding one CTC in 22.5 ml of blood. In addition a study to quantify CTCs in the neoadjuvant setting also found that the response of CTCs during the first chemotherapy treatments predicted the final response of the tumour and so could improve treatment decisions based on the efficacy of the drug against the CTC population (122). This has yet to be confirmed in other studies or using the CellSearch system. The reasons why these findings have not previously been found may be due to the lack of standardisation of methods and the lack of sensitivity to detect cells (118, 123).

There have been more studies investigating CTCs in early breast cancer using molecular PCR-based methods than immunocytochemical methods due to the problems of detection already discussed. The detection of *CK-19* mRNA transcripts in the blood has been found to be an independent prognostic indicator of poor clinical outcome in patients both before and after adjuvant chemotherapy (84, 124, 125). More recently, the use of multiple markers to detect CTCs have been used to improve detection rates. One study used CK-19, mammaglobin A and HER2 to detect CTCs in early breast cancer patients (87) and another group used *CK-19*, mammaglobin and *CEA* mRNA transcripts in the blood to detect CTCs (77). Both panels of markers predicted poor disease-free survival or association with relapse. This demonstrates the advantages of qPCR; being able to detect many markers at the same time and also increasing the likelihood of detecting CTCs with differing phenotypes. Very few studies have set out to use both molecular and immunocytochemical methods (126, 127). Our laboratory has previously shown that RT-PCR of *CK-19* is a more sensitive detection method than ICC, however the concordance of the methods was low (127, 128). This may be evidence of the different sensitivities and specificities of the assays and/or the detection of cells of differing viabilities. Immunocytochemical assays

may be able to detect both intact viable or non-viable cells whereas molecular assays detecting mRNA may only detect cells that are in an active state producing mRNA.

Evidence for CTCs in different activity states comes from the work on the EPISPOT assay, where viable CTCs are found to secrete proteins, in particular CK19, which can be detected in the bone marrow of breast cancer patients and is associated with a reduced survival. This suggests that their detection could indicate the presence of a subset of occult tumour cells that have a high biologically active, metastatic potential (129). An additional method for detecting live cells used an adenovirus that only replicates in live cancer cells using a telomerase-specific process which also had a green fluorescent protein tag to visualise the cells (71). Furthermore, a recent study found a proportion of CTCs in blood samples from breast cancer patients had expression of the apoptosis marker M30. This demonstrated the potential use of M30 as a biomarker for patients on therapy to determine more accurately if the treatment is working and causing occult cells to be undergoing apoptosis and cell death (130). Evidence therefore exists for viable cells and cells undergoing apoptosis, however proof for cells in a dormant quiescent state circulating in the blood has yet to be found.

1.18 Tumour dormancy in breast cancer patients

It is known that many breast cancer patients relapse a long time after removal of their primary tumour but it does not correlate with the growth kinetics of tumour cells. This phenomenon of dormancy in micrometastases is still poorly understood. A mechanism must be occurring that either puts individual or groups of cells into a dormant state (Figure 1.6). This may be due to cellular dormancy or tumour mass dormancy. For example angiogenic dormancy is when inefficient vascularisation occurs keeping the tumour mass in check with an equal amount of tumour apoptosis to proliferation. Similarly, immunosurveillance can keep tumour cells from proliferating (131). The study of these processes in breast cancer patients is difficult and hence there is a lack of studies in this area. However, in a group of breast cancer patients who were candidates of this so–called cancer dormancy, persistent CTCs were detected years after removal of the primary tumour. Intriguingly, this is despite the fact that these CTCs had a half-life of a few hours, which suggests that they originated from micrometastases in tissues elsewhere in the

body that were not causing overt disease. A balance between tumour replication and cell death over very long periods of time has therefore been suggested (132).

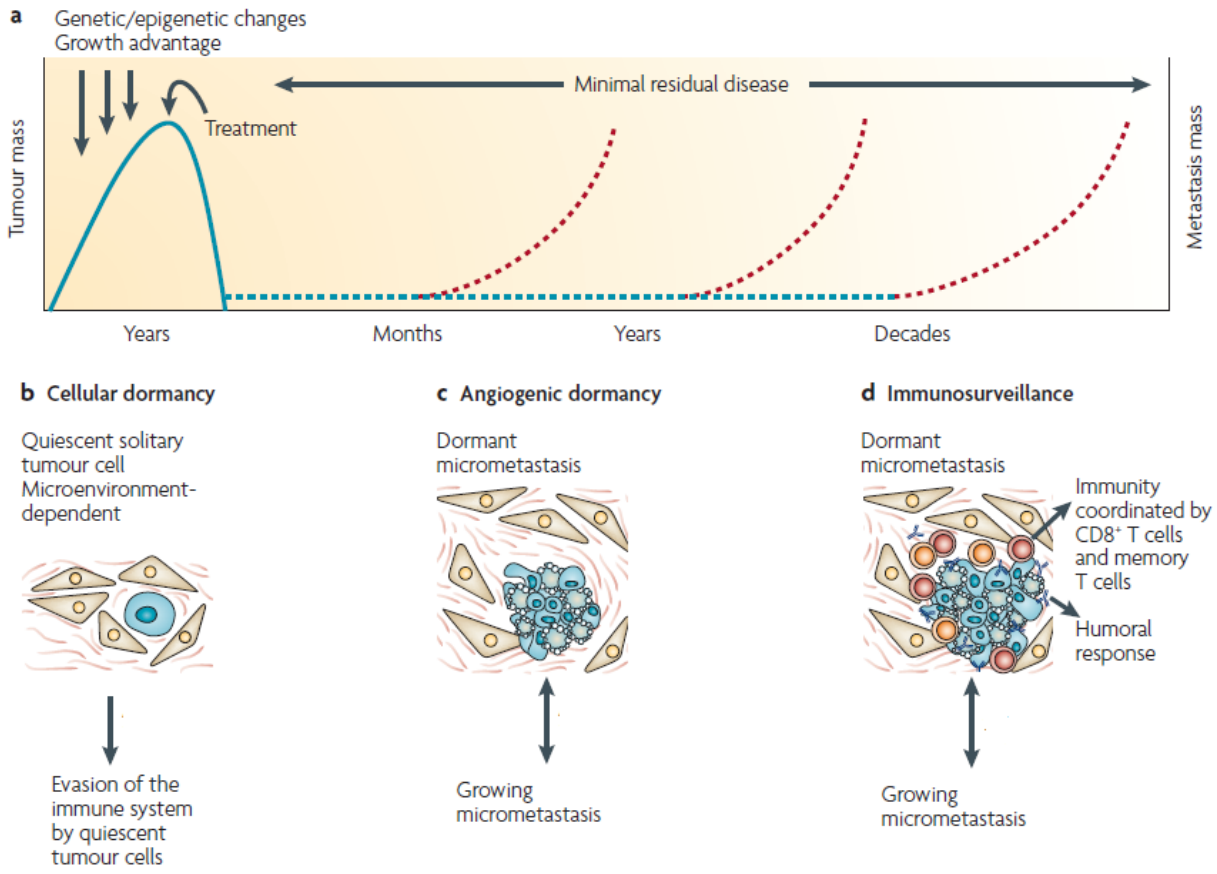


Figure 1.6: Proposed cancer dormancy mechanisms. Tumour cells have a growth advantage forming a primary tumour (solid blue line). After treatment and tumour regression, residual disease is detected for long periods of time (dashed blue line) with occasional increases in tumour mass from secondary sites (dashed red line), (a). Dormancy mechanisms may include cellular dormancy (b), angiogenic dormancy (c) or dormancy from immunosurveillance (d), adapted from (131).

Another theory is that of a steady, low number of CTCs over years, which is in agreement with the hypothesis of resting, dormant cells that re-circulate. These could have stem cell and chemotherapy resistant characteristics (133) (134). These two hypotheses are most likely not mutually exclusive but both play a role in tumour dormancy (131). For example, recent work

suggests that cells are dormant for a time before undergoing a switch to a fast-growing angiogenic-dependent type with gene transcription of angiogenic factors being most prominent (135). Evidence of single disseminated and circulating tumour cell detection also points to single cell dormancy along with studies showing CTCs do not express the Ki-67 proliferation marker (136). As the technology allows it, more research can be done towards characterising CTCs which will in turn allow the discovery of the mechanisms of tumour dormancy. In addition, the contribution of processes such as EMT and the role of cancer stem cells can be unravelled.

The theory that cancer has sub-populations of cancer stem cells has gained much attention in the last few years following the work by Al-Hajj's group (112). They found that a distinct population of cancer cells that had EpCAM positive, CD44-positive and CD2-negative, or low levels, were able to form tumours in mice whereas cells with differing phenotypes could not. Markers of stem cells such as aldehyde dehydrogenase 1 (ALDH1) have also been found to be present in blood samples with CTCs (89) as well as in DTCs in the bone marrow (137). Furthermore, one study found the presence of ALDH1 expressing CTCs to be associated with drug resistant proteins and a worse progression-free survival (138). This area of research is still very new and the characterisation of CTCs is required to find novel methods of targeting this sub-population of cells especially if they are resistant to therapies or have dormant phases.

1.19 DTC detection versus CTC detection

The comparison of DTC detection with CTC detection is very important to determine if blood sampling for CTCs can be performed in favour of painful bone marrow aspiration. Two areas of interest include the relationship of the two variables, and whether they are positively correlated. Also, are they phenotypically and genetically similar or do they represent different elements or points of evolution of the metastatic cascade? The correlation of CTCs to DTCs is not clear as several studies show differing conclusions. Some studies found no correlation (118, 139), one found a correlation when measuring *CK19* mRNA in the blood and bone marrow (140), other studies only showed a concordance if sometimes weak (123, 141). One study found concordance but DTCs were still a better prognostic indicator than CTCs, however the improved detection of

CTCs and their characterisation may help in this regard (119). These discrepancies may be due to the differing sensitivities of the methods used along with the lower incidence of CTCs.

This study attempts to investigate CTCs using the best available methods in patients with primary breast cancer on long-term follow up to find out their prognostic significance. This study also combines other markers of MRD, for example DTCs detected in the two most commonly studied ways (ICC and qRT-PCR) and also the measurement of cell-free DNA in the plasma. These measures of MRD will attempt to detect both viable and non-viable DTCs and CTCs with a measure of tumour cell death (cfDNA) so that we can distinguish patients with long term tumour dormancy from those who are vulnerable to relapse and require further treatment. We also investigated alternative advanced technologies for detecting CTCs in the most sensitive and specific way whilst gaining as much information about them as possible. This was to achieve an insight into the metastasis and progression processes and to phenotype a real-time marker of disease to find the best therapies. Intensive monitoring of a cohort of patients on follow-up, aims to identify the best method or combination of methods for detecting and characterising impending metastatic disease. In addition the increased volume and frequency of blood sampling used to detect CTCs may increase the sensitivity to find more cells. To our knowledge such an intensive monitoring programme has not been undertaken for primary breast cancer patients on long-term follow up. This would enable not only the characterisation of the period of tumour dormancy in a proportion of patients but also a test to detect impending metastatic disease. The administration of secondary adjuvant endocrine or cytotoxic therapy could then be considered at a stage capable of eliminating disease with a curative intent.

1.20 Studies of CTCs in metastatic breast cancer

The importance of detecting CTCs in early stage breast cancer is clear; however their detection and characterisation in the metastatic setting is also of great importance not only for individualised therapy but also as a representative model system for their study in the early stage when cancer spreads. The characterisation of CTCs in the metastatic setting also provides more material to study and could be applied to the primary setting to prevent metastatic spread. The study of cancer in its advanced form is crucial to understand it but also to be able to treat it

effectively. For example we know that resistance to therapy occurs, so novel strategies targeting alternative pathways may be of benefit. Finding out the specific pathways to target in CTCs may be beneficial in the management of metastatic disease.

1.21 HER2 measurement in CTCs

As mentioned previously, HER2 is an important target in breast cancer that has an effective therapy against it when HER2 amplification has been detected in the primary tumour. The detection of HER2 in tumour biopsies performed at a later date is not commonly performed which means any patients who have acquired HER2 overexpression during the course of their illness will rarely receive trastuzumab. By measuring HER2 amplification in CTCs from patients with metastatic breast cancer it was found that 38% of those who were negative for amplification in their primary tumour acquired it in their CTCs (132) and these findings have later been confirmed (142, 143). Treatment of 4 out of the 9 metastatic breast cancer patients with HER2 positive CTCs with trastuzumab-containing therapy showed 2 patients with a partial response and 1 with a complete response (132). Although these proof-of-principle studies have used a very small number of patients, they illustrate the importance of characterising an individual patients' 'cancer phenotype' in real-time to be able to treat them in the most effective way. This also demonstrates the contribution of HER2 over-expression in progressed breast cancer.

1.22 EGFR measurement in CTCs

It has long been known that the presence of EGFR confers a worse prognosis to breast cancer (144). It has also been found to be more common is breast cancers that are not only ER negative but also triple negative for ER, PR and HER2 or tumours that have the basal – like phenotype (145-147). In addition, EGFR over-expression is known to be implicated in resistance to several types of therapies including endocrine and anti-HER2 therapies although the mechanisms are still unclear (148, 149). These findings have been consistent in many studies despite the lack of consensus in the measurement of EGFR (150). The further investigation and measurement of EGFR in breast cancer is therefore of great importance, particularly in CTCs. This is so that the mechanisms of resistance to therapies can be elucidated and anti-EGFR therapies can be given.

The question of why the presence of EGFR over-expression does not always predict response to anti-EGFR therapies remains unanswered. Immunohistochemistry (IHC) is the most commonly used method to measure EGFR and has made some progress to being standardised with two methods in wide use - The Dako EGFR PharmDX kit (DakoCytomation) and the Clone 31G7 antibody (Zymed Laboratories). When these were compared, the Zymed antibody was found to identify 10% more EGFR over-expressing tumours than the Dako PharmDX kit (151). IHC is also prone to user interpretation and subjectivity and so cannot be completely standardised. There may also be problems with sample preparation and storage, for example epitope instability in formalin fixed paraffin embedded sections may bias results (152). There is also evidence that IHC measurement of EGFR will not always identify patients who will respond to anti-EGFR therapies (153). Tumour heterogeneity in EGFR expression may be a factor here when only one small fraction of the tumour is stained (154). Other technologies attempt to provide improved methods of detection with more objective ways of measurement like ligand binding assays mentioned previously and fluorescence in situ hybridization of EGFR gene copy number gains (155). These methods have not yet been fully validated and so are not widely used.

Other factors that may play a role include the varying treatment protocols and different types of anti-EGFR inhibitor. An example of this is the dual tyrosine kinase inhibitor lapatinib that targets both EGFR and HER2; EGFR expression was not found to correlate with benefit from Lapatinib (156). In this case HER2 is such a strong predictive factor of response to Lapatinib it may mask any affect EGFR has on the response to lapatinib. Similarly the other HER receptors may modify the signalling potential of EGFR (22). Other factors affecting EGFR signalling function include Met kinase activity which compensates for any loss of EGFR kinase activity (157). The discovery and investigation of other mechanisms that cause aberrant expression of EGFR may uncover the reasons why the measurement of EGFR alone cannot always predict response to therapy.

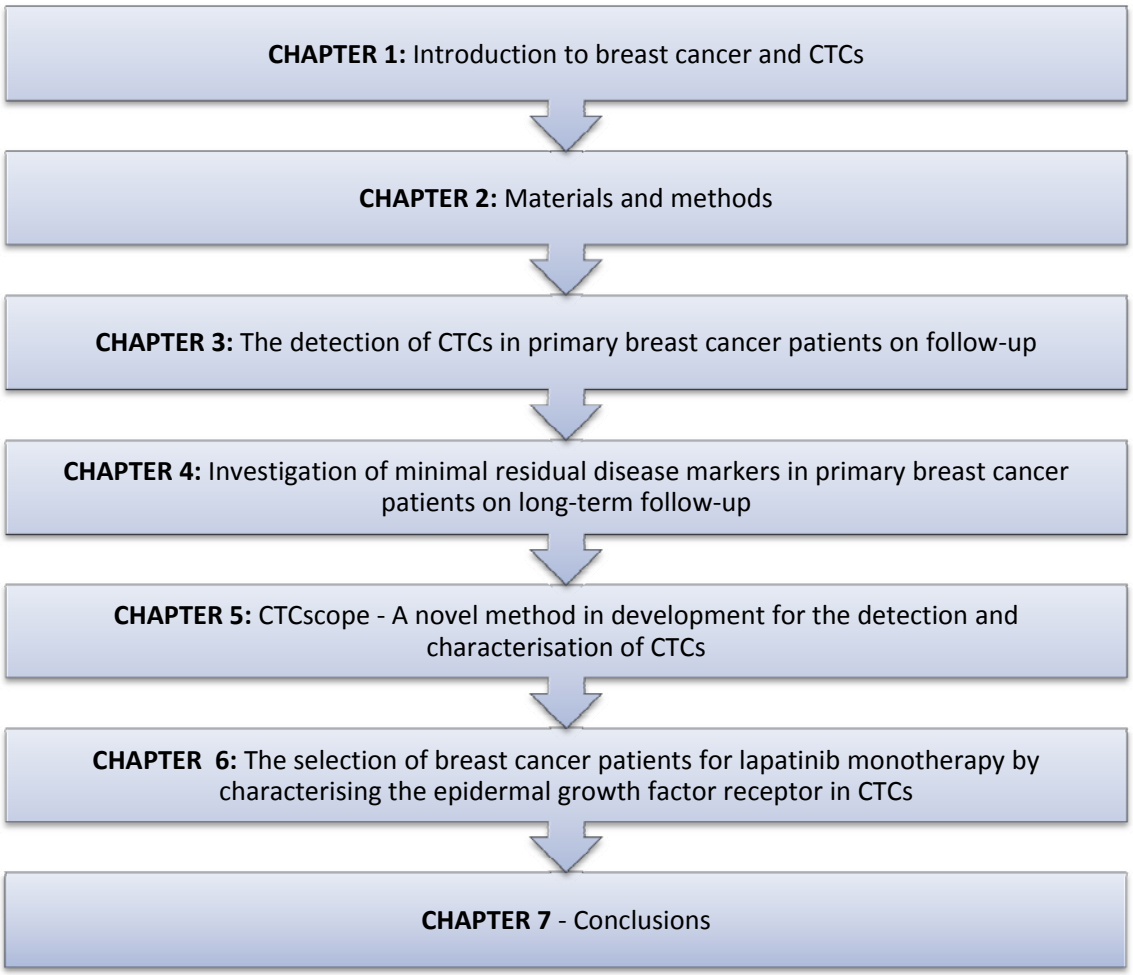
The characterisation of known tumour markers on CTCs that are the targets for therapies is very important for monitoring but also to gain understanding for processes such as resistance to therapies and metastasis. In this study I intend to investigate the measurement of EGFR in CTCs from patients with metastatic breast cancer. The aim being to identify patients with HER2

negative primary tumours who may respond to lapatinib therapy, this may broaden the population of patients that lapatinib may be given to. This is therefore a proof of principle study to firstly determine if EGFR can be measured to predict response to lapatinib, and secondly to determine the use of CTCs to monitor patients on a therapy. This may have implications for my study in primary breast cancer patients as phenotyping CTCs may provide information on the best possible therapies for cure or disease management. The improved detection technologies and the greater information gained from single CTCs could be translated to the clinic to enable the better stratification of patients in terms of prognosis and for the prevention of relapse.

1.23 Aims of the thesis

- To determine the use of CTCs measured by the CellSearch system as a test for impending metastatic disease in patients with primary breast cancer on long–term follow up as an alternative to measuring DTCs in the bone marrow
- To study the combined use of several markers of minimal residual disease; CTCs, DTCs and cfDNA as a test of impending metastatic disease in primary breast cancer patients on follow-up
- To establish the relationship of viable and non–viable CTCs and DTCs with measures of tumour cell death (cfDNA in the blood) as a means to characterise long term tumour dormancy in patients with primary breast cancer on long–term follow-up
- To investigate a novel method (CTCscope) to detect and characterise CTCs by *in situ* hybridisation of tumour markers including newly discovered EMT markers to improve CTC detection
- To investigate the phenotyping of CTCs for an important growth receptor tumour marker – EGFR by testing measures of EGFR phosphorylation, total protein and mRNA (by CTCscope) in CTCs and comparing these methods with each other to find the best method of EGFR characterisation in CTCs
- To determine the prognostic use of CTCs for monitoring patients with metastatic breast cancer on lapatinib (a dual tyrosine kinase inhibitor of EGFR and HER2) using both the CellSearch system and CTCscope

1.24 Flow diagram of circulating tumour cell studies



2.0 Materials and Methods

2.1 Cell lines and Cell Culture

Breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-453, T-47D, ZR-75-1 and SKBR-3 (Table 2.1) were obtained internally within the Department of Oncology, Imperial College, London (originally sourced from the American Type Culture Collection) and cultured in DMEM (Dulbecco's Modified Eagles Medium) supplemented with 10% FCS (Foetal Calf Serum) and 100U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine and maintained at 37°C, 5% CO_2.

Table 2.1: Breast cancer cell line phenotype and origin

Breast cancer cell line	Origin	Phenotype	Reference
MDA-MB-231	Pleural effusion	ER-negative	(158)
MDA-MB-468	Pleural effusion	ER and PR-negative, amplified EGFR	(158)
SK-BR-3	Pleural effusion	ER and PR-negative, amplified HER2 and EGFR positive	(159)
MCF-7	Pleural effusion	ER, PR and EGFR positive	(160)
T47-D	Pleural effusion	ER and PR positive. Low level amplification of HER2	(161)
ZR-75-1	Ascites fluid	ER and PR positive, HER2 amplified, low EGFR	(162)
BT-474	Solid tumour	ER, PR, EGFR positive, HER2 amplified	(163)

2.2 Validation experiments for the CellSearch System

Cell spiking experiments

The following cell lines were used to spike blood samples from healthy volunteers; MDA-MB-231, ZR-75-1, MCF-7 and SKBR-3 cells. Cells were trypsinised, harvested and washed in PBS. Cells were

resuspended in DMEM-supplemented media and counted using a haemocytometer. This was repeated three times and the average reading was taken to ensure as accurate reading as possible. Blood samples were then spiked in triplicate with 10, 100 and 500 cells. Samples were then put through the CellSearch System and enumerated using the CellTracks analyser (method described below).

2.3 Patients and controls

Patients were recruited for these projects from Charing Cross Hospital, London with ethical approval from the Riverside Research Ethics Committee (RREC numbers 0975 and 06/Q0401/94). All patients gave written informed consent in accordance with the declaration of Helsinki, and understood that the results would not be made available to them. The clinical characteristics are shown for 51 primary breast cancer patients studied in Chapter 3 (Table 2.2), 64 primary breast cancer patients studied in Chapter 4 (Table 2.3), 51 benign breast disease patients studied in Chapter 3 and 4 (Table 2.4), 45 advanced/metastatic breast cancer patients studied in Chapter 5 (Table 2.5) and finally 33 advanced breast cancer patients studied in Chapter 6 (Table 2.6).

Table 2.2: Clinical characteristics of primary breast cancer patients for the CTC study (Chapter 3)

	Lymph node positive >2 (n=33)	%	**Lymph node negative** (n=18)	%
Tumour type				
Invasive Ductal	26	81%	15	83%
Invasive Lobular	3	9%	2	11%
Other	4	12%	1	6%
T-stage				
T1[a]	11	33%	18	100%
T2[b]	16	50%	0	0%
T3[c]	5	16%	0	0%
unknown	1	3%	0	0%
Grade				
I[d]	1	3%	4	22%
II[e]	11	34%	8	44%
III[f]	20	63%	6	33%
unknown	1	3%	0	0%
ER status				
positive	20	61%	14	78%
negative	8	3%	4	22%
unknown	5	16%		
PR status				
positive	16	48%	13	72%
negative	10	31%	4	22%
unknown	7	22%	0	0%
Therapy				
Nil	19	59%	17	94%
Arimidex	11	34%	0	0%
Tamoxifen	2	6%	1	6%
Chemotherapy (FEC)[g]	1	3%	0	0%

[a]Tumours 2.0 cm or less in the greatest dimension, [b]tumours more than 2.0 cm but not more than 5.0 cm in the greatest dimension, [c]T3 are tumours more than 5.0 cm in the greatest dimension
[d]Grade - well-differentiated (low grade) tumour, [e]moderately differentiated (intermediate grade) tumour, [f]poorly differentiated (high grade) tumour
[g] FEC - Fluorouracil, Epirubicin and Cyclophosphamide

Table 2.3: Clinical characteristics of primary breast cancer patients for the MRD study (Chapter 4)

	Lymph node positive patients (n=42)	%	Lymph node negative patients (n=22)	%
Menopausal Status				
Premenopausal	11	26%	7	32%
Postmenopausal	31	74%	15	68%
Histology				
Invasive Ductal	31	74%	16	73%
Invasive Lobular	7	17%	2	9%
Other/Mixed Invasive	4	9%	4	18%
Tumour Size				
T1[a]	15	36%	20	91%
T2[b]	16	38%	2	9%
T3[c]	7	17%	0	0%
Unknown	4	9%	0	0%
Tumour Grade				
I[d]	1	2%	5	23%
II[e]	20	48%	9	41%
III[f]	20	48%	8	36%
Unknown	1	2%	0	0%
Hormone Receptor Status				
ER Positive	29	69%	17	77%
ER Negative	10	24%	5	9%
Unknown ER status	3	7%	0	0%
PgR Positive	17	40%	13	59%
PgR Negative	19	45%	7	32%
Unknown PgR Status	6	14%	2	9%
HER2 Status				
Positive	13	31%	3	14%
Negative	26	62%	17	77%
Unknown	3	7%	2	9%
Endocrine therapy				
Yes	41	98%	17	77%
No	1	2%	5	23%
Chemotherapy				
Yes	35	83%	8	36%
No	7	17%	14	64%
Total number of patients	42	66%	22	34%

[a]Tumours 2.0 cm or less in the greatest dimension, [b]tumours more than 2.0 cm but not more than 5.0 cm in the greatest dimension, [c]T3 are tumours more than 5.0 cm in the greatest dimension

[d]Grade - well-differentiated (low grade) tumour, [e]moderately differentiated (intermediate grade) tumour, [f]poorly differentiated (high grade) tumour

Table 2.4: Clinical characteristics of women with benign breast disease (Chapters 3 and 4)

	Benign breast disease patients (n=51)	%
Menopausal Status		
Premenopausal	31	61%
Postmenopausal	20	39%
Benign Breast Disease		
Breast lump	13	25%
Breast Pain	4	8%
Fibroadenosis/fibrocystic change	27	53%
Atypical ductal hyperplasia	2	4%
Other benign	5	10%
Total number	51	

Table 2.5: Clinical characteristics of advanced breast cancer patients for CTCscope vs CellSearch comparison (Chapter 5)

	Advanced Breast Cancer Patients (n=45)	%
Age		
Median and Range	60 (43 – 78)	100%
Histology		
Invasive Ductal	39	87%
Invasive Lobular	5	11%
Not known	1	2%
Hormone Receptor Status		
ER Positive	37	82%
ER Negative	8	18%
PgR Positive	32	71%
PgR Negative	11	24%
PgR unknown	2	4%
HER2 Status		
Positive	9	20%
Negative	32	71%
Unknown	4	9%

Table 2.6: Clinical characteristics of advanced breast cancer patients for measurement of EGFR on CTCs (Chapter 6)

	Advanced Breast Cancer Patients (n=33)	**%**
Age		
Median and Range	62 (40 – 81)	100%
Histology		
Invasive Ductal	25	25%
Invasive Lobular	8	8%
Hormone Receptor Status		
ER Positive	26	79%
ER Negative	7	21%
PgR Positive	20	61%
PgR Negative	13	39%
HER2 Status		
Positive	2	6%
Negative	31	94%

Healthy controls

Twenty-nine women were recruited from the Medical Oncology Department at Charing Cross Hospital and the Department of Surgery and Cancer at Hammersmith Hospital for measuring CTCs and cfDNA in the blood (studied in Chapters 3 and 4). Further healthy controls used in optimisation experiments (Chapter 5 and 6) were recruited from the Department of Surgery and Cancer, Imperial College London. All gave written, informed consent.

2.4 Preparation of bone marrow samples

Bone marrow samples were taken either from the left or right posterior iliac crest every 6 months for the high risk patients and every year for the low risk patients. The skin was incised before the aspirates were taken to minimize the risk of epithelial contamination. Between 2 and 5 ml of bone marrow was aspirated from each side using disposable 15-gauge (1.8 mm) marrow-gauge bone marrow aspirate needles (Rocket Medical) into syringes primed with preservative-free heparin (CP Pharmaceuticals). The whole bone marrow was layered onto 20 ml of Ficoll (GE Healthcare) and centrifuged at 1200 g for 30 min (brake off). After removal of the plasma the interface cells were then removed and washed in 50 ml PBS (Sigma Aldrich) and centrifuged at 800 g for 10 min. After removal of the supernatant the cell pellet was resuspended in 3-5 ml of

erythrocyte lysis buffer (Sigma Aldrich) for 3 min, this is made up to 50 ml with PBS and re-centrifuged. The cell pellet was resuspended in PBS and the cells counted using a haemocytometer, and $2.5x10^6$ cells removed for qRT-PCR (quantitative reverse transcriptase polymerase chain reaction) for CK19 and $2.5x10^6$ cells for ICC (immunocytochemistry). The pellets were resuspended in 350 µl guanidine thiocynate lysate (containing 4M guanidine thiocynate, 5mM EDTA, 25mM sodium citrate pH 7, 0.5% sarcosyl (Sigma Aldrich)) containing 7.1µl/ml β-mercaptoethanol (Sigma Aldrich) for qRT-PCR and stored at -20°C and in PBS for ICC. Cells for ICC were cytocentrifuged onto glass slides at 110g for 5 min at a concentration of $5x10^5$ cells per area ($240mm^2$). The samples are air dried overnight and then frozen at -20°C. Operators undertaking the ICC were blind to the clinical status and identity of the patients and their previous assay results.

2.5 Immunocytochemistry

Cells were cytocentrifuged at a concentration of 5×10^5 per cytospin and air dried. A total of 6 areas on the glass slides were stained, four were stained for the presence of cytokeratin positive cells and 2 were isotype controls. Slides were thawed for 20 min and the cytospins were blocked with 200 µl of 10% AB Serum (Biotest) in PBS for 20 min. The primary antibody (A45-B/B3 Micromet) raised against a common epitope of cytokeratin, (80) was used at 2 µg/ml in 10% AB serum on the cytospins for 45 min. The MCF-7 cell line was used as a positive control. An isotype IgG1 mouse myeloma antibody MOPC-21 (Sigma Aldrich) served as the isotype negative control. The slides were washed three times for 3 min in PBS. The rabbit anti mouse antiserum (Dako) was diluted 1 in 20 with 10% AB serum and added to the cytospins for 30 mins. These were washed three times for 3 min with PBS. The alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (Dako) was diluted 1 in 100 with 10% AB serum and added to the cytospins for 30 min and washed three times for 3 min in PBS. This reaction was developed by new Fuschin as follows: 45 mg of Levamisole (Sigma Aldrich) was dissolved in 31 ml 0.2M Tris Base (Sigma Aldrich), warmed to dissolve with 94 ml water added. Another solution of 25 mg of $NaN0_2$ dissolved in 625 µl water with 250 µl of 5% New Fuschin in 2N HCL (Sigma Aldrich) was mixed with it. A third solution was added consisting of 62 mg Napthol AS-B1 (Sigma Aldrich) dissolved in

750 µl N,N-Dimethyl-formamide (Sigma Aldrich), to a pH of 9.32. The solution was filtered through Whatman number 1 filter paper and added to the slides followed by washing with PBS three times for 3 min followed by 3 min with water. The cytospins were counterstained with haematoxylin (Dako) for 20 seconds, washed in water followed by addition of 0.8 % NH_4OH for 30 seconds. Slides were washed three times for 3 min with water and mounted using coverslips and Kaiser's glycerol gelatine (Merck). Slides were screened using the Automated Cellular Imaging System (ACIS, Carl Zeiss Ltd). Samples that were isotype positive were deemed uninterpretable and therefore excluded from the results.

2.6 RNA Extraction and cDNA synthesis

RNA was extracted from the interface cells from the bone marrow by using the E.Z.N.A Total RNA kit (Omega Bio-tek). Briefly, following washes with ethanol and wash buffers, RNA was eluted from the reversible binding HiBind Matrix mini column in 24 µl of DEPC treated water. The RNA was heated at 65 °C for 10 min followed by cDNA synthesis. The cDNA mix was made using 6.3 µl (200 u/µl) M-MLV reverse transcriptase (Invitrogen) and 3.2 µl (40 u/µl) RNasin (Promega) per 100 µl cDNA mix. cDNA mix consisted of 380 µl 5X M-MLV Buffer (Invitrogen), 19 µl 0.1M DTT (Invitrogen), 76 µl 25 mM 4X dNTPs (Invitrogen), 40 µl 5 mg/ml random hexamers (Amersham) and 372 µl water. Twenty-one µl were added per tube with 19 µl of RNA and pulse centrifuged and incubated at 37 °C for 2 h followed by 65 °C for 10 mins and stored at -20°C.

2.7 Quantitative Real-Time PCR

All pre-PCR work was performed in a PCR-only laminar flow cabinet with filter tips. Two negative controls were used in each run and were prepared last. One positive control of cDNA from MCF7 cells was also used in each run. All patient cDNA samples had *CK19* and *ABL* quantified by performing qRT-PCR on the LightCycler™ (Roche Diagnostics) and a ratio was calculated. More than 1 *CK* to 1000 *ABL* transcripts (expressed as a ratio of CK:ABL being greater than or equal to 0.1%) was deemed positive to minimise the detection of illegitimate *CK* transcripts in healthy individuals. The reaction for the amplification of *CK19* was made up with 10 µl Sybr green mix (Sigma Aldrich), 0.8 µl 25 mM magnesium chloride (Roche), 2.5 µl 10 pmol CK19S 5'-GCGGGCAACGAGAAGCTAA-3', 2.5 µl 10 pmol CK19Do 5'-CTCATGCGCAGAGCCTGTT-3', 4.2 µl

water and 2.5 µl of cDNA mix (20 µl total volume). The reaction for the amplification of *ABL* was made up with 10 µl Sybr green mix (Sigma Aldrich), 2.5 µl 10 pmol A2N 5'-CCCAACCTTTTCGTTGCACTGT-3', 2.5 µl 10 pmol A4- 5'-CGGCTCTCGGAGGAGACGTAGA- 3', 5 µl water and 2.5 µl of cDNA mix (20 µl total volume). Standards were developed previously (83). These were used in duplicate in the concentrations 10^1, 10^2, 10^3, 10^4 transcripts per 2.5 µl, for *CK19* detection and 10^3, 10^4, 10^5, 10^6 transcripts per 2.5 µl for *ABL* detection. Examples of standard curves for *ABL* (A) and *CK19* (B) are shown in Figure 2.1. Standard curves of reactions that are 100 % efficient have a gradient of -3.32. Only data generated from reactions with standard curve gradients between -3.2 and -3.45 (indicating 95 - 105 % efficiency) and correlation coefficients (r^2) of at least 0.99 were used. If the QPCR reactions were outside these limits, the data was discarded and the reactions repeated.

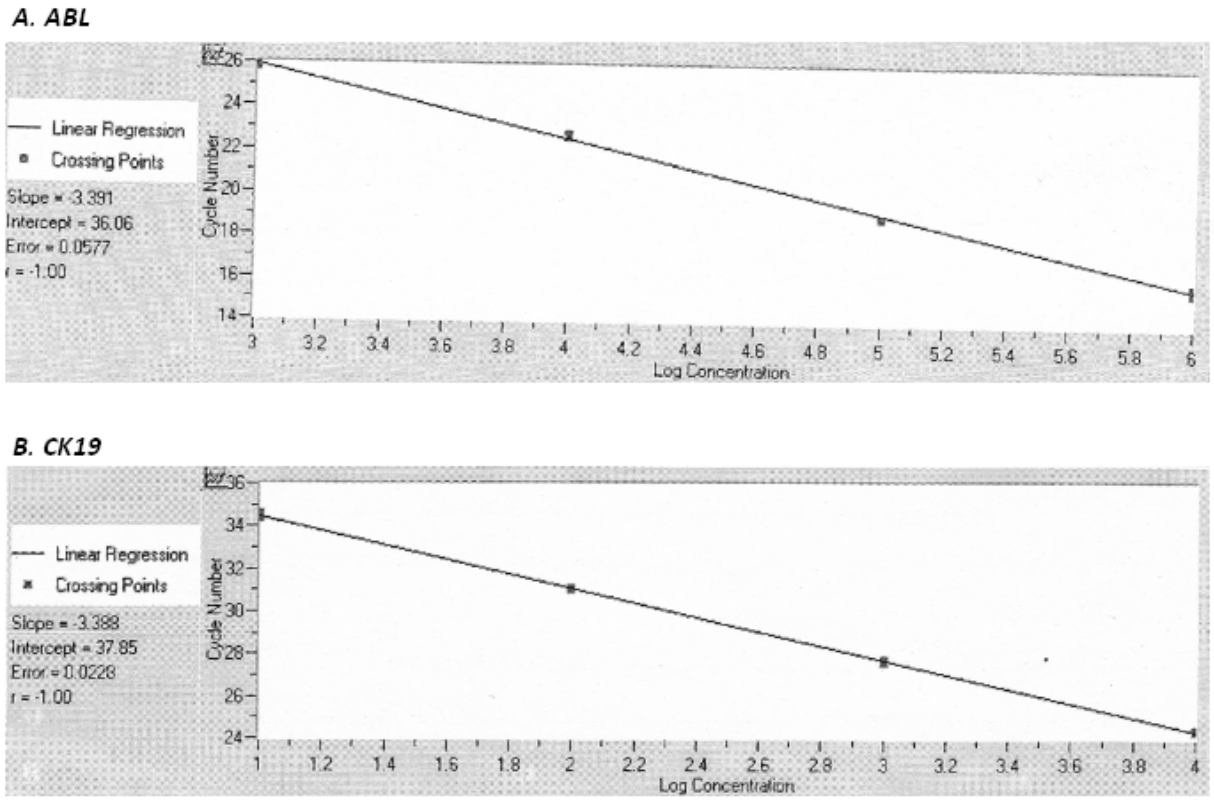


Figure 2.1: Standard curves for the detection of *ABL* (A) and *CK19* (B) transcripts in bone marrow aspirate samples

2.8 Sample preparation for isolation of epithelial cells from blood using the CellSearch system

Blood samples were collected in CellSave preservative tubes (Veridex LLC) containing EDTA as an anticoagulant and a cellular preservative. Samples were kept at room temperature and processed within 72 hours of collection. The CellSearch™ system (Veridex LLC) consists of an autoprep system to process the blood samples and a CellTracks analyser II to scan the samples (Figure 2.2). The autoprep system uses the reagents from the circulating tumour cell kit (Veridex LLC) to enrich and stain CTCs. Ferrofluid (magnetic iron particles 60 nm in radius) conjugated to antibodies against the epithelial cell adhesion molecule (Ep-CAM) were used to immunomagnetically enrich epithelial cells. The cells were labelled with the fluorescent nucleic acid dye 4',6-diamidino-2-phenylindole (DAPI); a mixture of two phycoerythrin-conjugated antibodies that bind to cytokeratins 8, 18 and 19 and a allophycocyanin-conjugated CD45 antibody (to distinguish CTCs from leukocytes). Two phenotyping reagents that were used in the system were FITC-conjugated antibodies against HER-2 and EGFR. A control consisting of SKBr3 cells was also used in each run to ensure the efficiency and specificity of detecting a low and high population of CTCs (Veridex LCC). Briefly, each 7.5 mL sample of blood was mixed with 6.5 mL of buffer and centrifuged at 800 *g* for 10 min, and then placed in the Autoprep system. After aspiration of the plasma and buffer layer by the system, ferrofluids were added. After incubation and subsequent magnetic separation, unbound cells and the remaining blood were aspirated and discarded. The staining reagents were added with a permeabilisation buffer to fluorescently label the enriched cells. After further incubation the magnetic separation step was repeated, and excess staining reagents were aspirated. The cells were then aspirated into the MagNest cell presentation device (Veridex LCC). This device is made up of a chamber and two magnets that hold the immunomagnetically labelled cells to the surface of a cartridge for analysis using the CellTracks Analyser.



Figure 2.2: The components of the CellSearch™ system (Veridex LLC)

2.9 CellTracks Analysis

The CellTracks Analyser is a semi-automated fluorescent microscope. Image frames of the entire surface of the analytical surface of the cartridge are taken using each of the four fluorescent filters. Captured images are then presented in a gallery for analysis. The criteria to define a CTC include: round or oval morphology, a nucleus contained within the cytoplasm which is stained by positive CK 8, 18 and 19 and that has no expression of CD45. In addition, if a tumour phenotyping marker has been added to the autoprep machine an additional column for images of FITC-conjugated to HER-2 or EGFR will appear in the gallery for review.

2.10 Cell-free DNA in the plasma – blood processing, DNA extraction and cfDNA analysis

All performed by N. Hava, Imperial College, London (164) and K. Page, J. Brown and D. Guttery, University of Leicester.

Blood samples were collected in EDTA tubes and processed within three hours of venesection. Plasma was separated by centrifugation at 1200 *g* for 10 minutes, and plasma was taken from the upper phase and decanted into fresh polypropylene tubes. Tubes were then spun again to

remove any contaminating leukocytes (165), and the resulting supernatant was aliquoted into sterile tubes and stored at -80 °C. Plasma samples were allowed to thaw to room temperature and re-spun in a bench-top centrifuge to remove any remaining cell debris as recommended by Page *et al.* (166), and cfDNA was extracted from 1 ml plasma using the QIAamp Blood DNA Kit (Qiagen) following the blood and body fluids protocol. DNA was eluted in 2 x 60 µl elution buffers AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) provided with the kit and stored at 4 °C.

2.11 Calculating cfDNA quantity and integrity

To estimate the cfDNA quantity the concentration of a 96-bp amplicon of *GAPDH* was measured, this was a measure of DNA amplicons below the apoptotic limit. The quantity of a 291-bp *GAPDH* amplicon measured cfDNA amplicons greater than the apoptotic limit, a measure believed to represent necrosis or lysis of tumour cells (99). The cfDNA integrity value was calculated from the quantity of the 291bp fragment divided by the total amount of the two amplicons. Primers and MGB probes were from the 14th intron of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, GenBank Accession No J04038), (164). QPCR reactions were carried out in triplicate on a TaqMan 7900 thermal cycler (Applied Biosystems). Each reaction was 10 µl in volume, made up of 5 µl TaqMan Universal Fast Mastermix (2X) (Applied Biosystems), 6 pmol of each primer, 2 pmol of minor groove binder (MGB) probes with FAM fluorescent dye attached, and 3.6 µl DNA template or double distilled water. The thermal cycling programme consisted of an initial activation step at 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 40 s. Absolute quantitation of cfDNA was obtained by interpolation of the experimental data on standard curves generated from normal human genomic DNA (Roche). Seven concentrations were used ranging from 25 ng to 0.006 ng in triplicate, and generated from the same QPCR plate (Figure 2.3).

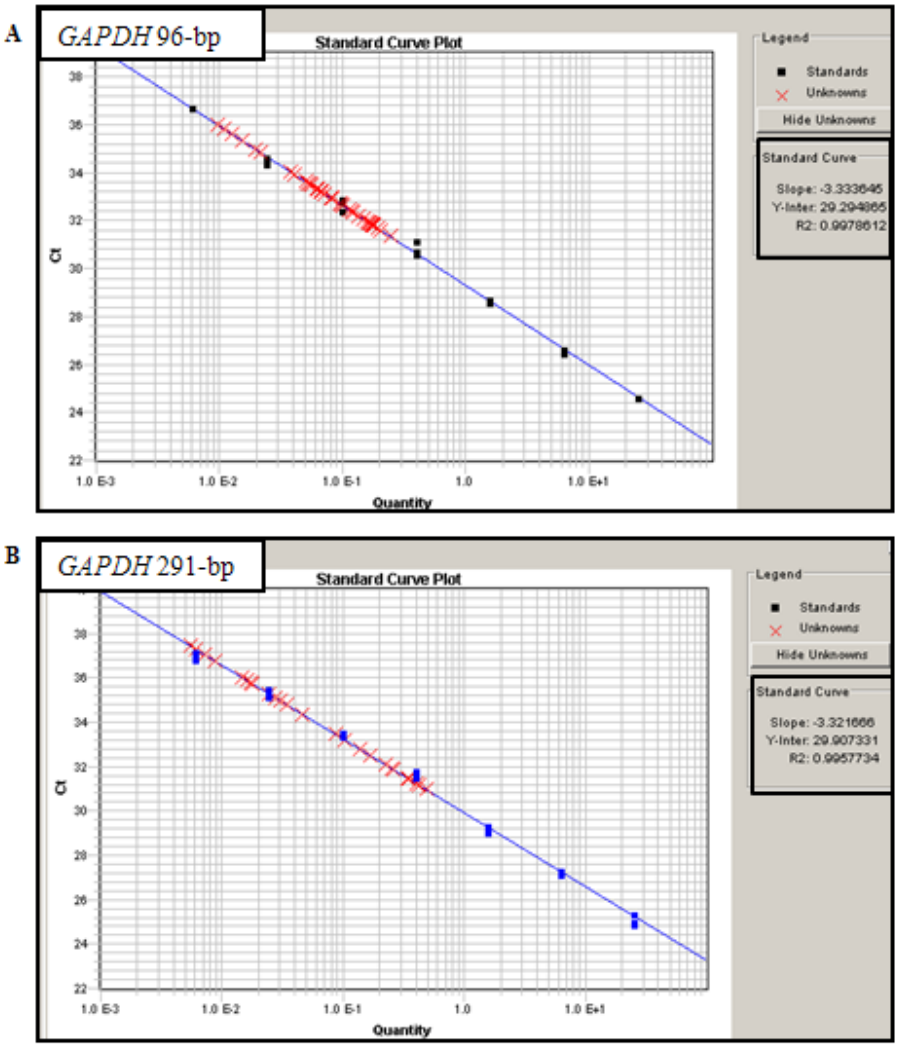


Figure 2.3: Standard curves for the 96-bp (A) and the 291-bp (B) GAPDH cfDNA amplicons

2.12 Optimisation of CTCscope

Assays were performed by F. Wang and N. Su, Advanced Cell Diagnostics, California, USA. Sample collection and help with optimisation of sample preparation by B. Ward and R. Payne, Imperial College, London. The RNAscope/CTCscope method is described in Chapter 5 and summarised in Figure 2.4.

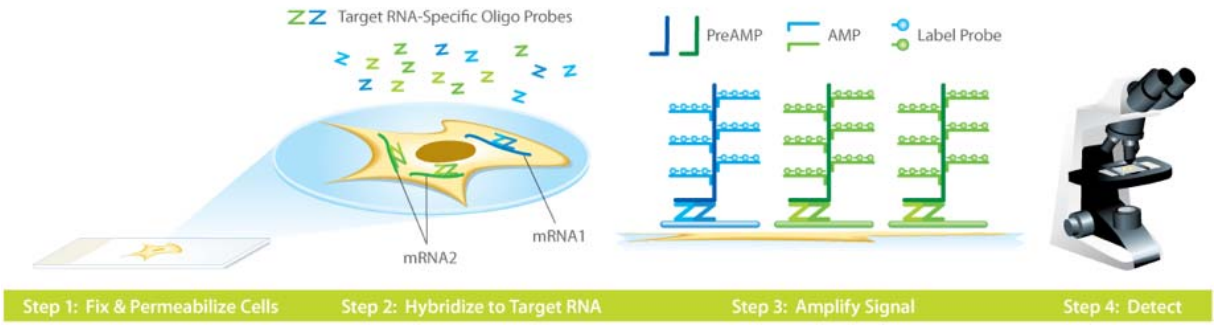


Figure 2.4: Overview of RNAscope method

Optimisation of cell preparation for CTCscope protocol

This is described in more detail in Chapter 5 and explains the optimisation process for the effective detection of CTCs from blood using an RNA *in situ* hybridisation method of tumour markers. Different cell enrichment methods were employed including the following: the CellSearch system, CPT™ cell preparation tube (Becton, Dickinson), Accuspin™ system-histopaque®-1077 tubes (Sigma-Aldrich). These were all tested using the rare-cell spike-in method described below. MDA-MB-468 breast cancer cell line cells were used and spiked into 7.5 ml blood samples donated from healthy volunteers. Between 40 and 100 cells were spiked into blood to test the recovery rates using different protocols. A cell-depletion antibody called RosseteSep, (StemCell Technologies) was tested to find out if depleting the PMBC fraction would aid CTC detection. The cytospins of PBMCs were performed using the Shandon EZ megafunnels (Thermo Scientific) which allowed large quantities of fluid up to 6 ml to be cytospun onto glass slides. Later protocols did not require the use of this step since samples were sent to Advanced Cell Diagnostics, Inc, (CA, US) in 70% ethanol. The samples were then cytospun using a Hettich centrifuge. A wash buffer (0.4% BSA + 1 mM EDTA in 1 X PBS buffer) was used to prevent cell clumping in the final protocol.

Optimised protocol - CTC detection and characterization

For CTC detection by CTCscope, 7.5 ml of peripheral blood was drawn into a vacutainer with EDTA. Samples were processed as soon as possible to prevent RNA degradation. The blood sample was split into two Accuspin™ system-histopaque®-1077 tubes (Sigma-Aldrich) and

centrifuged at room temperature in a horizontal rotor for 20 minutes at 800 *g* with minimal acceleration/break. In the meantime, the wash buffers were prepared for the sample. Eppendorfs of concentrated wash buffer were prepared at Advanced Cell Diagnostics, Inc and contained 1 ml phosphate buffered saline (PBS) with 0.4% bovine serum albumin (BSA) and 1 mM EDTA. This was added to 20 ml PBS and inverted 5 times for the working concentration. The upper plasma phase of the centrifuged blood samples were removed with a pipette leaving approximately 0.5 cm above the PBMC (peripheral blood mononucleocyte) layer. A pipette was then used to transfer the PBMC layer to a 15 ml polypropylene tube containing 10 ml of the wash buffer. Cell loss was minimised by pipetting up and down several times in the wash buffer. The PBMC layer from both ACCUSPIN tubes was combined into this tube. This was centrifuged at room temperature at 250 RCF for 10 minutes. The supernatant was then aspirated away leaving 100 – 150 µl liquid above the cell pellet. The cell pellet was then resuspended in the wash buffer by gently pipetting up and down 10 times until the cell pellet was broken up. Nine ml of the wash buffer was then added and centrifuged again at 250 *g* for 10 minutes and the supernatant removed as before. The PBMC pellet was resuspended in 500 µl of 10% buffered formaldehyde solution by gently pipetting up and down 10 times to break apart the pellet. This suspension was transferred to a 2 ml micro-centrifuge tube with 500 µl of the 10% buffered formaldehyde solution used for collecting any remaining cells in the tube, this was also transferred to the micro-centrifuge tube. This was inverted several times and then incubated at 37◦C for 40 minutes with rotation. Following this, the tube was centrifuged at 5000 *g* for 1 minute to pellet the cells. The supernatant was then removed very carefully by tipping the tube at an angle so that the pellet was not disturbed (Figure 2.5).

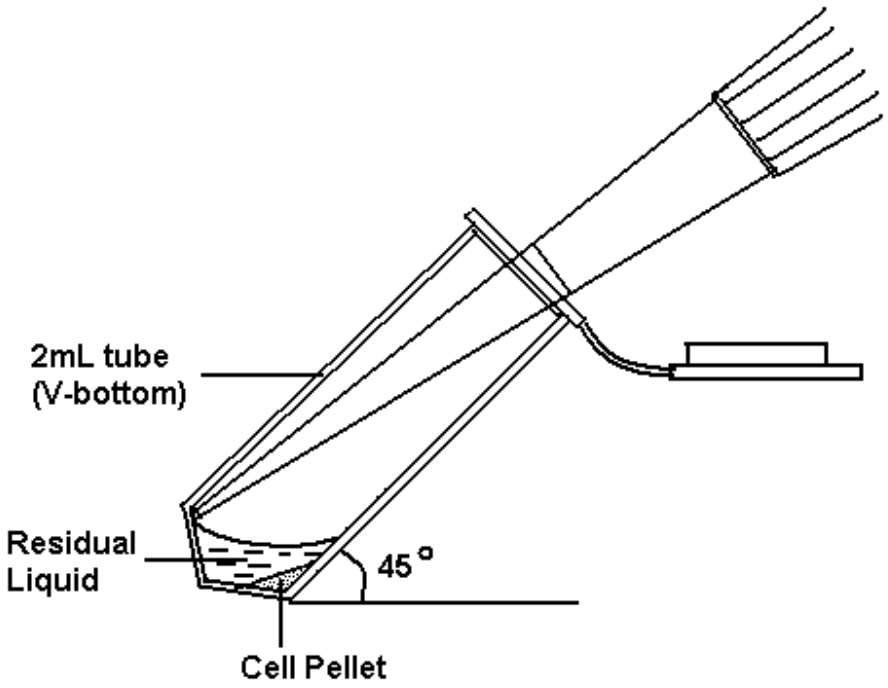


Figure 2.5: Method to remove supernatant without disturbing the PBMC pellet containing rare CTCs or spiked-in cells.

One ml of post–fixation buffer was then added followed by mixing on a vortex at 1/3 of the maximum output for 3 seconds. The tube was then inverted 3 times and vortexed for another 3 seconds. The tube was centrifuged again at 5000 *g* for 1 minute and the supernatant removed. The wash was repeated and centrifuged at the same conditions. The supernatant was removed and 700 µl of 80% ethanol/PBS was added. The pellet was resuspended by vortexing. The cap was tightly closed and parafilm was wrapped around the tube. The tube was then kept at 4°C for up to 7 days prior to shipping at room temperature to Advanced Cell Diagnostics, Inc.

2.13 Method for rare - cell spiking

To ensure a more accurate spike of very low cell numbers (1 – 60 cells) a different technique was used. The cell lines were prepared in the same way as above. A 40X22 glass coverslip was wiped

clean using 70% ethanol. A suitable concentration of cultured cancer cells (MDA-MB-648) were resuspended (around 50,000 cells/ml) in DMEM-supplemented media and 1 µl was transferred to the corner of the glass coverslip. This was performed to ensure the drop was neither too thick to allow inaccurate enumeration or too think to allow evaporation (Figure 2.6).

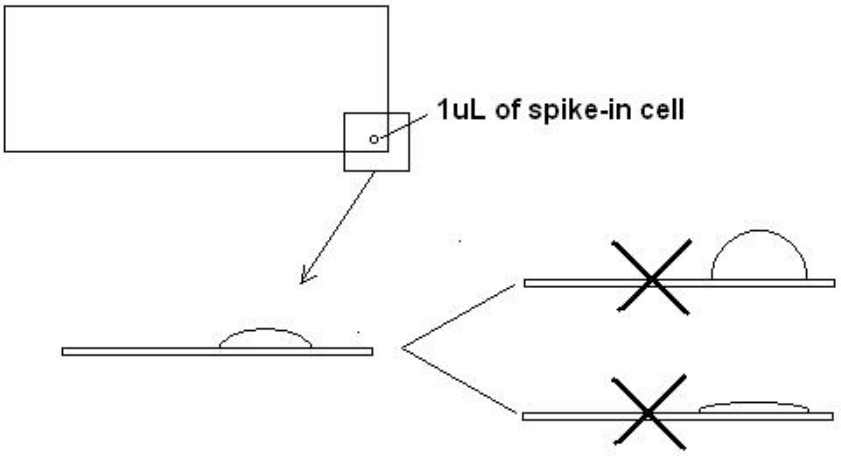


Figure 2.6: The rare cell counting method for spiking low numbers of cultured cells into blood

The cells were then counted under a 10X magnification on a bright field microscope with the entire 1 µl drop in one field of view. The cells were then counted using a tally counter. The corner of the coverslip was then placed next to the inner wall of the blood tube and then the drop of cells is then washed into the blood sample. This was achieved by washing the front side of the coverslip 4 times and the back once with 50 µl 1 X PBS with enough flushing force from the pipette to wash the cells into the sample. All the residual PBS on the slide was also placed in the sample. The blood tube was then inverted 5 – 10 times with the lid on (Figure 2.7).

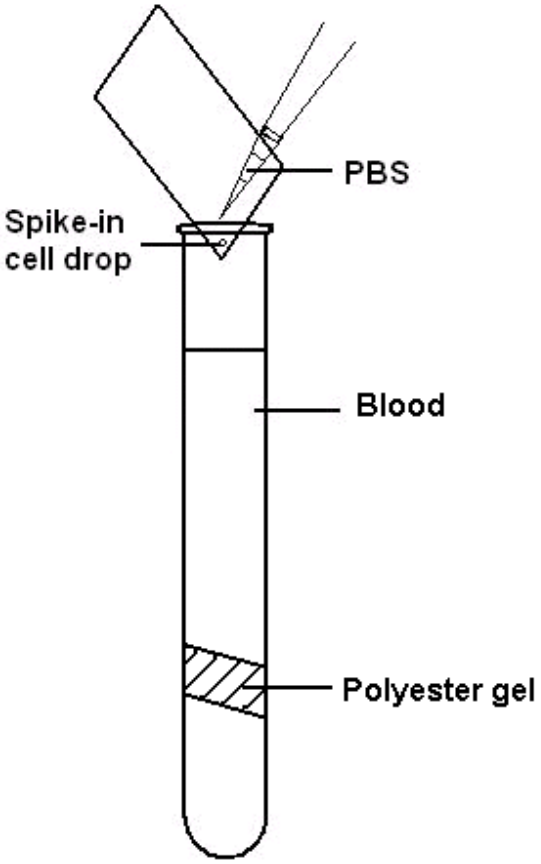


Figure 2.7: The rare cell spiking method for spiking low number of cultured cells into blood

2.14 Target probe design and the signal amplification system

Target probes were generated using modified ProbeDesigner Software (167). Each target probe contains between 18 and 25 bases of complementary sequence to the target mRNA and are screened *in silico* against nonspecific hybridization with other human genes. Initial experiments used target probes for *CK8*, *CK18* and *CK19*. Further optimisation incorporated more tumour markers and those known to be important in metastasis, these included *CK14, CK17, CK20, EpCAM, MUC1, TWIST, N-Cadherin* and *Fibronectin*. Target probes were also developed for the detection of *EGFR* transcripts (All target probe sequences are detailed in the Appendix). The two 14-bp tails of each target probe pair form a hybridization site for the signal amplification system

consisting of PreAmplifier (PreAMP), Amplifier (AMP), and Label Probe (LP). The PreAMPs are hybridized at a temperature that only allows hybridization to both tails of a target probe pair but not to the tail of individual target probes. Each PreAMP contains 20 AMP hybridization sites, and each AMP has 20 LP hybridization sites. For a typical mRNA target, 20 target probe pairs are designed spanning a 1kb region resulting in the concentration of up to 8,000 LPs onto a single target. For fluorescent detection, LPs are conjugated with Alexa fluorescent dyes. For chromogenic detection, alkaline phospatase or horseradish peroxidase is conjugated to LPs for detection with FastRed or DAB, respectively.

2.15 CTCscope assay

For the CTCscope staining of cell lines or PBMCs, cells were placed on slides and fixed in 4% formaldehyde followed by protease digestion. Next, cells were incubated at 40 ^{o}C with the following solutions in this order: target probes in Hybridization Buffer A containing 6X SSC (saline-sodium citrate), 25% formamide, 0.2% LDS (lithium dodecyl sulfate), blocking reagents] for 3 hrs; PreAMP in Hybridization Buffer B (20% formamide, 5X SSC, 0.3% LDS, 10% dextran sulfate, blocking reagents) for 30 min; AMP in Hybridization Buffer B at 40^{o}C for 15 min; and LP in Hybridization Buffer C (5X SSC, 0.3% LDS, blocking reagents) for 15 min. Following each hybridization step, slides were washed with wash buffer (0.1X SSC, 0.03% LDS) three times at room temperature. For multiplex detection, equimolar amounts of Target Probes, PreAMPs, AMPs, and LPs of each amplification system were used.

2.16 Detection of mRNA transcripts in FFPE tumour tissues by RNAscope

Tissue sections were cut and subjected to deparaffinization in Histoclear (National Diagnostics,) followed by dehydration in an ethanol series. Tissue sections were then boiled in citrate buffer followed by protease digestion and hybridization with target probes, using the protocol above. A probe set targeting a housekeeping gene: Ubiquitin C (UbC) was used as a positive control to ensure mRNA was present in each tissue section. A no-probe control was used as a negative control for each section and EGFR probes were used to stain tumour tissue sections with an EGFR positive tumour section stained with each run.

2.17 QuantiGene 2.0 analysis

This was performed by F. Wang and N.Su at Advanced Cell Diagnostics, Inc, CA, US. MCF-7, MDA-MB-231 and MDA-MB-468 cells were trypsinized to get a cell count and then lysed for QuantiGene 2.0 (Affymetrix/Panomics, Fremont, California). This was performed as per the manufacturer's instructions. The samples were thawed and diluted as appropriate. The working probe set was prepared (EGFR). These were dispensed into capture plates with the samples and the controls. These were left to hybridise overnight. Unbound material was then washed away and the following signal amplification probes were sequentially hybridised: 2.0 Pre-Amplifier, 2.0 Amplifier and the label probe. 2.0 Substrate was then added and incubated followed by reading of the chemiluminescence signal on a microplate luminometer. EGFR copy numbers in the lysate were determined by using a standard curve of *in vitro* transcribed RNA. Per cell copy number was determined by dividing the number of mRNA molecules in the lysate by the number of cells used in the assay.

2.18 Microscopic imaging

For the assay validation of RNAscope, images were obtained using an Olympus IX71 fluorescent microscope and a PXL37 CCD camera (Photometrics, Tucson, Arizona). For multiplex RNAscope staining and the studies with patient samples, images were acquired using a Zeiss Axioplan M1 microscope and Nuance Multispectral Imaging System (CRI, Cambridge, Massachusetts). Overlapping signals from different fluorophores were separated by comparing composite signals to a reference spectral library generated with single colour stained samples.

2.19 Validation of the tumour phenotyping reagent EGFR

One hundred cells from a cell line known to be low in EGFR expression (MCF-7) and 100 cells known to be high in EGFR expression (MDA-MB-468) were spiked in blood from healthy volunteers in duplicate and put through the CellSearch™ System. The tumour phenotyping reagent EGFR was added to the machine and the cells were enumerated and analysed for EGFR staining. The criterion for positivity was the presence of staining with a clear distinguishable edge to the cell, staining that was faint or hard to define was deemed negative. The protein expression

levels of EGFR in MCF-7 and MDA-MB-468 cells were confirmed by western blot as detailed below.

2.20 EGFR measurement in metastatic breast cancer patients

Thirty-three patients with metastatic breast cancer (recruited with appropriate ethical approval) had sequential blood samples taken in CellSave tubes (Veridex LLC) (74 samples in total) and processed through the CellSearch System. Five patients who had measureable CTCs also had repeat samples taken. The number of CTCs was enumerated and the number staining for EGFR was analysed. The criteria for an EGFR positive cell was a clear image with a distinguishable edge to the cell in the marker reagent channel, along with a dark background.

2.21 Protein analysis

Preparation of cell lysates

For the evaluation of EGFR and HER2 expression, cell lines were trypsinised, harvested and washed with PBS and 100-200 µl of lysis mix was added. Lysis mix consisted of 100 ml lysis buffer (150 mM NaCl, 0.1% sodium dodecyl sulphate, 5 mM EDTA, 10 Mm Tris (pH 7.2), 1% Triton-X and 1% deoxycholate), 1 ml of 100 mM phenylmethanesulfonylfluoride in methanol and 0.5 mL protease inhibitor cocktail (Sigma P8340: AEBSF 104 mM, Aprotinin 0.8 mM, Leupeptin 2 mM, Bestatin 4 mM, Pepstatin A 1.5 mM, E-64 1.4 mM). Samples were kept on ice for 10 min and vortexed every 2 min followed by shearing of DNA using a needle and syringe. Samples were centrifuged at 4°C, 500 *g* for 10 min. The supernatant was aspirated and frozen at -80°C for western blot analysis.

Determination of protein concentration

Bradford reagent (Pierce Biotechnology) was used to stain protein lysates. Bovine serum albumin (BSA) (Sigma Aldrich) at 0, 3.125, 6.25, 12.5, 50 and 100 µg/ml in deionised water with 10 µl/ml western lysis buffer (previously described) were used as standards. Samples were diluted 1/50, added to the Bradford reagent and analysed at 595 nm on a microtitre plate reader.

Western blotting

Protein extracts (20 μg/μl) were heated at 100°C for 10 min. Samples were loaded onto a 15% SDS-TRIS polyacrylimide gels. Biorad kaleidoscope ladder was run as a molecular weight marker and the gel was run at 60 volts through the stacking gel, and 100 volts though the running gel. Proteins were transferred to nitrocellulose filters at 100 volts for 1 hour. Ponceau staining of the membrane was used to check efficient transfer. Membranes were washed in 0.1% TBS-Tween (TBST), and incubated in 5% BSA in 0.1% TBST on a shaker at 4°C for 1 hour. Membranes were probed with Total EGFR and HER2 (Cell signaling Technologies) antibodies diluted in 5% BSA in 0.1% TBST overnight. Probed membranes were washed in 0.1% TBST and secondary antibodies (anti-goat IgG- GE Healthcare Ltd; 1/1000 in 5% BSA-0.1% TBST) were incubated for 1 hour at 4˚C. Protein bands were visualized using chemiluminescence ECL reagents (Amersham Bioscience), according to the manufacturer's protocol.

2.22 Treatment with lapatinib in breast cancer cell lines

MDA-MB-468 and MDA-MB-231 cells were plated and treated with 100ng/ml of EGF (Sigma-Aldrich, Poole, UK) for 10 minutes followed by lapatinib (also known as Di-*p*-Toluenesulfonate Salt, LC laboratories) in differing concentrations of 0.5 mM, 2.5 mM and 5 mM for 6 hours along with a DMSO vehicle control. A western blot was performed as above for EGFR (Cell Signalling) and actin.

2.23 Immunofluourescence staining of pEGFR in EGF and lapatinib treated cell lines

Cells were cytospun onto glass slides. Cells were washed in PBS, fixed with 4% formalin, permeabilised with 0.2% Triton-X and blocked with 5% human antibody serum (hAbS). FITC conjugated p1068 (R&D) was diluted in 5% hAbs (2.5 units/ml), and incubated at room temperature for 30 minutes. Cells were stained and mounted with Vectorshield 4',6-diamidino-2-phenylindole (DAPI) to stain cell nuclei.

2.24 Immunohistochemistry of EGFR staining

Staining of total EGFR in FFPE primary breast cancer section biopsies was performed by D. Peston, and scoring was performed by S. Sousha, (Charing Cross hospital). Paraffin sections of 5 µm thickness were de-paraffinised by incubating sections in three washes of xylene (Sigma-Aldrich, Poole, UK) for 5 minutes each, followed by incubation in two washes of 100% ethanol (Sigma-Aldrich, Poole, UK) for 10 minutes each and lastly, incubation in two washes of 95% ethanol (Sigma-Aldrich, Poole, UK) for 10 minutes each. Slides were then washed in dH_2O twice for 5 minutes each, followed by washing in phosphate buffered saline (PBS) (10x PBS: 0.58M sodium phosphate dibasic, 0.17M sodium phosphate monobasic, 0.68M NaCl; pH 7.4) for 5 minutes. Sections were then incubated with 3% hydrogen peroxide for 10 minutes and washed again in dH_2O followed by PBS.

For antigen retrieval, enzyme digestion was performed. Tissue sections were incubated with 2-4 drops of pepsin (Digest-All 3, Zymed Laboratories Inc) for 10 minutes at 37^{o}C. The slides were allowed to cool to room temperature and then washed in dH_2O followed by PBS. Each section was blocked with 400 µl of blocking serum (5% normal goat serum) for 30 minutes to block non-specific staining. Sections were washed with PBS and incubated with 400 µl of primary antibody (Zymed total anti-EGFR, Zymed Laboratories Inc) overnight at 4^{o}C. The primary antibody was removed and sections washed in PBS three times for 5 minutes each followed by 400 µl of the secondary and tertiary (horseradish peroxidase) antibody system (ChemMate EnVision/HRP, rabbit/mouse; DAKO Cytomation) for 30 minutes at room temperature. Sections were washed with PBS and stained with DAB (3'3'3' diamino benzamidine tetrahydrochloride) reagent and hydrogen peroxide buffer (DAKO Cytomation), until they turned brown. To intensify the DAB, sections were immersed in 0.5% $CuSO_4$ in 0.9% NaCl for 2 minutes. Following washing in dH_2O the sections were counterstained in Coles Haematoxylin (Pioneer Research Chemicals Ltd) for 30 seconds, and washed in tap water. Samples were dehydrated in 95% ethanol (two times for 10 seconds each) and 100% ethanol (2x10 seconds), followed by xylene (2x10 seconds) and then mounted on glass coverslips with Pertex mounting medium (CellPath Plc).

2.25 Statistical methods

Chapter 3 - Due to small numbers exact two-sided nonparametric tests were used throughout at 5% level of significance. The exact Pearson chi-square test was used for testing association of two categorical variables, the Mann-Whitney test for the comparison of continuous variables between the two groups, and the Spearman correlation for correlations of two continuous variables. (performed by Elena Kulinskaya, Statistical advisory service, Imperial College London).

Chapter 4 - Non-parametric statistical tests were used for analysis: Spearman Rank correlation (corrected for ties) was used to examine the associations between continuous or ordinal factors; the Mann-Whitney and Kruskal-Wallis test were used to compare continuous or ordinal scores between two groups and the chi-square test or Fisher's exact test was used to assess associations in categorical data. The two-sample t test and one way analysis of variance test were used for normally distributed data to compare two groups and more than two groups, respectively. Measurements were frequently skewed, e.g. some patients consistently had no 291-bp amplicons in cfDNA or very low levels of cfDNA (performed by the author and Roger Ahern, Institute of Cancer Research, London).

Chapter 5 – The Fisher's exact test was used to evaluate the relationship between CTC detection by the CellSearch system and by CTCscope. Non-parametric tests were used to examine the associations of CTCs with continuous or ordinal factors (performed by the author).

Chapter 6 – The paired t test was used to determine an association of two methods for EGFR measurement. The Fisher's exact test was used to evaluate the concordance between EGFR detection by IHC and by RNAscope and for EGFR Measurement in the primary tumour to EGFR detection in CTCs (performed by the author).

2.26 Lapatinib monotherapy, phase II clinical study

This study is a multicenter open-label, phase II study to evaluate the efficacy and safety of daily dose of lapatinib in advanced breast cancer patients with HER-2 non amplified primary tumours and positive HER2 or EGFR circulating tumour cells.

Trial design and patient selection

Patients enrolled in this study were treated with oral lapatinib at the dose of 1,500 mg daily on day 1 to 28 every 4 weeks. A daily dose of lapatinib is six 250 mg tablets taken approximately at the same time each day. Lapatinib must be taken either at least 1 hour before or after meal.

The primary objective of this study is to evaluate the efficacy of a daily dose of lapatinib in advanced breast cancer patients with HER-2 non-amplified primary tumours and EGFR positive CTCs.

The secondary objectives of the study are:

- To evaluate antitumour activity of lapatinib
- To correlate response to lapatinib with HER2 and EGFR protein levels and amplification on CTCs as part of the translational research
- To evaluate the safety of lapatinib

Endpoints:

The primary endpoint for the analysis is to evaluate the overall response rate (ORR) according to RECIST criteria.

The secondary efficacy endpoints are:

- Clinical Benefit Rate (CBR = CR+PR+SD≥24 weeks)
- Duration of response (DoR) and of Clinical Benefit (DoCB)
- Time to Tumour Progression (TTP)
- Time to Best Response (TBR)

The translational research endpoints

- Evaluation of biological effects on EGFR and HER-2 in CTCs.
- Comparison between CTC and tumour samples from the primary and/or from the

metastatic site in terms of biological marker expression profiles.

The study will consist of three study periods: screening, treatment and follow-up.

- Screening Period: The screening period will include time from the time point in which eligible subjects sign the informed consent form until confirmation of eligibility from central laboratory based on CTCs EGFR and HER2 status. The screening phase can last up to 28 days prior to 1st dose.
- Treatment Period: The treatment period will start from the 1st study drug dose intake until disease progression, unacceptable toxicity or death whichever occurs first.
- Follow up Period: This period will commence 28 days from the time of last study drug dose intake. Post- study survival follow up status will be collected every 12 weeks and can be collected by phone if the patient cannot attend the hospital.

Inclusion criteria

This study attempted to prove the concept that patients who would never be treated with anti-HER2 compounds can indeed be considered for this option. From the ethical point of view this option is acceptable even as first-line treatment for metastatic disease because patients will receive other treatments after progression. In clinical practice breast cancer is always treated sequentially with various agents.

1. Signed written informed consent
2. Female patients, age ≥ 18 years
3. Histologically confirmed diagnosis of HER2 negative (i.e. no gene amplification by FISH or IHC 2+ and no amplification by FISH or IHC 0/1+) infiltrating primary breast cancer.
4. Evidence of EGFR positive circulating tumour cells in a 7.5 ml peripheral blood sample taken at screening visit.
5. Metastatic disease with at least one measurable lesion as per RECIST criteria classification (168).
6. No brain metastasis requiring local therapy. Stable or asymptomatic subjects are

allowed.

7. Availability of tumour block or at least six unstained 4 µ sections of the primary tumour to be sent to the central lab after eligibility confirmation for HER-2 central testing by FISH.
8. Eastern Cooperative Oncology Group (ECOG) score for performance status 0 to 2 (Chapter 6, Table 6.8).
9. Life expectancy > 12 weeks
10. Adequate baseline organ function at screening visit.
11. Normal left ventricular ejection fraction (LVEF) by echocardiogram or MUGA.
12. Negative serum pregnancy test (only in childbearing potential female).
13. Patients with reproductive potential need consistent and correct use of adequate non-hormonal methods of birth control (barrier methods or intrauterine device).
14. Previous treatment with anthracyclines and/or taxanes in the neo-adjuvant, adjuvant or advanced setting.

Exclusion Criteria

Subjects meeting any of the following criteria must not be enrolled in the study:

1. Any unstable systemic disease including active infections, significant cardiovascular disease, as well as myocardial infarction within the previous year, any significant hepatic, renal or metabolic disease, metabolic dysfunction, physical examination finding, or clinical laboratory finding that contraindicates the use of study medication or that might affect the interpretation of the results or render the patient at high risk from treatment complications.
2. Dementia, altered mental status, or any psychiatric condition that could prohibit the understanding or rendering of informed consent
3. Lack of physical integrity of the upper gastrointestinal (GI) tract, poor absorption of the GI tract or inability to take oral medication
4. Other co-existing malignancy or malignancies within the last 5 years with the exception of basal cell carcinoma or in-situ carcinoma.
5. Concurrent anti-cancer therapies (chemo or hormonal therapy) other than study drug.

6. Concurrent treatment with an investigational agent or participation in any investigational drug study within 4 weeks preceding treatment start.
7. Concurrent radiotherapy to the only target lesion or concurrent bisphosphonates if bone metastases are the only target lesions.
8. Previous treatment with anti HER-2 or anti-EGFR therapies.
9. Protocol specified treatment regimens that would be inappropriate for the management of the subject.

3.0 The detection of circulating tumour cells in primary breast cancer patients on follow-up

3.1 Introduction

Over the last decade, clinical trials evaluating adjuvant endocrine and chemotherapy in breast cancer patients on long-term follow-up have indicated improved relapse-free and overall survival. This is particularly apparent in studies assessing novel combinations and sequencing of therapies compared to single therapies (169-171). A monitoring system to assess whether a patient needs further sequential therapy is urgently required to prevent therapies given when it is not necessary. Also, the real-time observation of minimal residual disease may help clinicians switch to a more appropriate therapy sooner, reducing the chance of relapse. A test to assist in the optimal type and duration of therapy is critical in these patients who may have prolonged disease-free intervals but who have MRD and are at risk of clinical relapse.

The detection of DTCs in the bone marrow of primary breast cancer patients is associated with worse progression-free and overall survival (59). Their presence however, does not always indicate that relapse will occur (82). This, along with the painful nature of bone marrow aspiration makes this method of monitoring patients problematic. The detection of tumour cells circulating in the blood may be a practical alternative since blood samples are much more acceptable to patients for regular monitoring. The prognostic implications for the detection of CTCs in blood samples from patients have not been fully explored partly due to the relative rarity of CTCs compared to DTCs as well as the lack of a standardised, sensitive method. For this study, we used the CellSearch system which is the only currently available automated and FDA validated method for detecting CTCs. The system was acquired by our department in 2007 for this purpose and also for an additional study for monitoring CTCs in metastatic breast cancer patients on a targeted therapy (discussed later in Chapters 6 and 7). Before initiation of these studies, the CellSearch system was tested by performing spiking experiments with four breast cancer cell lines to determine the efficacy of enriching and detecting tumour cells from the blood. CTC

testing in control groups of healthy volunteers, benign breast disease patients and advanced breast cancer patients was also undertaken to compare with numbers found in primary breast cancer patients.

3.2 Aims and objectives

- To establish the sensitivity of the CellSearch system at detecting a range of phenotypically diverse breast cancer cell lines spiked in blood
- To determine the number of CTCs found in healthy controls, patients with benign breast disease and patients with advanced breast cancer and to compare with the numbers present in patients with primary breast cancer on follow-up
- To investigate the use of CTCs as a test for impending metastatic disease in patients with primary breast cancer on long–term follow up as an alternative to measuring DTCs (by ICC and qRT-PCR) in the bone marrow

3.3 Patients and sampling

The cohort of disease-free breast cancer patients we studied included a high risk group who had more than 2 involved lymph nodes (LNs) positive at surgery. This group was compared to patients at very low risk of relapse with small tumours and no involved LN to determine the differences in CTC detection, because LN involvement is one of the most important current indicators of a poorer prognosis (9). Many studies have focused on the period straight after surgery whereas few studies have studied patients 4 years post- surgery. Around 1% of patients relapse per year in this period accounting for a small but significant group of patients who develop incurable metastases between clinic visits (172). The detection of CTCs in sequential samples in these patients was investigated, the hope being that larger sample volumes (22.5 ml of blood) taken regularly every 3 months over a period of 6 – 34 months would detect more CTCs and provide an indicator of impending metastatic disease. Disseminated tumour cell detection by both ICC and qRT-PCR from bone marrow aspirates was also performed in the same patients to compare to the CTC measurements. At the time of initiation no other study had performed such an intensive monitoring programme for CTC detection in primary breast cancer patients. This study was published in 2009 (173), (appendix).

The CellSearch system was used to detect EPCAM positive, Pan-CK positive, DAPI-positive and CD45-negative cells from the blood. Each sample run also had a control run through it consisting of a breast cancer cell line with a marker to detect low numbers and high numbers to ensure sensitivity and specificity.

3.4 Results

3.5 Detection rates of breast cancer cell lines using the CellSearch system

Four different breast cancer cell lines were spiked in 7.5 ml of blood from healthy volunteers at three concentrations in triplicate to assess the efficacy and reproducibility of the CellSearch system in our laboratory. A diverse range of cell lines was used to reflect the different phenotypes of breast cancers in patients, for example two were oestrogen receptor positive (MCF-7 and ZR-75-1 cells) and two were oestrogen receptor negative (MDA-MB-231 and SKBR-3 cells) (158, 160, 162, 174). These cell lines also had widely differing expression levels of HER2, for example SKBR-3 cells demonstrated high expression and MCF-7 and MDA-MB-231 have very low expression, while ZR-75-1 cells have intermediate expression (43). A range of spiking concentrations was also used (10, 50 and 500 spiked cells respectively, per 7.5 ml blood) to reflect the diverse number of CTCs found in patient samples (117). Recovery rates varied considerably from 27 – 69%. The ER-negative SKBR-3 cell line had the highest mean recovery rate of any of the cell lines (69%) and the ER positive MCF-7 cell line had the lowest mean recovery rate of 27%. Mean rates of recovery of ZR-75-1 cells and MDA-MB-231 cells were intermediate at 33% and 46%, respectively (Figure 3.1). The rates of recovery obtained with SKBR-3 cells were similar to previous reports (116).

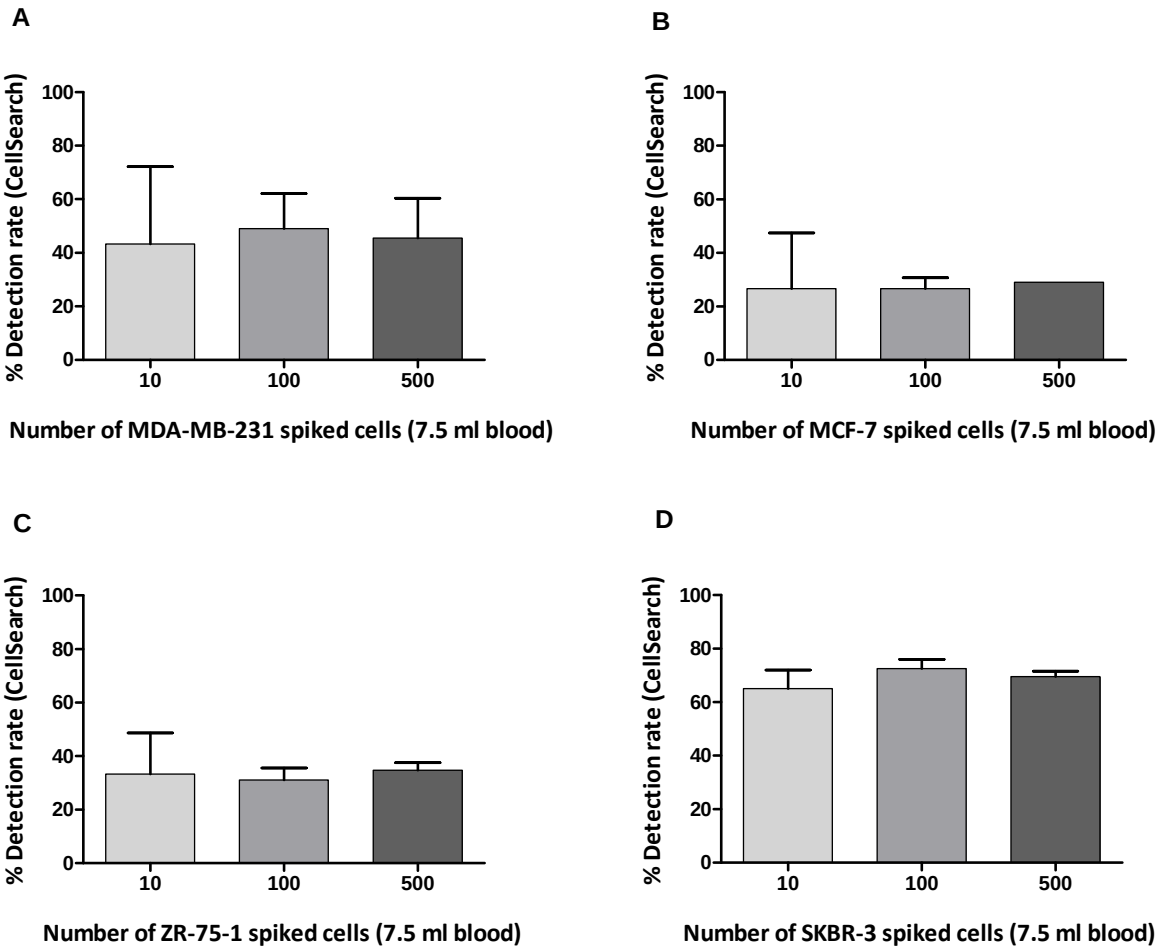


Figure 3.1: Detection rates of four phenotypically diverse breast cancer cell lines spiked in 7.5 ml blood and processed by the CellSearch system. Spiking was performed in triplicate with three concentrations of cells. *A*, MDA-MB-231; *B*, MCF-7; *C*, ZR-75-1 and *D*, SKBR-3 cells. SKBR-3 cells had the highest mean recovery rate of 69%. Error bars are shown (SEM).

3.6 Patients and samples

Fifty-one patients who were being routinely followed up 5 – 14 years (mean 9.9 years) after diagnosis for primary breast cancer were included in this study. These patients were recruited from a subset of an initial cohort of patients recruited to our long-running minimal residual disease studies (82, 83). No patient had any sign or symptom of metastatic breast cancer. The group consisted of eighteen patients who had small tumours and no evidence of spread to the

LNs at the time of surgery and 33 patients who had more than 2 involved LNs at surgery. Fourteen LN positive patients and one LN negative patient were taking adjuvant endocrine therapy. No patient changed treatment during the course of the monitoring period. The higher risk patients (LN positive) had two bone marrow aspirations taken 6 - 12 months apart. For ethical reasons, patients with negative LNs had one bone marrow sample taken yearly. All patients had between 1 - 7 samples of peripheral blood taken (three tubes or 22.5 ml of blood at each time point) for immunocytochemical evaluation using the CellSearch system approximately 6 - 34 months apart. In total, 235 blood samples were taken. These patients also had an isotopic bone scan, liver ultrasonography and chest radiology, as well as blood evaluation for full haematological and biochemical screen to exclude overt metastatic disease. All but two patients had no evidence of metastatic disease during the study. For ethical reasons, only patients with primary breast cancer had bone marrow aspirates taken. The number of CTCs and DTCs present in blood and bone marrow, respectively, were compared in the LN positive (high risk of relapse) and negative (low risk of relapse) primary breast cancer patient groups. Statistical analysis was performed by Elena Kulinskaya (Statistical Advisory Service, Imperial College).

3.7 CTC measurement in control groups

The investigation of the number of CTCs found in healthy controls and benign breast disease patients was important to determine if their presence was significant in the high risk primary breast cancer group. We hypothesised that either nil or low numbers of CTCs would be present in the healthy, benign and low risk groups whereas evidence of CTCs in the high risk, LN positive group was expected. Three control groups were recruited to measure CTCs in the blood; these consisted of healthy controls (n = 29), benign breast disease (BBD) patients (n = 28) and metastatic breast cancer patients (n = 12). The amount of blood taken for CTC measurement was 22.5 ml in the healthy controls and benign breast disease patients and 7.5 ml in the metastatic breast cancer patients. One CTC was found in one out of 29 (3%) healthy controls. For the BBD patients one CTC was present in 3 patients and 5 CTCs were found in one. A total of 4 patients were positive out of 28 (14%). The patient who had 5 CTCs detected in 22.5 ml of blood had a fibroadenoma diagnosed and three patients with one CTC in 22.5 ml of blood had a diagnosis of

breast pain, breast lumps and breast cysts, respectively. These results were similar to previous reports of CTCs in these control groups when taking into account the higher quantity of blood (22.5 ml) we used (73, 117). For the patients with primary breast cancer, 46 of the 235 (20%) samples were positive for CTCs. Finally, 7 metastatic breast cancer patients had CTCs present out of 12 (58%). The number of CTCs detected in primary breast cancer patients was significantly different to both healthy controls ($p=0.03$) and metastatic breast patients ($p<0.0001$) but not to patients with benign breast disease ($p=0.48$). When a cut-off of positivity was used of 2 or more CTCs per sample, the number of CTCs in these groups was still not significantly different ($p=0.73$). (Table 3.1).

Table 3.1: CTC measurements in healthy controls, benign breast disease patients and primary and metastatic breast cancer patients

		Healthy controls (n = 29)	**Benign breast disease patients (n = 28)**	**Primary breast cancer patients (n = 51)**	**Metastatic breast cancer patients (n = 12)**
Circulating tumour cells[a]	Volume of blood measured	22.5 ml	22.5 ml	22.5 ml	7.5 ml
	No. of samples positive for CTCs per test[b]	1/29 (3%)	4/28 (14%)	46/235 (20%)	7/12 (58%)
	Mean	0.03	0.29	0.46	51.33
	Median	0	0	0	12.5
	95% confidence interval	0.04 – 0.11	0.09 – 0.66	0.18 – 0.63	5.21 – 108.48
	Range	0 - 1	0 - 5	0 - 23	0 - 301

[a] Detected with the CellSearch system
[b] Samples were considered positive for CTCs when at least one cell was detected per total sample volume

3.8 Comparison of CTCs in LN positive versus LN negative primary breast cancer patients

Clinical characteristics and results for all patients with primary breast cancer are shown in tables 3.3 and 3.4. All 51 patients were tested for CTCs using the CellSearch system at 1 - 7 time points (mean 4.6). In total, 30 out of 51 (59%) showed evidence of 1 or more CTCs. There was no difference in the

number of tests between the LN positive and negative groups (p=0.812). There were some differences both in the time from diagnosis to start of testing (p=0.0001) (median 97 months, IQR (Inter-quartile range) 91 – 110 in the LN negative group versus 116 months, IQR 98 – 134 in the LN positive group) and in the interval of testing between the groups (p=0.014) (median 16 months, IQR 6 – 27 in the LN negative versus median 29 months IQR 12.5 - 34 in the LN positive group), but these time variables were not associated with the results of the CTC tests within the groups (p= 0.274 and p=0.602).

Patients with LN positive disease at surgery had significantly more CTCs (at least 1 positive) than patients with LN negative disease with 22 out of 33 (70%) positive compared to 7 out of 18 (39%) respectively, (p=0.042, exact chi-square test). Since we occasionally find 1 positive cell in controls, we then analysed our results taking more than 1 cell as positive: 2 out of 18 (11%) LN negative patients versus 10 out of 33 (30%) LN positive patients were positive using this cut-off point. This was not statistically significant (p=0.174, exact chi-square test) but this may be due to the small number of patients with 2 or more CTCs present (Figure 3.2 and Table 3.2).

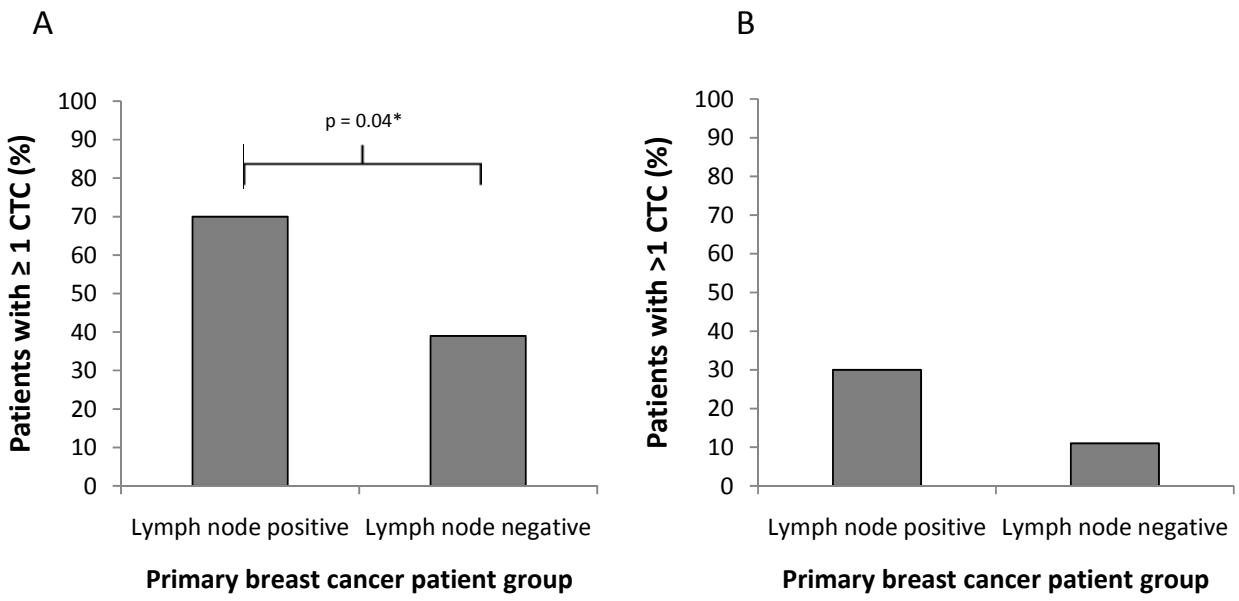


Figure 3.2: Primary breast cancer patients with lymph nodes positive at surgery have significantly more CTCs present on follow-up than lymph node negative patients. The percentage of LN positive (n = 33) and LN negative (n = 18) primary breast cancer patients with CTCs present (1 or more) or absent in any blood sample taken (1 – 7 X 22.5 ml taken at 3 - monthly intervals) is shown in A. The percentage of patients with more than 1 CTC in any blood sample taken is shown in B. (*statistically significant, exact chi-square test).

3.9 Proportion of tests positive for CTCs

Because the number of tests varied among individual patients, a calculation of the proportion of positive tests was also carried out for comparisons across the groups. CTC > 0 proportions were significantly higher in LN positive patients (p=0.031), but for a more stringent criterion CTC > 1 proportions did not differ (p=0.124, Mann-Whitney test). Six out of 18 (33%) LN negative patients showed evidence of CTCs in less than half of the tests performed versus 15 out of 33 (45%) in LN positive patients. Only 1 patient from this group compared with 8 out of 33 (24%) LN positive patients showed CTCs on 50% or more of the tests (p=0.064).

3.10 Lymph node positive patients have more sequential blood samples positive for CTCs

Of the 18 LN negative patients, 5 (28%) patients were positive at one time point, and 2 (11%) at 2 time points. In contrast, 13 (39%) LN positive patients were positive at one time point, 6 (18%) at two time points and 4 (12%) at three time points, and therefore had significantly more sequential samples positive (p=0.034), (Figure 3.3). In addition, persistently positive samples were found 3 – 34 months apart (mean of 15.3 months apart) in all patients. This provides evidence of micrometastatic disease persisting for up to 10 years post surgery, often at random intervals with one patient having CTCs present 34 months apart but remaining disease-free.

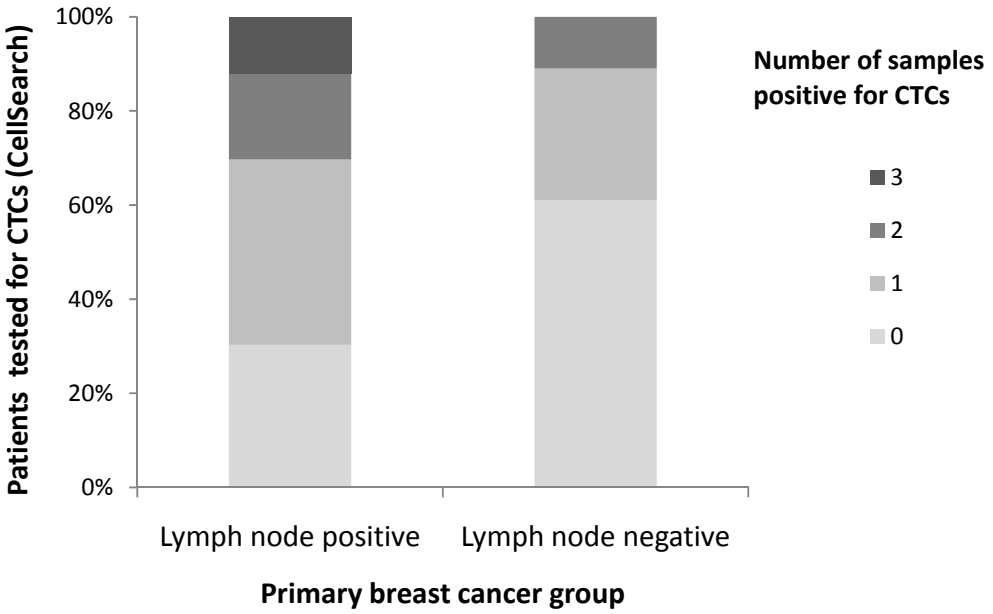


Figure 3.3: Primary breast cancer patients with positive lymph nodes at surgery have more sequential blood samples positive for CTCs than lymph node negative patients. The number of blood samples positive for CTCs is shown in 51 primary breast cancer patients as a percentage (33 high risk and 19 low risk) who had 1 – 7 X 22.5ml blood samples taken at 3 monthly intervals. The difference between the two groups is statistically significant (Mann-Whitney test, p=0.034).

3.11 Disseminated tumour cell detection in bone marrow aspirates from primary breast cancer patients

Over this period of sampling for CTCs, we also obtained 68 bone marrow aspirate samples for DTC detection by ICC and qRT-PCR; 13 LN negative patients and 1 LN positive patient were tested once annually for ethical reasons, and 27 LN positive patients at two time points annually. For bone marrow, 1 out of 13 (8%) LN negative patients had DTCs detected by ICC (≥ 1) in contrast to 19 out of 28 (68%) in LN positive patients ($p<0.001$). Only 8 out of 27 (30%) LN positive patients were negative at both time points, 16 out of 27 were positive once, and 3 out of 27 positive at two time points, but none had 2 or more cells at both time-points. For bone marrow qRT-PCR, none of the 13 LN negative patients were positive in contrast to only 11 negative of 27 (41%) LN positive patients tested at two time points ($p<0.001$); 7 patients were positive at one and 16 at both time points (Table 3.2).

Table 3.2: Comparison summary of CTC and DTC detection in lymph nodes positive and lymph node negative primary breast cancer patients

	Lymph node negative breast cancer patients N = 18 (%)	**Lymph node positive breast cancer patients N = 33 (%)**	**p value[e]**
Positive for CTCs at any time point[a,b]	7 (39%)	23 (70%)	0.042*
Positive for CTCs in > 1 time point[a]	2 (11%)	10 (30%)	0.174
Positive for > 1 CTC in one test[a]	2 (11%)	10 (30%)	0.174
Positive for DTCs in bone marrow (qRT-PCR)[c]	0/13 (0%)	15/28 (54%)	0.001*
Positive for ≥ 1 DTC in bone marrow (ICC)[d]	1/13 (8%)	19/28 (68%)	0.001*
Positive for > 1 DTC in bone marrow (ICC)[d]	0/13 (0%)	7/28 (25%)	0.077

[a] Detected with the CellSearch system
[b] Samples were considered positive for CTCs when at least one cell was detected per 22.5 ml blood
[c] Calculated as the percentage ratio of CK19:ABL by qRT-PCR
[d] Detected by immunocytochemistry
[e] Exact chi-square test and Mann-Whitney test
*Statistically significant

3.12 Effects of endocrine therapy

The chances of finding positive results did not seem affected by whether the patients were receiving endocrine therapy at the time of sampling (p=0.389 and p=0.693).

3.13 Variability in CTC and DTC detection

There was evidence of variability using both tests but this may be due to variability in the disease or the assays. Thus, the ICC for DTC was consistently negative in 8 and consistently positive in 3 of 27 instances. The CellSearch was consistently negative in 17 of 24 cases but no patients were consistently positive.

3.14 Relationship between CTCs and DTCs

We also studied the relationship between the CTC and DTC results. Patients with consistently negative ICC had positive CTCs (5 out of 12 in LN negative patients and 4 out of 8 in LN positive patients), but among the patients with positive ICC results, there was a high proportion of patients with positive CTCs (1 out of 1 in LN negative and 16 out of 19 (84%) in LN positive patients). Those with positive DTCs by qRT-PCR also had a high proportion positive for CTCs (10 out of 16 (63%) LN positive and none in the LN negative patients). These numbers were too small to reach statistical significance (p=0.145). This relationship is analysed in more detail in Chapter 4 where the DTC results of all follow up results are correlated with CTCs. This confirms a statistically significant positive correlation of DTCs (ICC) to CTCs.

3.15 Relapsed patients

Two patients developed evidence of overt metastases during the course of the study (patients 28 and 44, Table 3.3). Both of these patients had positive results by the CellSearch system and in each patient DTCs were detected by ICC in one of the two bone marrows and by qRT-PCR in two of the bone marrows. Both had CTCs and DTCs detected before relapse occurred. DTCs (both by qRT-PCR and ICC) were detected before CTCs were detected in one patient and the other patient only had one CTC test performed. Since blood samples were not taken at exactly the same time as the bone marrows and relapse only happened in two patients it was not possible to statistically compare

which was the more effective monitoring tool. Further analysis of the relapsed patients is detailed in Chapter 4.

Table 3.3: Patient Characteristics, details of therapy and timing of bone marrow and peripheral blood sampling in low risk, LN negative patients with primary breast cancer (173)

Patient no.	Date of diagnosis	Tumour type	Size (mm)	Grade	No. of LN	ER status	PR status	Therapy during study	Time from diagnosis BM1 (months)	QPCR	ICC	Time from diagnosis PB1 (months)	CTCs	Time from diagnosis PB2 (months)	CTCs	Time from diagnosis PB3 (months)	CTCs	Time from diagnosis PB4 (months)	CTCs	Time from diagnosis PB5 (months)	CTCs	Time from diagnosis PB6 (months)	CTCs	Time from diagnosis PB7 (months)	CTCs
1	03/03/1998	IDC	20	2	0/15	2+	3+	Nil	89	0.046	0	94	0	100	0	110	0	114	0	118	1	121	0		
2	23/03/1998	IDC	9	2	LN-	3+	2+	Nil	96	0	0	96	ND	102	0										
3	19/05/1998	IDC	10	2	0/26	2+	3+	Nil	90	0	0	92	0	98	0	109	0	112	0	117	0				
4	26/05/1998	IDC	7	1	LN-	3+	Neg	Nil								108	0	111	0	114	0	117	0	120	0
5	21/07/1998	2 foci and tubulo-lobular	10	1	0/22	3+	3+	Nil	87	0	0	90	0	96	0	106	1	109	0		ND	114	1	118	0
6	03/11/1998	IDC	10	2	0/20	3+	3+	Nil								101	0	104	0	107	0	110	0	114	0
7	03/11/1998	IDC	17	1	0/15	3+	3+	Nil	92	0	0	92	0		ND										
8	25/05/1999	Multifocal ILC	10	2	0/34	Pos	Pos	Nil								100	1		ND	106	0				
9	06/06/2000	IDC	15	3	0/27	Neg	Neg	Nil								81	0	84	0	88	0	91	0	94	0
10	24/10/2000	IDC	10	1	0/27	3+	3+	Nil	62	0.032	0		ND	68	0	78	0	81	0		ND	87	2		
11	26/02/2001	ILC	18	2	0/29	3+	1+	Nil	52	0	0	60	0	66	0	76	0	79	0	82	0	85	1		
12	06/10/2003	IDC	1.5	3	0/4	Neg	—	Nil	17	0.002	0	29	0	35	0										
13	12/07/2002	IDC	12	3	0/17	Neg	Neg	Nil	33	0.023	0	43	0	49	0	59	0		ND	66	0	69	0		
14	10/12/2002	2 areas and IDC	20 and 11	1 and 2	0/28	3+	3+	Tam								54	0	57	0	60	0	63	0		
15	07/01/2003	IDC	19	3	0/31	3+	1+	Nil	29	0	1	36	0	42	0	53	1	56	2	59	0	63	0		
16	25/02/1994	IDC	17	3	LN-	Neg	Neg	Nil	135	0	0	135	ND	141	0	159	0	162	0		ND	169	1		
17	22/12/1997	IDC	17	3	0/16	2+	3+	Nil	90	0.006	0	96	0	102	0										
18	07/03/2000	IDC	10	2	0/23	3+	1+	Nil	60	0.018	0	68	0	74	0	88	0	91	0	94	0	97	0		

BM = bone marrow; CTCs = circulating tumour cells; DTCs = disseminated bone marrow tumour cells; ER = oestrogen receptor; ICC = immunocytochemistry; IDC = infiltrating ductal carcinoma; ILC = infiltrating lobular carcinoma; ND = not done; Neg = negative; PB = peripheral blood; Pos = positive; PR = progesterone receptor; QPCR = quantitative PCR; Tam = patient receiving tamoxifen at the time of analyses, all other patients were receiving no therapy. A CK-19 : ABL ratio of ≥ 0.1 by RT–PCR is taken as positive. Results for CTCs and DTCs in 18 group II T_1N_0 primary breast cancer patients.

Table 3.4: Patient Characteristics, details of therapy and timing of bone marrow and peripheral blood sampling in LN positive, high risk patients with primary breast cancer (173)

Patient no.	Date of diagnosis	Tumour type	Size (mm)	Grade	No. of LN	ER status	PR status	Therapy during study	Time from diagnosis BM1 (months)	QPCR	ICC	Time from diagnosis BM2 (months)	QPCR	ICC	Time from diagnosis PB1 (months)	CTCs	Time from diagnosis PB2 (months)	CTCs	Time from diagnosis PB3 (months)	CTCs	Time from diagnosis PB4 (months)	CTCs	Time from diagnosis PB5 (months)	CTCs	Time from diagnosis PB6 (months)	CTCs	Time from diagnosis PB7 (months)	CTCs
19	09/01/1999	IDC	30	3	4\13	3+	—	Arim	78	0.12	0	84	0.25	2	77	2		ND	99	0		ND	105	0	108	0	111	1
20	09/01/1995	IDC	30	3	12\17	1+	1+	Nil	126	0.18	1	132	0.11	1	125	0	132	0	149	0	152	1	156	2	159	0		
21	07/01/1994	ILC	>50	3	12\16	—	—	Nil	137	0.10	0	143	0.47	7	135	5	140	0	162	0	165	0	167	1	170	0		
22	05/01/1997	IDC	27	3	6\19	Neg	Neg	Nil	102	0.22	3	108	0.08	0	102	1	108	0	125	1	128	1		ND	134	0		
23	01/01/1996	IDC	33	3	4\20	—	—	Nil	117	0.03	0	123	0.00	0	119	0	125	0	138	0	141	1	144	0	147	0		
24	09/01/1996	ILC	55	2	5\29	3+	2+	Nil	129	0.02	1	135	0.10	1	119	0	124	0	138	0	142	0		ND	148	0		
25	23/12/1997	Tubular	11	-	2\13	Pos	Pos	Tam											113	0	117	0	121	0	123	0		
26	12/03/1997	IDC	16	2	16\36	1+	1+	Arim	92	0.14	0	104	0.00	0	98	0	100	0	122	0	126	0	129	0	132	0		
27	08/01/1999	IDC	10	2	5\15	3+	3+	Arim	68	0.22	1	74	0.04	0	76	0	82	0	100	0	104	0	107	0	112	0		
28	01/01/1997	IDC	20	3	8\21	Neg	Neg	Nil	107	0.20	3	113	0.20	0	106	0	112	2	Relapsed with metastatic disease 115 months after diagnosis									
29	11/01/1998	IDC	30	3	6\18	Neg	Neg	Nil	78	0.12	1	84	0.20	1	84	0	90	0										
30	02/01/2000	IDC	13	3	9\21	Pos	Neg	Arim	70	0.18	1	76	0.32	0		ND	76	0	89	0	92	0	94	1	97	0	100	0
31	05/01/1998	IDC	25	3	6\11	Pos	—	Nil	91	0.03	6	97	0.09	0	91	0		ND	113	0	116	0	119	0	121	0	124	1
32	03/01/1997	IDC	60	3	4\31	Neg	Neg	Nil	104	0.03	0	110	0.52	1	104	0	110	0	125	1	128	0	131	0	133	0		
33	27/09/1994	IDC	23	3	9\29	—	—	Nil											151	0	154	2	157	0	161	0	164	0
34	03/01/1995	Both IDC	2 × 16 and 22	3	9\24	1+	1+	Nil	126	0.06	0	132	0.13	3	132	8	136	0										
35	05/01/1997	IDC	50	3	4\22	Neg	Neg	Nil	95	0.02	0	101	0.00	0	95	0	101	0	125	0		ND		ND	132	0		
36	09/01/1997	T2 N1	25	2	11\20	2+	2+	Nil	90	0.06	0	96	0.06	1	96	0	109	0	124	0	127	0	130	1	133	1	136	0
37	10/01/1995	ILC	35	2	9\21	—	—	Nil	122	0.01	0	128	0.07	0	123	0	127	0	149	0	152	0		ND				
38	06/01/1999	IDC plus DCIS	25	3	4\29	2+	2+	Arim	78	0.01	0	84	0.05	0	78	0	84	0	100	1	103	1	107	23	112	0		
39	09/01/1997	IDC	40	3	10\21	1+	1+	Nil	93	0.02	1	99	0.00	0	99	0	103	0	126	1		ND	130	0	133	0		
40	02/01/1999	IDC	45	3	6\14	Pos	Pos	Arim	84	0	0	90	0.13	0	84	0	90	0	105	0	108	1	111	0				
41	03/01/1998	IDC	40	2	7\27	Pos	Pos	Arim	94	0.07	0	100	0.07	0	93	0	100	0	115	0	118	0	120	0				
42	08/12/1997	Multifocal IDC	>50	2	9\20	2+	3+	Arim											112	0	115	1	119	0	122	1	125	2
43	09/01/1997	TX N4	—	3	14\25	Neg	Neg	Nil	95	0.04	1	101	0.03	0	101	1	105	0										
44	04/01/1998	IDC	60	2	8\19	—	—	Tam	74	0.11	0	86	0.23	1		ND	96	7			Relapsed with metastatic disease 101 months after diagnosis							
45	05/09/1995	IDC	18	2	8\13	2+	Neg	Nil											144	0	147	0	151	0				
46	08/01/1997	IDC	20	2	7\19	3+	3+	Nil	85	0.12	1	97	0.26	0	101	0	106	0	126	0	129	1	132	0	135	0		
47	29/12/2003	IDC	15	3	45\45	Neg	Neg	CT (FEC)											41	0	44	0		ND	49	0	53	0
48	16/12/1998	Mucoid carcinoma	50	1	8\19	2+	3+	Arim	72	0.03	0	84	0.05	0	84	3	97	0	103	0	106	0	109	1	112	0		
49	08/01/1999	IDC	20	3	4\26	Neg	Neg	Nil	61	0.33	2	72	0.53	0	77	1	83	0	99	1		ND	105	0	108	0	111	0
50	02/01/2001	IDC	14	2	20\39	3+	3+	Arim	49	0.00	0		ND	ND	61	0	65	0	77	1	81	1	84	0	88	1		
51	01/01/2000	Multifocal lobular carcinoma	17	3	6\18	3+	3+	Arim	37	0.08	1	51	0.03	0	47	0	51	0	87	0	90	0	93	0	97	0	100	1

Arim = arimidex; BM = bone marrow; CT = chemotherapy; CTCs = circulating tumour cells; DCIS = ductal carcinoma *in situ*; DTCs = disseminated bone marrow tumour cells; ER = oestrogen receptor; FEC = fluorouracil, epirubicin and cyclophosphamide; ICC = immunocytochemistry; IDC = infiltrating ductal carcinoma; ILC = infiltrating lobular carcinoma; ND = not done; Neg = negative; PB = peripheral blood; Pos = positive; PR = progesterone receptor; QPCR = quantitative PCR; Tam = tamoxifen. High-risk patients therapy and monitoring details. Results for CTCs and DTCs in 33 group III high-risk primary breast cancer patients.

3.16 Discussion

The CellSearch system effectively enriched four phenotypically different spiked breast cancer cell lines from healthy blood, albeit with widely varying recovery rates of between 27% and 69%. This may demonstrate differing expression levels of EpCAM and CK in these cell lines that form the basis for enrichment and detection used by the CellSearch system. An alternative method of detection using mRNA *in situ* hybridisation of multiple tumour markers is discussed in detail in Chapter 5. Studies have shown heterogeneity in EpCAM expression in single tumour cells with lower levels present in CTCs in the blood than in the primary tumour (175, 176). Recent work has also shown that subsets of normal- like breast tumour cells are not picked up by the CellSearch system (177). Furthermore, another study also found limitations of a solely EpCAM–based capture system due to lower recovery rates of the basal-like tumour cells due to low EpCAM expression (178). A CTC-chip method used EpCAM-coated posts and special laminar flow conditions and captured CTCs more efficiently than the CellSearch system (66), although this has yet to be proven in prospective trials looking at prognosis, the recent improvements to the method mean this may surpass the CellSearch system in the future (67). The CellSearch therefore is not detecting a proportion of CTCs with low EpCAM expression. High levels of EpCAM expression however, have been found to be associated with poor prognosis in node-positive breast cancer (179), so those CTCs that are recovered may be those associated with poorer prognosis anyway. A validation study that found five or more cells in 7.5 ml of blood to be associated with a shorter overall survival also proves the efficacy of the system in the metastatic setting at identifying these poor prognosis patients (117).

Ultimately the purpose of this study was to identify those patients with a worse prognosis which the CellSearch has been proven to do. Certainly the improved sensitivity to detect more CTCs is an important factor in the primary setting where very few CTCs are found. One study added CD146 ferrofluid into the CellSearch system which was found to detect more of the normal-like breast cancer cells (180). When CTC counts were tested in advanced breast cancer patients in this study, 8 out of 20 (40%) patients had a higher CTC count than using the standard

CellSearch system reagents. The prognostic implications of this have yet to be determined and show that improvements to the sensitivity of this system are possible.

The family of cytokeratins also used as detection markers in the CellSearch system shows variation in expression in tumours (181) and even absence in aggressive tumours (182), so the use of a pan-cytokeratin antibody in the CellSearch system attempts to minimise this limitation. This shows that despite the CellSearch system detecting CTCs using markers known to confer a poor prognosis (59, 179, 183), the heterogeneous population of CTCs actually present in a patient's blood sample may not all be recovered. This selection process must therefore be taken into account when studying CTCs further as they may only be a subset of the original tumour cell load circulating in the blood. This has particular implications for the sensitivity of the system particularly in studies to detect very low levels of CTCs such as this one. Despite this, the CellSearch system remains the most standardised and reliable method available.

CTCs were detected in healthy people and BBD at similar rates to other studies, with 3% of healthy people and 14% of BBD patients with evidence of CTCs (73). One patient with BBD did however have 5 CTCs in 22.5 ml of blood. This meant CTC detection was not significantly different to the patients with a primary breast cancer diagnosis on follow-up (14% of BBD patients positive for CTCs compared to 20% primary patients), although this may be due to low statistical power due to the low numbers of CTCs detected. However when compared to previous findings, the mean number of CTCs per 7.5 ml blood sample in BBD patients was the same at 0.1 (73). It is also known that BBD patients have a greater risk of breast cancer depending on the pathology (184). The patient who had 5 CTCs in 22.5 ml of blood had a diagnosis of fibroadenoma, which confers a risk of breast cancer around 2.17 times the general population (185). This may mean this patient had an undiagnosed breast tumour, since the presence of 5 false positives is unlikely, although it cannot be ruled out. Three other BBD patients had one CTC present, but as we find one CTC occasionally in a healthy patient, this may represent false positive results.

This study, at the time of publication, showed for the first time that LN positive patients at a higher risk of relapse had significantly more CTCs present (1 or more) than LN negative patients

at multiple time points on follow-up. Although this was not statistically significant when a cut-off of 2 or more CTCs was used, this is likely to be due to the low occurrence of CTCs in this group of patients. Again, the significance of finding one per 22.5 ml of blood in this study may represent a false positive. The number of patients with sequentially CTC positive samples was however, significantly higher in LN positive patients so a positive finding of even one CTC in sequential samples is more likely to be a true positive and thus a possible predictive indicator of prognosis. Evidence for this comes from the on-going SUCCESS trial in Germany that has investigated the role of CTCs in the peripheral blood of primary breast cancer patients at diagnosis and during adjuvant treatment. Recent findings from this study showed one or more CTCs in 23 ml of blood in 1489 high risk primary breast cancer patients before adjuvant chemotherapy is associated with a poor disease-free and overall survival. Those with CTCs present after treatment only predicted a worse disease free survival with borderline significance. The study also found that those patients persistently negative for CTCs had a better disease-free and overall survival than those persistently positive (186). This provides evidence that patients in this study with persistent CTCs may have a worse prognosis.

Considering the patients in our study were sampled many years after diagnosis (mean of 9.9 years), the clinical significance of CTCs might be different to those found in patients in the SUCCESS trial who had a recent breast cancer diagnosis. For example, soon after surgery and during systemic treatment, any remaining CTCs are likely to be those resistant to therapy and therefore are likely to be more aggressive and non-responsive to treatment. In contrast, CTCs found in patients years after surgery are likely to have undergone a phase of little or no proliferation (or a balance with cell death) followed by a phase of exponential growth. This is because the kinetics of tumour growth can only explain early relapses and not late relapses (187). Therefore, the CTCs we find in these patients may represent a tumour cell in a different state of quiescent growth or aggressiveness and may not be as prognostically relevant. Additional characterisation of these CTCs and the dormancy mechanisms of cancer may uncover their biological and clinical significance. This is discussed further in Chapter 4.

In this study we found 70% of the LN positive and 39% of the LN negative patients had evidence of CTCs over 1 – 7 sequential blood samples (a mean of 4.6 samples or 103.5 ml of blood tested

per patient). This is a much greater volume of blood tested for CTCs than previous studies (119, 136, 139, 186, 188) and is therefore one of the reasons why more CTCs were detected along with the use of the CellSearch system to standardise detection. This increased the sensitivity of detection and also reduced sampling variability which is an inherent problem of studies to detect rare cells. This greater volume of blood per clinic visit (22.5 ml) was still a relatively small amount for the patient and did not cause much more discomfort than taking 7.5 ml of blood. It is also considerably less painful than a bone marrow aspirate and as previously mentioned would provide a much more convenient way of monitoring a patient for MRD.

The first study to find a prognostic use for CTCs, found that the presence of *CK19* mRNA in the blood predicted a worse progression-free survival (183). Despite qRT-PCR DTC data predicting prognosis better than ICC (128), clinicians are not convinced about using it for widespread monitoring of patients. With the limitations in specificity for qRT-PCR, the problem of false negatives and the lack of long-term large-scale prospective studies to fully validate this method, the prognostic implications of this method are still not fully realised. This is in contrast to ICC detection which has been validated in multi-centre trials (59). Immunocytochemical evidence with characterization is more likely to provide enough confirmation along with prospective data of their prognostic use. Many studies have proven the tumour origin of occult cells by detecting tumour markers or known tumour mutations (113, 189, 190). It is only recently through the CellSearch system that immunocytochemical evidence of CTCs provided enough sensitivity and standardisation to enable accurate detection from the blood. This system can also screen larger quantities of blood without being laborious to analyse (a problem with ICC evaluation of cells in amongst many PBMCs on a slide). The recent use of panels of tumour markers for qRT-PCR analysis however, may improve the issue of false positives in analysis of CTCs based on mRNA detection (77, 87).

The results of this study show that approximately 76% of the fifty-one primary breast cancer patients we studied had some evidence of micrometastatic disease during follow-up even if this was one CTC or DTC present. This was despite most of the patients having no clinical sign of metastases. Two patients from the high risk group relapsed whilst on the study, both CTCs and DTCs were present in their blood and bone marrow prior to relapse. Although this confirmed

the efficacy of both methods for monitoring patients, the relapses occurred early in the study making any comparisons difficult to interpret.

This study showed that there were significantly more DTCs in the high risk group measured by both ICC and qRT-PCR than in the low risk group (p=0.001). This is in line with previous studies that also showed the presence of DTCs in the bone marrow to be an independent prognostic indicator in primary breast cancer patients (59). The presence of CTCs in the blood has only very recently been proven to be of use in prospective clinical trials analysing progression-free survival and survival data, but needs to be verified (186). The variability in the findings of CTCs and DTCs may be due to the differences in the methods used to detect them and/or their prevalence. The CellSearch system uses EpCAM enrichment followed by staining of CTCs by pan-cytokeratin whereas the bone marrow uses Ficoll separation followed by ICC or qRT-PCR. Either that or CTCs and DTCs represent two different levels of risk, for example CTCs may only provide a "snap shot" of tumour cell dissemination whilst DTCs are homed to the bone marrow and are often in a dormant state (191). It has been shown that CTCs have a half-life of only 1 – 2.4 hours and must be replenished regularly (132, 192), however it has also been shown that both CTCs and DTCs are non-replicating, implying similarity (136). Our study also showed that in those high risk patients with DTCs measured by ICC, 84% also had CTCs present.

Studies mentioned here provide some verification that tumour cell dissemination starts early during tumour progression. The observation of DTCs and CTCs being present in patients in this study despite no clinical signs of metastases also point to this theory. The monitoring of patients following surgery and during adjuvant therapy may be of most use due to the chance of cure before overt metastases occur. This may prevent the seeding of tumour cells to the bone marrow. One study measured CTCs during adjuvant therapy in primary breast cancer patients using laser scanning microfluorimetry following centrifugation and magnetic bead separation. This showed that an increase in the number of cells at the end of adjuvant treatment was predictive of early relapse (193). They found that the dynamics of CTC measurement was important so that the effects of therapy were monitored closely and changed if necessary.

It is not clear if monitoring CTCs in primary breast cancer patients will be of more use than monitoring DTCs. Each have their own limitations, for instance some patients with DTCs may not ever have clinical relapse (59). On the other hand some metastatic patients do not have CTCs so both methods may exclude groups of patients (117). Large prospective studies to monitor patients using both methods may help to ascertain the relevance of these monitoring tools. This, in conjunction with further adjuvant therapy for those patients with detectable DTCs or CTCs may cure a higher proportion of patients at risk of relapse. The improvement of current technologies for more sensitive and specific detection of cells may also help towards this goal.

Other methods of CTC enrichment are being studied and have shown promise (74, 193, 194), one of note mentioned previously, uses a microfluidic device with herring-bone, microvortex shaped channels to ensure the most contact of CTCs with the antibody-coated chip surface. This was found to have higher mean rates of CTC recovery from metastatic breast cancer patients than its predecessor and also therefore, the CellSearch system. This may be due to the one-step nature of this technique resulting in decreased loss of rare cells. The slow flow rate of blood through the chip may also facilitate increased exposure to the anti-EpCAM antibody, thus capturing more CTCs even on cells with few EpCAM antigens (66, 67). This method is still in its early stages however, until it is developed further and capable of standardised routine testing in large prospective trials, its potential cannot be determined.

In conclusion we confirm here that LN positive breast cancer patients on long-term follow-up have significantly more CTCs than lower risk, LN negative patients demonstrating their possible prognostic use. In addition, two patients who relapsed had CTCs present a few months before overt disease. We provide evidence that testing greater quantities of blood at regular 3-monthly intervals ensures more CTCs can be detected in these patients, and may provide a possible monitoring system to detect MRD at a time when the eradication of micrometastatic lesions is possible. Although the meaning of detecting one CTC can be scrutinised, repeated sampling may provide confirmation of a positive result. In addition, the characterisation of these rare cells may help to determine if they definitely are CTCs as well as to determine their potential at causing relapse. This, together with the more sensitive detection to prevent false

positives may help in this regard. We do not know the significance of CTCs in apparently disease-free patients many years after surgery and primary treatment, but the long term study in a larger population of patients with other indicators of MRD may help to determine their prognostic importance.

4.0 Investigation of minimal residual disease markers in primary breast cancer patients on long-term follow up

4.1 Introduction

DTCs detected in the bone marrow and CTCs present in the peripheral blood of primary breast cancer patients on follow-up provide useful prognostic information. Both are more prevalent in the blood and bone marrow of high risk LN positive primary breast cancer patients and are present prior to clinical relapse. Our study discussed in Chapter 3 demonstrated a surprising 59% (30 out of 51) of primary breast cancer patients on long-term follow-up had CTCs detected in their blood at one or more time-points, yet only 4% relapsed during the study period. Their presence therefore does not necessarily mean a patient will relapse or have a worse prognosis. Treatment decisions based on MRD detection are therefore not straightforward for clinicians since additional therapy would only be given if it was thought it would provide benefit, otherwise it may cause overtreatment, undue illness and anxiety to the patient. The additional characterisation of MRD is needed to establish the potential for relapse and to determine the necessity for further adjuvant treatment. Currently, around 70% of patients with breast cancer receive adjuvant chemotherapy or endocrine therapy following surgery and no attempt is made to establish if they are successfully eradicating any remaining MRD in each patient.

The processes involved in long-term cancer dormancy where DTCs and/or CTCs are present with no sign of overt disease is present in patients are poorly understood. The investigation and characterisation of this period may help towards treating patients more appropriately. Several theories exist including the dormancy of single cells or dormancy of micrometastases that undergo immunosurveillance or different angiogenic states or other unknown mechanisms that keep the tumour cells in check by cell death (131). Evidence for this in patients is not confirmed due to the difficulties in sampling for multiple measures of MRD over the long follow-up period required and as a result, little has been reported on the subject.

A project running alongside the studies of DTCs and CTCs investigated the use of circulating-free DNA in the blood of patients as an additional marker for MRD. Studies examining cfDNA have

demonstrated increased quantities in the blood of cancer patients, and have been proven to be derived at least partially from the primary tumour (94, 95). CfDNA found in healthy people is thought to derive from apoptosis of lymphocytes and other nucleated cells and represents DNA fragments of around 185-200 bp corresponding to the internucleosomal cleavage of chromatin (195). The mechanisms by which greater quantities and more variable lengths of cfDNA are released in the blood of cancer patients are still inconclusive. Much of the DNA is longer and so may represent other cell death mechanisms such as lysis or necrosis (99). Additional blood samples were taken in this study at the same time as the bloods for CTC analysis and the bone marrows for DTC detection. This was to compare different measures of MRD to find out if cfDNA may supplement or provide more prognostic information. Blood sampling is also much more acceptable to patients than bone marrow aspirates so a test for CTCs and cfDNA detection would be preferable.

We set out to determine if increased levels of cfDNA correlated with the presence of micrometastatic disease. For example, the presence of intact DTCs and CTCs implies a micrometastatic lesion may be active somewhere in the body. The presence of tumour-derived cfDNA on the other hand, implies tumour cells are releasing DNA by a form of cell death from these lesions. We set out to observe any possible relationship of these processes to find out if tumour dormancy relies on cycles of cell growth and death or if it relies on single quiescent cells with low activity and low cfDNA levels. This in turn may help to elucidate the reasons for relapse many years after diagnosis and determine the nature of dormant micrometastatic disease.

The long-running studies of MRD in our laboratory provide us with a huge amount of data of bone marrow DTCs using both ICC and qRT-PCR methods. In addition, the sub-study of CTC measurement as discussed in Chapter 3 was conducted along with pilot studies of cfDNA analysis. This involved the detection of 96-bp amplicons, representing total, apoptotic cfDNA that is found in healthy people, as well as 291-bp amplicons, representing larger fragments of DNA thought to be mostly derived from the cell lysis, necrosis and death of tumour cells. All this data has been compiled to enable an in-depth analysis. The hope is that one or more measures of MRD provide enough information to form the basis of a test for impending metastatic

disease. Chapter 3 examined the detection of CTCs and DTCs in a time period of up to 2.8 years, this study examined 64 patients with primary breast cancer on long-term follow-up up to 11.5 years post surgery. The purpose was to investigate long-term trends in multiple MRD measurements in relapsed and non-relapsed patients to find their prognostic value.

4.2 Aims and Objectives

- To determine the use of cfDNA as a marker of MRD by comparing the quantity in the plasma of primary breast cancer patients with healthy controls, benign breast disease and metastatic breast cancer patients
- To confirm the concordance of DTC detection by two methods: qRT-PCR and ICC
- To confirm the relationship between the presence of DTCs in the bone marrow and CTCs in the blood from samples taken at the same clinic visit in primary breast cancer patients
- To measure DTCs in the bone marrow, CTCs in the blood and cfDNA in the plasma of primary breast cancer patient to determine the relationship to the most important current prognostic factor: the presence of involved lymph nodes
- To investigate the relationships between the presence of MRD markers with other known prognostic factors (ER, PR, HER2, triple negative status, menopausal status, tumour size and grade of the primary tumour).
- To determine the relationship of MRD markers to each other to find out if cfDNA adds further information to DTC and CTC detection
- To compare MRD values in relapsed and non-relapsed patients
- To study the patterns of DTC, CTC and cfDNA detection in relapsed patients

4.3 Results

Patients and samples

Sixty-four patients with a breast cancer diagnosis and who were clinically disease-free were recruited and followed up for 1 - 11.5 years after initial surgery (mean 7.9 years). The majority of these patients continued on follow-up from earlier studies carried out at in our department. Twenty-two patients continued on our minimal residual disease studies (82, 83). Twenty-nine patients were recruited from a trial of adjuvant high dose chemotherapy (196) and were selected because they had a high risk of relapse. Thirteen patients were selected as additional patients. Thirty-five (83%) of the LN positive patients had received adjuvant chemotherapy and, if the tumours were oestrogen receptor positive, adjuvant endocrine therapy for at least 5 years; eight (36%) of the LN negative patients had also received adjuvant chemotherapy and 17 (77%) of the LN negative patients had received adjuvant endocrine therapy. Table 2.3 (Chapter 2) shows patient characteristics and adjuvant systemic therapy details. Patients all agreed to repeat bone marrow sampling at the outset of the study followed by repeat blood sampling for further tests to measure MRD. These studies investigated CTCs and cfDNA and were reported separately (164, 173). In total, 20 patients had tumours measuring less than 20 mm with no involved lymph nodes detected (T_1N_0) and therefore had a low risk of relapse and a good prognosis. Four patients had 1 – 3 involved lymph nodes detected, 2 patients had tumours measuring more than 20 mm with no involved LNs detected and 38 patients had ≥3 involved lymph nodes at surgery and so were deemed to have a high risk of relapse. For the LN analysis, patients were grouped into LN positive (n = 42) or LN negative at surgery (n = 22). None had any sign or symptom of distant metastatic disease when recruited to the study.

The LN positive group of patients had bone marrow aspirate samples taken at 0, 3, 6 and 12 months followed by 6 months thereafter for a period of up to 11.5 years. The low risk group had bone marrow samples taken at 0, 3, 6 and 12 months and every 12 months thereafter for up to 11.5 years. Fewer were taken due to ethical reasons. Blood sampling took place post-operatively over a follow-up period of 2 – 11.5 years and took place every 3 – 6 months. Ethical and technical restraints prevented us from taking samples for all tests from all patients at the

same time points. An intensive study was initiated in 2007 for 15 months, this involved blood sampling for CTC and cfDNA analysis at the same time points as well as yearly or 6-monthly bone marrow aspirates. Prior to this, pilot studies had been performed for cfDNA analysis (3 monthly samples for 24 months in 2004 and 2005) and CTCs (6 monthly samples taken for a year in 2005 – 2006). In total 2 - 13 (mean 7.4) plasma samples were analysed per patient for cell-free DNA with a total of 474 analysed. Two amplicons were measured, one below the apoptotic limit at 96-bp to represent total cfDNA and one above (291-bp) to represent DNA derived from mostly lysis or necrosis of tumour cells. Up to 7 (mean 3.5) blood samples were analysed per patient for CTC analysis with a total of 225 analysed from all patients using the Cell Search system. Up to 21 (mean 9.5) bone marrow aspirates were taken from each patient for DTC analysis (both for ICC and qRT-PCR if the volume was sufficient) with a total of 608 analysed from all patients.

All samples for CTC analysis also had corresponding samples for cfDNA analysis, unless there was a problem with venipuncture. CTC analysis for the pilot study (the first two samples from each patient) was performed by S. Reithdorf at The University Medical Centre, Hamburg-Eppendorf, Germany. The remaining 5 samples were all analysed at Hammersmith Hospital, Imperial College London, as were all of the samples for cfDNA and DTC analysis. One-hundred and 68 bone marrow aspirates were taken at the same time points as the blood sampling and used in the direct comparison of DTCs, CTCs and cfDNA. All samples for CTC analysis also had corresponding samples for cfDNA analysis. Total measurements of DTCs, CTCs and cfDNA were used when comparing primary tumour characteristics or other clinical prognostic factors.

Eight out of 64 (12.5%) patients relapsed with either overt metastatic disease (6 patients) or with primary contralateral breast cancer (2 patients) over the course of the follow-up period. These patients relapsed at 5 – 11 years post surgery (mean 7.8 years).

4.4 Minimal residual disease in control groups

The measurement of MRD in control groups was important to distinguish patients with primary disease to healthy, benign and metastatic controls. This was to enable CTC and cfDNA tests to

be effective at detecting very rare tumour cells or tumour DNA in the blood and bone marrow with a high level of sensitivity. CTC detection in control groups including non-cancer controls, patients with benign breast disease and patients with metastatic breast cancer are as described in Chapter 3 and also shown here in Table 4.1 for comparative purposes. DTCs in the bone marrow were not measured in these control groups due to ethical reasons. To find out the use of cfDNA as a marker of MRD, the quantity of the 96-bp and 291-bp amplicons were measured in the same control groups as the CTC measurements in addition to our cohort of primary breast cancer patients. The cfDNA was extracted from 20 ml of blood for all controls and patients (performed by N. Hava, Imperial College, London).

Higher mean 96-bp cfDNA levels were detected in primary breast cancer patients than in healthy volunteers ($p<0.001$) and women with BBD ($p<0.001$) (mixed effect ANOVA, FDR corrected). A higher quantity of the 291-bp amplicon was also seen in patients with primary breast cancer than in healthy controls ($p=0.032$ Wilcoxon Rank Sum test, with FDR correction). However, no differences were detected between patients with primary breast cancer and women with BBD ($p=0.51$), or patients with primary and metastatic disease ($p=0.34$, Wilcoxon signed rank test with FDR correction) as shown in Table 4.1.

Table 4.1: CTC and cfDNA measurements in patient control groups**.** CTCs from blood and cfDNA quantity in plasma are lower in healthy controls and benign breast disease patients than in primary and metastatic breast cancer patients.

		Healthy controls (n=34)	Benign breast disease patients (n=54)	Primary breast cancer patients (n=64)	Metastatic breast cancer patients (n=31)
Total circulating-free DNA 96-bp (ng/ml)	Number analysed	34	51	474	31
	Mean	0.24	0.54	16.97	3.5
	95% CI	0.09 – 0.63	0.27 – 1.07	3.83 – 30.10	1.48 – 8.29
	Median	0.86	1.27	1.41	2.45
	Range	0 – 4.52	0 – 8.36	0 - 2626	0.03 – 1044
Circulating-free DNA 291-bp (ng/ml)	Number analysed	34	51	471	31
	Mean	0.24	0.44	3.78	3.5
	95% CI	0 – 0.20	0.1 – 0.40	0.39 – 7.17	0 – 1.5
	Median	0	0.22	0.21	0.44
	Range	0 – 1.37	0 – 1.94	0 - 785	0 – 602
Circulating tumour cells[a]	Number analysed	29	28	235	12
	Volume of blood measured	22.5 ml	22.5 ml	22.5 ml	7.5 ml
	No. of controls with CTCs[b]	1 (3%)	4 (14%)	46 (20%)	7 (58%)
	Mean	0.03	0.29	0.46	51.33
	95% CI	0.04 – 0.11	0.09 – 0.66	0.18 – 0.63	5.21 – 108.48
	Median	0	0	0	12.5
	Range	0 - 1	0 - 5	0 - 23	0 - 301

[a] Detected with the CellSearch system

[b] Samples were considered positive for CTCs when at least one cell was detected per total sample volume

4.5 DTC detection by qRT-PCR is strongly correlated with the presence of DTCs by ICC

DTC measurement in the bone marrow was performed by two methods to enhance the specificity (by ICC) and sensitivity (by qRT-PCR) of detection. This was achieved by splitting a bone marrow aspirate sample in two and measuring pan-CK staining on cytospins by ICC and *CK19* mRNA by qRT-PCR from lysates. The concordance of the two methods is confirmed here with 72% of the DTC (ICC) positive samples also positive for DTCs by (qRT-PCR). The median qRT-PCR value was significantly higher in the ICC positive samples than in the ICC negative samples (0.1 versus 0.04) with a p value of <0.0001, Mann Whitney test, (Figure 4.1 and Table 4.2).

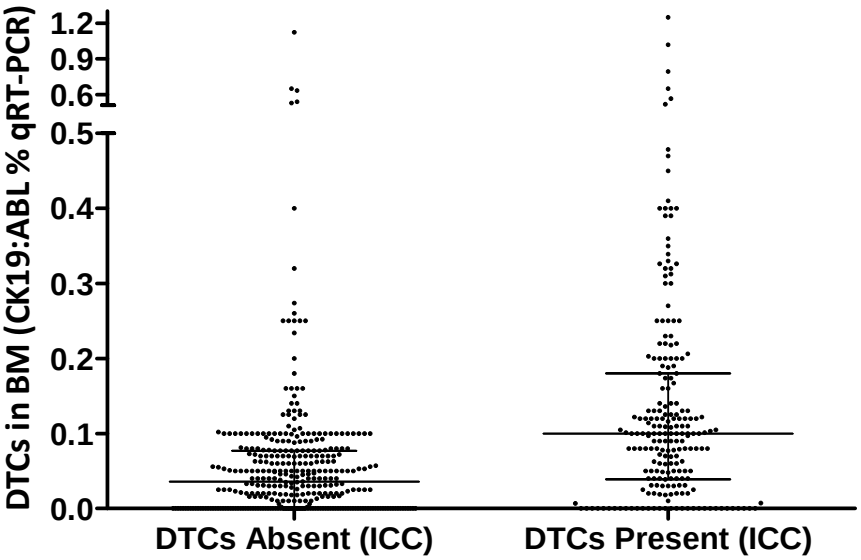


Figure 4.1: DTC qRT-PCR values of *CK19* are significantly higher in DTC positive samples by ICC. Median and inter-quartile ranges are shown, ($p<0.0001$, Mann Whitney test).

Table 4.2: DTC detection by qRT-PCR is strongly correlated with DTC detection by ICC

		Disseminated tumour cells (ICC)[a]	
		Positive	**Negative**
Disseminated tumour cells (qRT-PCR)[b]	No. samples positive	151/211 (72%)	61/353 (17%)
	Mean	0.14	0.06
	95% CI	0.11 – 0.16	0.05 – 0.07
	Median	0.1	0.04
	Range	0 – 1.25	0 – 1.12
	No. samples	211	353
	p value	<0.0001*	

[a] Detected by pan-CK antibody

[b] Calculated as the percentage ratio of qRT-PCR *CK19:ABL* (0.1 or more is considered positive)

*Statistically significant difference of median values by Mann Whitney test

4.6 CTCs in the blood are positively correlated with DTCs in the bone marrow as assessed by immunocytochemistry

Since patients with a high risk of relapse based on tumour stage are more likely to harbour both DTCs and CTCs (59, 173), it was important to determine their relationship with each other. Forty-one out of the 64 patients on follow-up for primary breast cancer had both DTC and CTC values measured in matched samples taken at the same clinic visit. CTCs were positively correlated with DTCs measured by ICC only (p=0.01). This confirms our previous findings in Chapter 3; 84% (16 out of 19) of LN positive primary breast cancer patients with DTCs present by ICC also had CTCs present (173). However, there was no statistically significant correlation between CTCs and DTCs measured by *CK19* qRT-PCR (Table 4.3).

Table 4.3: CTCs in the blood are positively correlated with DTCs in the bone marrow as assessed by ICC. This was measured in 41 primary breast cancer patients on follow up with paired blood and bone marrow samples.

		Matched CTC negative samples [a]	**Matched CTC positive samples** [a]	**p value**
DTCs (ICC) [b]	No. of samples positive	37/90 (41%)	12/18 (67%)	0.01*
	Mean	0.78	2.11	
	95% CI	0.50 – 1.05	0.38 – 3.84	
	Median	0	1.5	
	Range	0 - 7	0 – 15	
	No. of samples	90	18	
DTCs (qRT-PCR) [c]	No. of samples positive	14/82 (17%)	4/16 (25%)	0.34*
	Mean	0.04	0.056	
	95% CI	0.029 – 0.059	0.009 – 0.10	
	Median	0	0	
	Range	0 – 0.47	0 – 0.25	
	No. of samples	82	16	

[a] Samples were considered positive for CTCs when at least one cell was detected in 22.5 ml of blood by the CellSearch system

[b] Detected by immunocytochemistry

[c] Calculated as the percentage ratio of *CK19:ABL* by qRT-PCR

*Statistically significant correlation, Mann-Whitney test.

This was also confirmed by comparing the median and maximum values of DTCs and CTCs from each patient taken over time; this took into account DTC results from 390 bone marrow aspirate samples and 225 CTC samples. CTCs were positively correlated with DTCs by ICC (p=0.002, Spearman's rank) but not by qRT-PCR (p=0.72) in Table 4.4.

Table 4.4: Median values of CTCs in the blood are positively correlated with median values of DTCs in the bone marrow as assessed by ICC in breast cancer patients. Median and maximum values of CTCs per patient were compared to the median and maximum DTC (qRT-PCR and ICC) values in 41 primary breast cancer patients, p values are shown for correlations between variables.

	Median DTC (ICC)[b]	**Median DTC (QPCR)**[c]
Median CTC (CellSearch)[a]	0.002*	0.72
Max CTC (CellSearch)[a]	0.05	0.52

[a]Samples were considered positive for CTCs when at least one cell was detected in 22.5 ml of blood by the CellSearch system
[b]Detected by immunocytochemistry
[c] Calculated as the percentage ratio of *CK19:ABL* by qRT-PCR
*Statistically significant correlation, Spearman's rank

4.7 CTCs and DTCs are more prevalent in lymph node positive patients

To confirm previous findings, the relationship of DTCs and CTCs to lymph node status was measured. The mean numbers of DTCs and CTCs present per sample was greater in LN positive patients than in LN negative patients. LN positive patients had a higher number of DTCs (ICC) with a mean of 0.94 DTCs in all bone marrow samples compared to 0.79 in the LN negative patients but this did not reach statistical significance (p=0.057). DTCs detected by qRT-PCR were significantly higher in LN positive patients with a mean of 0.09 compared to 0.07 in the LN negative patients (p=0.0007). CTCs were also more common in LN positive patients with a mean of 0.55 CTCs in each 22.5 ml blood sample taken compared to 0.15 in the LN negative patients, this did not reach statistical significance in this cohort of patients due to the lower numbers (Table 4.5).

4.8 CfDNA levels are negatively correlated with the number of lymph nodes positive at surgery

We also compared values of cfDNA quantity in LN negative versus LN positive patients to find out if they correlated with this important prognostic factor. The median values were compared due to the skewed nature of the cfDNA data with extreme outliers. The LN positive patients unexpectedly had a lower median cfDNA quantity (both the 96-bp and 291-bp amplicons) than the LN negative patients. The mostly cancer-derived amplicon 291-bp, had a median of 0.17 in the LN positive compared to 0.38 in the LN negative patients (p=0.048, Mann-Whitney test). Plasma samples from LN positive patients contained a lower median level of cfDNA but a much broader range of 0–728 mg/ml of the 291-bp amplicon with a standard deviation of 45 compared to a range of 0–78 ng/ml with a standard deviation of 8.83 in the LN negative patients (Table 4.5 and Figure 4.2).

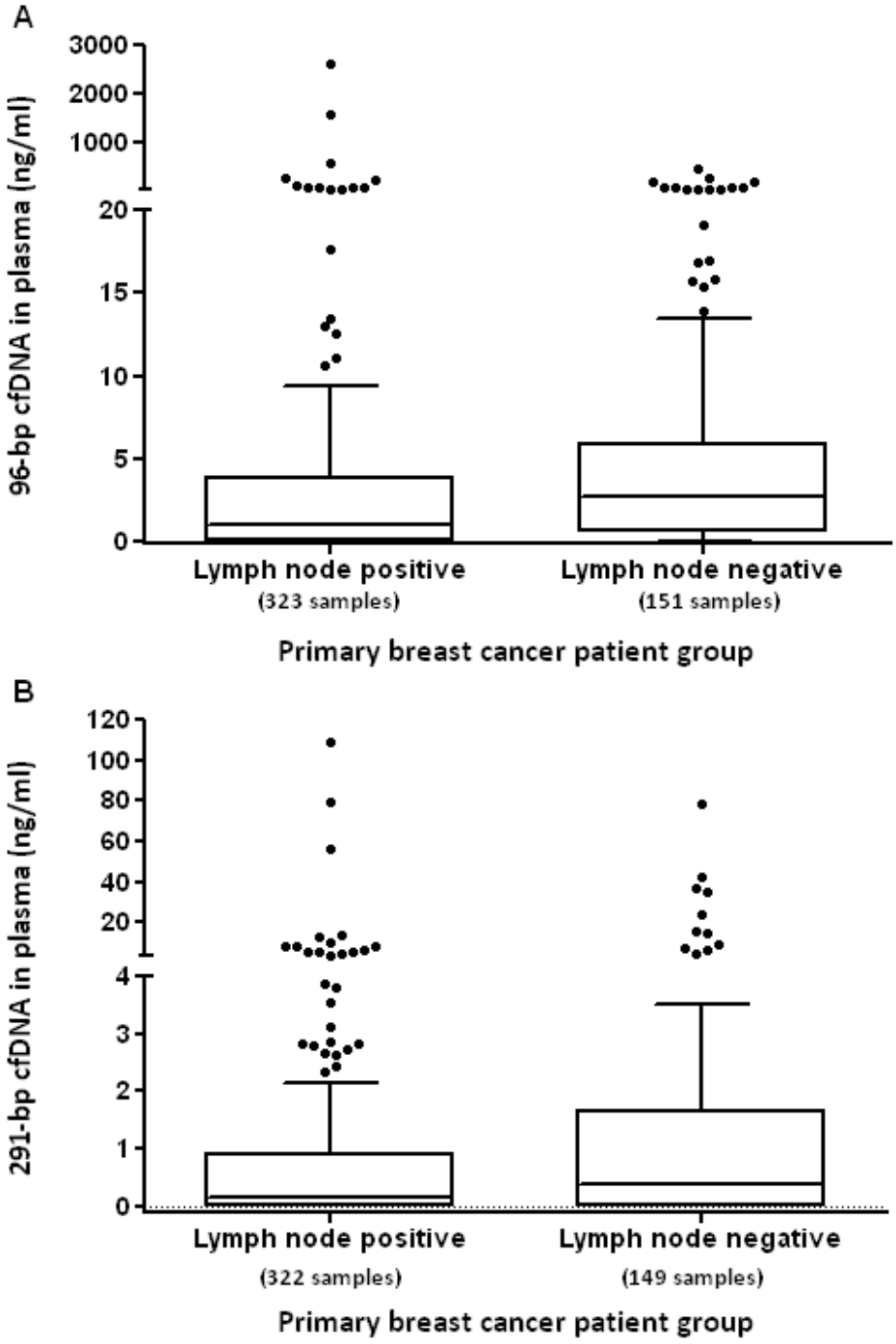


Figure 4.2: Primary breast cancer patients on long-term follow-up with no lymph node involvement at surgery have higher levels of the 96-bp and 291-bp cfDNA amplicons in plasma than lymph node positive patients at surgery. Graphs show the median, inter-quartile range and outlier values of the 96-bp cfDNA quantity, (A) and 291-bp cfDNA quantity, (B) in 64 primary breast cancer patients on follow-up (a mean of 7.4 samples per patient). 476 plasma samples were tested over time.

Table 4.5: Comparison of minimal residual disease marker values between lymph node positive and lymph node negative primary breast cancer patients. Measurements included; cfDNA (96-bp and 291-bp amplicons), DTCs (by qRT-PCR and ICC) and CTCs taken over time.

		Lymph node positive patients at surgery (n = 42)	Lymph node negative patients at surgery (n = 22)	p value
Total circulating-free DNA 96-bp (ng/ml)	Mean	19.19	12.21	0.016*†
	95% CI	0.19 – 38.18	4.83 – 19.60	
	Median	1.06	2.76	
	Range	0 - 2626	0 – 440	
	Number of samples	323	151	
Circulating-free DNA 291-bp (ng/ml)	Mean	4.35	2.54	0.048*†
	95% CI	-0.58 – 9.27	1.14 – 3.94	
	Median	0.17	0.38	
	Range	0 - 785	0 – 78	
	Number of samples	322	149	
Disseminated tumour cells (qRT-PCR)[a]	Number positive	159/484 (33%)	29/133 (22%)	0.007*
	Mean	0.09	0.07	
	95% CI	0.08 – 0.10	0.05 – 0.10	
	Median	0.053	0.04	
	Range	0 – 1.25	0 – 1.12	
	Number of samples	484	133	
Disseminated tumour cells (ICC)[b]	Number positive	197/474 (42%)	45/139 (32%)	0.057
	Mean	0.94	0.79	
	95% CI	0.78 – 1.08	0.51 – 1.07	
	Median	0	0	
	Range	0 - 15	0 – 12	
	Number of samples	474	139	
Circulating tumour cells[c]	Number positive	33/153 (22%)	9/72 (12%)	0.13
	Mean	0.55	0.15	
	95% CI	0.16 – 0.94	0.05 – 0.25	
	Median	0	0	
	Range	0 - 23	0 – 2	
	Number of samples	153	72	

[a] Calculated as the percentage ratio of qRT-PCR CK19:ABL (0.1 or more is positive)

[b] Detected by pan-CK antibody

[c] Samples were considered positive for CTCs when at least one cell was detected in 22.5 ml of blood by the CellSearch system

*Statistically significant difference of median values by Spearman's rank or Mann-Whitney test

†Negative correlation

4.9 Comparisons of markers of MRD with established prognostic factors of 64 breast cancer patients on long-term follow-up

Total MRD values were compared to the presence or absence of different prognostic factors from the primary tumour of 64 breast cancer patients. The total cfDNA 96-bp amplicon value was significantly higher in patients with ER negative and HER2 negative disease and consequently it was also higher in patients with triple negative ER, PR and HER2 status. A median 96-bp value of 2.49 ng/ml was present in triple negative patients compared to 1.21 ng/ml in those who were not triple negative, (p=0.004). The DTC (qRT-PCR) values were higher in PR negative patients and triple negative patients with a *CK19:ABL* % ratio of 0.08 compared to 0.05 in patients with tumours not triple negative, this did not reach statistical significance (p=0.06). DTCs (ICC) and CTCs were also more prevalent in triple negative patients but this also did not reach statistical significance (Table 4.6).

Next, MRD marker values were compared with menopausal status. The 291-bp cfDNA amplicon was higher in post-menopausal women with a median of 0.3 ng/ml compared to 0.05 ng/ml in pre-menopausal women (p=0.007, Mann Whitney test). No other associations were found (Table 4.7).

MRD markers were then correlated with tumour size and grade of the primary tumour. All MRD markers increased with higher tumour grade, however only the 96-bp cfDNA amplicon was significantly higher with a median value of 0.55 ng/ml in grade 1 tumours increasing to 1.25 ng/ml in grade 2 and up to 1.9 ng/ml in grade 3 tumours (p=0.03). Tumour size showed an inverse correlation with both cfDNA amplicons although this was not statistically significant. Tumour size was positively correlated with DTCs measured by qRT-PCR with a median *CK19:ABL* % ratio of 0.4 in patients with small primary tumours compared to 0.5 with larger tumours (Table 4.7).

Table 4.6: Comparison of minimal residual disease markers in primary breast cancer patients with hormone receptor and HER2 receptor status of the primary tumour

		ER Status		HER2 Status		PR Status		Triple Negative Status	
		Positive	**Negative**	**Positive**	**Negative**	**Negative**	**Positive**	**Yes**	**No**
Total Circulating-free DNA 96-bp (ng/ml)	Mean	21.67	4.82	21.2	30.23	15.14	9.50	5.29	20.43
	95% CI	3.42 – 39.92	3.14 – 6.51	-4.81 – 47.2	-2.54 – 63	-1.81 – 32.10	4.11 – 14.9	3.15 – 7.43	3.34 – 37.53
	Median	1.2	2.29	0.89	1.35	1.91	1.70	2.49	1.21
	Range	0 – 2626	0 – 53.71	0 – 1581	0 – 2626	0 – 2626	0 - 440	0 – 53.71	0 – 2626
	No. samples	341	105	128	187	308	220	81	364
	p value (medians)	p=0.004*†		p=0.013*†		p=0.71		p=0.004*	
Circulating-free DNA 291-bp (ng/ml)	Mean	4.46	4.46	8.08	6.85	1.79	2.08	2.84	4.21
	95% CI	-0.28 – 9.20	0.79 – 3.87	-4.11 – 20.28	-1.53 – 15.22	1.01 – 2.57	0.68 – 3.47	0.86 – 4.83	-0.22 – 8.63
	Median	0.23	0.19	0.1	0.16	0.28	0.38	0.24	0.23
	Range	0 – 785.4	0 – 56	0 – 785.4	0 – 785	0 – 78.03	0 - 124	0 – 56	0 – 785.4
	No. samples	336	106	129	188	305	215	82	360
	p value (medians)	p=0.792		p=0.188		p=0.23		p=0.50	
Disseminated tumour cells (qRT-PCR)[a]	Mean	0.087	0.096	0.87	0.10	0.09	0.07	0.09	0.089
	95% CI	0.07 – 0.10	0.07 – 0.11	0.07 – 0.11	0.08 – 0.12	0.08 – 0.10	0.06 – 0.09	0.07 – 0.11	0.08 – 0.10
	Median	0.05	0.07	0.05	0.05	0.048	0.05	0.08	0.05
	Range	0 – 1.25	0 – 0.79	0 – 0.79	0 – 1.25	0 – 1.25	0 – 1.12	0 – 0.65	0 – 1.25
	No. samples	440	135	171	261	399	273	106	476
	p value (medians)	p=0.11		p=0.39		p=0.02*		p=0.06	
Disseminated tumour cells (ICC)[b]	Mean	1.02	0.86	0.82	0.98	0.93	0.81	1.15	0.88
	95% CI	0.67 – 1.36	0.72 – 1.00	0.60 – 1.03	1.82	0.75 – 1.11	1.47	0.70 – 1.60	0.74 – 1.02
	Median	0	0	0	0	0	0	0	0
	Range	0 – 15	0 – 12	0 – 9	0 – 15	0 – 15	0 - 12	0 - 15	0 – 12
	No. samples	136	435	175	260	389	272	102	474
	p value (means)	p=0.34		p=0.45		p=0.23		p=0.16	
Circulating tumour cells[c]	Mean	0.36	0.67	0.54	0.51	0.38	0.40	0.91	0.34
	95% CI	0.08 – 0.64	-0.15 – 1.49	-0.09 – 1.17	-0.03 – 1.06	0.08 – 0.67	0.03 – 0.77	-0.25 – 2.08	0.08 – 0.61
	Median	0	0	0	0	0	0	0	0
	Range	0 – 23	0 – 19	0 – 23	0 – 19	0 – 19	0 - 23	0 – 19	0 – 23
	No. samples	166	48	74	72	135	125	34	178
	p value (means)	p=0.38		p=0.60		p=0.73		p=0.15	

[a] Calculated as the percentage ratio of qRT-PCR *CK19:ABL* (0.1 or more is considered positive)

[b] Detected by pan-CK antibody

[c] Samples were considered positive for CTCs when at least one cell was detected in 22.5 ml of blood by the CellSearch system

*Statistically significant difference of median values by Mann Whitney test or mean values by t test

†Inverse correlation

Table 4.7: Comparison of minimal residual disease markers in primary breast cancer patients with menopausal status and with tumour size and grade of the primary tumour

		Menopausal status		Tumour Size			Grade		
		Post	**Pre**	**T1**	**T2**	**T3**	**1**	**2**	**3**
Total Circulating-free DNA 96-bp (ng/ml)	Mean	23.51	5.39	9.74	33.22	3.22	13.84	7.28	27.73
	95% CI	3.01 – 44.02	2.71 – 7.98	1.16 – 15.31	-1.85 – 68.3	1.62 – 4.82	-9.53 – 37.21	3.47 – 11.09	-0.89 – 56.36
	Median	1.42	1.33	1.91	1.4	1.06	0.55	1.25	1.90
	Range	0 – 2626	1 - 198	0 – 439.6	0 - 2626	0 – 45.41	0 – 439.6	0 - 264	0 – 2626
	No. samples	303	170	202	175	68	38	216	214
	p value (medians)	p=0.70			p=0.10			p=0.03*	
Circulating-free DNA 291-bp (ng/ml)	Mean	5.10	1.48	2.79	6.37	0.68	1.45	1.78	5.99
	95% CI	-0.23 – 10.42	0.55 – 2.41	1.18 – 4.40	-2.59 – 15.34	0.25 – 1.11	0.35 – 2.53	0.39 – 3.17	- 1.31 – 13.3
	Median	0.3	0.05	0.45	0.17	0.1	0.03	0.17	0.29
	Range	0 – 785	0 - 56	0 – 124.2	0 – 785.4	0 – 13.68	0 – 16.04	0 – 124.2	0 – 785.4
	No. samples	299	171	199	175	67	36	214	215
	p value (medians)	p=0.007*			p=0.13			p=0.28	
Disseminated tumour cells (qRT-PCR)[a]	Mean	0.09	0.09	0.068	0.096	0.104	0.078	0.082	0.097
	95% CI	0.08 – 0.10	0.07 – 0.11	0.05 – 0.08	0.08 – 0.11	0.07 – 0.14	0.05 – 0.10	0.07 – 0.10	0.08 – 0.11
	Median	0.053	0.046	0.04	0.06	0.05	0.04	0.05	0.05
	Range	0 – 1.12	0 – 1.25	0 – 1.24	0 – 1.02	0 – 1.25	0 – 0.4	0 – 1.25	0 - 0.02
	No. samples	379	237	184	264	113	49	258	290
	p value (medians)	p=0.99			p=0.019*			p=0.56	
Disseminated tumour cells (ICC)[b]	Mean	0.85	0.99	0.81	0.84	0.96	0.91	0.88	0.93
	95% CI	0.69 – 1.00	0.75 – 1.23	0.58 – 1.04	0.66 – 1.02	0.61 – 1.31	0.31 – 1.52	0.69 – 1.07	0.72 – 1.13
	Median	0	0	0	0	0	0	0	0
	Range	0 – 12	0 - 15	0 - 12	0 - 11	0 – 15	0 - 12	0 - 9	0 – 15
	No. samples	382	228	188	254	119	47	263	284
	p value (means)	p=0.31			p=0.72			p=0.95	
Circulating tumour cells[c]	Mean	0.36	0.53	0.15	0.51	0.87	0.35	0.15	0.68
	95% CI	0.04 – 0.69	0.05 – 1.01	0.07 – 0.24	-0.04 – 1.06	-0.15 – 1.89	0.01 – 0.68	0.07 – 0.23	0.12 – 1.24
	Median	0	0	0	0	0	0	0	0
	Range	0 – 23	0 - 19	0 - 2	0 - 23	0 – 19	0 - 3	0 - 2	0 – 23
	No. samples	144	81	97	84	39	23	95	107
	p value (means)	p=0.55			p=0.16			p=0.17	

[a] Calculated as the percentage ratio of qRT-PCR *CK19:ABL* (0.1 or more is considered positive)

[b] Detected by pan-CK antibody

[c] Samples were considered positive for CTCs when at least one cell was detected in 22.5 ml of blood by the CellSearch system

* Statistically significant by one way analysis of variance, Kruskall-Wallis, Mann Whitney test or t test

4.10 Relationship of cfDNA quantity to DTCS and CTCS

Results shown here find that patients with a high risk of relapse based on tumour stage are more likely to have both DTCs and CTCs. It was therefore important to establish the relationship between the presence of cfDNA to DTC and CTC detection. This relationship was measured in two ways, firstly by analysing all the matched patient samples that had bone marrow aspirates and blood samples performed at the same clinic visit for a direct comparison. The second approach was by comparing the median cfDNA values with the median and maximum DTC and CTC values taken over time from each patient.

4.11 The presence of DTCs in the bone marrow as assessed by CK19 qRT-PCR is inversely related to the quantity of the 291-bp amplicon in the blood

We investigated the relationship between cfDNA with DTCs and CTCs by analysing all the matched patient samples with bone marrow aspirates and blood samples performed at the same clinic visit or within a month of either sample being taken. One hundred and sixty-eight matched bone marrow and blood samples had both DTC analysis (ICC and qRT-PCR) and qPCR measurement of 96-bp and 291-bp amplicons in cfDNA. Eight out of 64 patients had no matched samples; the rest had both a mean and median of 3 per patient. Up to 179 matched samples were analysed for both CTCs and cfDNA 96-bp and 291-bp amplicon quantities.

Interestingly, an inverse correlation was found between the presence of DTCs as measured by *CK19* qRT-PCR and the quantity of the 291-bp cfDNA amplicon, p=0.04 (Figure 4.3 B and Table 4.8). The box plots demonstrate that those patients with DTCs (qRT-PCR) had lower median and inter-quartile ranges of the 291-bp cfDNA amplicon than the group with no DTCs present. No other strong correlations were evident (Table 4.8).

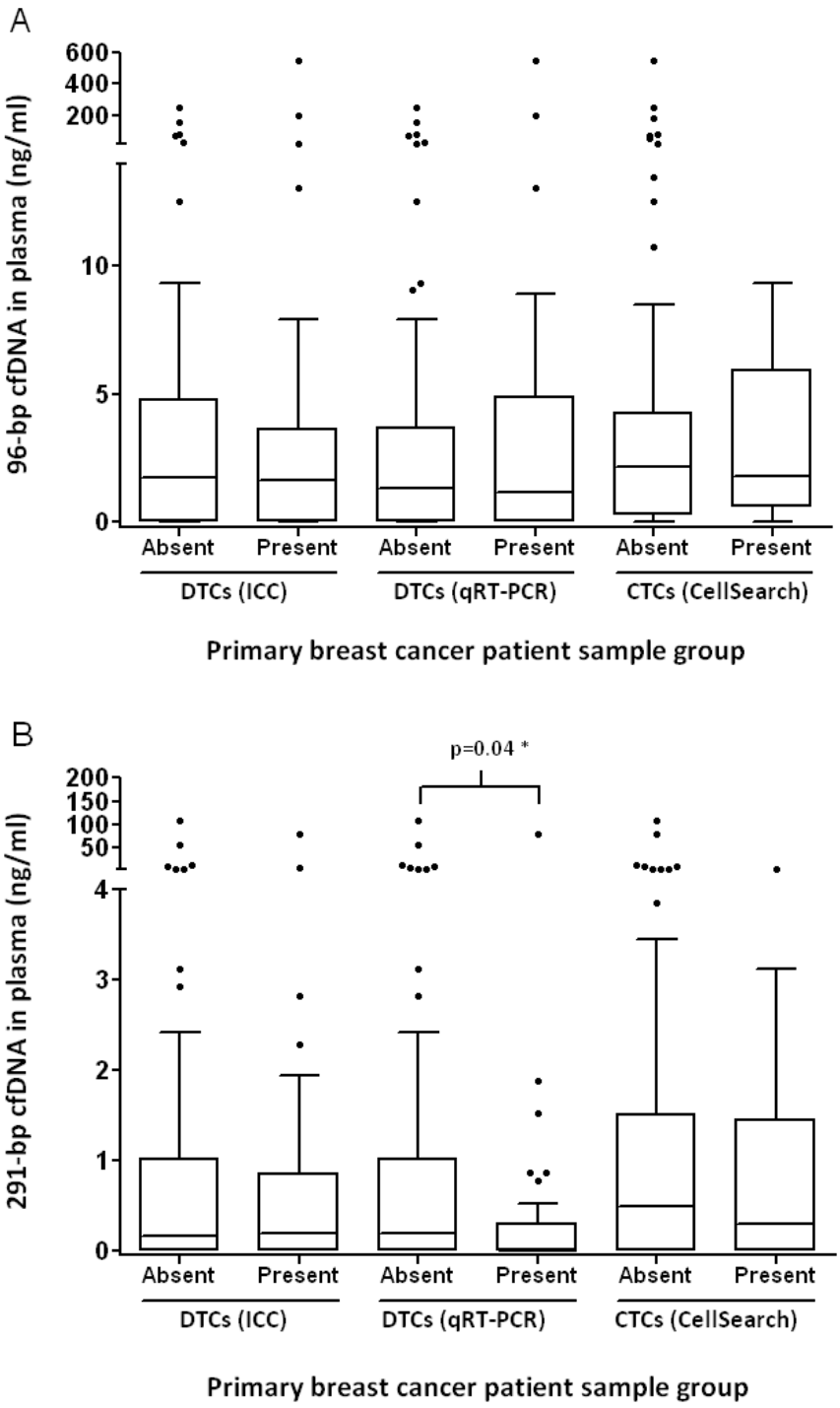


Figure 4.3: The largely cancer specific 291-bp cfDNA amplicon in plasma is inversely related to the presence of DTCs by qRT-PCR. The box plots show the comparison of A, 96-bp cfDNA, B, 291-bp cfDNA in patient samples grouped by the presence or absence of DTCs (ICC and qRT-PCR) and CTCs (CellSearch) in paired bone marrow aspirate and blood samples from 64 primary breast cancer patients on follow-up. The box-plots show the median and inter-quartile ranges. and the whiskers indicate 1.5 x the inter-quartile range, (*statistically significant, Mann-Whitney test).

Table 4.8: The 291-bp cfDNA amplicon is inversely related to the presence of DTCs in the bone marrow by qRT-PCR

		Disseminated tumour cells (qRT-PCR)[a]		Disseminated tumour cells (ICC)[b]		Circulating tumour cells[c]	
		Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
	Mean	24.22	7.23	12.94	8.85	3.12	1.97
Total circulating-	95% CI	-10.1 – 58.5	1.81 – 12.65	-3.5 – 29.38	2.04 – 15.65	0.05 – 4.19	1.97 – 19.86
free DNA	Median	1.15	1.31	1.62	1.73	1.77	2.15
96-bp (ng/ml)	Range	0 - 547	0 - 247	0 - 547	0 – 247	0 – 9.34	0 – 547
	No. samples	34	112	70	89	35	139
	p value (medians)	0.98		0.69		0.65	
	Mean	2.56	2.35	1.75	2.73	0.82	2.48
Circulating-free	95% CI	-2.18 – 7.30	0.16 – 4.54	-0.55 – 4.05	-0.03 – 5.50	0.43 – 1.22	0.53 – 4.42
DNA	Median	0	0.19	0.19	0.16	0.29	0.53
291-bp (ng/ml)	Range	0 - 79	0 – 109	0 - 79	0 – 109	0 – 4.71	0 – 109
	No. samples	34	111	69	88	35	137
	p value (medians)	0.04*†		0.64		0.51	

*Statistically significant difference of median values by Mann Whitney test
†Inverse correlation

4.12 Correlations between cfDNA, DTC and CTC detection as markers of MRD using the median and maximum values of samples from individual patients

It was important to establish that the inverse correlation of the 291-bp cfDNA quantity to DTCs (qRT-PCR) was seen in the median and maximum values for samples from each patient over time, and not just in the 142 individual samples matched at one time point. This took all the results into account from 474 plasma samples and 608 bone marrow samples taken over the follow up period of all patients. The important variations apparent in each patient were then included and taken into consideration, as well as providing a large-scale analysis to confirm our findings. Both the median and maximum DTC (qRT-PCR) values were confirmed to be inversely correlated with the median quantity of cfDNA 291-bp for each patient (p=0.035 and 0.0082 respectively, Spearman's Rank), (Table 4.9).

Table 4.9: Correlations between cfDNA, DTC and CTC detection as markers of MRD in 64 primary breast cancer patients on follow up. Median values of cfDNA were compared to the median and maximum DTC and CTC values for each patient. Spearman's rank was used to compare the results of two tests with the p values shown.

	Median DTC (qRT-PCR)[a]	Median DTC (ICC)[b]	Median CTC (CellSearch)[c]	Max DTC (qRT-PCR)[a]	Max DTC (ICC)[b]	Max CTC (CellSearch)[c]
Median cell-free DNA 96-bp (ng/ml)	0.093†	0.48†	0.33	0.065†	0.42†	0.39
Median cell-free DNA 291-bp (ng/ml)	0.035†*	0.9†	0.53†	0.0082†*	0.56†	0.66†

[a] Calculated as the percentage ratio of qRT-PCR CK19:ABL (0.1 or more is considered positive)
[b] Detected by pan-CK antibody
[c] Samples were considered positive for CTCs when at least one cell was detected in 22.5 ml of blood by the CellSearch system
*Statistically significant. Spearman's Rank was used to compare the results of two tests
†Negative correlation

4.13 MRD values in relapsed versus non-relapsed patients

Eight of 64 patients on follow up for primary breast cancer relapsed with either metastatic disease (n=6) or contralateral breast cancer (n=2). These patients relapsed at 5 – 11 years post surgery (mean 7.8 years). There was no evidence that a 'lead interval', where a rise of one marker ante-dated overt metastatic relapse or local recurrence in this small group of patients. CTCs and cfDNA were only sampled at limited time points during the follow up period and therefore trends could not be fully evaluated. Due to this, pooled MRD values were compared between the relapsed and non-relapsed patients. The median quantity of the 291-bp cfDNA amplicon was lower in the relapsed patients (0.01 compared to 0.26 ng/ml) but this was not statistically significant (p=0.085), Table 4.10. As expected, DTC detection by qRT-PCR but not by ICC was significantly higher in relapsed patient samples (p=0.003), Table 4.10.

Table 4.10: Comparison of minimal residual disease markers in the blood and bone marrow of relapsed and non-relapsed breast cancer patients

		Relapsed	
		No	**Yes**
Total Circulating-free DNA 96-bp (ng/ml)	Mean	17.64	9.43
	95% CI	3.38 – 31.9	-1.55 – 20.41
	Median	1.45	1.09
	Range	0 - 2626	0 – 198
	No. samples	436	37
	p value (medians)	0.70	
Circulating-free DNA 291-bp (ng/ml)	Mean	3.94	1.97
	95% CI	0.25 – 7.64	-0.41 – 4.36
	Median	0.26	0.01
	Range	0 - 785	0 – 42
	No. samples	432	38
	p value (medians)	0.085	
Disseminated tumour cells (qRT-PCR)[a]	Mean	0.08	0.14
	95% CI	0.07 – 0.09	0.09 – 0.19
	Median	0.05	0.07
	Range	0 – 1.12	0 – 1.25
	No. samples	541	75
	p value (medians)	0.003*	
Disseminated tumour cells (ICC)[b]	Mean	0.898	0.904
	95% CI	0.75 – 1.04	0.55 – 1.26
	Median	0	0
	Range	0 - 15	0 – 7
	No. samples	537	73
	p value (means)	0.98	
Circulating tumour cells[c]	Mean	0.43	0.25
	95% CI	0.15 – 0.71	-0.34 – 0.84
	Median	0	0
	Range	0 - 23	0 – 2
	No. samples	217	8
	p value (means)	0.81 (numbers too small for analysis)	

[a] Calculated as the percentage ratio of qRT-PCR CK19:ABL (0.1 or more is considered positive)
[b] Detected by pan-CK antibody
[c] Samples were considered positive for CTCs when at least one cell was detected in 22.5 ml of blood by the CellSearch system
*Statistically significant difference of median values by Mann Whitney test or mean values by t test

4.14 Trends of DTC detection in eight relapsed patients with corresponding CTC and cfDNA values over time

In general, we observed 3 trends of DTC (qRT-PCR and ICC) detection; a fluctuating pattern in 2 patients, consistently low levels in 2 patients and an initial rise, drop and subsequent rise in 4 patients over a period of years (Figures 4.4 – 4.6). All 8 patients (100%) who relapsed showed evidence of DTCs by qRT-PCR, whereas 6 of 8 (75%) had DTCs present detected by ICC.

Eight patients out of 64 primary breast cancer patients on follow up relapsed with either metastatic disease or contralateral breast cancer and were subsequently taken off the study. No measurements were obtained after clinical relapse. Three trends of DTC detection were found in this small set of patients. The first was a fluctuating pattern over a period of years of DTC detection both by ICC and qRT-PCR, in two patients (Figure 4.4). The second was DTC detection at consistently low levels by ICC with evidence of low levels of DTC detection by qRT-PCR (Figure 4.5). The third trend showed a rise in DTC detection by one or both methods after surgery followed by a fall and then a subsequent rise by one or both methods (except patient 3b who showed no final rise), (Figure 4.6).

The CTC and cfDNA values were only sampled at limited time points during the follow-up, particularly in patients who relapsed early on in the study and therefore trends could not be fully evaluated. Five out of 8 patients did however, show evidence of cfDNA quantity in their plasma decreasing prior to relapse. In addition, all 8 patients had 291-bp cfDNA values that were all within the range of values found in the healthy controls prior to relapse (0 – 1.37 ng/ml). This may indicate that tumour cell death is not occurring (low release of cfDNA) at a rate to counter-balance proliferation thus causing disease progression and relapse. There was also evidence of cfDNA quantity rising after DTCs were detected (patient 1a and 3c, Figures 4.4 and 4.6). One of the two patients who had CTC analysis performed showed 2 CTCs present in 22.5ml of blood 12 months prior to relapse with metastatic disease (1b, Figure 4.4).

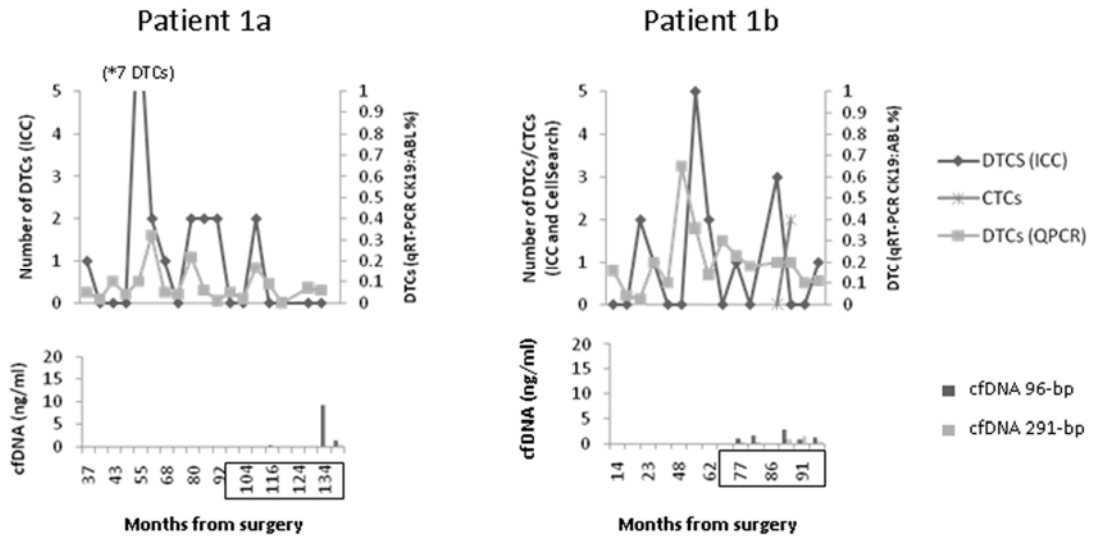


Figure 4.4: Trend 1 – DTC detection shows a fluctuating pattern over a period of years in two relapsed patients. Graphs 1a and 1 b represent two primary breast cancer patients on follow up with DTC, CTC and cfDNA measurements over time from surgery. The upper graphs show DTC (ICC and qRT-PCR) and CTC measurements and the lower graphs show cfDNA quantities over time (boxed months illustrate when plasma samples were taken with available cfDNA measurements). Relapse was at the end of the sampling period.

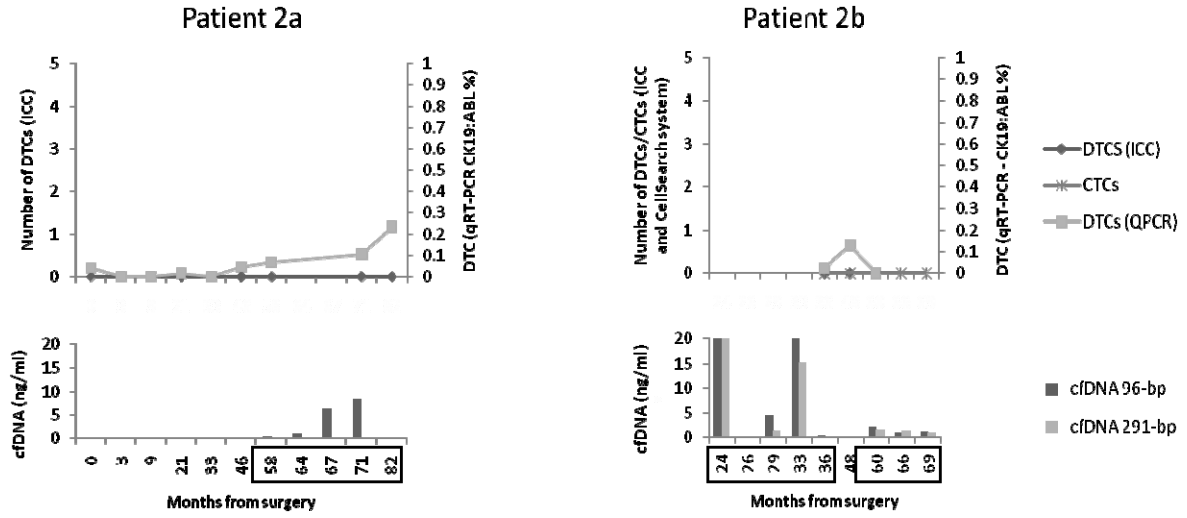


Figure 4.5: Trend 2 – DTC (ICC) and CTC detection demonstrate consistently low levels in two relapsed patients. Graphs 2a and 2b represent two primary breast cancer patients on follow up with DTC, CTC and cfDNA measurements over time. The upper graphs show DTC (ICC and qRT-PCR) and CTC measurements and the lower graphs show cfDNA quantities over time (boxed months illustrate when plasma samples were taken with available cfDNA measurements). Relapse was at the end of the sampling period.

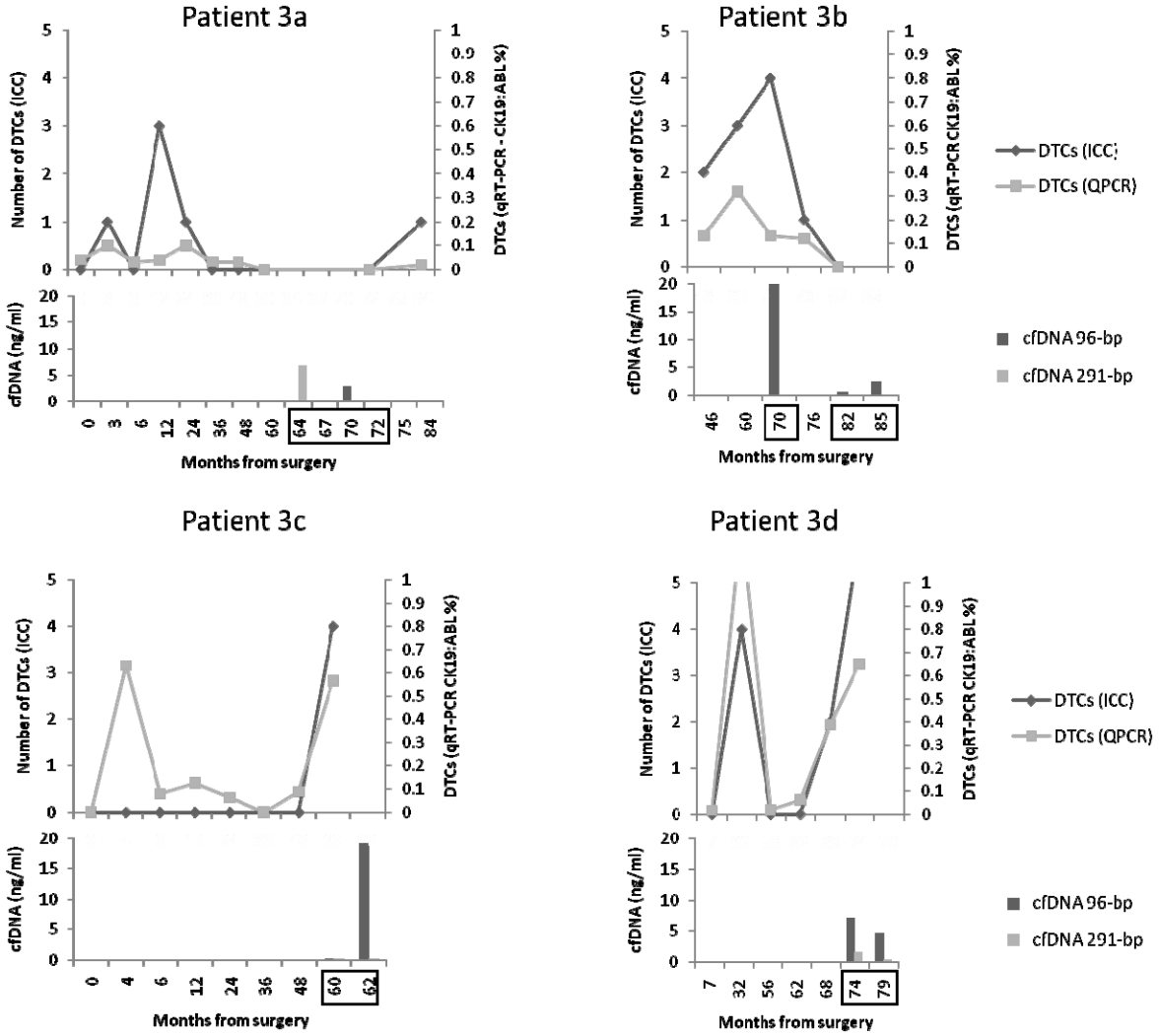


Figure 4.6: Trend 3 – DTC detection increases after surgery followed by a decrease with a further rise in DTCs prior to relapse (except 3b which shows no rise prior to relapse) in four patients. Graphs 3a – 3d represent four primary breast cancer patients on follow-up with DTC and cfDNA measurements taken over time. The upper graphs show DTC (ICC and QPCR) measurements and the lower graphs show cfDNA quantities over time (boxed months illustrate when plasma samples were taken with available cfDNA measurements). Relapse was at the end of the sampling period.

4.15 Trends of DTC, CTC and cfDNA detection in patients on follow up

Due to limited blood sampling in the relapsed patients it was not possible to ascertain a clear pattern of CTC or cfDNA detection over the course of the follow up. It was possible however, to look at patterns in the LN positive and negative patients who had not relapsed and were more fully evaluated. There was evidence in some patients that after DTC detection there is a subsequent increase in cfDNA quantity. One example showed anecdotal evidence in Figure 4.7. One hypothesis is that the replication of the disseminated tumour cells is then balanced by cell death occurring some months later manifesting as cfDNA quantity. DTC and cfDNA detection shown in this patient over time demonstrates a pattern similar to trend 1 (Figure 4.4) although the 291-bp quantity shows greater variation than those patients who have relapsed (Table 4.10). These fluctuations may be due to cycles of cell death from the tumour cells remaining in the bone marrow.

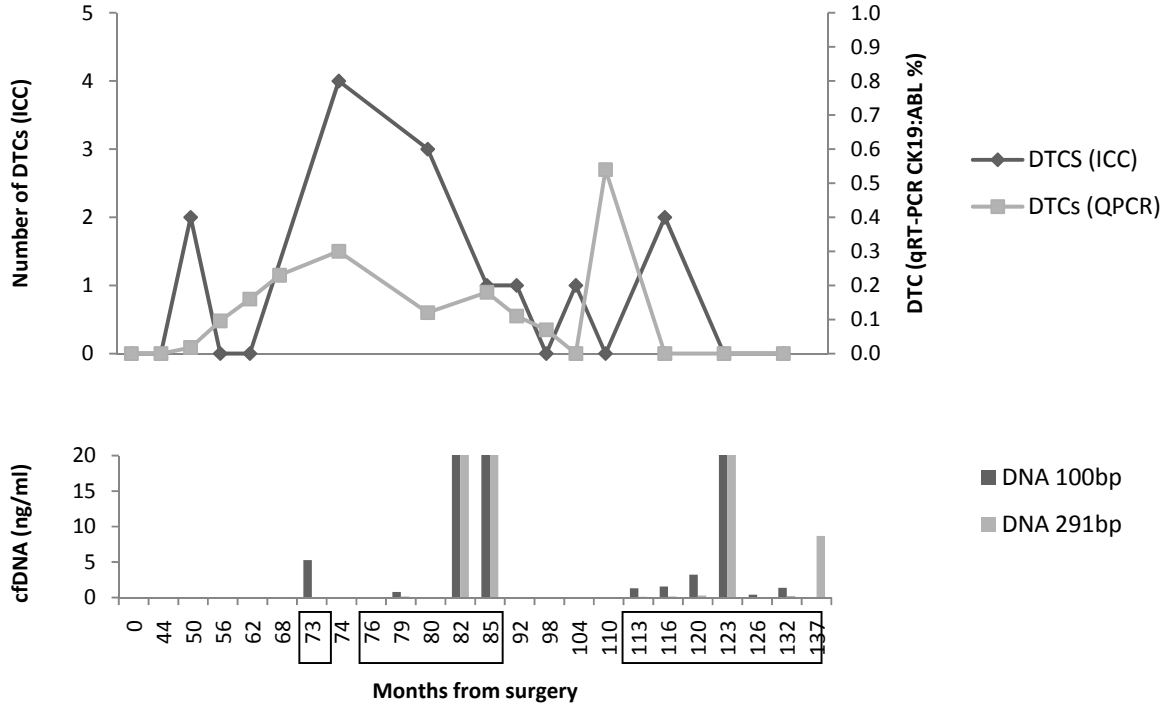


Figure 4.7: DTC and cfDNA detection in a breast cancer patient at high risk of relapse on follow up. The upper graphs show DTC (ICC and qRT-PCR) measurements and the lower graphs show cfDNA quantities over time (boxed months illustrate when plasma samples were taken with available cfDNA measurements). The presence of DTCs is followed by a decrease in number with a parallel increase in cfDNA quantity of both the 96-bp and 291-bp amplicons up to 6 months later.

4.16 Limitations of the study

Sampling was not consistent in all MRD measures due to technical and funding constraints. Bone marrow aspirates were taken over the course of the whole study with pilot studies for cfDNA and CTC analysis performed at different times. Despite this, the study is still the first of its kind to intensively study a cohort of patients with three measures (cfDNA, CTCs and DTCs) of MRD. The inconsistency in sampling was dealt with by performing analyses of paired samples where blood and bone marrow samples are taken at the same clinic visit or within a month of either test. Many of the values for MRD were negative or very low making analysis difficult due to the possibility of statistical errors. The 291-bp quantity of cfDNA was not only negatively correlated with lymph node status but also to DTCs by qRT-PCR which adds confidence to this finding since DTCs are correlated with lymph node status in previous studies. Sampling errors are likely in tests to detect such small quantities of mRNA or single cells and are an inherent problem in studies such as these. DTC detection using qRT-PCR of *CK19* and also cfDNA detection relies on the effective and timely sample processing within 4 hours to limit RNA degradation. We used a control to minimise this problem by only including samples with 1000 transcripts of *ABL* detected, since we take one or more *CK19* transcripts per 1000 *ABL* to indicate a positive result for the detection of DTCs.

4.17 Discussion

This study provides evidence of the presence of one or more different measures of MRD, in all 64 primary breast cancer patients on long-term follow-up of up to 11.5 years post-surgery. Measures of both viable and non-viable MRD were detected in multiple samples taken over time in this cohort of patients. Examining MRD trends over time helps us to understand the possible signs of disease relapse and a possible hypothesis of cancer dormancy.

Detection of MRD in control groups

In Chapter 3 CTCs were demonstrated to be more prevalent in primary breast cancer patients than in healthy controls, this is also true of cfDNA of both the total, apoptotic 96-bp amplicon and the mostly cancer-derived 291-bp amplicons which were increased in the blood of breast cancer patients, both primary and advanced. The total cfDNA measurement of the 96-bp amplicon quantity in plasma was significantly higher in cancer patients compared to benign breast disease patients, although overlap of values still occurred along with low levels being present in healthy controls. The median quantities of the 291-bp amplicon were not significantly different to benign conditions due to a large overlap of values meaning this test alone would not be suitable for detecting micrometastatic disease. A study of circulating nucleosomes (a proportion of cfDNA packaged with histones that are released by apoptosis in 180-bp lengths) found the quantity also could not distinguish benign from malignant patients. When additional characterisation was performed of apoptotic protease activity, this was linked with breast cancer progression (197). Low levels of cfDNA are also a problem in other conditions like many autoimmune diseases such as systemic lupus and rheumatoid arthritis (97, 198). This illustrates the need for further characterisation of specific tumour-derived factors to prove they are of cancer origin.

The use of cfDNA quantity in plasma used alone is therefore not specific enough to be used as a test of MRD, the additional problems with this assay that may affect the detection of small quantities of DNA are also numerous. This includes sampling and assay issues such as the delay in blood processing causing increased cfDNA clearance, freeze-thawing and temperature

changes that influence the integrity and yield of cfDNA (199). In our studies, a robust and standardised method helped to reduce these problems, with an extra centrifuge step to eliminate any cellular DNA contamination (166).

The measurement of DTCs was not possible in control groups due to ethical reasons, however this method has been validated previously in our laboratory. In addition the concordance was high between the two methods of detection by ICC and qRT-PCR at 72%, this was consistent with our laboratory's previous concordance of 71% of DTCs detected by both methods (128). A proportion of DTCs detected by ICC are not picked up by qRT-PCR and vice versa. This may be due to the different markers used or the detection of protein versus mRNA, for example the ICC method stains for pan-CK (CK8, 18 and 19) positive DTCs whereas the qRT-PCR method detects *CK19* mRNA. DTC detection by qRT-PCR may be more sensitive in some cases but may be less sensitive if low levels of mRNA are produced by individual DTCs.

Correlation of CTCs in the blood with DTCs in the bone marrow

The presence of DTCs (measured by ICC) in the bone marrow was correlated with the presence of CTCs in the blood in our cohort of 64 patients with a diagnosis of breast cancer, studied over a period of 11.5 years. Matched patient blood and bone marrow samples confirmed that in those samples with CTCs detected, a mean of 2.11 DTCs were present in the bone marrow compared to a mean of 0.78 DTCs in the bone marrow of patients with no CTCs detected. Since there were only 18 matched samples that were CTC positive, we also looked at the median values of both CTCs and DTCs in 41 patients taken over time, this also revealed this correlation. Our findings imply these occult tumour cells are related in some way, and the simplest explanation is that bone marrow DTCs are occasionally released into the blood stream as CTCs. Many studies have shown different concordances with no consensus; this is due to the different methodologies and the different quantities of blood and bone marrow taken. This makes it difficult to compare studies accurately.

The prognostic importance of DTCs detected in the bone marrow of breast cancer patients is well known and was validated in a large, multi-centre study (59). This indicates that CTCs may have a similar prognostic value although the detection level is still lower than DTCs. This study

showed 39% of all bone marrow aspirates taken had DTCs by ICC present compared to 20% of blood samples positive for CTCs. Many studies have also showed this trend of lower CTC detection (118, 119, 123, 127, 141). This may be due to the varying methods used that may not be sensitive enough, the low quantities of blood measured or that DTCs are more prevalent. One study found an almost equal detection rate of CTCs in the blood and DTCs in the bone marrow when *CK19* mRNA was detected by RT-PCR in 20 ml of blood and 3 ml of bone marrow in paired samples from stage 1 - 3 breast cancer patients. There was also a high concordance of detection of 94% before chemotherapy was given (140). This reveals the importance of using specific and sensitive methods to detect occult tumour cells effectively along with greater quantities of blood. As mentioned in Chapter 3 however, the specificity of RT-PCR methods still requires validation.

The concordance between DTCs and CTCs is determined by the detection method used; our study showed that CTCs measured by an ICC-based method (the CellSearch system) was correlated with DTCs detected by ICC but not by qRT-PCR. Similarly, the study mentioned above used RT-PCR to detect mRNA of *CK19* in the blood and bone marrow and showed a correlation (140). Two other studies revealed the detection of CTCs and DTCs (both by ICC) were concordant or conferred an especially poor prognosis to primary breast cancer patients (119, 136). Studies using a combination of methods showed only a weak concordance between CTCs detected by multi-marker qPCR (Adnatest) with DTCs detected by ICC (123). Our study showed no concordance with DTCs by qRT-PCR to CTCs tested by the CellSearch. The population of tumour cells detected by either tumour mRNA markers or by protein tumour markers may be different since they are not correlated, for example those detected by ICC may be in a non-proliferative state as shown by the lack of the Ki67 proliferation marker in CTCs in one study (136). More recently, heterogeneous levels of Ki67 were found in CTCs from prostate cancer patients, many with only 1% of the total CTC population positive for Ki67 (192). In addition, the apoptosis marker M30 was found in 15 out of 19 (79%) CTC–positive breast cancer patients providing evidence these cells may not all be viable (130).

Evidence therefore suggests that DTCs are correlated to CTCs depending on the method used, although their prognostic use, either alone or in combination still needs to be validated. The further characterisation of these occult tumour cells is therefore crucial. Recent studies have looked at the role of EMT in cancer progression and suggested that a sub-population of these cells may be cancer stem cells (89). Furthermore, the detection of DTCs and CTCs in patients in long remissions may show a different prognostic relevance to those less than 5 years after surgery when the risk of relapse is higher. The distinction between these may be crucial to understand the processes of tumour dormancy and relapse.

MRD marker correlations with primary tumour prognostic predictors

The measurement of the 96-bp amplicon representing total cfDNA was the only marker significantly higher in both ER and PR negative patients and subsequently in triple negative patients. This is logical since patients with triple-negative or basal-like tumours have a worse prognosis (145, 200). DTCs and CTCs were also higher in patient samples with a triple negative tumour, although this did not reach significance. DTCs measured by qRT-PCR were significantly higher in patient samples with PR negative tumours. The 96-bp amplicon was significantly higher with increasing tumour grade ($p=0.03$), conversely however, the value was lower with increasing tumour size but this was not statistically significant. DTC detection by qRT-PCR was associated with increasing tumour size though as previously shown (59). The largely cancer associated 291-bp amplicon was higher in post-menopausal patients, the reasons for this are not clear. This demonstrates that patients with smaller tumours, triple negative tumours, high grade tumours and no local spread detected are more likely to have higher 96-bp amplicons present in their blood on follow-up.

The correlation of DTCs and CTCs to one of the most important predictors of prognosis; lymph node status is confirmed here, although DTCs detected by ICC had borderline significance ($p=0.057$) and due to the lower patient numbers the detection of CTCs was not correlated with LN status although we have confirmed this in a larger population in Chapter 3. Interestingly, DTCs detected by qRT-PCR correlated to lymph node status better than DTCs detected by ICC. It is unclear why this is but *CK19* is a known predictor of a worse prognosis (183) and so this

method may simply be more sensitive at detecting prognostically relevant DTCs that are more aggressive. One study suggested CK19 may not simply be a structural marker but also a biologically relevant marker involved in metastasis with stem cell properties. Evidence for this was found with the detection of full length CK19 protein being released only from viable DTCs from breast cancer patients, this was associated with a worse prognosis (129). ICC therefore may not detect low levels of CK19 expression or it may detect two populations of cells that are both viable and non-viable. Evidence for this was found in this study, DTCs detected with ICC were detected in 39% of the 608 bone marrow aspirates compared to 30% detected by qRT-PCR showing ICC is detecting more cells that are not as prognostically relevant. However, this may be due to the specificity and the sensitivity of the assays in our laboratory.

A surprising finding was that both the 96-bp and the 291-bp amplicons of cfDNA (found to be increased in breast cancer patients compared to healthy controls), were inversely related to lymph node status. Our hypothesis of increasing cfDNA in more advanced stages of cancer was therefore incorrect. The comparison of cfDNA levels in breast cancer patients with metastatic disease was also not significantly different to that of primary breast cancer patients. These difference between the LN positive and negative patients are subtle and are only shown in the median values, in fact the lymph node positive patients demonstrated a wider range and variation of values as revealed by the 95% CI 291-bp amplicon values of -0.58 – 9.27 compared to 1.14 – 3.94 in the LN negative patients. This wide variation is also apparent in individual LN positive patients as depicted in Figure 4.7. Those patients whose cancer has not spread locally unexpectedly have more cfDNA present on long-term follow-up. The reasons for these lower levels of cfDNA in LN negative patients are examined below when we also look at the relationship of DTCs to cfDNA.

Inverse correlation of the cfDNA 291-bp amplicon with DTCs measured by qRT-PCR

An additional surprising finding was the significant inverse correlation of the cancer-associated 291-bp amplicon of cfDNA with DTCs measured by qRT-PCR ($p=0.04$). Some caution must be taken when analysing the cfDNA data with the DTC qRT-PCR data since many values are zero, this may bias the data towards an inverse correlation when we include data that is below the

threshold of detection by qPCR. Both assays always included standards as a positive control to ensure accurate results though. Even so, when we take this into account, these results are still in line with the inverse correlation to LN status and each finding is therefore backed up because cfDNA is likely to be inversely correlated to both since DTCs are more prevalent in LN positive patients. This adds confidence that these results are not artifactual. These results also suggest that tumour-derived and total cfDNA are in a greater proportion of primary breast cancer patients than previously thought.

At first these results may appear counterintuitive. However if the vast majority of patients have the mostly cancer-derived 291-bp amplicon of cfDNA indicating dying micrometastases, then those who also have viable DTCs may show lower levels of tumour cell death, necrosis or lysis due to cells proliferating rather than dying. This means cycles of tumour cell replication followed by cell death may be occurring in many of these patients. Evidence for this is shown in Figure 4.7 and by the inverse correlation of the cancer-associated 291-bp amplicon with DTCs by *CK19* mRNA – a viable measure of DTCs since the active transcription of genes is occurring. The finding that a proportion of CTCs and DTCs (detected by CellSearch and ICC), have low proliferative potential and express a marker of apoptosis as alluded to earlier, mean they could represent a measure of cell death, and may be the reason why this is not also inversely correlated with cfDNA – also a measure of cell death, necrosis or lysis. Patient bone marrow samples with and without DTCs (ICC) showed similar levels of the 291-bp amplicon.

Few studies have been performed comparing the presence of cfDNA in the blood with DTCs in the bone marrow. One group found no correlation of tumour induced genomic alterations in the blood with the presence of DTCs measured by ICC. A correlation was found however, of increased quantities of total cfDNA in DTC-positive patients. This is in contrast to the inverse correlation in our study and may be due to using a cohort of patients close to surgery as much higher quantities of cfDNA were also detected (201). The cfDNA may be derived mostly from the primary tumour whereas in our study, patients were studied up to 11.5 years post surgery and so the cfDNA present is likely to be from the cell death/necrosis or lysis of micrometastases. Our study found no correlation of cfDNA with CTCs and may be the same

reason why no relationship was found with DTCs (ICC). The study mentioned above also found a correlation of allelic imbalances in cfDNA to the presence of CTCs (201). This provides evidence that cfDNA is indeed derived from micrometastases and could be used as a surrogate marker. Our studies did not perform characterisation of cfDNA to find these relationships although work in our laboratory is now looking at single nucleotide polymorphism profiles of cfDNA in primary breast cancer patients at risk of relapse.

Tumour dormancy

Evidence to support the hypothesis that immunosurveillance or angiogenic dormancy keeps micrometastases in check, is seen from the consistent or fluctuating levels of the mostly cancer derived cfDNA amplicon in the plasma of a large proportion of primary breast cancer patients on follow-up. This may represent cycles of tumour cell death from micrometastases. This is in terms of tumour mass dormancy where proliferation is counterbalanced by tumour cell apoptosis, lysis or necrosis (131). Our study describes the relapse of 8 patients 5 – 11 years after surgery to remove their tumour, with different patterns of DTC detection throughout this period and at least 4 patients showing a period of time where no DTCs are detected in a number of sequential time points (Figure 4.5 and 4.6). In contrast, two patients showed constant fluctuations in DTC detection. These findings, plus the long, disease-free periods in these patients suggest that there may be intermissions in disease progression in some patients explained by different mechanisms of tumour dormancy depending on a myriad of factors including the tumour microenvironment, angiogenic factors, growth factors and by immune responses (131, 187).

Our data indicates a dormancy process that may act systemically in some patients since the inverse correlation of cfDNA to DTCs and LN positive status is so apparent, along with the evidence for fluctuations of MRD in individual patients. The phases of proliferation and cell death/necrosis or lysis may be distinct as shown by a high risk patient with high levels of DTCs followed by a rise in cfDNA (Figure 4.7). However, this is only anecdotal evidence and these phases may not all be distinct in all patients who harbour MRD with no signs of disease. Also,

sampling errors and incomplete data may also mean this is not shown explicitly in many patients. In addition, sampling for MRD has only taken place in the blood and bone marrow, other organs not as accessible to sampling may harbour micrometastases. A further observation of cfDNA in the relapsed patients is that levels are very low, implying cell death is not occurring at a high rate and is being surpassed by proliferation of cells causing relapse. Since immune responses are systemic, this may reveal why the inverse correlation of DTCs with cfDNA is so evident, an immune response may be causing tumour cell death, necrosis or lysis in a sustained period of time, followed by periods of dormancy or proliferation when the micrometastases remains 'under the radar' of immune attack or the immune system may prevent the expansion of these cells. Evidence for this has been shown in a mice model of murine B cell lymphoma, where mice were immunized against the antigen on the lymphoma cells and then challenged with a B cell lymphoma which then entered a dormant state. A steady number of cells were found to be replicating and dying (202). Another study used a mouse model of spontaneous melanoma and found dormancy of disseminated tumour cells to the lung had reduced proliferation partly due to the immune mediated $CD8^+$ T cell response (203). This hypothesis fits with data shown here although caution must be taken when comparing mouse models with clinical data.

Although animal models are useful and have provided important information on the mechanisms of metastasis, the shorter dormancy time-frames do not recapitulate the processes that occur in humans accurately. A number of different mechanisms could be occurring at different times, for example, tumours in a dormant state may start proliferating upon a switch to an angiogenic phenotype that allows the growth of blood vessels, this may then be kept in check by immunosurveillence and/or anti-angiogenic factors. This may be before or after periods of cell quiescence (131, 187). Evolutionary theories point to dormancy as a phenomenon that pauses a cell to ensure the conditions are optimal for growth. This allows a selective advantage for the replication and perpetuation of tumour cells (204), which may also be happening in the bone marrow. In addition, a recent theoretical model of cancer dormancy found that long-term breast cancer dormancy can be maintained by a small subset of

growth-restricted, dangerous micrometastases (205). This agrees with the observations described here of the persistence of CTCs and DTCs in a fluctuating pattern observed from growth restricted, and not necessarily relapse-inducing, micrometastases.

In conclusion, we have shown here the use of multiple methods for measuring MRD over a long period of time in 64 breast cancer patients, many of whom are thought to harbour occult tumour cells. For the first time, we have shown patterns of DTC, CTC and cfDNA detection over time and how this may relate to tumour dormancy. Evidence not seen before shows dormancy processes may be occurring in breast cancer patients. This is in the form of an inverse correlation of cell death manifesting as cfDNA in the plasma to measures of viable cells in the bone marrow. This indicates a possible balance of proliferation with cell death over many years keeping micrometastases in check by unknown mechanisms. These may be dictated by the microenvironment or by systemic immune or anti-angiogenic responses. Further characterisation of MRD is warranted to enable the mechanisms of dormancy to be maintained so that relapse in breast cancer does not occur. CK19 mRNA found in the bone marrow and blood is prognostically relevant so characterisation of viable CTCs may be an important avenue to explore. One method discussed in the next study describes a novel technique to detect and phenotype viable CTCs.

5.0 CTCscope - a novel method in development for the detection and characterisation of CTCs

5.1 Introduction

Previous studies have shown that CTCs are not detected by the CellSearch system in all metastatic breast cancer patients with around 20 – 30% having no measureable CTCs (116, 117). The CellSearch system uses EpCAM enrichment followed by staining for pan-cytokeratin (CK8, 18 and 19) and has the option of staining for tumour markers such as HER2 and EGFR. There is evidence to suggest this method does not detect all CTCs present in a blood sample and may miss low EpCAM expressing CTCs (66, 178). Further evidence suggests CTCs of the 'normal-like' breast tumour type that are ER negative and express a putative tumour stem cell phenotype (often associated with aggressive disease), are not detected (177). Since it is hoped that CTCs will provide information for personalised therapy, it is important to be able to detect the full heterogeneity present in a CTC population by detecting a number of tumour markers. This is particularly important in early stage cancer where CTCs are very rare and their characterisation could enable the potential eradication of micrometastases by the use of specific therapies. Single cell detection and characterisation is limited due to the technical difficulties in isolating adequate numbers of cells from the blood as well as the problems (both cost and optimisation time) in multiplexing antibodies against multiple tumour markers (206). Antibody staining is also prone to low specificity and user subjectivity when interpreting the staining (207). Despite this, a recent study was successfully able to phenotype four markers simultaneously using immunofluorescence for future staining of DTCs (206).

The use of qRT-PCR for detecting multiple tumour marker mRNA transcripts in the blood is possible. One study looked at patient blood samples with more than 100 CTCs and extracted RNA to create a global gene expression profile for each patient; this was able to detect CTC and cancer-specific genes (208). One drawback is the detection of illegitimate transcripts present in blood cells, it also does not account for tumour heterogeneity. The search for sub-populations of tumour cells is also important, for example the recent interest in breast cancer stem cells

may hold important information of prognosis or even the dormancy mechanisms involved in breast cancer.

Methods for single cell detection and characterisation include laser microdissection, DNA fluorescent *in situ* hybridisation (DNA FISH), enzyme-linked immunosorbant assays (ELISA), immunofluorescence and IHC. Laser microdissection involves cutting out single cells from cytospins or tissue for further analysis of DNA, RNA or protein. This has shown promise for phenotyping CTCs with one study able to detect mRNA transcripts of tumour markers such as *CK19* and *HER2* using qRT-PCR from advanced breast cancer patients (209). This method is technically challenging and time consuming though and requires effective enrichment prior to analysis along with very sensitive and specific qRT-PCR assays to pick up rare mRNA transcripts. An increasing number of studies have been published that have performed FISH analysis on single CTCs for tumour marker amplification, such as the androgen receptor in prostate cancer and *HER2* in breast cancer (178, 192, 210, 211). Although FISH provides useful information, the method is only able to study limited numbers of markers at a time.

For breast cancer patients with metastatic disease, the phenotyping of CTCs is important to enable personalised therapy to minimise toxicity and to maximise efficacy of treatments. A system to detect and phenotype CTCs more accurately would therefore be of benefit to both early and advanced stage breast cancer patients. In addition, the ability to phenotype several markers at the same time is imperative. One area of particular interest is the study of EGFR as a tumour marker. The overexpression of EGFR is not correlated to patient response to anti-EGFR therapies (153), which makes the selection of patients for treatment very difficult. The measurement of mRNA transcripts of EGFR in CTCs could overcome this problem and is discussed in more detail in Chapter 6.

The use of single cell expression profiling using RNA *in situ* hybridisation has been studied previously (212, 213) but not in CTCs. Here, we describe the development of a new method to detect CTCs, incorporating a panel of epithelial and tumour markers with a novel system to detect mRNA transcripts in single cells *in situ*. This method is called 'CTCscope' and uses a system to amplify signals from target mRNA transcripts; it is able to intensify signals while

suppressing background noise simultaneously. Up to 20 oligonucleotides complimentary to each target mRNA are used with further oligonucleotides attached to create a 'scaffold' for further amplifier molecules and the attachment of fluorophores for visualisation (Figure 5.1). This method therefore aims to detect CTCs both sensitively and specifically with a high signal to background ratio. This technology is based on an assay called QuantiGene, created by Panomics (an Affymetrix, Inc. company). A spin-off from this company called Advanced Cell Diagnostics (ACD), Inc (CA, US) developed this technology and in collaboration with our department at Imperial College, optimised the CTC preparation for this method.

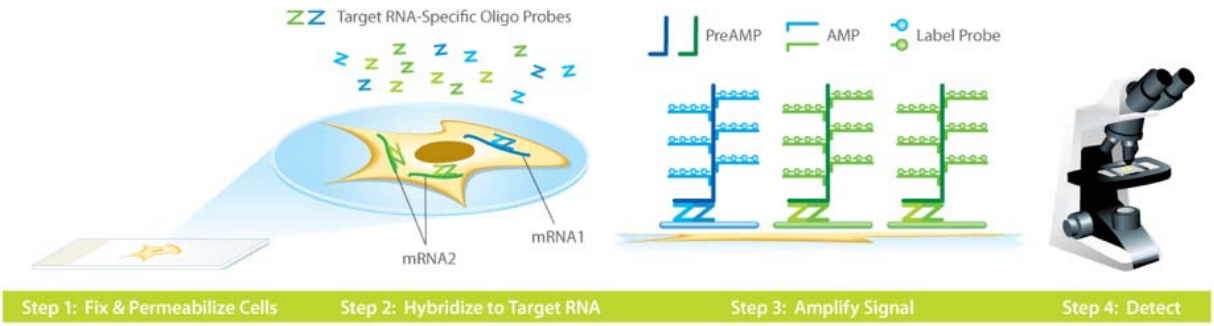


Figure 5.1: Overview of the CTCscope method

5.2 Aims and Objectives

- To optimise different cell recovery and fixation methods from the blood to recover the most CTCs possible whilst ensuring compatibility with the CTCscope assay
- To test the method on spiked-in breast cancer cell lines in healthy blood
- To compare the optimised CTCscope method to the CellSearch system by testing paired blood samples for CTCs from advanced breast cancer patients
- To correlate the number of CTCs detected by CTCscope and the CellSearch system to prognostic indicators such as clinical response and the CA 15-3 tumour marker

5.3 Optimisation of the enrichment of CTCs from blood samples

Unlike antibody-based assays, CTCscope has no established cell preparation and staining protocol for analysing patient blood samples, Therefore, It was first necessary to find the best method for separating and preparing the CTCs from blood prior to the CTCscope assay. The two major steps required before the assay is first of all, the blood cell enrichment and secondly, the preservation of RNA inside the enriched blood cell/CTC samples. This was achieved by spiking breast cancer cells (MDA-MB-468 cell line) into blood samples from healthy volunteers in triplicate and trying different enrichment and fixation protocols. The aim was to find a method to separate the blood cell constituents from the rare CTCs whilst minimising CTC loss in combination with a fixation method to give maximum retention of RNA in individual cells. Protocols and methods are summarised in Table 5.1. The most common method was tested first; the Ficoll gradient, centrifugation method. A special proprietary tube called a CPT™ cell preparation tube (Becton, Dickinson, NJ, USA) was used which contained Ficoll and a polyester gel to help separate the blood components. This system provided a tube to take the blood in, combined with a standardised method to separate the PBMC fraction from the red blood cells. The separated cells were cytospun using Shandon EZ megafunnels (Thermo Scientific). The recovery rates using this method was between 0 – 44%. Further optimisation was required to recover more cells so the next step was to use the CellSearch system. The CellSearch profile kit was used as it allows further molecular characterisation by enriching the cells using EpCAM, without permeabilising and staining them for DAPI, Pan-CK or CD45. The recovery rates for this method ranged from 0 – 50%.

The Accuspin™ system-histopaque®-1077 method (Sigma-Aldrich) was another method using Ficoll in a tube to separate the blood and was tested to find out if recovery rates could improve further. This method was also an economical alternative to the previous methods tested. This was indeed found to recover more cells from the blood with rates of 35 – 53%. The Shandon EZ megafunnels cytospin step was also removed at this stage to prevent further loss of cells. Instead, cells were resuspended in 70% ethanol (700 µl 80% ethanol with 100 µl cell suspension) for transportation to ACD. The next protocol used a CD45 depletion antibody

(RossetteSep, StemCell Technologies, Grenoble, France), used to deplete the leukocytes from the CTC fraction to make it easier to stain and visualise the cancer cells. This was added to the Sigma-Aldrich Ficoll tubes before centrifugation and despite no longer doing a cytospin step the recovery rates fell to 24 – 50 %. This step was then removed due to the loss of cells. Keeping the same protocol, a final optimisation step used a wash buffer (0.4% BSA + 1 mM EDTA in 1 X PBS buffer) to prevent cell clumping (full protocol in materials and methods, Chapter 2). This method was the optimum technique for CTC enrichment with recovery rates of 65 – 73% (Table 5.1).

The probes used to detect CTCs were *CK8, 18 and 19* in this optimisation process. In addition, the optimisation of the processing times was also undertaken (not shown). To avoid the degradation of mRNA, processing of blood samples was performed as soon as possible after blood collection.

Table 5.1: Optimisation of protocols for the most efficient recovery of spiked cancer cells from healthy blood. Recovery rates of spiked MDA-MB-468 cells are shown using different protocols to enrich and prepare the cells from blood.

Protocols				Recovery rates of spiked cells using CTCscope
Protocol	**Enrichment method**	**Cell preparation method**	**Depletion antibody or other variables**	
1	CPT ficoll tube (Beckton, Dickinson)	0.25% formaldehyde fixation, Shandon cytospin	No depletion antibody	0 – 44%
2	CellSearch system profile kit (Veridex LLC)	0.25% formaldehyde fixation, Shandon cytospin	No depletion antibody	0 – 50%
3	Accuspin™ system-histopaque®-1077 ficoll tube (Sigma-Aldrich)	0.25% formaldehyde fixation, Shandon cytospin	No depletion antibody	35 – 53%
4	Accuspin™ system-histopaque®-1077 ficoll tube (Sigma-Aldrich)	10% formaldehyde fixation, no cytospin, shipped in 80% ethanol	CD45 depletion antibody (Stemcell technologies)	24 – 50%
5	Accuspin™ system-histopaque®-1077 ficoll tube (Sigma-Aldrich)	10% formaldehyde fixation, no cytospin, shipped in 70% ethanol	No depletion antibody. Washes with EDTA/BSA/PBS to stop cell clumping	65 – 73%

5.4 Comparison of recovery rates using the CellSearch system versus CTCscope

The number of CTCs detected in paired 7.5 ml blood samples from 20 metastatic breast cancer patients was compared using CellSearch with CTCscope (using Protocol 1, Table 5.1). We tested paired samples from the early stages of the development of the method and found that CTCscope detected CTCs in the half the number of patients than the CellSearch system (Table 5.2).

Table 5.2: CellSearch recovered twice as many CTCs as early-stage CTCscope using the early cell preparation and CTCscope method

	No. of patients positive for CTCs[b] (n = 20)	**% of patients with CTCs**
Early stage CTCscope[a]	7	35%
CellSearch	14	70%

[a] This method used the CPT Ficoll tube (Beckton Dickenson) to enrich the cells, 0.25% formaldehyde fixation and the Shandon cytospin
[b] Positive for CTCs when 1 or more CTCs present in 7.5 ml blood

5.5 Summary of the optimisation process for CTCscope by Advanced Cell Diagnostics, Inc

Optimisations of the CTCscope methodology were performed by Advanced Cell Diagnostics, Inc (CA, USA). The fixation process was optimised to use a 10% formaldehyde/PBS solution to retain RNA quality. The number of markers for CTC detection was increased from using pan-cytokeratin 8, 18 and 19 to using an array of markers including; *cytokeratins 8, 14, 17, 18, 19* and *20*, *EpCAM*, *MUC-1*, *Twist*, *N-Cadherin* and finally *fibronectin* (probe sequences in Appendix Tables 8.1 - 8.4). All these markers were independently tested by staining spiked-in cancer cell lines (MDA-MB-468 and MRC-5) in blood and were found to be specific to the cancer cell line and not visualised in peripheral blood mononucleocytes (Figure 5.2 and 5.3). A negative selection marker was also developed to ensure that CTCs were not leukocytes. All samples were

therefore also stained for *CD45* (a leukocyte marker) using a panel of probes. The method is very specific for detecting CTCs in amongst many leukocytes in the blood (Figure 5.4).

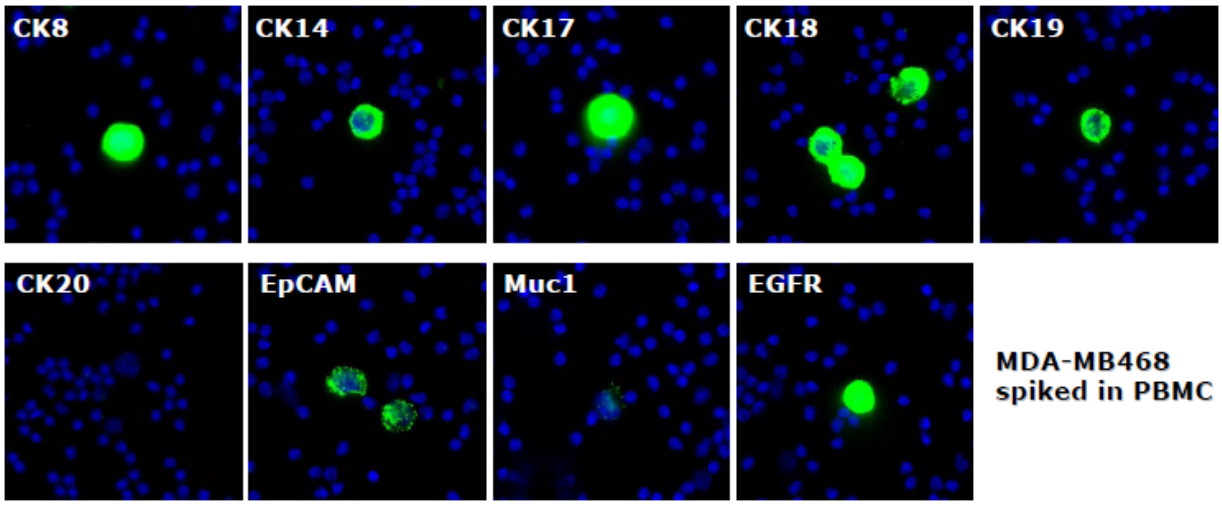


Figure 5.2: Visualisation of mRNA transcripts of epithelial markers using CTCscope show high specificity in a spiked breast cancer cell line in the blood

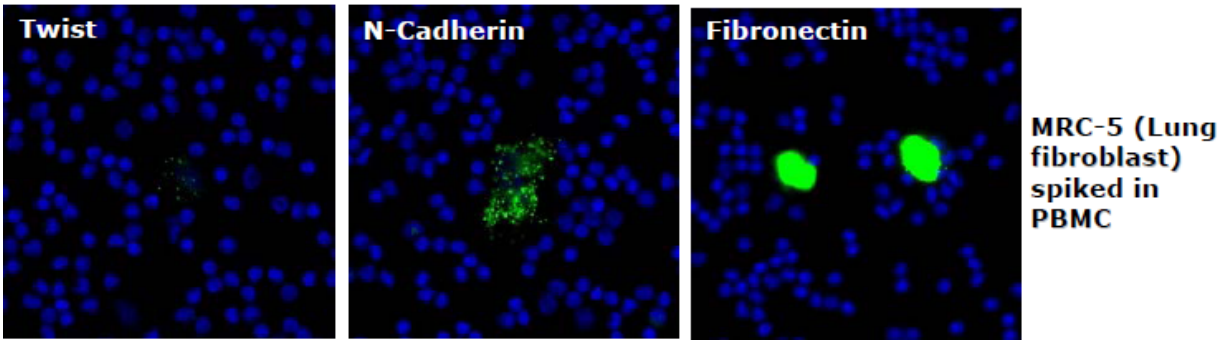


Figure 5.3: Visualisation of mRNA transcripts of EMT markers using CTCscope show high specificity in a spiked lung fibroblast cell line in blood

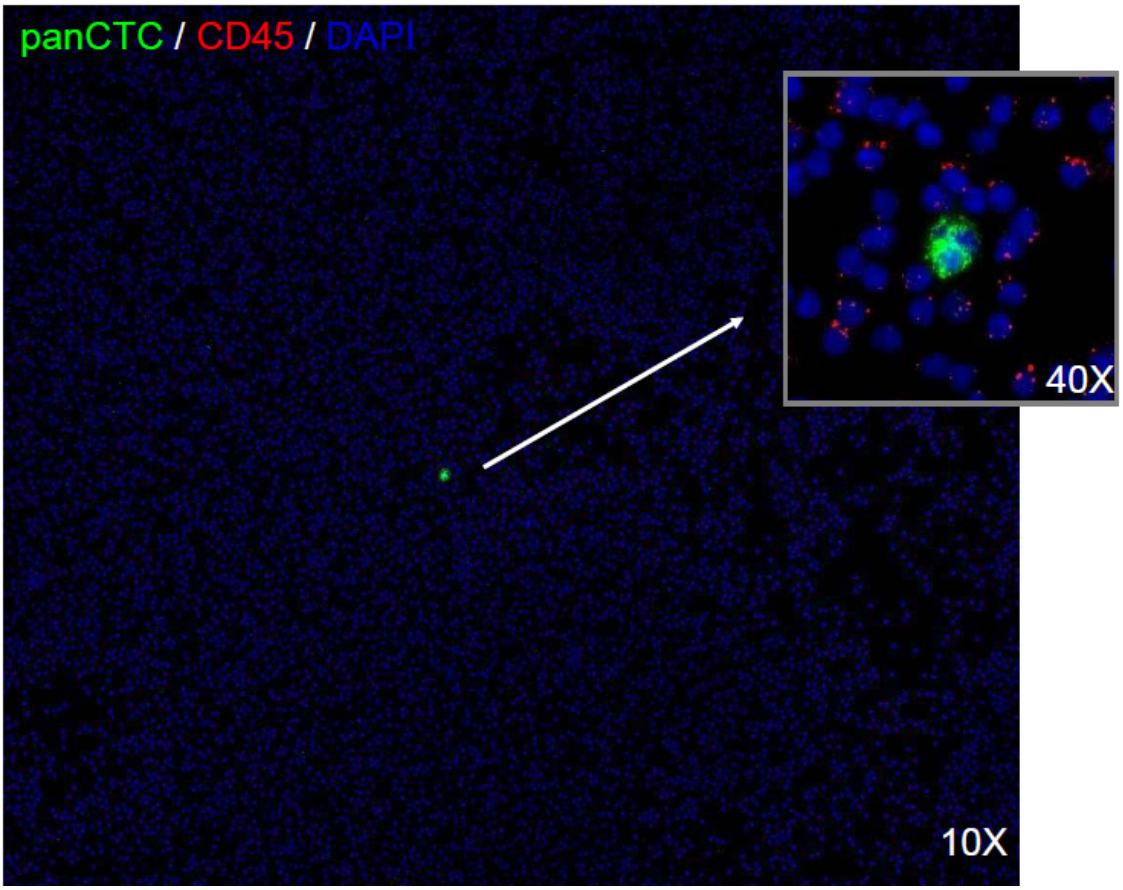


Figure 5.4: CTCscope is very specific for detecting rare CTCs amongst PBMCs in a patient with metastatic breast cancer. The visualisation of one CTC using pan-CTC mRNA probes (green) is shown. PBMCs are visualised with CD45 mRNA probes (red), Cell nuclei are detected by DAPI (blue).

5.6 Optimised CTCscope method tested using cell recovery experiments

The optimised CTCscope method was tested by spiking a breast cancer cell line (MDA-MB-468) at different concentrations into blood from healthy donors, to determine the recovery rate. The samples were prepared using the new protocol (Protocol 5, Table 5.1). F. Wang and N. Su performed these experiments at Advanced Cell Diagnostics, Inc (CA, USA). Two spiked samples were performed at Imperial College to test the new protocol at a different site. MDA-MB-468 cells were used and 48 and 49 cells were spiked in two blood samples, the recovery rates were 35 (73%) and 32 (65%) respectively. This was within the range found at ACD (65 - 80%) with a mean of 71% of cells recovered, (Figure 5.5).

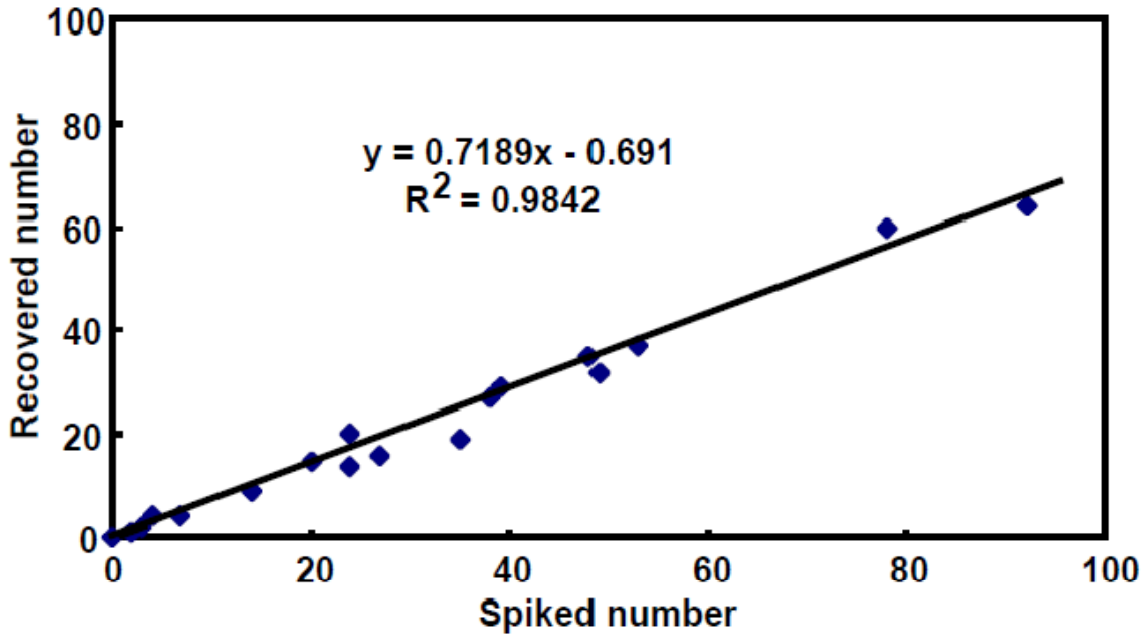


Figure 5.5: The mean recovery rate of spiked tumour cells (MDA-MB-468) in 7.5 ml blood using the optimised CTCscope method is 71%

5.7 Measurement of CTCs in healthy controls and in advanced breast cancer patients using CTCscope

No CTCs were identified in 13 healthy individuals. Once the cell enrichment and preparation had been optimised with a recovery rate of 71%, we compared CTC detection using both CTCscope and the CellSearch system in 45 patients with metastatic breast cancer. Patients had paired 7.5 ml blood samples taken to detect CTCs by both methods. It was hoped CTCscope would detect more cells due to the detection of many tumour markers bundled together. The number of patients positive for CTCs (one or more in 7.5 ml of blood) was similar for both methods with CTCs detected in 47% of patients using CTCscope and 51% of patients using the CellSearch system (Table 5.3).

Table 5.3: The optimised CTCscope method detects CTCs in metastatic breast cancer patients in comparable numbers to the CellSearch system

	No. of patients positive for CTCs[b] (n = 45)	Patients with CTCs (%)
Optimised CTCscope[a]	21	47%
CellSearch	23	51%

[a] This method used the Accuspin™ system-histopaque®-1077 ficoll tube (Sigma-Aldrich) to enrich the cells followed by a 10% formaldehyde fixation
[b] Positive for CTCs when 1 or more CTCs present in 7.5 ml blood

5.8 CellSearch and CTCscope show concordance for CTC detection

The concordance of the two methods was high with 31 out of 45 patients (69%) demonstrating a positive or negative result for CTC detection by both methods (p = 0.017 Fishers exact test). Eight patients (18%) had CTCs detected by the CellSearch and not by CTCscope. Similarly, six patients (13%) had CTCs detected by CTCscope and not by the CellSearch system (Table 5.4).

Table 5.4: CTCscope and the CellSearch system show concordance for the detection of CTCs in 45 patients with metastatic breast cancer

		CellSearch system		
	No. patients [b]	Positive	Negative	P value
CTCscope[a]	Positive	15	6	0.017*
	Negative	8	16	

[a] This method used the Accuspin™ system-histopaque®-1077 ficoll tube (Sigma-Aldrich) to enrich the cells followed by a 10% formaldehyde fixation
[b] Positive for CTCs when 1 or more CTCs present in 7.5 ml blood
*Statistically significant (Fishers exact test)

5.9 The CellSearch system detects more CTCs in patients with metastatic breast cancer than CTCscope

The number of CTCs detected by the CellSearch system (mean of 19.53, 95% CI 6.33 – 32.73) was greater than the optimised CTCscope assay (mean of 1.56, 95% CI 0.4 – 2.71). The median detection values were much lower with 1 CTC detected by the CellSearch system and 0 CTCs by CTCscope (Figure 5.6 and Table 5.5).

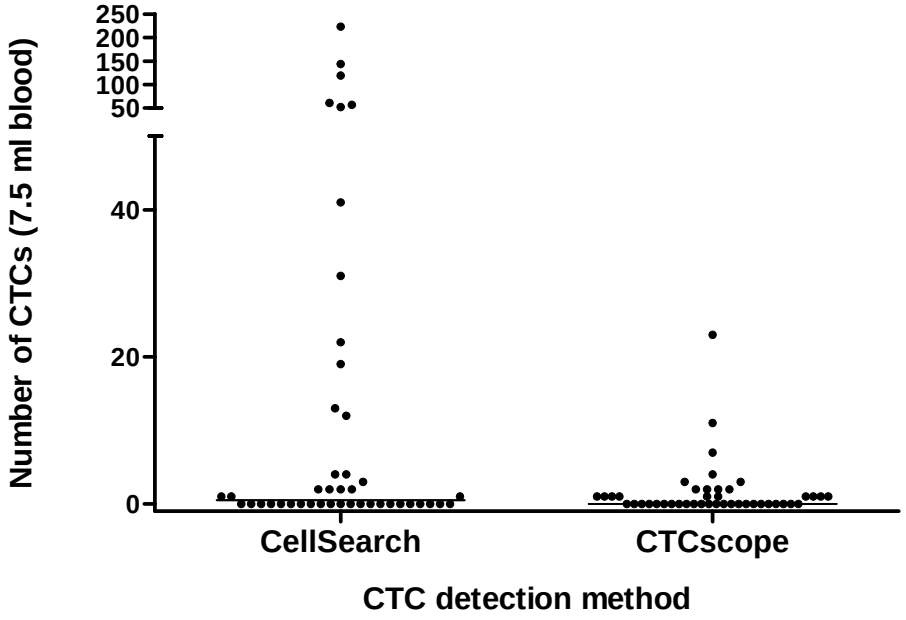


Figure 5.6: The CellSearch system detects more CTCs than CTCscope in paired blood samples from 45 patients with metastatic breast cancer. The median value is depicted by a line (1 for CellSearch and 0 for CTCscope).

Table 5.5: The CellSearch system detects more CTCs than CTCscope in paired blood samples from patients with metastatic breast cancer

		CTCscope[b]	CellSearch system
CTCs[a]	Number of samples	45	45
	Mean	1.56	19.53
	95% CI	0.4 – 2.71	6.33 – 32.73
	Median	0	1
	Range	0 - 23	0 - 223

[a] Positive for CTCs when 1 or more CTCs present in 7.5 ml blood
[b] This method used the Accuspin™ system-histopaque®-1077 ficoll tube (Sigma-Aldrich) to enrich the cells followed by a 10% formaldehyde fixation

5.10 Correlation of CTC detection to clinical response in patients with metastatic breast cancer

Next, the clinical response to therapy for each patient was compared to the number of CTCs detected by both CTCscope and the CellSearch system. Progression-free survival and overall survival data was not available due to the short time period since this study was performed. The clinical response to therapy at the time of each CTC sample was therefore studied in 45 patients with metastatic breast cancer. The overall clinical response was measured by RECIST criteria from CT imaging (168). Patients were grouped by progressive disease or non–progressive disease (this included stable or partially responding disease), (Table 5.6 and Figure 5.7). A greater number of CTCs was detected by the CellSearch system than by CTCscope in patients with progressing metastatic breast cancer (median of 12 versus 1 CTC respectively). A similar number of CTCs was detected by both methods in patients with non - progressing metastatic disease (median of 0 by both methods and a mean of 1.3 versus 0.95 CTCs). CTCs detected by the CellSearch system better reflected the clinical response of each patient at the time of the sample with 78% of patients with progressive disease having CTCs present compared to 23% in patients with non–progressive disease. CTCscope detected CTCs in 61% and 32% of patients with progressing and non–progressing disease respectively (Table 5.6).

Table 5.6: The number of CTCs detected by the CellSearch system corresponded more closely to clinical response than CTCs detected by CTCscope

		CTCscope	CellSearch system
CTCs in patients with progressive disease (patients = 23)	Number of patients with CTCs	14 (61%)	18 (78%)
	Mean no. CTCs	2.13	36.96
	95% CI	0.04 – 4.22	12.53 – 61.39
	Median	1	12
	Range	0 - 23	0 - 223
CTCs in patients with non – progressing disease[a] (patients = 22)	Number of patients with CTCs	7 (32%)	5 (23%)
	Mean no. CTCs	0.95	1.31
	95% CI	-0.49 – 2.40	-0.75 – 3.39
	Median	0	0
	Range	0 - 11	0 - 22
P value		P=0.088	p<0.0001*

[a]This included patients with stable disease or patients with a partial response
*Statistically significant difference (Mann-Whitney test)

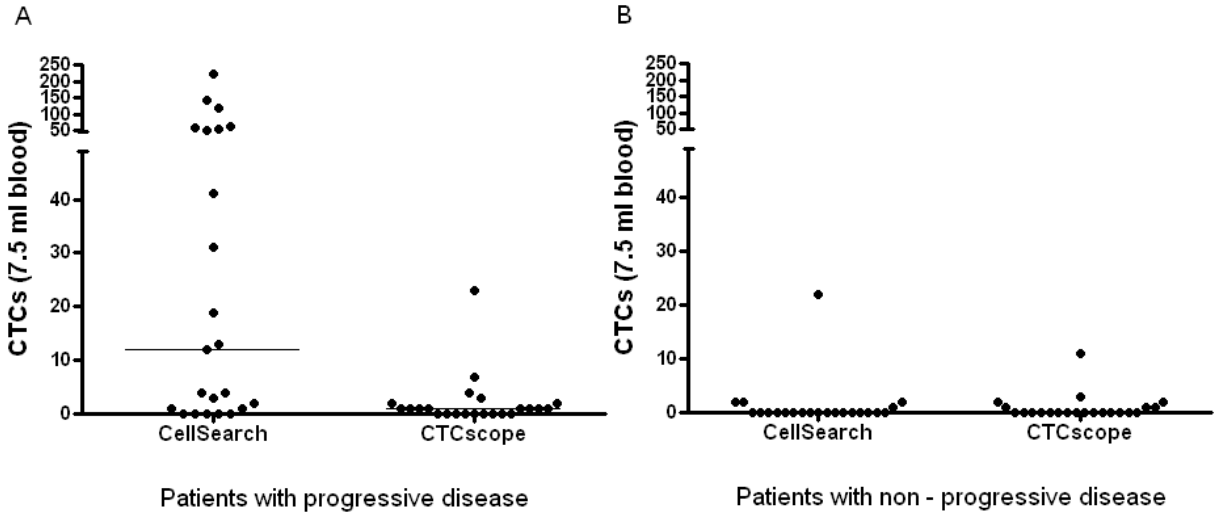


Figure 5.7: The CellSearch system detects more CTCs in patients with clinically progressing metastatic breast cancer than CTCscope (A). Few CTCs are detected by either method in patients with non-progressive disease, (B). The mean value of each group is depicted by a line. Disease was assessed by CT scan.

5.11 CTC detection by the CellSearch system but not by CTCscope is correlated with CA 15-3 tumour marker levels at the time of blood sampling

Next we compared the CA 15-3 tumour marker value from blood samples taken at the same time as the samples for CTC detection. This marker is often used in conjunction with imaging to assess the response to therapy. The CA 15-3 values did not correspond to CTC detection by CTCscope (p=0.091, Spearman's rank) although there was an observable correlation with CTCs (p=0.0003, Spearman's rank) detected by CellSearch (Figure 5.8).

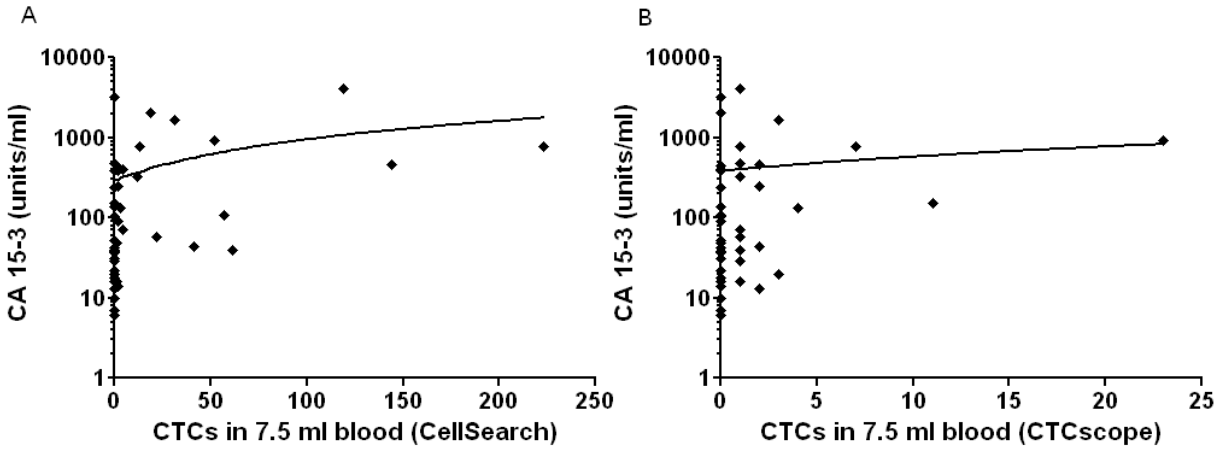


Figure 5.8: CTC detection by the CellSearch system showed a marginal correlation to CA 15-3 tumour marker values (A), whereas CTC detection by CTCscope did not (B). CTCs were measured in paired blood samples from 45 patients with metastatic breast cancer.

5.12 Discussion

This study describes the optimisation of CTCscope for enriching and detecting CTCs from the blood of breast cancer patients. Although this data is preliminary, it shows the promise that this method may hold in the future. The data shown here also highlights the difficulties faced in the sensitive and specific detection of CTCs from the blood. Our findings show the best gradient centrifugation enrichment method we tested was a commercially available tube containing Ficoll (Accuspin™ system-histopaque®-1077 ficoll tube, Sigma-Aldrich). The recovery rate was 71% and provided a simple, standardised and relatively inexpensive way of enriching CTCs.

This system was compared to the CellSearch system for detecting CTCs in paired blood samples from advanced breast cancer patients. Our results showed CTCscope detected CTCs in 47% of patients compared to 51% of patient with CTCs by the CellSearch system. The concordance was high with 31 out of 45 (69%) patients with the two methods both positive or negative for CTCs. This confirmed the efficacy of CTCscope for detecting CTCs in breast cancer patients, however the CellSearch system detected many more CTCs than CTCscope with a mean of 19.53 compared to 1.56 and a median of 1 compared to 0 respectively. The spiking experiments showing 71% recovery rates however, and should therefore theoretically detect similar numbers of CTCs as the CellSearch system. This discrepancy may be due to several reasons; firstly, the mRNA transcript levels of the pan-CTC markers may not be as high in patient CTCs as in spiked breast cancer cell line cells. Secondly, the processing method may be damaging the patient CTCs, whereas breast cancer cell lines may be more robust and homogeneous. Thirdly, mRNA does not always correspond to protein expression (214). Lastly, a proportion of CTCs in breast cancer patients may be dead, dying or in a quiescent or dormant state, because transcription is low or not occurring. Evidence for this includes heterogeneity of the proliferation marker Ki-67 in CTCs, and also the finding of a short half-life of CTCs from prostate cancer patients suggesting a dying phenotype in the blood (192). The presence of an apoptotic marker has also been found in CTCs suggesting a dying phenotype and may explain the discrepancy between the number of CTCs detected by CTCscope and by the CellSearch system (130).

The clinical response to therapy (according to RECIST criteria) at the time of CTC testing was correlated with the presence and number of CTCs detected by the CellSearch and CTCscope. Patients either had progressing disease or non-progressing disease. The number of CTCs detected by the CellSearch system was significantly higher in the progressive disease group of patients than the non-progressing group ($p<0.0001$). The number of CTCs detected by CTCscope, on the other hand was not significantly different between the groups ($p=0.088$). The CellSearch system therefore predicts response better than CTCscope. In addition the levels of CA 15-3 were also measured at the same time as CTC detection, the results showed that CTCs detected by the CellSearch system positively correlated with the CA 15-3 marker but CTCs detected by CTCscope did not.

Results shown here reveal that the CellSearch system is more effective at detecting CTCs relevant to prognosis and response to therapy than CTCscope. This may be due to several reasons; firstly, the enrichment of CTCs for the CTCscope assay has the potential for improvement so that a greater number of CTCs are recovered than 71%. Secondly, as mentioned, CTCs in patient blood may be more fragile than breast cancer cell lines, this may make enrichment, fixation and detection more difficult due to cell damage and RNA degradation. Thirdly, the detection of CTCs that are heterogeneous in terms of viability may all be prognostically relevant since viable or not, they are still being shed from micrometastases somewhere in the body and represent part of a tumour mass. Since CTCs only live for a matter of hours it is not likely CTCscope will detect all of them at a given time. The concordance of the two methods was high at 69% with a small proportion of patients having CTCs detected exclusively by one or the other method. Six patients had CTC detected by CTCscope and not by the CellSearch system. This may provide evidence of increased sensitivity and specificity in some patients, the same is also true for the CellSearch system however.

The use of CTCscope needs to be confirmed by studies with longer-term survival data to fully realise its potential as a prognostic tool. The detection of cells by this method may identify those with a more aggressive phenotype with cellular functions that are intact and able to withstand the circulatory environment to colonise other sites. Furthermore, although the

CellSearch system detects prognostically relevant CTCs, it may also be detecting those CTCs that have been successfully targeted by therapies undergoing apoptotic cell death. CTCs detected by CTCscope on the other hand may represent CTCs that are viable and resistant to therapies. Both viable and non-viable cells are important, however CTCs with active transcription may be aggressive so survival analyses in the future will help in this regard.

Further optimisation of the enrichment of CTCs for this method, for example using a more effective and gentle approach to prevent damage to CTCs, may mean recovery of greater numbers of intact CTCs. In addition the search for relevant subpopulations of CTCs using additional markers may be of great importance, for example by improving detection of cancer stem cells. This method therefore holds promise in the future for the reliable detection of morphologically intact CTCs with the ability to bundle many tumour markers together for more specific and sensitive detection. This also allows visualisation of additional specific tumour markers of interest such as EGFR using different fluorophores as shown in Chapter 6.

6.0 The selection of breast cancer patients for lapatinib monotherapy - characterising the epidermal growth factor receptor in CTCs

6.1 Introduction

The accurate identification and measurement of molecular targets for therapy is becoming increasingly important so that eligible patients are given the correct therapy and those not eligible are spared toxic effects or ineffective treatment. The use of CTCs to measure these targets will give a real-time assessment to tailor therapies to attempt to maximise response. The measurement of these targets needs to be robust and standardised to be used in the clinic. There are limited numbers of clinically useful markers that have emerged in part due to the inconsistent data using differing methodologies. A sensitive and specific method for detecting CTCs with detectable EGFR expression is clearly required due to the increasing number of anti-EGFR therapies.

A clinical trial utilising the CellSearch system was set up to select patients with advanced breast cancer with EGFR-expressing CTCs for lapatinib monotherapy. Lapatinib is a small molecule, dual tyrosine kinase inhibitor of HER2 and EGFR (Commercial name, Tyverb by GlaxoSmithKline). A unique cohort of advanced breast cancer patients was selected that have not been studied before: those who were negative for HER2 amplification in their primary tumour with EGFR expressing CTCs. The premise being that a wider population of patients could be treated with lapatinib, not solely those with HER2 amplification known to respond to lapatinib (38, 40). Although EGFR is part of a critical pathway in cancer, trials of anti-EGFR therapies have not always used EGFR expression as the basis for patient enrolment (33, 34). This may be due to many reasons including the lack of consensus and differing methods used to detect it or due to the low expression levels required for function (31). The EGF pathway can also be triggered beyond the status of the target by multiple mechanisms, for example by increased ligand expression, mutations in activated intracellular kinases or other pathway members like KRAS, although this has not been confirmed in breast cancer. Additional

mechanisms include epigenetic or miRNA regulation (215-217). The activation status of EGFR has also shown to be important, with the presence of phosphorylated EGFR by IHC staining of tumours associated with a poor prognosis in non-small-cell lung cancer as well as in breast cancer (218, 219).

The most effective method for measuring response to lapatinib by EGFR detection in CTCs was investigated. This examined the measurement of total EGFR protein staining, and phosphorylated-EGFR using the CellSearch system. Any tumour marker antibody that is pre-conjugated to FITC can be added to the system to phenotype CTCs, in addition to the standard detection markers. The detection of EGFR mRNA transcripts in CTCs using CTCscope was also examined in CTCs as an alternative method for EGFR characterisation. Since the CellSearch system is standardised, it was used to identify patients, it was crucial to investigate how many metastatic breast cancer patients had raised EGFR expression levels in CTCs and to see how it varied over time. It was also important to see if the measurements were reproducible using the CellSearch system and to prove their specificity to EGFR. The levels of EGFR in breast cancer CTCs had also never been quantified over time before using this system. This small, focused study was published in 2009 (Appendix).

Lapatinib is approved for use in combination with therapies for improved clinical response. One example is the combination with the chemotherapy drug capecitabine (Xeloda, Roche), used for patients with advanced HER2 positive disease who have received previous therapies, and for use with letrozole in post-menopausal patients with ER and HER2 positive disease (39, 220). It is also known to have efficacy in trastuzumab refractory disease and those patients with a truncated form of HER2 (p95HER2) (221, 222). Studies have shown that HER2 is the main predictive factor with the presence of EGFR showing either an additive, predictive role in breast cancer cell lines or none at all depending on the methods used for measurement (43, 223). The correlation of the presence of EGFR (HER1) with clinical response to lapatinib is less well known and is the focus of study here.

The measurement of EGFR in the primary tumour does not always predict the efficacy of anti-EGFR therapies. The use of CTCs used as a ‘real time’ biopsy could prove to be more useful

since it is known that the phenotype of a tumour is known to change over time (190). This is also very important in the metastatic setting since progressive disease becomes increasingly aggressive and resistant to therapies, and so individualised therapy is even more important to ensure the least toxic and most effective therapy to target metastatic lesions. The detection and characterisation of CTCs may provide not only information on the best possible treatment option but also may help to gain further understanding of the processes of metastasis. This study is a proof of principle study designed to select advanced breast cancer patients with HER2 non-amplified primary tumours for anti-EGFR lapatinib monotherapy based on the presence of EGFR positive CTCs in their blood.

6.2 Aim and Objectives

- To test the use of the CellSearch system for measuring EGFR expression in CTCs by testing spiked-in breast cancer cell lines with known EGFR expression levels
- To identify cell lines with different levels of protein expression of EGFR and HER2 to study the effects of Lapatinib in cells with high expression of EGFR and low HER2 expression
- To identify a marker of pEGFR conjugated to FITC that shows decreased pEGFR status upon Lapatinib treatment and to test this pEGFR marker in the CellSearch system to phenotype patient CTCs
- To determine the use of CTCscope as a method to measure EGFR mRNA transcripts in CTCs as an alternative to EGFR protein measurement by the CellSearch system
- To determine the use of RNAscope for detecting EGFR in retrospective FFPE primary tumour sections from advanced breast cancer patients
- To correlate EGFR mRNA in the primary tumour with CTCs detected in advanced breast cancer patients to determine concordance
- To determine the number of patients with advanced breast cancer with detectable EGFR positive CTCs
- To determine the patterns of EGFR positivity in CTCs over time in these patients
- To identify advanced breast cancer patients for lapatinib monotherapy by testing for EGFR positive CTCs using the CellSearch system
- To measure CTCs and EGFR expression in CTCs before and after lapatinib monotherapy in advanced breast cancer patients
- To correlate CTC measurements with clinical response to lapatinib and assess the use of EGFR as a predictive marker
- To compare the use of the CellSearch system with CTCscope for measuring CTCs and EGFR positive CTCs before and after lapatinib monotherapy in advanced breast cancer patients

6.3 Patients and samples

Forty-three patients with advanced breast cancer who met the inclusion criteria for the lapatinib monotherapy clinical trial were screened for EGFR positive CTCs. Briefly, these patients all had a histologically confirmed HER2 negative primary, infiltrating tumour (i.e. no gene amplification by FISH or IHC 2+ and no amplification by FISH or IHC 0/1+), at least one measureable metastatic lesion measured by the RECIST (response evaluation criteria for solid tumours) guidelines (168), and at least 12 weeks life expectancy. All patients gave written informed consent approved by a local ethics committee. Sixteen patients with the presence of EGFR positive CTCs in 7.5 ml blood were then recruited onto the study, 12 are reported here due to RECIST reporting not yet completed for all patients. These patients were given oral lapatinib at the dose of 1500 mg a day (250 mg 6 times a day) for 28 days. Their CTC and EGFR measurements were performed at baseline and after each 28 day cycle of treatment. Due to study cost limitations as well as ethical considerations, only one 7.5 ml blood was tested for EGFR at each time point. HER2 testing was also only performed once, prior to lapatinib administration.

6.4 Study Design

This clinical trial was a multicenter, open-label, phase II study to evaluate the efficacy and safety of a daily dose of lapatinib in advanced breast cancer patients with HER2 non amplified primary tumours and positive HER2 or EGFR CTCs.

This study was part of a multi-centre trial with patients allocated into one of the following two strata:

- UK study group: Advanced breast cancer patients with HER2 non-amplified and EGFR positive CTCs
- Italian study group: Advanced breast cancer patients with HER2 non-amplified primary tumours and HER2 positive CTCs

Objectives

The primary objective of this study was to evaluate the efficacy of a daily dose of lapatinib in advanced breast cancer patients with HER2 non-amplified primary tumours and EGFR positive CTCs.

The translational research endpoints

- Evaluation of biological effects on EGFR in CTCs.
- Comparison between CTC and tumour samples from the primary and/or from the metastatic site in terms of biological marker expression profiles.
- Correlation between EGFR, both total and phosphorylated baseline levels evaluated on CTCs and response to lapatinib.

6.5 Results

There is currently no consensus for a standardised method to detect EGFR effectively in primary tumour biopsies or CTCs. Three different methods of EGFR detection and measurement were investigated to determine the best predictor of lapatinib response.

6.6 EGFR measurement on CTCs by the CellSearch system

The EGFR antibody used in conjunction with the CellSearch system is a fluorescein conjugated, mouse monoclonal (clone 225) antibody, that detects the extracellular domain of the receptor with proven reactivity and clinical significance. For example, the humanised, therapeutic monoclonal antibody cetuximab (Merck KGaA) is derived from this clone and is proven for use in patients with colorectal cancer (224). It was first necessary to test the efficacy of this EGFR antibody by using breast cancer cell lines known to have high EGFR expression (MDA-MB-468 cells) or very low/undetectable levels of EGFR expression (MCF-7), (43). This was confirmed by an immunoblot of EGFR protein in these cell lines (Figure 6.1B). One hundred cells from each cell line were spiked into healthy 7.5 ml blood samples each in duplicate and processed through

the CellSearch system using the EGFR reagent. The CK-PE staining was very bright in approximately 80% of all cells, with bleed-through of this marker to the EGFR-FITC and CD45-APC channels; however the EGFR staining was much more evident in the MDA-MB-468 cells than the MCF-7 cells. An example of cells with no CK-PE bleed-through is shown in Figure 6.1A. This demonstrated the efficacy of EGFR detection using the CellSearch system.

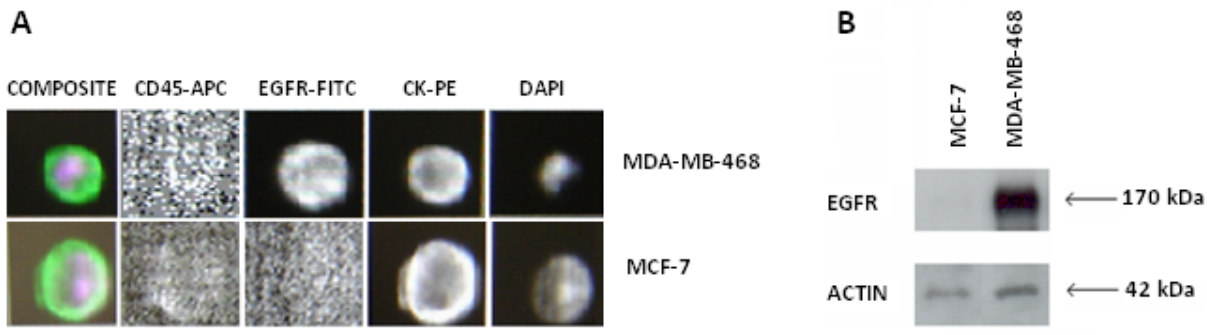


Figure 6.1: CellSearch gallery of an EGFR positive breast cancer cell line, MDA-MB-468 and an EGFR negative EGFR breast cancer cell line, MCF-7 (A). An Immunoblot to confirm high EGFR protein levels in MDA-MB-468 cells and undetectable levels in MCF-7 cells (B).Spiked samples put through the CellSearch system in duplicate. Immunoblot performed once.

6.7 EGFR and HER2 protein analysis in breast cancer cell lines

Since the measurement of EGFR protein in the primary tumour is not known to correlate with the response of anti-EGFR therapies, it was important to investigate other measurements that may predict response. Two possibilities were explored, the first was to look at the activation status of EGFR and the second (discussed later) was to explore the use of CTCscope to detect EGFR transcripts and to determine if lapatinib had any effect on EGFR at the transcription level.

To study the effects of lapatinib on EGFR signalling it was necessary to identify breast cancer cell lines that had differing expression levels of both EGFR and HER2. Cell lines high in EGFR expression and low in HER2 expression were required to test the effect of lapatinib on EGFR without the influence of high HER2 expression which is known to be the predominant indicator

of lapatinib response (43). The expression levels of EGFR and HER2 proteins in a number of breast cancer cell lines were determined by immunoblots. The MDA-MB-468 cell line showed high levels of EGFR and no apparent HER2 expression (as did MDA-MB-231 and ZR-75-1 cells) whereas MCF-7 cells showed low levels of both EGFR and HER2 expression. One cell line, BT-474, showed high levels of HER2 and EGFR whereas MDA-MD-453 cells showed high HER2 expression but no EGFR protein. Finally T-47D cells showed little expression of either protein (Figure 6.2). This identified cell lines for future experiments to explore markers of response to lapatinib.

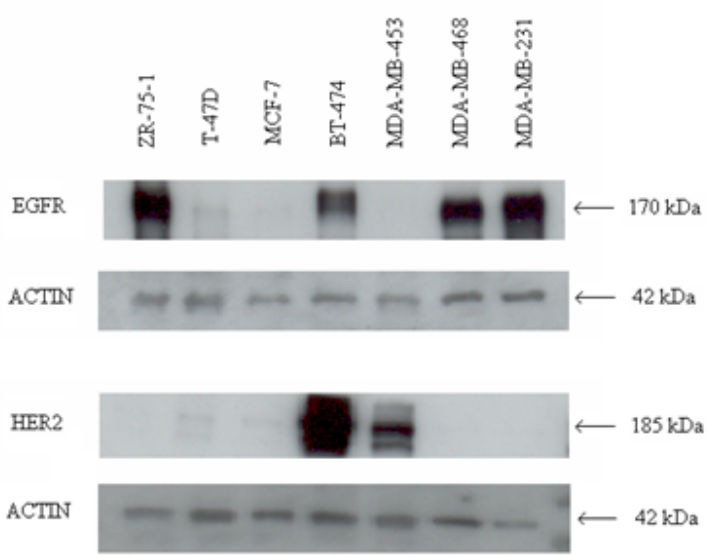


Figure 6.2: Expression of EGFR and HER2 protein expression in breast cancer cell lines. Immunoblots showing total EGFR (top panel) and total HER2 (lower panel). Beta-actin expression was used as a loading control. The molecular mass is indicated with an arrow on the right. Immunoblot performed once.

Two cell lines, MDA-MB-468 and MDA-MB-231, were selected due to the high levels of EGFR with little or no HER2 expression present on the immunoblot. These were used for treatment with different concentrations of lapatinib to assess markers of response in the EGFR signalling pathway without the influence of the HER2 receptor.

6.8 Investigation of phosphorylated EGFR as a marker of response to lapatinib

An antibody against a phosphorylated tyrosine kinase residue of EGFR was required to test the effect of lapatinib on activated EGFR. The tyrosine kinase phosphorylation site, 1068 (p1068) antibody was available conjugated to FITC (R&D systems, Minneapolis, USA) and was chosen because the p1068 site directly binds the Grb2, an adaptor protein involved in signal transduction (225). It has also been shown to be a marker of response to an anti-EGFR therapy, erlotinib, in pancreatic cancer (226). In addition, because it was pre-conjugated to FITC, it meant it could be used in the CellSearch system as an extra marker in the FITC channel.

The p1068-FITC antibody was tested first of all by treating MDA-MB-468 cells with and without EGF, the ligand for EGFR. Immunofluorescent staining shows brighter visualisation of the EGF treated cells confirming the efficacy of the antibody since EGFR was activated by its ligand (Figure 6.3).

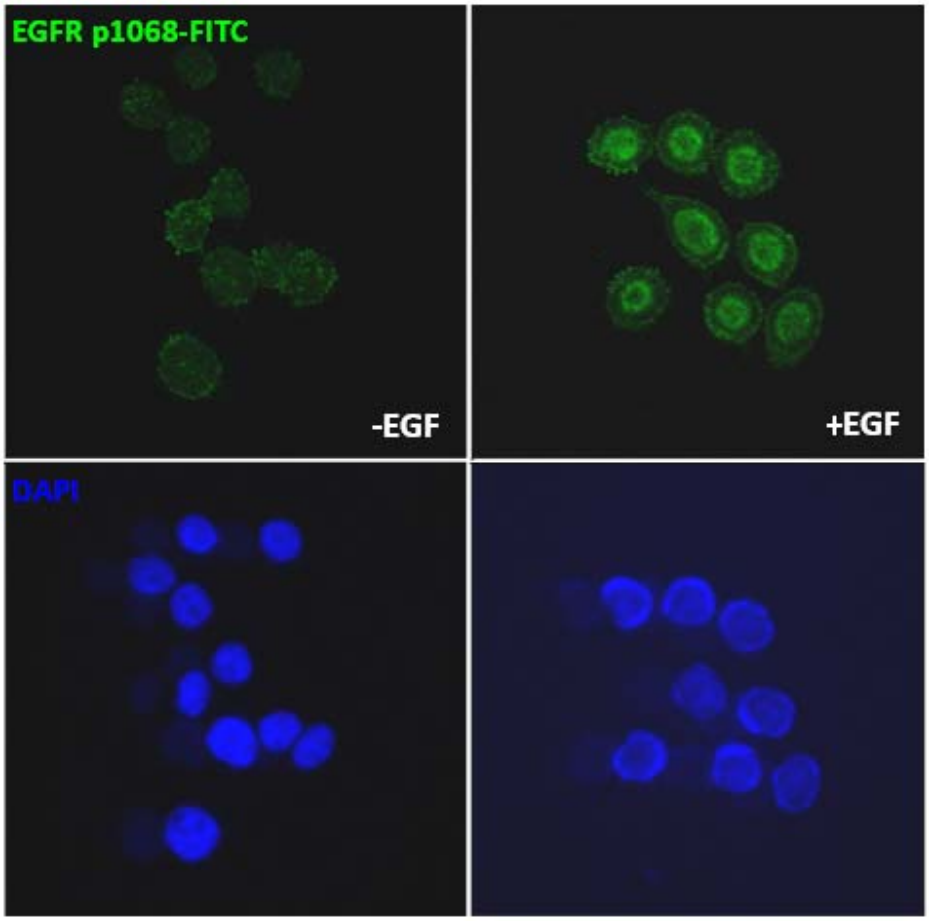


Figure 6.3: EGFR p1068 is visualised by immunofluorescence when cells are treated with 100 mM EGF for 10 minutes compared to cells with no EGF treatment. EGFR p1068-FITC is visualised in green and nuclear staining with DAPI is blue.

Next, the p1068-FITC EGFR antibody was tested on lapatinib treated cells to find out if there was an expected decrease in the activated EGFR at this phosphorylation site. The treatment concentration was based on the IC_{50} of lapatinib in MDA-MD-468 and MDA-MB-321 cells (43). This was tested previously at 2 µM and 7 µM respectively, so the cells were treated above and below the IC_{50} concentrations at 0, 1 and 10 µM. The breast cancer cell lines were treated with EGF for 10 minutes followed by lapatinib treatment for 6 hours. A western blot of the p1068 EGFR protein (Cell Signaling antibody) was performed in addition to the Immunofluorescent staining using the EGFR p1068-FITC conjugated antibody. Both provided evidence of a decrease of p1068 following lapatinib treatment (Figure 6.4 and 6.5).

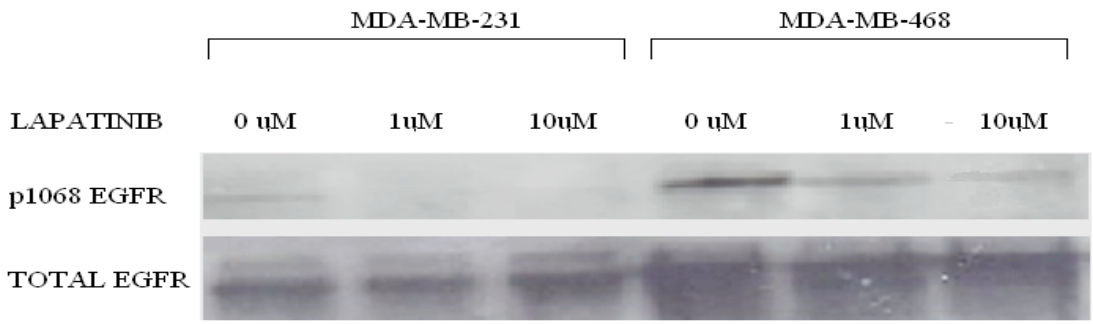


Figure 6.4: The p1068 EGFR tyrosine residue protein is decreased with lapatinib treatment in two breast cancer cell lines, MDA-MB-231 and MDA-MB-468. Cells were treated for 10 minutes with EGF followed by 6 hours of lapatinib treatment at different concentrations; 0, 1 and 10 µM. (Experiment performed once).

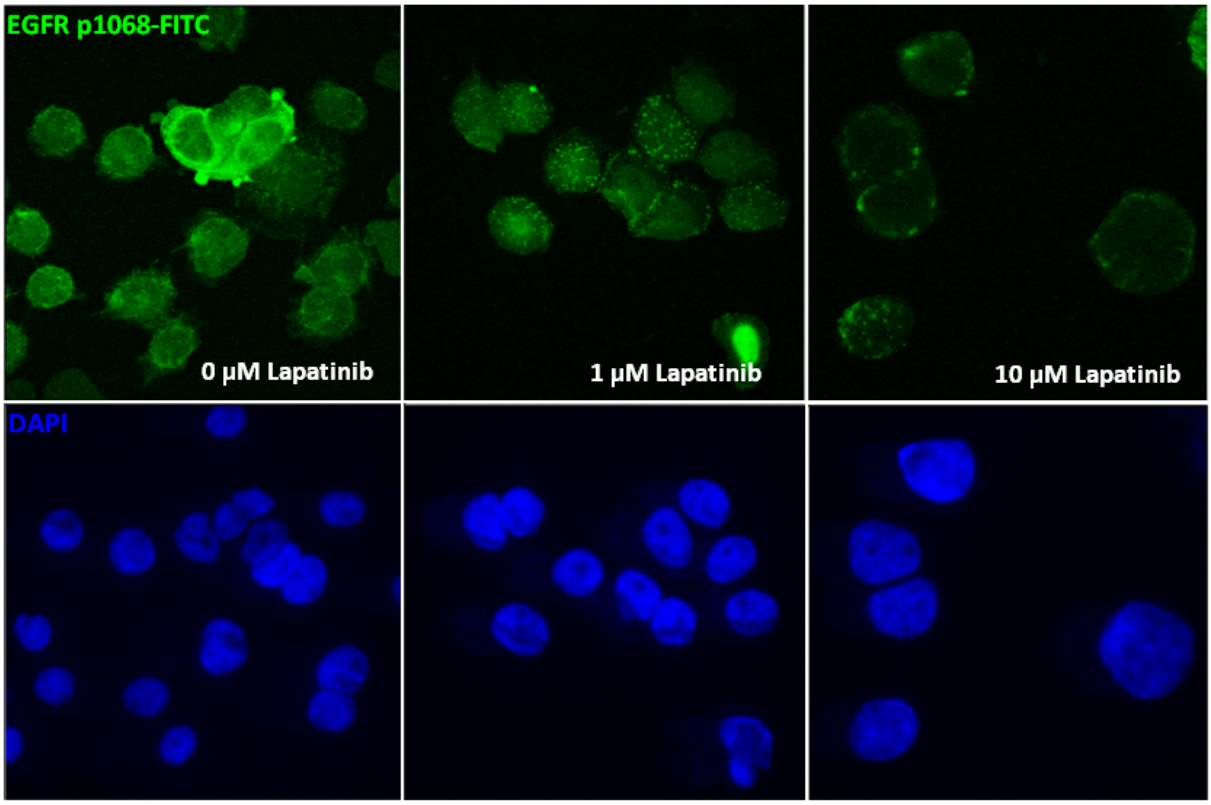


Figure 6.5: EGFR p1068 is inhibited with increasing treatment with lapatinib. Immunofluorescent staining of MDA-MB-468 cells, treated with EGF for 10 minutes followed by lapatinib treatment at different concentrations (0, 1 and 10 µM) for 6 hours. (Experiment performed once).

6.9 Measurement of EGFR p1068 in spiked breast cancer cells processed by the CellSearch system

The efficacy of EGFR p1068 was confirmed, the next step was to test this antibody out in breast cancer cells spiked in healthy bloods processed through the CellSearch system. To do this, cells were treated with and without EGF prior to spiking in blood, as controls to test the efficacy of staining cells using the EGFR p1068-FITC antibody added to the reagent carousel of the CellSearch system. The optimised antibody concentration was used and around 100 MDA-MB-468 cells (both EGF treated and untreated) were spiked in healthy blood samples and processed. The background staining in the EGF treated cells was found to be too high. Further optimisations (summarised in Table 6.1) also did not provide evidence that the antibody could stain cells effectively in the CellSearch system. The difference between the p1068 staining of EGF treated and untreated cells was not enough to provide evidence this antibody was specific. Due to funding constraints and the cost of CellSearch reagents, this study was limited in the number of CTC tests that could be performed. The complexity of this staining required many more optimisations including further titration of the antibody conjugate, optimisation of the exposure time to visualise staining and the assessment in staining in more EGFR over-expressing cell lines. This line of enquiry was therefore not continued and the detection of EGFR transcripts was investigated instead.

Table 6.1: Summary of the optimisation of EGFR p1068 measurement in spiked breast cancer cells detected by the CellSearch system

Method of cell treatment prior to spiking in blood and processing using the CellSearch system	**Plus EGF (% cells EGFR p1068 positive)**	**No EGF (% cells EGFR p1068 positive)**
Treat cells with 200 ng/ml EGF 15 mins or no EGF 1 in 400 p1068-FITC	Background too high	29%
Treat cells with 200 ng/ml EGF 15 mins or no EGF 1 in 2000 p1068-FITC	22%	11%
Treat cells before trypsinising with 200 ng/ml EGF 15 mins or no EGF 1 in 2000 p1068-FITC	30%	25%
Treat cells before trypsinising with 200 ng/ml EGF 15 mins or no EGF 10µl phosphatase inhibitor 1 in 1250 p1068-FITC	47%	35%
Same as above new EGF and p1068-FITC 1 in 1000 p1068-FITC	10%	7%
Same as above new EGF and p1068-FITC 1 in 400 p1068-FITC	3%	0%

6.10 *In Situ* detection of EGFR mRNA transcripts in CTCs

Due to the technical difficulties involved with measuring phosphorylated EGFR in CTCs, an alternative option was the use of CTCscope (discussed in Chapter 5) to measure transcripts of *EGFR in situ* in CTCs. This assay is capable of using multiple fluorescent probes to detect more than one mRNA, enabling the detection of several markers of response to lapatinib simultaneously. The detection of HER2 and EGFR as well as tumour markers could feasibly be performed at the same time.

Target probes were identified and produced for EGFR (Appendix, Table 8.4). This work was carried out at the same time as the optimisation work for CTCscope (Chapter 5). The number of

EGFR mRNAs was quantified in three breast cancer cell lines (in a known number of cells) using the Quantigene 2.0 assay which can measure mRNA directly from lysates and is based on the same principle as CTCscope. *EGFR* mRNA was measured *in situ* by CTCscope assay to ensure correlation (Figure 6.6). This was confirmed and was also related to the EGFR protein levels in these cell lines (Figure 6.2). The MCF-7 cell line had the lowest number of transcripts as shown by the few fluorescent signals of EGFR mRNA. This was also shown by the Quantigene assay (Panomics) which gives an approximate value of mRNAs per cell using the same technology in cell lysates. An average of approximately 0.2 transcripts per cell was shown in MCF-7 cells. MDA-MB-468 cells showed a much larger number of transcripts per cell (559) along with many more fluorescent signals for CTCscope. The MDA-MD-231 cell line showed an average of 317 transcripts per cell. The CTC images also showed heterogeneity in transcript numbers in single cells among homogeneous cell lines.

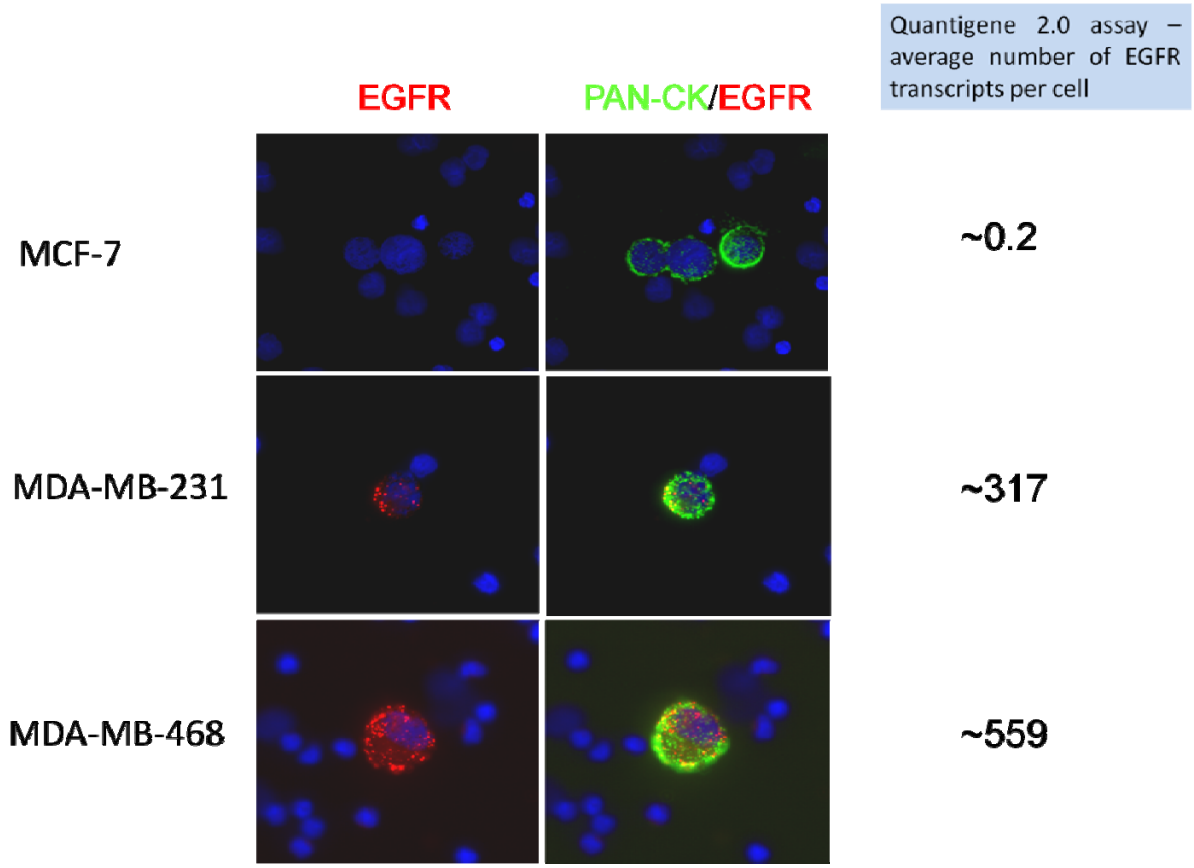


Figure 6.6: CTCscope and Quantigene assays of EGFR mRNAs correlate in three breast cancer cell lines (performed by Fay Wang, Advanced Cell Diagnostics, Inc). EGFR mRNA signals are shown in red, Pan-CK in green (to show epithelial origin) and nuclear staining in blue (DAPI). Images show representative cells from each cell line with differing levels of EGFR mRNA. The Quantigene 2.0 assay was used from a known number of cells in lysis mix, followed by quantification by measuring luminescence.

6.11 EGFR mRNA measurement in CTCs using CTCscope

EGFR transcripts were detected in CTCs from advanced breast cancer patients using both the early-stage CTCscope method and the optimised method (Chapter 5). A comparison of EGFR measurement in CTCs by the CellSearch system and by CTCscope was performed in 20 paired blood samples from advanced breast cancer patients. The early-stage CTCscope method identified CTCs in 4 patients out of 20 (20%) compared to 8 out of 20 (40%) detected by the CellSearch system. Concordance was high with all 4 patients positive for EGFR positive CTCs by

CTCscope also positive by the CellSearch system. The proportion of patients with CTCs who showed EGFR positivity was the same with CTCscope and the CellSearch system (57%), Table 6.2.

Table 6.2: The CellSearch system detects more EGFR positive CTCs in paired blood samples from advanced breast cancer patients than early-stage CTCscope

	No. of patients with EGFR positive CTCs[b] (n = 20)	**(%)**	**No. CTC positive patients with EGFR positive CTCs**	**(%)**
Early stage CTCscope[a]	4	20%	4/7	57%
CellSearch	8	40%	8/14	57%

[a] This method used the CPT Ficoll tube (Beckton Dickenson) to enrich the cells, 0.25% formaldehyde fixation and the Shandon cytospin
[b] Positive for CTCs when 1 or more CTCs present in 7.5 ml blood

EGFR measurement in CTCs was also compared using the optimised CTCscope method with the CellSearch system in 42 advanced breast cancer patients recruited from the clinics at Charing Cross hospital. In this comparison, even though CTC detection by CTCscope was improved, this assay only detected 1 (2%) patient with EGFR positive CTCs compared to 12 (29%) by the CellSearch system. The proportion of CTC positive patients with EGFR detected in their CTCs was 60% for the CellSearch system, similar to the previous comparison. CTCscope had a much lower proportion of only 5% (Table 6.3). The numbers of patients positive for CTCs detected by the CellSearch system in this cohort was much lower compared to the previous comparison (48% versus 70%), which may account for the lower presence of EGFR positive CTCs particularly by CTCscope. This may show advanced breast cancer patients with more CTCs have greater expression of *EGFR* mRNA in their CTCs.

Table 6.3: The CellSearch system detects more EGFR positive CTCs in paired blood samples from advanced breast cancer patients than the optimised CTCscope method

	No. of patients with EGFR positive CTCs[b] (n = 42)	(%)	No. CTC positive patients with EGFR positive CTCs	(%)
CTCscope[a]	1	2%	1/20	5%
CellSearch	12	29%	12/20	60%

[a] This method used the Accuspin™ system-histopaque®-1077 ficoll tube (Sigma-Aldrich) to enrich the cells followed by a 10% formaldehyde fixation
[b] Positive for CTCs when 1 or more CTCs present in 7.5 ml blood

Examples of CTCs with varying levels of EGFR mRNA in advanced breast cancer patients are shown in Figure 6.7. Individual CTCs also showed heterogeneity in EGFR mRNA signal per cell and this was always much lower than in the EGFR positive breast cancer cell lines tested (Figure 6.6).

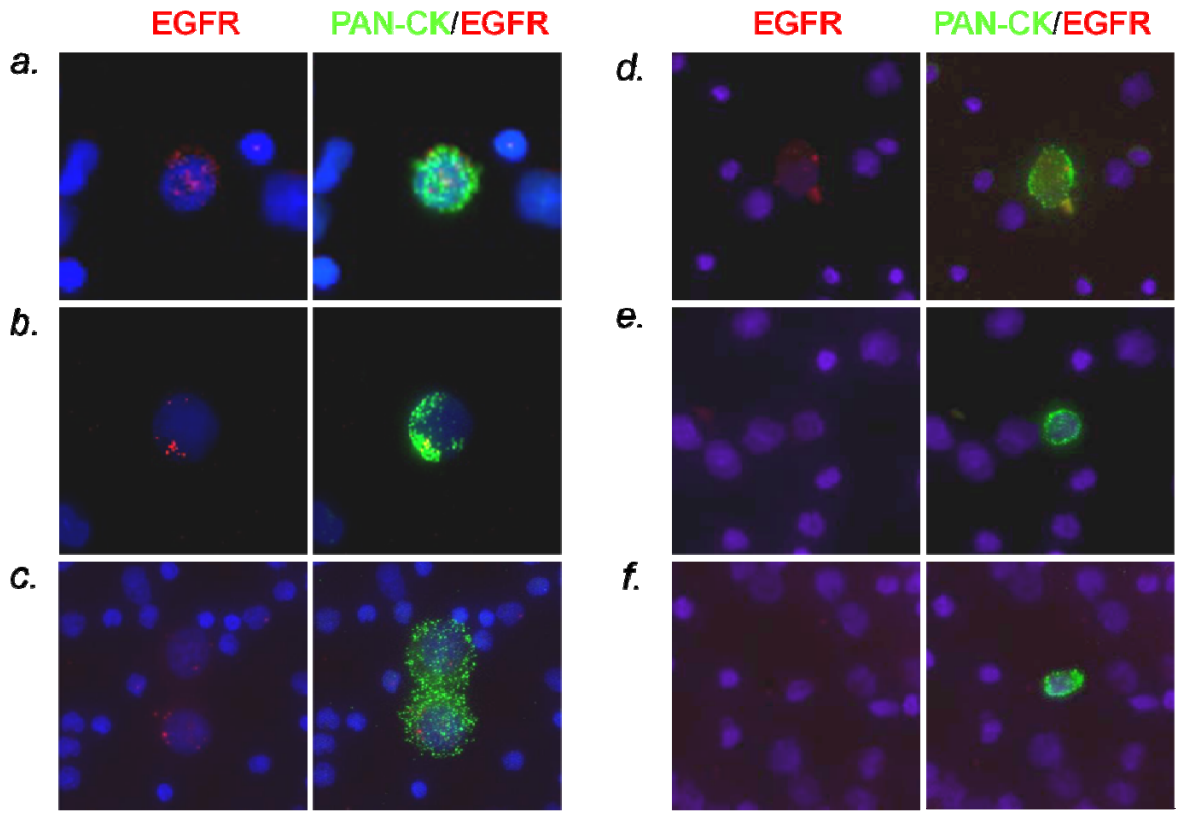


Figure 6.7: Examples of EGFR detection in CTCs from advanced breast cancer patients using CTCscope (*a-f*). Images *a-d* show EGFR positive CTCs with varying staining intensities and *e* and, *f* show EGFR negative CTCs.

6.12 EGFR in FFPE tumour sections – comparison of IHC EGFR protein and RNAscope EGFR mRNA

Since the measurement of EGFR by IHC in tumour tissue sections does not predict efficacy of anti-EGFR therapies it was of interest to detect EGFR transcripts in primary tumour biopsies by the same method as CTCscope. Advanced Cell Diagnostics, Inc, CA, USA, have a commercial kit recently available for this purpose, called RNAscope. This study was conducted as part of their final development work into this method. Tumour sections from 18 advanced breast cancer patients were collected for dual EGFR staining. These were retrospective biopsies from patients from Charing Cross Hospital, screened for recruitment onto the lapatinib trial, and who had

CTCs and EGFR tests performed. All patients gave written informed consent. We compared EGFR protein measurement using the standard IHC protocol (using the EGFR 31G7 clone antibody from Zymed Laboratories, Inc), as used in the Charing Cross Histopathology Department with RNAscope staining of EGFR mRNA. Both assays tested positive control tumour tissue to ensure efficacy per staining run (Figure 6.8). RNAscope also measured Ubiquitin C (*UbC*) mRNA as an additional control for each FFPE tumour section to ensure mRNA was present along with a no probe control to ensure EGFR probe binding was specific.

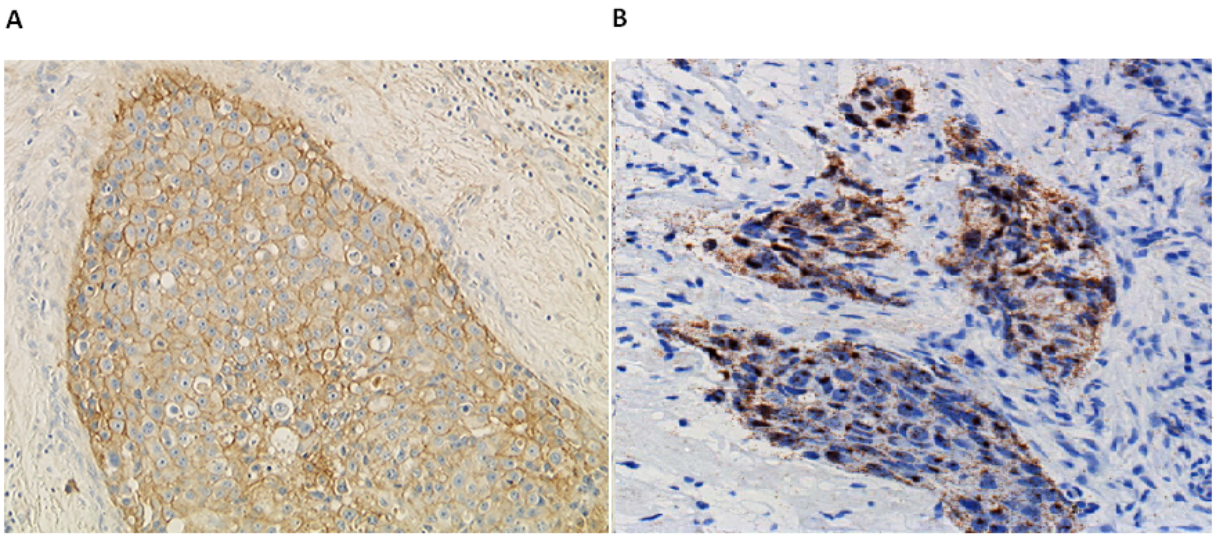


Figure 6.8: Examples of positive controls for both EGFR IHC (A) and EGFR RNAscope (B) staining show high levels of EGFR protein present.

6.13 RNAscope is more sensitive at detecting EGFR in FFPE tumour sections than standard IHC

In this preliminary study, RNAscope staining confirmed more FFPE tumour sections were positive for EGFR mRNA than EGFR protein using IHC. Only 2 out of 18 (11%) were positive for EGFR protein compared to 8 out of 18 (44%) for EGFR mRNA, this was a statistically significant difference ($p=0.0096$, t test). A positive result represented the presence of staining in more than 10% of the cells for both EGFR mRNA and protein. A scoring system was used for EGFR

mRNA based on the ability to visualise the transcripts at different magnifications. This is because low levels of punctuated EGFR mRNA signals present are difficult to see under X40 magnification. Zero represented under 10% or no staining, 1 = detectable under X60, 2 = detectable under X40 and 3 = detectable under X20 magnification. This assay was therefore less subjective and more sensitive at detecting low levels of EGFR mRNA in the primary breast tumour FFPE sections (Table 6.4).

Table 6.4: RNAscope detects EGFR mRNA in more FFPE sections than IHC detects EGFR protein in paired FFPE breast tumour sections from 18 metastatic breast cancer patients. CTC/ EGFR measurements are also shown.

	EGFR staining result				
FFPE tumour section number	**Immunohistochemistry – EGFR protein**	**RNAscope – EGFR mRNA**	**Staining score[a]**	**CTC result[b]**	**CTC EGFR+ (%)[b]**
1	70-80% positive (small focus)	20% Positive (small focus)	1	99	5 (5%)
2	80% positive	90% Positive	2	1	1 (100%)
3	Negative	Negative	0	1	0
4	Negative	Negative	0	33	7 (21%)
5	Negative	>80% Positive	1	3	3 (100%)
6	Negative	10% Positive	1	137	32 (23%)
7	Negative	Negative	0	48	12 (25%)
8	Negative	Negative	0	529	9 (2%)
9	Negative	Negative	0	15	1 (7%)
10	Negative	Negative	0	2	0
11	Negative	Negative	0	5	2 (40%)
12	Negative	Negative	0	19	4 (21%)
13	Negative	Negative	0	32	3 (9%)
14	Negative	50% Positive	1	1	1 (100%)
15	Negative	50% Positive	1	1	1 (100%)
16	Negative	30% Positive	1	1217	410 (34%)
17	Negative	20% Positive	1	8	2 (25%)
18	Negative	Negative	0	12	1 (8%)
Total positive for EGFR	2 (11%)	8 (44%)			16 (89%)
p value	0.0096*				

[a] Scoring system was based on the ability to visualise EGFR mRNA easily by magnification. 0 – little or no staining, 1- detectable under X60, 2 – detectable under X40, 3 – detectable under X20 magnification.
[b] CTCs were measured in 7.5 ml blood by the CellSearch system.
* Statistically significant difference, paired t test.

An example of very low numbers of EGFR transcripts and very high levels in two patient FFPE tumour sections is shown (Figures 6.9 and 6.10). EGFR protein is not detectable in one tumour section; however EGFR transcripts are detectable at X60 magnification (Figure 6.9). A tumour section with high levels of EGFR protein demonstrates correspondingly high levels of EGFR mRNA clearly seen at X60 (Figure 6.12) but also visualised at X40 and therefore given a score of 2.

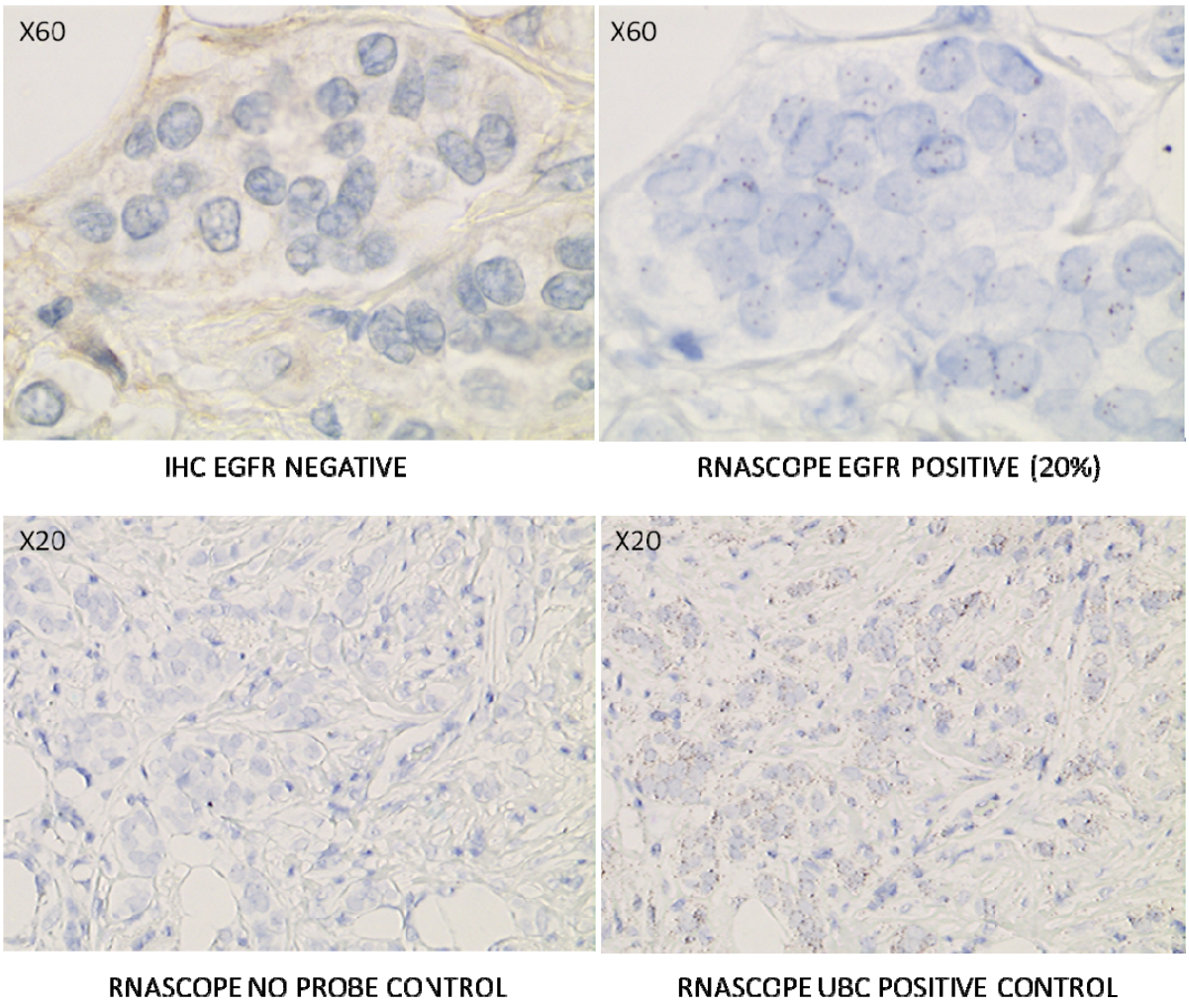


Figure 6.9: Comparison of IHC and RNAscope EGFR staining from a FFPE primary breast tumour section. EGFR mRNA is present by RNAscope but no EGFR protein is detected by IHC from the same cut section. Positive controls include a no probe control for RNAscope and a ubiquitinase C positive control to show the presence of mRNA is retained. Sections were scored based on the percentage of cells positive for staining. (Tumour section number 17 in Table 6.4).

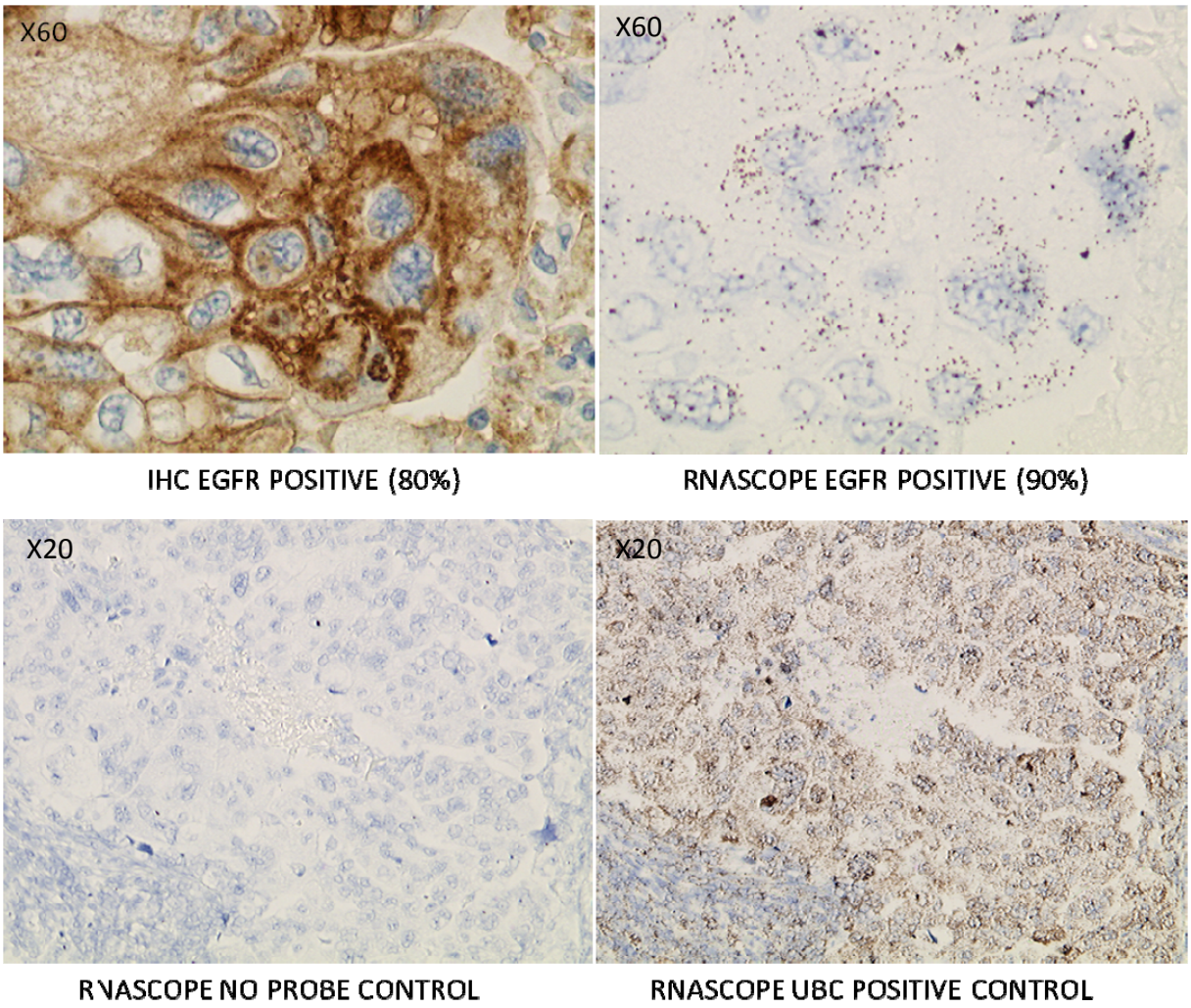


Figure 6.10: Comparison of IHC and RNAscope EGFR staining from a FFPE primary breast tumour section. EGFR mRNA is present by RNAscope and also EGFR protein is detected by IHC from the same cut section. Positive controls include a no probe control for RNAscope and a ubiquitinase C positive control to show the presence of mRNA is retained. Sections were scored based on the percentage of cells positive for staining. (Tumour section number 2 in Table 6.4).

The concordance of the two methods was limited due to the low numbers of tumour sections positive for EGFR by IHC with only 2 out of 18 showing presence of EGFR protein. These were also positive for EGFR mRNA, however 6 tumour sections showed the presence of EGFR mRNA with no EGFR protein staining, therefore the methods were not significantly concordant at 67% (p=1.0), Table 6.5.

Table 6.5: The concordance of the presence of EGFR protein or mRNA in paired FFPE tumour sections from breast cancer patients

		RNAscope EGFR		
	No. patients	Positive	Negative	p value
IHC EGFR[a]	Positive	2	0	1.0
	Negative	6	10	

[a] This method used the 31G7 clone, EGFR antibody (Zymed Laboratories, Inc)
*Statistically significant (Fishers exact test)

6.14 EGFR over-expression can be acquired with disease progression

Investigations so far have revealed that EGFR can be measured in CTCs using the CellSearch system more effectively than CTCscope. Conversely, in FFPE tumour sections, EGFR can be measured more sensitively by RNAscope than by IHC. We compared the measurements of EGFR in primary tumour sections by both IHC and CTCscope with EGFR measurement in CTCs by the CellSearch system. Both tumours positive for EGFR by IHC also had CTCs positive for EGFR. Eight tumour sections positive for EGFR by RNAscope also had EGFR positive CTCs. Interestingly many more patients had CTCs positive for EGFR implying EGFR expression may be gained in disease progression. Fourteen patients who had no EGFR protein expression measured by IHC had EGFR positive CTCs later on. Eight patients with tumours negative for EGFR by RNAscope had EGFR positive CTCs (Table 6.4 and 6.6).

Table 6.6: The presence of EGFR measured by IHC (protein) or RNAscope (mRNA) in early and late stage breast cancer is not concordant with EGFR detected in CTCs by the CellSearch system

		EGFR in advanced breast cancer			
		EGFR positive CTCs	EGFR negative CTCs	Total	Concordance (p value)
Primary FFPE tumour section	Positive for EGFR by IHC	2 (11%)	0 (0%)	18 (100%)	1.0
	Negative for EGFR by IHC	14 (78%)	2 (11%)		
	Positive for EGFR by RNAscope	8 (44%)	0 (0%)	18 (100%)	0.48
	Negative for EGFR by RNAscope	8 (44%)	2 (11%)		

* Statistically significant by Fishers exact test

6.15 Measurements of EGFR expression in circulating tumour cells over time in metastatic breast cancer patients

The CellSearch system was previously confirmed to detect EGFR expression in a breast cancer cell line specifically. This was then used to assess the presence and reproducibility of CTC/EGFR measurements in advanced breast cancer patients. A total of 74 samples were taken from 33 individuals with metastatic breast cancer. Of these, 51 of the 74 samples (69%) had measurable CTCs. Twenty-six of the 33 patients (79%) had CTCs present in 7.5 ml of blood. Of these, a mean of 44% of CTCs (95% CI 34 – 54%) were positive for EGFR protein with a range of 2 – 100% positivity. Staining intensity varied but was mostly very weak in patient CTCs, with examples shown in Figure 6.11. Of the samples with measureable CTCs, 44 out of 51 had EGFR positivity (86%), with 44 out of 74 (59%) samples with EGFR positive CTCs present. EGFR expression was analysed in all 74 samples and those patients with the presence of CTCs in sequential samples were included in our analyses.

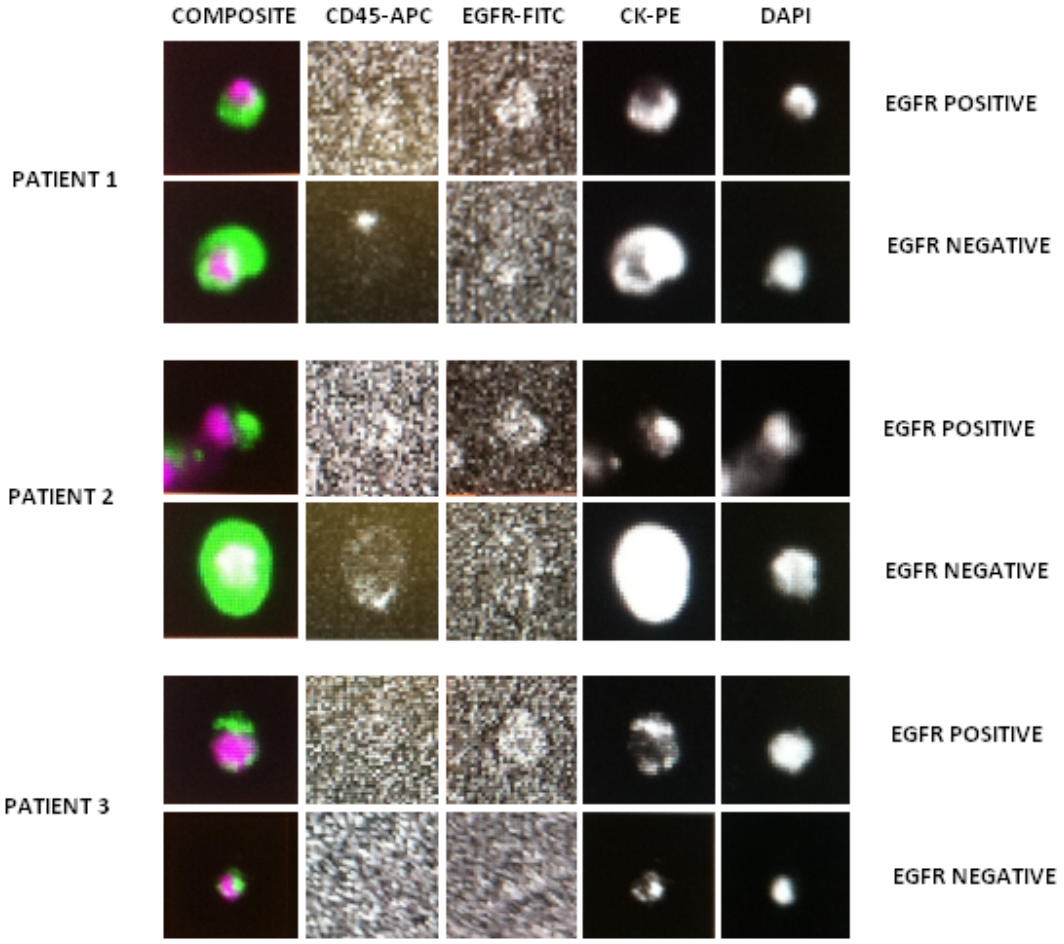


Figure 6.11: Patients with advanced breast cancer have variable expression of EGFR on their CTCs as measured by the CellSearch system. This gallery of images shows representative examples of EGFR positive and negative CTCs from the same sample in three advanced breast cancer patients. Patients demonstrated heterogeneity of EGFR expression in their CTCs.

CTC and EGFR measurements were taken over time from six advanced breast cancer patients with detectable CTCs (Figure 6.12, A - F). Samples were taken up to 14 weeks apart and ranged from 2 – 5 samples taken per patient. Four patients (B, D, E and F) show a consistent percentage of EGFR positive CTCs with one showing a fluctuation of 25% between samples (graph E). One patient (graph D) had stable disease during CTC sampling which is reflected by a consistent number of EGFR positive and total CTCs over time. Two patients (graphs A and C)

demonstrated fluctuations in EGFR positivity with an increase in both CTCs and EGFR positive CTCs at the last time point from 21 – 58% and from 21 - 45% respectively, suggesting the EGFR positive ratio may increase with disease progression in some patients. One patient had a substantial increase in CTCs with no increase in the ratio positive for EGFR (graph B). The percentage of CTCs positive for EGFR staining, are shown above each bar graph. This data demonstrates EGFR measurements in CTCs are reproducible over time.

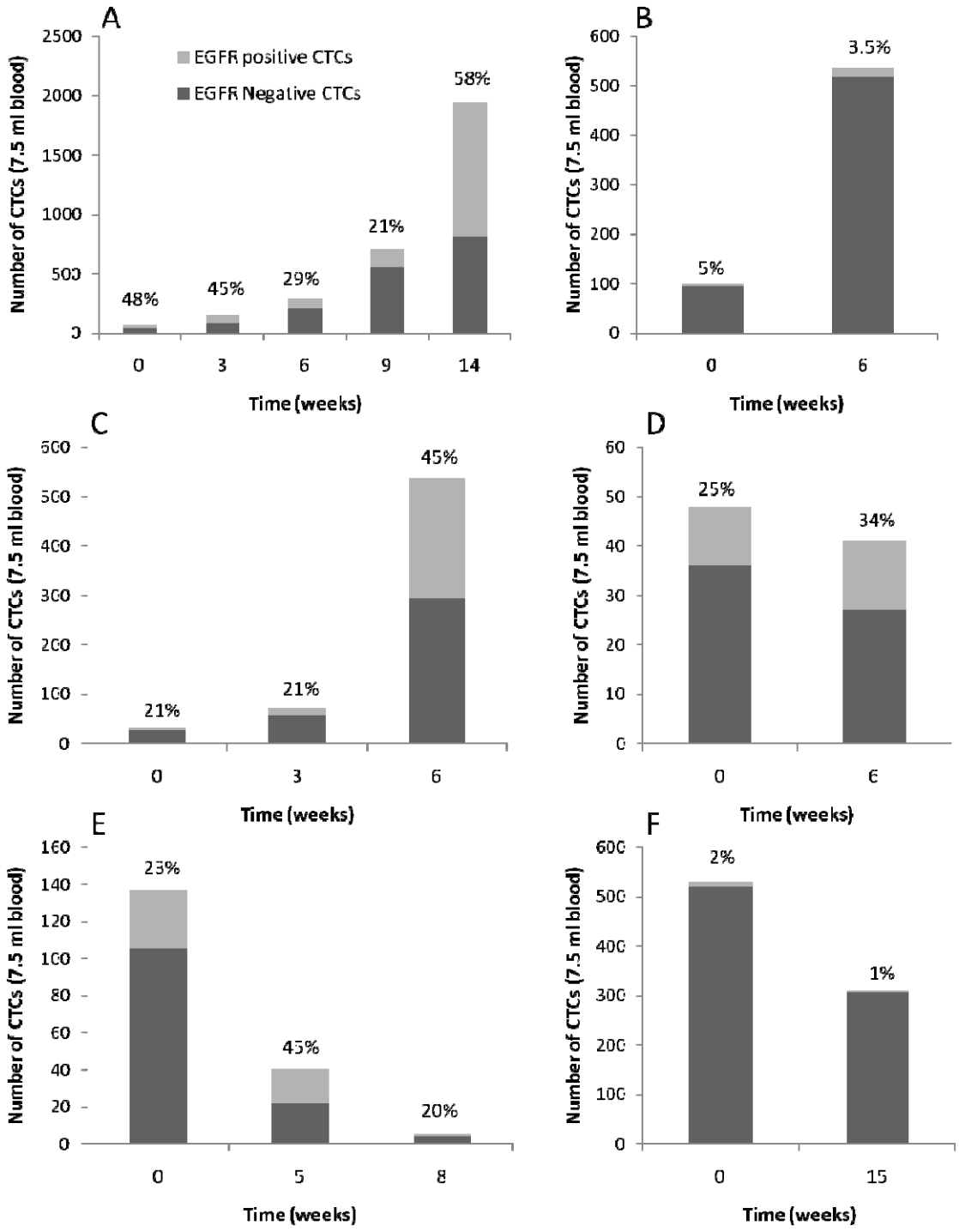


Figure 6.12: EGFR measurements in CTCs are reproducible over time in advanced breast cancer patients. Each graph represents CTC and EGFR measurements (as assessed by the CellSearch system) from one patient over time (A – F). Four patients show a consistent percentage of EGFR positive CTCs (B, D, E and F). Two patients (A and C) show an increase in the percentage of EGFR positive CTCs at the last time point. The percentage of CTCs positive for EGFR is shown above each bar graph.

6.16 CTC and EGFR measurements in advanced breast cancer patients on Lapatinib monotherapy

Screening

Out of 43 patients screened for EGFR positive CTCs, 16 were eligible (12 of which are recorded here, 4 are yet to have RECIST criteria reported) with detectable, EGFR positive CTCs and also for meeting the inclusion criteria for the study. A mean of 42 CTCs (95% CI 3.5 – 80.4) were detected in 7.5 ml of blood in the screening samples. These CTC samples had a mean EGFR positivity rate of 22% of the total CTC population (95% CI 13 – 30.7). All twelve patients had 5 or more CTCs in 7.5 ml of blood (range 5 – 221 CTCs), and were considered to have a poor progression-free survival and overall survival (117), (Tables 6.7 and 6.8).

Table 6.7: Numbers of CTCs and EGFR positive CTCs in 43 advanced breast cancer patients screened for recruitment for lapatinib monotherapy

	Lapatinib trial screening
Number of metastatic breast cancer patients	43
Number positive for CTCs [a]	29 (67%)
Mean number of CTCs	42 (95% CI 3.5 to 80.4)
Range of CTCs detected	5 - 221
Number positive for EGFR positive CTCs	20 (47%)
Mean % of CTCs EGFR positive	22% (95% CI 13.0 to 30.7%)

[a] Positive for CTCs when 1 or more CTCs present in 7.5 ml blood assessed by the CellSearch system

6.17 Clinical characteristics of advanced breast cancer patients

All 12 patients had a life expectancy of over 12 weeks and had a confirmed HER2 negative primary tumour. Three patients who had a primary tumour section available had EGFR staining performed, 3 patients had surgery at another hospital and 6 patients had FFPE tumour sections archived at a separate site, these samples will all be stained for EGFR using both IHC and

RNAscope at a later date. Those patients with available tumour biopsies all had a negative EGFR IHC result in their primary tumour. Two of these patients were also negative for EGFR transcripts by RNAscope, and one showed positivity of 10% with a score of 1 (patient 2, Table 6.8). These results suggest EGFR expression may be increased or gained in CTCs during the metastatic process. All twelve patients were heavily pre-treated with a range of treatments. All but one patient was positive for ER in their primary tumour (Table 6.8).

6.18 HER2 positivity in CTC samples at screening or baseline

The twelve patients recruited to the trial also had HER2 CTC measurements taken at either screening or baseline. This was not a prerequisite for the trial but was necessary to find out the status as lapatinib also targets HER2. Ten patients (one patient had difficult veins to bleed, one sample was aborted from the CellSearch machine so no HER2 result was recorded) had HER2 positive CTCs present measured by the CellSearch system (a mean of 68% of the total CTC population was HER2 positive, range 39 – 100%). This was despite all patients having a negative HER2 primary tumour status (Table 6.8).

Table 6.8: Primary tumour characteristics, prior treatments and CTC screening measurements of 12 patients with advanced breast cancer. Twelve patients were recruited to the trial of a daily dose of lapatinib monotherapy. All patients had one or more EGFR positive CTCs. Ten patients were assessed for HER2 positivity in their CTCs and all had a proportion HER2 positive.

Patient	Age	ECOG[a] status	Primary tumour characteristics (All HER2 negative)				Previous therapies				CTCs[c] (% EGFR positive)	CTCs[c] (% HER2 positive)
			TYPE[b]	ER	PR	EGFR	CHEMO	HORMONAL	RADIO	TARGETED		
1	47	1	IDC	POS	N/K	N/K	X3	X4	X1	X1	3/5 (60%)	-
2	52	1	ILC	POS	N/K	NEG	X4	X3	X2		2/8 (25%)	5/5 (100%)
3	63	2	IDC	POS	POS	N/K	X5	X5	X2		16/49 (33%)	6/8 (75%)
4	72	2	IDC	POS	NEG	N/K	X2	X2		X2	2/13 (15%)	32/62 (52%)
5	68	1	ILC	POS	POS	NEG	X2	X3		X3	9/34 (26%)	28/33 (85%)
6	67	1	IDC	POS	NEG	N/K	X3	X3	X1	X2	37/221 (17%)	334/513 (65%)
7	45	1	IDC	POS	POS	NEG	X2	X3		X1	1/12 (8%)	15/18 (83%)
8	39	2	IDC	NEG	NEG	N/K	X3		X5	X1	44/66 (67%)	26/41 (63%)
9	61	1	IDC	POS	POS	N/K	X3	X4	X3	X2	1/6 (12%)	14/26 (54%)
10	52	0	IDC	POS	POS	N/K	X2	X3	X1	X2	3/14 (21%)	-
11	48	0	IDC	POS	POS	N/K	X3	X5	X2	X4	1/13 (8%)	7/12 (58%)
12	51	0	IDC	POS	NEG	N/K	X1	X2	X3	X3	5/62 (8%)	30/76 (39%)

[a]Eastern Cooperative Oncology Group Status. 0 - Fully active, able to carry on all pre-disease performance without restriction, 1- Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work, 2 - Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.

[b]IDC – Invasive ductal carcinoma, ILC – Invasive lobular carcinoma

[c] CTCs present in 7.5 ml blood at screening or baseline as assessed by the CellSearch system

6.19 EGFR detection in CTCs before and after Lapatinib monotherapy

Patient results were grouped according to the CTC response after one cycle of lapatinib, by either an increase or decrease in their CTCs. Six out of twelve patients (50%) had an initial decrease in the number of CTCs after the first cycle of lapatinib treatment, 4 of which also had a reduction in the number of EGFR positive CTCs. Despite this, two patients had progressive disease and were taken off treatment at 4 weeks (graphs D and F, Figure 6.13). The remaining 4 patients all had an increase in CTC load at the 8 week time point after 2 cycles of lapatinib therapy and were taken off the study either at 8 or 12 weeks due to disease progression (graphs A, B, C and E Figure 6.13).

The remaining 6 patients had an increase in the number of CTCs in 7.5 ml blood following the first cycle of lapatinib treatment with all but one also having an increase in the number of EGFR positive CTCs. Four out of the 6 had disease progression after the first cycle and were taken off the study (graphs A, C, E and F, Figure 6.14). The remaining two patients had an increase of CTCs after two cycles of lapatinib and had progressive disease and were taken off the study (graphs B and D, Figure 6.14).

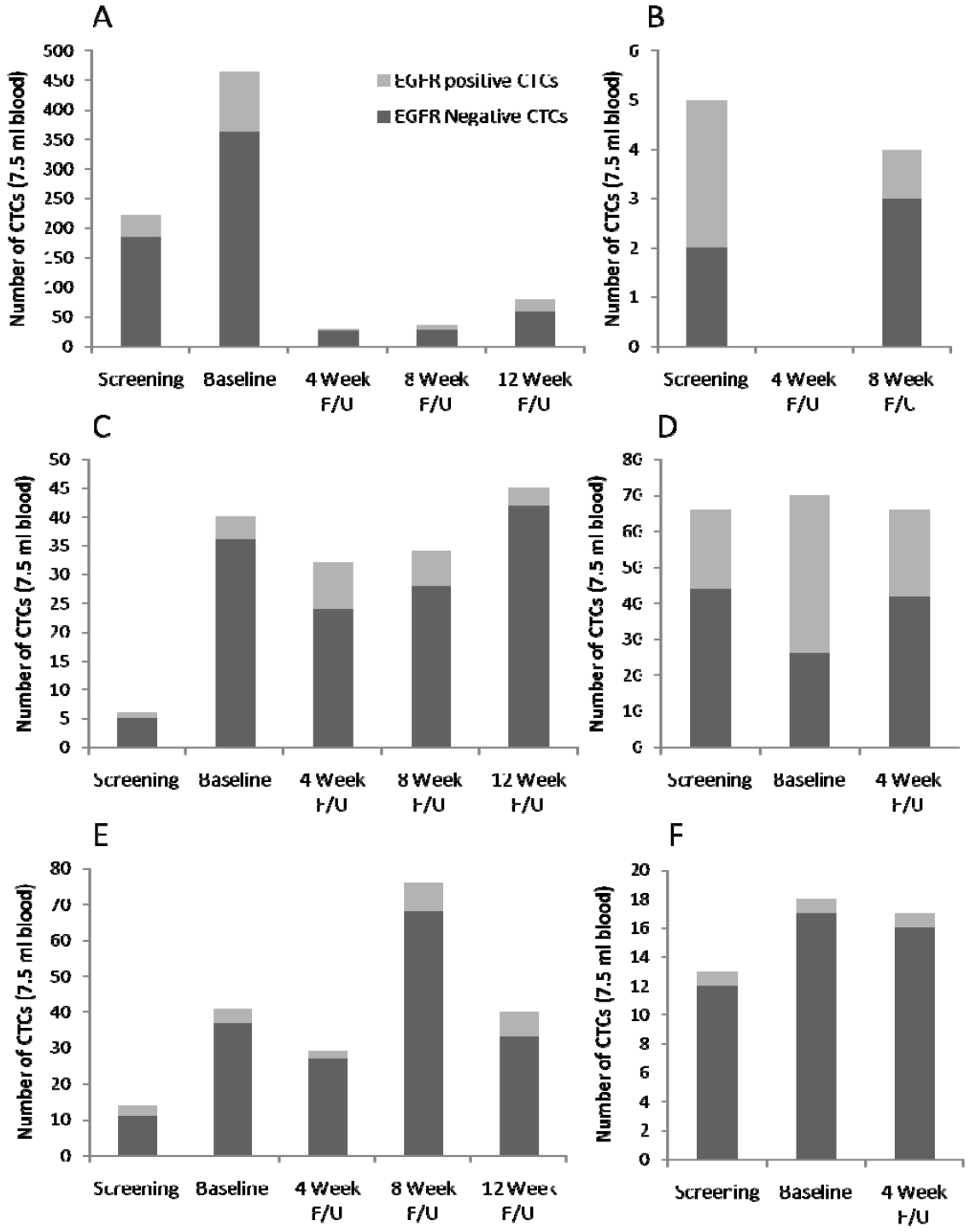


Figure 6.13: Six out of twelve patients with advanced breast cancer demonstrated a decrease in the quantity of CTCs in 7.5 ml blood following the first cycle of Lapatinib treatment. Graphs show CTC measurements and EGFR positivity at screening, baseline and at follow-up after Lapatinib treatment in each patient (A – E). Four of these also had a decrease in EGFR positive CTCs (A, B, D and E).

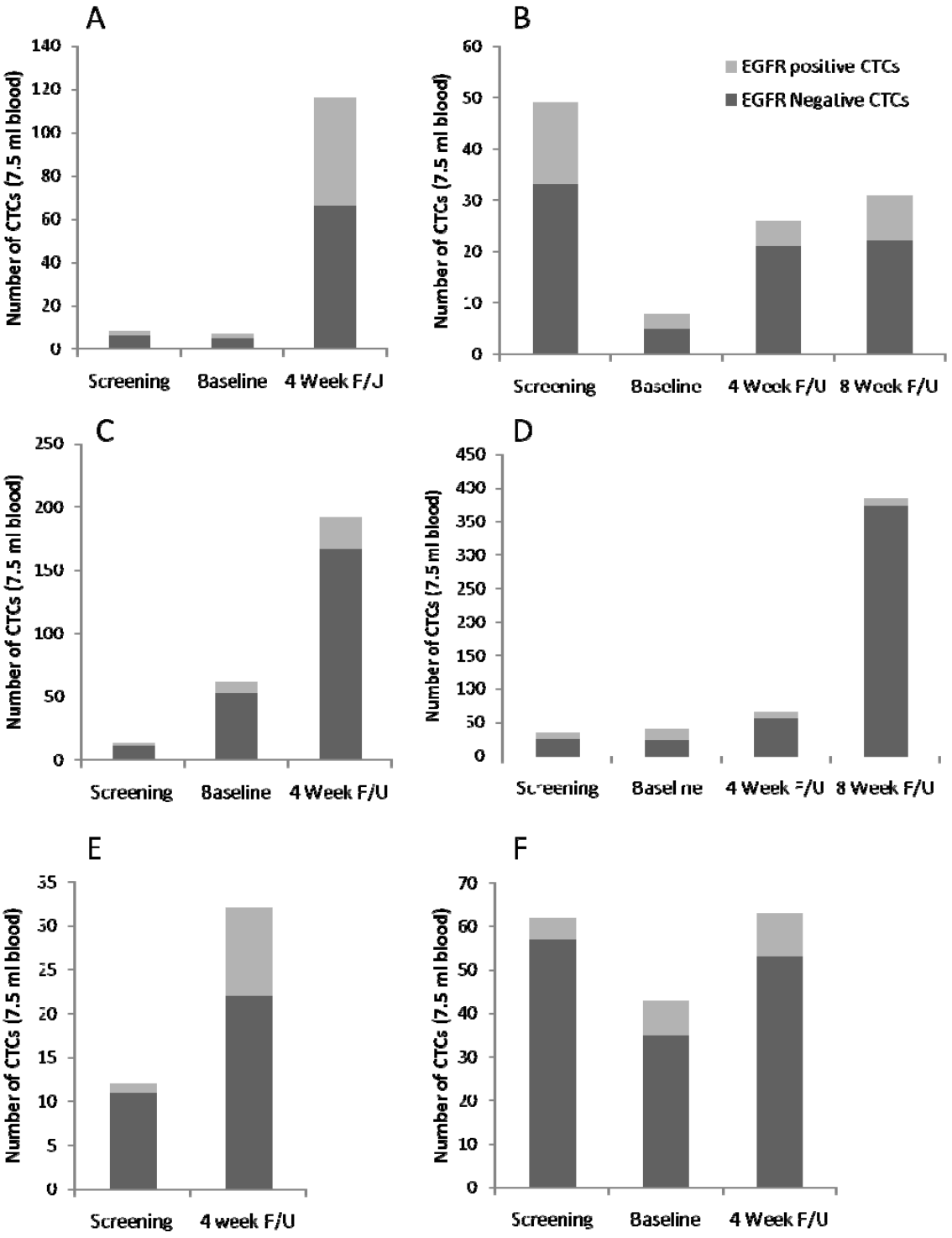


Figure 6.14: Six out of twelve patients with advanced breast cancer demonstrated an increase in the quantity of CTCs in 7.5 ml blood following the first cycle of Lapatinib treatment. Graphs show CTC measurements and EGFR positivity at screening, baseline and at follow-up after Lapatinib treatment in each patient (A – F). Patient D demonstrated a decrease in the number of EGFR positive CTCs.

6.20 Disease response

All twelve patients had disease progression after 12 weeks at the first disease staging assessment. Also, the final 4 patients, not yet reported, also had disease progression therefore the trial did not progress to the next stage and lapatinib monotherapy in this trial was declared to be insufficiently active. The increase in the target lesions is shown in Table 6.9.

6.21 CTCs and disease response

The number of CTCs increased post therapy (from the baseline samples to the last measurement performed) in 9 out of 12 (75%) patients and remained at similar levels in 3 out of 12 (25%) patients. This demonstrates that the quantity of CTCs correlated with progressive disease in these patients. The proportion of CTCs positive for EGFR decreased in 8 of the 12 (67%) patients and increased in 4 out of 12 (33%) patients overall from the baseline sample to the last CTC measurement for each patient. This demonstrates that overall, the measurement of the proportion of EGFR positive CTCs did not correlate closely to disease response. It is worth noting however, that patient A (Figure 6.13 and Patient 6 in Tables 6.8 and 6.9) responded to Lapatinib therapy after the first cycle of 4 weeks, stable disease was evident as assessed by CT scan at that time point, and the number of CTCs and EGFR positive CTCs also decreased from 465 (22% EGFR positive) to 29 (10% EGFR positive). By the 12 week time point, the patient had an increase in their target lesions along with an increase in their CTCs. In addition, patient B (Figure 6.13 and Patient 1 in Tables 6.8 and 6.9) showed a decrease in their CTC count from 5 (60% EGFR positive) to 0 after the first cycle of treatment, clinically their ascites were well controlled. By the second cycle however their CTC count had gone back up to 4 (25% EGFR positive) and they had progressive disease both on the CT scan and by the accumulation of ascites. This provides further evidence of the correlation of CTC number with disease response.

Table 6.9: Target lesion response in twelve metastatic breast cancer patients on Lapatinib monotherapy after 12 weeks

Patient	Weeks on therapy	Target Lesions[a]	Target Lesion Change at 12 Weeks	Disease Response[b]
1	8	OVARY 48MM	plus 11mm	PD
2	8	LIVER SEGS 19MM, 16MM, OVARY 55MM, 47MM	plus 7mm, others the same	PD
3	12	BREAST 23MM, COELIAC TRUNK 11MM	plus 5mm	PD
4	8	LIVER SEG 6 37MM, AXILLA 24MM	plus 38mm and plus 1mm	PD
5	8	STOMACH WALL 11MM	plus 6mm	PD
6	12	LIVER SEG 3 37MM	hepatomegaly	PD
7	14	BREAST 28MM, PARATRACHEAL LN 15MM	plus 21mm, 7mm	PD
8	4	MEDIASTINUM LN 20MM, SUBC 20MM, HILAR 13MM	plus 28mm,	PD
9	9	LIVER SEG 4A 30MM, 7 31MM, 6 40MM, 5 35MM, 5 52MM	plus 7mm, 11mm, 20mm, 10mm, 7mm	PD
10	12	LIVER SEG 7 19MM, SEG4 12MM, SEG3 10MM, SEG 5 13MM	plus 4mm, 7mm, 9mm	PD
11	4	LIVER SEGS 12MM	minus 1mm, new lesion	PD
12	3	MEDIASTINUM 73MM, LN HILAR 14MM, 26MM	Stable	PD

[a]Target lesions are measurable lesions up to 5 per organ and 10 in total. These were selected on the basis of their size (those with the longest diameter) and suitability for accurate repeat measurements as per RECIST criteria (168).
[b]Response criteria: PD – progressive disease as assessed by RECIST criteria .

6.22 Correlation of CTCs measured by the CellSearch system and CTCscope in patients on Lapatinib monotherapy

Due to the optimisation process of CTCscope, few patients had CTCs measured by both methods at multiple time-points before and during therapy. Three patients had at least one CTC measurement by both methods prior to Lapatinib therapy and during treatment. As previously mentioned, fewer CTCs were detected by CTCscope than by the CellSearch system in these patients. One of the three patients showed the same pattern of CTC detection (Patient A, Figure 5.15) with fewer CTCs detected by CTCscope. The other patients did not show obvious correlations of CTC numbers.

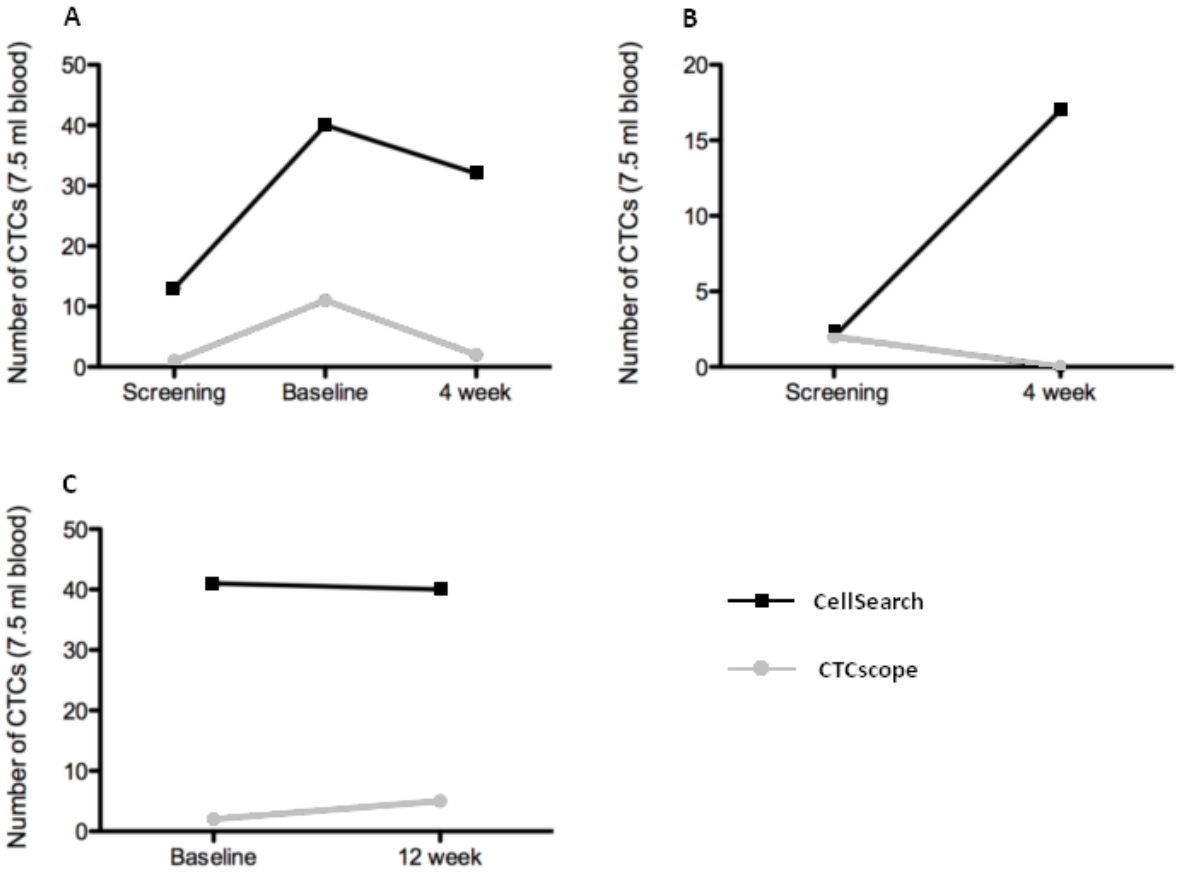


Figure 6.15: Correlation of CTCs measured by the CellSearch system and CTCscope in 3 patients on lapatinib monotherapy. CTCscope detects fewer CTCs than the CellSearch system and does not correlate with it in all patient samples measured.

6.23 Discussion

This study demonstrates the potential of CTC and EGFR characterisation for selecting patients for a specific biological therapy. Although the results of the trial did not show any significant clinical responses, lessons can be learnt about this specific target and the therapy against it. This study demonstrated the effective measurement of EGFR in patient CTCs using the CellSearch system. This was tested by using two breast cancer cell lines that were each low and very high in EGFR expression. These were spiked in blood and processed through the CellSearch system. Those with low or absent EGFR protein expression as verified by an immunoblot showed no EGFR detection whereas those high in expression showed distinct staining by the CellSearch system. This confirmed the efficacy of measuring EGFR in CTCs by the CellSearch system. Two other methods of EGFR detection in CTCs was explored but were not as effective as the CellSearch system (discussed later).

CTC testing was performed in blood samples from advanced breast cancer patients by the CellSearch system and showed EGFR staining was present and furthermore, the measurement was reproducible over time in some patients. One patient with stable disease showed a consistent number of CTCs and also a consistent proportion of CTCs positive for EGFR. All patients with CTCs demonstrated a wide range of EGFR positivity from 2 – 100% with a mean of 44% of the total CTC population detected. Out of 33 metastatic breast cancer patients, 18 (55%) had EGFR positive CTCs detected. This is in accordance with a study to detect EGFR mRNA in the blood that interestingly found a similar percentage (59%) of carcinoma patients had evidence of EGFR present (227), a greater proportion of patients than many studies of EGFR protein in primary tumour biopsies (31, 32). This wide range of values was also found to be evident in a study of EGFR positive CTCs in prostate cancer (211). Two out of three patients with sequential CTC sampling in our study had an increase in CTCs and also showed an increase in the proportion of EGFR positive CTCs. This strengthens the association of EGFR with a poor prognosis (31). In addition, those patients with CTCs who have a poor prognosis are more likely to have EGFR positive CTCs, as shown here with 86% of patients with CTCs present having EGFR

expression. Further evidence for this was the observation that CTCs are more prevalent in triple negative breast cancer patients, known to have a poorer prognosis (Chapter 4).

Various limitations were encountered with this method including the very low expression levels of EGFR in CTCs from advanced cancer patients. This plus the very subjective nature of assigning a positive score to a CTC showing an immunofluorescent signal for EGFR was a drawback. For this reason, specific criteria were employed: EGFR detection on cells was only called positive if staining had a clear, distinguishable edge to the cell and a dark background. Any staining that was very faint or hard to define was deemed negative. A study published after the preliminary experiments described in this thesis were performed tested the measurement of EGFR positive CTCs in non small-cell lung carcinoma patients using a scoring system similar to that used for ER staining (228). This may have been beneficial here although our findings showed EGFR was consistently weak in the breast cancer patient CTCs we tested. This study also found much heterogeneity of EGFR expression (both positive and negative cells present) in each patient sample in common with our findings (178). Evidence for this was also demonstrated when EGFR was measured in tumour biopsies by a sensitive radio-ligand binding assay in 2567 tumours. Wide ranging values were present in EGFR positive tumours of 10 – 11,000 fmol/mg (10 or over being positive). Most of these however, (90%) had under 100 fmol/mg which provides additional evidence of the low amounts of EGFR present (31).

Since detection of total EGFR is not always indicative of the activation of its pathways, other methods were investigated to do this. Breast cancer cell lines were identified to study the measurements of activated or phosphorylated EGFR to see if this may be an effective indicator of anti-EGFR therapy response. Two ER-negative breast cancer cells lines, MDA-MB-231 and MDA-MB-468 were found to have high EGFR expression but low or absent HER2 expression, so were suitable cell models to study the effects of Lapatinib therapy without the compounding influence of HER2 expression. An antibody was chosen that was available conjugated to FITC (Tyr1068) to enable CTC phenotyping by the CellSearch system as well as being a key mediator of EGF signalling.

A simple test to check the efficacy of the antibody was performed by treating cells with and without EGF, the ligand for EGFR. The p1068-FITC antibody effectively stained the EGF treated cells. Next, lapatinib treatment of cells crucially showed p1068-FITC staining was decreased upon Lapatinib treatment. This meant the antibody was suitable for testing Lapatinib response in patient CTCs. The optimisation for this was technically challenging due to the limiting conditions for antibody staining in the CellSearch system. For example, the assay runs for 2 – 3 hours at room temperature and extra staining needs to be compatible with staining of 3 other tumour markers using fluorophores in these conditions. Measuring the activated status of CTCs may also be difficult due to the transient nature of phosphorylation and the low levels of kinases compared to other proteins (229). The addition of a phosphatase-inhibitor cocktail to preserve phosphorylation by inhibiting phosphatases did not help retain p1068-FITC staining. In addition the high cost per sample run meant that optimisations were not as thorough as would be necessary to ensure staining efficacy. This route of investigation was therefore discontinued. There are also no known studies of staining CTCs with antibodies to detect phosphorylated proteins using the CellSearch system which may mean other groups have had difficulty with this.

It is of interest that phosphorylated EGFR in CTCs was successfully measured by a group that did not use the CellSearch system (230). Patients were identified by the presence of *CK19* mRNA in their blood. PBMCs were then collected and cytospin preparations made, ICC was performed and staining for pan-CK, EGFR, pEGFR (Tyr 1068), HER2, phospho-PI3K and phospho-Akt was carried out. Although the number of breast cancer patients in the study was small, pEGFR in CTCs was visualised in two (33%) early and six (86%) metastatic EGFR-positive breast cancer patients. In addition, dual staining was achieved of EGFR with HER2, pAkt and pPI3K demonstrating that activation of the signalling pathway was apparent in a proportion of CTCs and patients. This small study provided evidence that pEGFR and other down-stream markers can be visualised on CTCs and may be a possible method for studying effects of lapatinib therapy. The drawbacks of this method are the loss of CTCs from the Ficoll-gradient centrifugation step and the technical difficulty and time-consuming nature of the method which only allowed staining of CTCs in very few patients. If staining was optimised in the CellSearch

system and with biomarkers of lapatinib response, this would allow many more samples to be analysed with greater accuracy and standardisation.

One other interesting observation made by this group, found that EGFR expression did not correlate between the primary tumour and the CTCs from the same patient. EGFR overexpression was gained with disease progression, and, in addition, a higher proportion of advanced breast cancer patients had EGFR positive CTCs (230). Data presented here also revealed a higher proportion of EGFR/CTC positive patients than in tumour sections and two patients who had a higher number of CTCs also had a greater proportion EGFR positive. As previously mentioned, evidence exists for breast cancer to progress to an ER-independent, more aggressive EGFR positive phenotype with a poor prognosis (144, 231).

Due to the difficulties of staining for pEGFR, another technique for EGFR measurement in CTCs was explored. This used CTCscope by performing *in situ* hybridisation of EGFR mRNA in addition to the pan-CK mRNAs. The hope being that the measurement of EGFR transcription would provide a predictive measure of lapatinib response since the method provides sensitivity and specificity. The CTCscope assay was originally intended for this purpose and showed some promising results in the early optimisation work (discussed in Chapter 5). The detection of EGFR transcripts using CTCscope was tested and confirmed in breast cancer cell lines with low, medium and high levels of EGFR protein expression. This correlated with the protein immunoblots and with the Quantigene assay that quantified the EGFR mRNA signal from cell lysates of known numbers.

Both the CellSearch system and early CTCscope method detected EGFR positive CTCs in an equal proportion of patients despite CTCscope detecting far fewer CTCs. When the CTCscope assay was optimised, EGFR positive CTCs were only found in one patient out of 42, although this may be due to the population of patients that was tested. These patients were all unselected patients with advanced breast cancer who gave informed consent from the clinics of Charing Cross Hospital. These patients had relatively low numbers of CTCs therefore EGFR positivity was less likely to be found. As mentioned in Chapter 5, the cell preparation for the CTCscope assay of enriching, fixing and washing may affect the level of transcription and therefore mRNA

detection, particularly in cells with low levels of expression. Also, the stochastic nature of transcription and the heterogeneous and infrequent signals obtained when detecting many mRNAs in single cells mean detection may be difficult. In addition, bursts of transcription in active cells may be buffered by the slower degradation of proteins (232). This may be happening with the EGFR mRNA and protein in CTCs. Another reason may be due to the inactive or dying state of CTCs with evidence of low Ki67 expression and the presence of apoptotic markers (130, 192). Another possibility is the poor recovery rate of CTCs using the current method using a commercial tube containing Ficoll. Other more sensitive enrichment methods are required to fully realise the potential of this method.

Many therapies are given to patients on the basis of the expression of hormone or growth factor receptors in the primary tumour. The purpose of the studies here was to assess the use of CTCs for this purpose in a real-time manner. The problems of the detection of EGFR in CTCs have been described here, therefore the measurement of EGFR mRNA transcripts in primary tumours were tested to determine if this method may be an alternative tool for the selection of patients for lapatinib monotherapy. This method, called RNAscope was also in development at the same time as CTCscope and was performed in collaboration with Advanced Cell Diagnostics, Inc, US. This was based on exactly the same principle since mRNA is retained in FFPE tumour sections. Positive controls were performed by using sections known to be EGFR positive, along with an abundantly transcribed gene *Ubiquitin C*, to ensure mRNA levels were retained in each tumour section. A negative control was also used per tissue section with no EGFR probes to ensure the specificity of the staining reagents. This method was very sensitive at detecting EGFR transcripts and a greater proportion of sections were positive when compared with paired staining of sections for EGFR protein. EGFR mRNA was detected in four times as many tumour sections as EGFR protein even at very low levels. Sections with EGFR protein also had correspondingly high numbers of EGFR mRNA, confirming its efficacy. This may have implications for the selection of patients for anti-EGFR therapies and this shows significant potential to quantify EGFR transcription in primary tumours in a reliable and sensitive way with less subjectivity than protein IHC measurement. It may be a very useful technique for the

detection of gene products where protein measurement is inconsistent or difficult. Further validation of this method is required with comparisons to IHC assessment.

The concordance between EGFR positivity in the retrospectively stained primary tumours and CTCs in patients who had advanced breast cancer was low. EGFR was present in the CTCs of most patients when measured by the CellSearch system (16 out of 18) whereas EGFR positivity was only present in 2 of the primary tumours by IHC from these patients. Even tumours stained by the more sensitive RNAscope method showed fewer patients positive than those with EGFR positive CTCs. This demonstrated that depending on the accuracy of EGFR measurement by the CellSearch system, EGFR was acquired with disease progression as mentioned previously, and shows a phenotype associated with aggressive disease (31). This is also shown by our results from the lapatinib trial.

Lapatinib clearly relies on the presence of HER2 overexpression as well as EGFR to be effective, the combined blockade therefore is important. Most of the patients recruited to the trial to test Lapatinib monotherapy had HER2 positive CTCs, despite being negative for HER2 in their primary tumour. No clinical responses were found in this group of patients even with the presence of both receptors in CTCs of these advanced breast cancer patients. All of the patients had more than 5 CTCs positive at baseline indicating a poor prognosis at the outset of the study (117). They had also received many lines of therapy (a mean of over 9 different types) so the disease had managed to evade multiple treatment types. These patients may therefore represent very advanced disease with resistant and heterogeneous tumour phenotypes that are aggressive despite a range of therapies. There is evidence from an advanced breast cancer case with a HER2 positive tumour that was refractory to chemotherapy, of a response to lapatinib. This patient showed a partial response to lapatinib/capecitebine therapy along with a decrease in the CTC/EGFR pool (233). This shows anecdotal evidence of efficacy of lapatinib which is correlated with CTC response but in a patient with a HER2 positive primary tumour.

The mechanisms of intrinsic and acquired resistance to lapatinib are a current area of interest. One study looked at the close links with ER pathways, and demonstrated a restored sensitivity to anti-endocrine therapies after lapatinib treatment of two ER positive cell models (234).

Eleven of the 12 patients treated with lapatinib in our study had ER positive primary tumours suggesting at some stage, resistance to anti-endocrine therapies occurred. This raises the question of whether this should be explored as a therapeutic strategy- for example previous therapies could be given again or in novel combinations to combat resistance (19). Other resistance mechanisms include the possible concomitant activation of multiple receptor tyrosine kinase pathways to reduce the dependence on a single pathway (235). Evidence for this was found when cells resistant to lapatinib were successfully targeted by sorafenib, a multi-kinase inhibitor (236). In addition, one group targeted the downstream molecule PI3K/mTOR by using an mTOR blocker in breast cancer cells to inhibit growth (237). This represents a promising strategy to overcome resistance to drugs like lapatinib, particularly if used in combination. This also represents a possible biomarker of response that may prevent the problems associated with EGFR and HER2 measurement in CTCs (238). Many of these studies have used cell models and may not predict what is going on in advanced metastatic disease, however. The phenotyping of CTCs to examine oestrogen receptor status or downstream biomarkers of endocrine or lapatinib resistance may be crucial in future studies involving advanced breast cancer patients.

In terms of response to lapatinib, the number of CTCs present was indicative of the poor clinical response as 9 out of 12 (75%) patients showed an increase in the number of CTCs present in 7.5 ml of blood from baseline to the end of treatment. In addition, the number of EGFR positive CTCs increased in 4 out of 12 (33%) patients and decreased only marginally in the remaining patients with only one patient showing a reduction of all EGFR positive CTCs after a cycle of Lapatinib. This demonstrates the poor response of this population of CTCs to lapatinib monotherapy. This again reveals that EGFR expression measured by a widely available antibody (used in the CellSearch system) has little predictive effect of anti-EGFR therapies, a finding also shown in other studies (239, 240). The use of CTCs for assessing therapy response is therefore relevant but not the specific measurement of EGFR in CTCs.

This study provides an insight into the use of CTCs for monitoring patients on a therapy and their potential use for selecting patients for therapy. The population of patients studied here

are a group that are in most need of attention due to the aggressive nature of their disease. The characterisation of CTCs is crucial to study the mechanisms of resistance or lack of response to therapies such as lapatinib. This may then enable the switch of a therapy to one that is more suitable for the patient rather than waiting for scans to assess the disease response.

The study has particular issues due to the complexity of EGF signalling that is still not fully understood. It is not known if the very advanced nature of these patient's disease shows the presence of stem cell-like properties or the transition of CTCs to a more EMT phenotype. In turn this may create difficulties for detecting CTCs that have lost epithelial features. This may be an additional reason for such low detection on EGFR transcripts in the metastatic breast cancer patients. An alternative explanation may be that the cells detected have low proliferative potential and are not transcribing genes, because EGFR protein on CTCs was detected in high numbers. In addition this may show the metachronous nature of EGFR expression or the fact that it is a broadly expressed protein expressed at low or undetectable levels in all CTCs and many tissues with little predictive relevance for anti-EGFR therapies.

The study of EGFR and its role in breast cancer is therefore a complex one which needs further investigation. The detection of down-stream molecules of the EGF pathway may help in the quest to discover the most effective predictive markers of Lapatinib response. Multiple biomarkers of response in CTCs may therefore be required. The study of CTCs is a very useful tool for learning more about advanced breast cancer in a real-time approach.

7.0 Conclusions

These studies have shown the potential prognostic and predictive use of CTCs in both the early and advanced breast cancer settings. The importance and necessity of very sensitive and specific detection of CTCs is clear for the appropriate identification and stratification of patients who require further adjuvant therapy in the early breast cancer setting. It is also crucial for the predictive value of systemic therapies in progressive disease. The field is at a very exciting point in time with the combined advancement of sensitive technologies to detect cells along with promising new methods of molecular characterisation. This in turn will provide important clues to unravel the mechanisms involved in tumour dormancy and metastasis and enable suitable ways to target them.

Our long-running studies looking at minimal residual disease over time in patients with a past primary breast cancer diagnosis showed that a surprisingly high proportion of patients have evidence of residual cancer present. This is despite the majority being clinically disease-free and remaining so, over many years. The presence of CTCs is higher in patients with involved lymph nodes at surgery, demonstrating a possible prognostic role since lymph node involvement at surgery is currently the most important indicator of a worse prognosis. This discovery has been possible by using the current, most standardised method for detecting CTCs (The CellSearch system) and two crucial refinements: taking larger volumes of blood and frequent testing. We also find a correlation between CTCs and DTCs (ICC), the latter being an independent predictor of a worse prognosis. Importantly, this implies that CTCs are also relevant for prognosis and are released in a fairly consistent manner over time.

These findings have been confirmed in recent trials measuring CTCs in early breast cancer patients using a larger volume of blood. These studies assessed patients soon after surgery though and tested the levels of CTCs before and after adjuvant therapies (186, 188). Survival analyses of patients in these trials will give further insight into the prognostic use of CTCs present in apparently disease-free patients. As we have shown, the detection of CTCs many years after surgery may have a different relevance and may show more cells that are not

prognostically relevant. Future survival analyses from the set of patients studied here will also allow the prognostic relevance of these CTCs to be established, with possible additive information from other evidence of MRD. This may be particularly important with the finding of rare false positives or epithelial cells in healthy and benign controls.

The correlations of different measures of MRD in breast cancer patients showed an interesting inverse relationship between cell death (as assessed by the presence of circulating DNA in the blood), with tumour cells measured by qRT-PCR in the bone marrow. In addition we found an inverse correlation of cfDNA with positive lymph node status. Since the vast majority of patients had evidence of cfDNA at different points in time we were able to identify these inverse correlations. These relationships have not been identified before in primary breast cancer patients partly due to the lack of studies with long follow-up, and the lack of assessment of multiple sensitive methods to detect MRD. Our studies provided evidence of possible cycles of cell proliferation in the bone marrow, followed by cell death (cfDNA in the blood). This may represent micrometastases that are kept 'in check' by unknown systemic mechanisms. We demonstrate evidence that patients who developed metastatic disease, showed signs of MRD prior to relapse with a lowered level of cfDNA, a sign that tumour cell death was not occurring and dangerous metastases were able to proliferate. Long term survival data may help to confirm the predictive use of these measurements, along with characterisation of the mechanisms of cell death. This in conjunction with highly specific CTC detection may provide a useful aid to assessing the MRD load, phase of cell cycle (proliferation or cell death) and phenotype in a given patient with a past primary breast cancer. This represents a fascinating insight into the possible mechanisms of dormancy in breast cancer. By examining these processes, novel strategies to keep micrometastases in this dormant state may be possible.

We know that the presence of *CK19* mRNA in blood and bone marrow confers a worse prognosis to the patient. This still does not provide critical information such as the likelihood of relapse or when relapse may occur or even the phenotype of these cells. The importance of detecting viable, relapse-inducing occult tumour cells particularly with the specificity only given with ICC methods is therefore very important. This area is gaining prominence with the use of

new innovative ways to detect cells that are still viable (67, 129). This, along with the phenotyping of cells for cell death and proliferation markers will help to determine their prognostic and predictive potential. This is especially important since evidence from this study and others point to only a small proportion of micrometastases being relapse-inducing in the right conditions (205).

Our work to optimise the cell preparation for a novel method to detect viable CTCs by amplifying mRNA signals of CTCs from blood revealed the great promise this technique holds. This is a unique and specialised method that can detect CTCs easily in amongst leukocytes in a highly specific and sensitive way. The hope was that the grouping of several tumour markers, including the traditional cytokeratin epithelial markers with markers of EMT and metastasis would enable the superior detection of CTCs. The difficulty in optimising the cell preparation however, both in terms of enriching the cells and ensuring the RNA was not degraded was the main problem with this method. The other possibility is that the detection of viable CTCs is not a suitable avenue to explore since we know that CTCs detected by the CellSearch may be dead or dying and have a short half-life and yet still show prognostic relevance. We are currently looking into ways to assess and circumvent these issues. Our group and collaborators are currently exploring the use of CTC separation by size using filtration devices to ensure a more gentle method to detect CTCs. This, along with the further optimisation of fixation techniques may help to retain features that may be lost with fixation and washing cycles. This in turn should enable CTCscope to detect all the viable CTCs present in a blood sample along with important predictive markers for therapy efficacy.

The use of CTCs in the metastatic setting has been proven to be clinically useful by predicting a worse prognosis (117). Furthermore, CTC assessment before and after systemic therapies gives a more reproducible and earlier indication of disease status than traditional radiology scans (241). Future studies to assess a change in therapy based on CTC testing are critical to prove the use of CTCs in the clinic. Current ASCO guidelines still do not advocate their use, despite the burgeoning interest and number of studies studying them (115). Our study of lapatinib monotherapy in pre-treated advanced breast cancer patients confirmed the use of CTCs to

assess response to therapy. This study unfortunately found that all evaluable patients demonstrated progressed disease which was reflected in the increase in CTC number in most of these patients. The importance of characterising the changing nature of progressive disease by phenotyping CTCs was also demonstrated. Two patients showed a transient response which was followed with progression within weeks. Studies in breast cancer cell models looking at resistance to lapatinib point to important links of a co-dependence to ER pathways or other receptor tyrosine kinase pathways, this remains largely unexplored *in vivo*. The use of CTCs is therefore a very important tool for studying targets to overcome resistance to therapies. The assessment of multiple biomarkers may also be required to achieve this. Sophisticated modelling methods to find biomarker profiles in CTCs (and other blood markers) that are predictive of therapy response could be a valuable tool in the future (242).

CTC research is gaining momentum as increasingly novel technologies improve to detect tumour cells efficiently from the blood along with the capability to characterise them further. The recent collaboration of the two most promising technologies for CTC detection means the highly sensitive and specific detection of CTCs is on the horizon. This is anticipated to combine the standardisation and reliability of the CellSearch system with the sensitive, gentle and specific capture methods employed in CTC-microfluidic devices. This allows the characterisation of intact, viable CTCs. Many other groups are now hot on the heels of CTC-chip technology; the single cell proteomics group at Imperial College London are optimising these methods with additional proteomic analysis. Their routine prognostic and predictive use in the clinic is therefore likely to be forthcoming in the future. This in turn will enable the study of the processes of metastasis alongside clinical trials of novel combinations of drugs.

8.0 Appendix

Table 8.1: Target probe sequences for the detection of CTCs using CTCscope - CK8, CK14, CK17

CK8	**CK14**	**CK17**
cggtgatgcctcccatgc	gagacagacaggccgccc	accaccagagccaaagctgta
ggctctggttgaccgtaactg	cagaggagaagcgggaggat	caaagctgctgccatagcc
ggacaaaggggctcagca	cagcccgtaggctcccc	gcagcccatcaacacccc
atgttggggtccacctcca	tctttgatctcagcaggccg	gccttctcacctccagcca
gtgcgcacggcctgg	aatggtcttgaagtagggactgtag	gtcattgaggttctgcatggtg
ttgatctgctccttctcctgg	gagaatcttgttcctcaggtcctc	caggtaggaggccaggcg
ggcaaacttgttgttgagggtc	ggcattgtccactgtggctgt	cagggcacgcaccttgtc
gaaccgtaccttgtctatgaagga	ttgtcaatctgcagaaggacatt	cagctcagtgttggcctcctc
gcatcttgttctgctgctccag	gcggccagacgggca	accagtcacggatcttcacctc
gaggctccacttggtctcca	acttggtgcggaagtcatcc	cggggcctgcctctggt
ccgtcttctgctgctgcag	cgcaggttcaactctgtctcat	tcacgggcgggccc
atgttgtccatgttgcttcgag	tgtcggcttccacactcatg	caattgtcctgtagtactggctgtag
aggttgttgatgtagctctcgaac	cctgcgcaggccattga	aggatcttgttctgcagctcct
agtctccagctgccgccta	agggtcagttcgtccagcac	cattgtccacggtggctgtg
tcagcttctcctggcccag	ctccaggtcagctctggcc	gtcaatctgtagcaggatgttgg
caagctccgcctccagct	ccttcaggctctcaatctgcat	agcagccagacgggcatt
ccagcccctgcatgttgc	tcttcaggtaggccagctcct	aaacttggtgcggaagtcatc
tcatacttgttcttgaagtcctcca	ttcatctcctcctcgtggttct	gcagggcctgctctgtctc
ctctgtacgcttattgatctcatcc	acctggcctctcagggca	cggcctccacactcaggc
atgaggacaaattcgttctccat	tccacattgacatctccaccc	tgcgcaggccattgatgt
aagcttcatccacatccttcttg	ccaggtgcagcgtccatc	ggtcagctcatccagcaccc
actccagctctaccttgttcatgt	tgcggctcaggtccacg	caggtcggctctggccag
tcagcccttccaggcgag	ggtcacgcatctcgttcagaa	cttgaggttctcaatctgcatctc
tgaggaagttgatctcgtcgg	ttcttctctgccatcttctcatact	ttcaggtaggccagctcctc
ggatctcctcttcatatagctgcc	cctcggcatccttgcgg	catctcctcctcgtggttcttc
gatctgggactgcagctccc	cctctgtcttggtgaagaaccatt	ggcctcgcagggcgtt
cagcaccacagatgtgtccga	ccacctcgcggttcagct	acattgatctcaccacccacct
gcggctgttgtccatgga	ccagctcgctgttggtgg	ggggcagcgtccatctcc
atgctgtccatgtccaggga	cgctcttgccgctctgca	cggctcaggtccacgcct
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cggttggcaatatcctcgtac	ctccaggttctgcatggtgc	ttctctgccatcttctcatactgg
cagcctcagcccggctg	agctgggactgcagctcaat	cggcatccttgcggttc
ctcatacttgatctggtacatgctct	ccagggatgctttcatgctg	tgtcttgctgaagaaccaatcct
cagccaggctctgcagctc	tggtctcctccaggctgttct	cctcgcggttcagttcctc
ggtcatccccgtgcttcc	gctgcatgcagtagcgacctt	cagctcactgttggtggcca
cagtctttgtgcgccgca	atctcctggatctgggcca	tctcactcttgccactctgcac
gttccggttcatctcagagatct	ctcctccacgctgccaatc	cgccggagctccgaga
gcctggagccggctgat	cggagctgggccagctg	tccaaggcctgcatggtg
cctttgaggccctcaatctca	ctgctgctccatctcgcag	gagctgggactgcagctctatc
tccagggaagccctctgg	agcaggatcttgtactcctggtt	tccagggatgctttcatgct
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gctctccacgctgctcgg	tggcgatctcctgctcca	cacgcagtagcggttctctg
ttggcatccttaatggcca	ggatgactgcgatccagagga	ccctggatctgggacagctg
cagctcggacaacttggcg	ggctggaggaggtcacatctct	tccacgctgccaatcagc
	ccttggtgcggatttggc	gctgggccagctgctcc
	gccatcgtgcacatccatga	gctccatctcgcagcgaa
	cgtgggtggacaccacctt	ggattttgtattcctggttctgct
	ttggtgcgaaggacctgct	cgcgtcttcacatccagca
	ggctgggcagcctcagttc	ggcaatctcctgctccagc
	cctcctaggcctgagcgg	agcaggcggcggtaggt
	tgtgtccacacgggggg	tgggcatcctctccctcc
	ggggatcttccagtgggatc	cggttctttcttgtactgagtcagg
	tgaagtgcttgggcaggaga	gcacctgacgggtggtcac

Table 8.2: Target probe sequences for the detection of CTCs using CTCscope - CK18, CK19, CK20

CK18	**CK19**	**CK20**
cccattcctgccagaccc	tcatgcgcagagcctgttc	cactgttagacgtattcctctctcagt
tccttctcgttctggatgcct	tgatgtcggcctccacgc	caggccttggagatcagcttc
cgttcaggctttgcatggtc	ccctgcgcaggccgt	gggttaggtcatcaaagaccttatt
ggtaagaggccaggcggt	cagggtcagctcatccagca	tttgaatctccaaatctgttttatgta
ccaggctcctcactctgtcca	ctccaggtcggtcctggc	agctaggtctttattcagttcttcaa
agcctccggttctcggtct	ttcaggccttcgatctgcat	cctgatgctccttttgaggag
ctcccggattttgctctcc	tcaggtaggccagctcttcc	tgtgtaggccatcgacttcct
gggtcccttcttctccaagtg	tgatttcctcctcatggttcttct	tgacagtgttgcccagatgct
aatggctccagtctctgacctg	acttggcccctcagcgtac	gagcagcatcaacctccacat
caggtcctcgatgatcttgaagt	cacactgacctggcctccc	cgccaaggttcaggcctg
tttgcgaagatctgagccct	cggagcggaatccacctc	ctgcctcatttcattcatgatga
gcgggcattgtccacagta	ttggcgagatcggtgcc	ttctgggccatgacttcatactt
gcattgtcaatctgcagaacgat	gcttcgcatgtcactcaggatc	ttctttggcctcttgaaggttc
aagtcatcagcagcaagacgg	ctcggccatgacctcatattg	gaactgcagtctgtctctcaaactg
ccagctctgtctcatacttgactcta	tcagcatccttccggttctg	tcactgtgacctgttgctgca
ccacagactggcgcatgg	cggctggtgaaccaggct	cctcagttccttttaattcttcagtat
gagcccatggatgtcgttct	tcccggttcaattcttcagtc	cgtctcagctccgttagttgaa
ggtgtcatcaatgaccttgcg	ccgtgtggccagcgacc	tctcaaggctctgggaggtg
ccagctgcagtcgtgtgatatt	cctgctcatctggagctgct	gctgagatgggactggagttcta
cttgagagcctcgatctctgtct	cgcaggtcagtaacctcgga	agtgtgctccaaagactctttcat
ttcttcatgaagagcagctcctc	agaccctgaagggtgcgc	cgggccttggtctcctctag
ccttttacttcctcttcgtggttc	gctgtgactgcagctcaatctca	ttggctaactggctgctgtaa
tggcaatctgggcttgtagg	caaggcagctttcatgctca	agctcaacagcgactggagg
tccacggtcaacccagagc	ccgtttctgccagtgtgtcttc	catcagttgggcctccagag
tgagatttgggggcatctacc	gctccaaagcgcgcct	cgttccatgttactccgaatctg
ccatgatcttggcgaggtcc	tggatatgcgccagctgg	aggatatggtattcgttgttctgg
ttgggcccggatgtctg	aataccgctgatcagcgcc	gttcaagtcgagtctttatgtcaaga
tccgagccagctcgtcata	cgcccagctgggcttc	ggcggtaagtagcaatttcct
cttgtctagctcctctcggttct	cgctcactatcagctcgcacat	cgtcttctccttccagaaggc
tcctcaatctgctgagaccagta	cgctggtactcctgattctgc	ggtgcttaactgatattctgtagttttta
gtggtgaccactgtggtgctc	cgcgacttgatgtccatgagc	ggttttctttatatctctctcttccag
agctccaacctcagcagactgt	ggcaatctcctgctccagc	ttgcacgactgtcttaatcttcct
ctctgtgagcgtcgtctcagc	cgagcaggctgcggtaggt	cgaccttgccatccactacttc
caaggactggactgtacgtctcag	tgttgtagtgatcttcctgtccct	ccacctctttgacttcagatgaca
tcatggagtccaggtcgatctc	ggaccttggaggcagacaaat	ccttctggtagctatttagatattttctt
ccaagctggccttcagatttc	cagagcctgctgcctcaga	tttcaaaacctcagcagcatct
ccacctccctcaggctgttct	ccaaaggacagcagaagccc	gcaaagataagattatagccaaatttc
agggcgtagcgggcct	ccctctacccagaagacaccct	tggctgatttcttgcaggga
ccgttgagctgctccatctgt		actgcagagtattaatagtgctttctta
gactcaaggtgcagcaggatc		ccaccccacccctttctaatc
ccgggtctgtgccagctct		agagtctgataaaataggattcccg
tggcgctgtccctctgc		cctctgagtaaaacatttatattcaattac
ggcctcatactcctgggcc		ttgcaggcaatttgcagct
gcttgaccttgatgttcagcag		ttctagtgctcactggatttcattt

Table 8.3: Target probe sequences for the detection of CTCs using CTCscope - EpCAM, Muc1, Twist

EpCAM	**Muc1**	**Twist**
ccgctggtgctcgttgatg	cgctggctgcttgagagg	gggagttgggcgagagctg
cgggactgctcacctctgg	ggcagaacagattcaggcagg	agagcccgcgaggtgtct
gcctctcggccccttcc	tgaaatgggtggggaggg	acggtgccggtgctgc
gcgcgcggcgggg	ccggtgtcatggtggtgg	ccgccgccgctgctgc
ccgaaggactagcgctctgt	ggaagaaaggagactgggtgc	cgccaggcctcctggaa
gtcgaaggtgctcgctcg	cactgtgaggagcagcagca	gggacctccgcgggga
gggtccccggaccgc	ccagaacccgtaacaactgtaag	gcaggaggacggacggga
ccgaacgcgaggacctg	cctggggtagagcttgcatga	tcatctctcgagcggcga
ccgcggcaagcagaagc	gccgaagtctccttttctcca	cgagctggacacgtcctgca
ggcaaaagtcgccgtcg	gggcactgaacttctctgggta	gctgttgctcaggctgtcgt
cagacacattcttcctgagctgc	acaaagcattcttctcagtagagct	gccggtctggctcttcctc
acggccagcttgtagttttca	ggaaaaagaaagagaccccagtag	tcctcgtaagactgcggactc
gacgattattattcacaaagcagttt	ggaggtttgaaatgtgaaaagaca	cccgctgcgtctgcagc
caccaactgaagtacactggcatt	ggatcttccagagaggaattaaact	ccgcacgttggccatga
tttgagcaaatgacagtattttgtg	gctcttggtagtagtcggtgctg	cttgctcagcttgtccgagg
caaacatttggcagccagc	aacatttcagaaatgtctctctgca	cgccagcttgagggtctgaat
ccattcatttctgccttcatcac	ccccttgtttataaatctgcaaa	agaggaagtcgatgtacctggc
tgctcttctcccaagttttgag	tattggagaggcccagaaaac	tcgctctggaggacctggt
ggagggccccttcaggttt	cacagatcctggcctgaacttaa	ccatcttggagtccagctcg
gatcataaagcccatcattgttct	aaggccagagtcaattgtaccac	gagccacatagctgcagcttg
ccgctctcatcgcagtcag	ggacattgatggtaccttctcgg	cgtagctgagccgctcgt
cactgcttggccttaaagagc	ttgaactgtgtctccacgtcgt	catcctccagaccgagaagg
gcacatggaggtgccgttg	ggctgcttccgttttatactga	catggaccaggccccctc
cccagcagtgttcacacacca	ctgagatcgtcaggttatatcgaga	ctgctagtgggacgcgga
cagtgtccttgtctgttcttctgac	gcacatcactcacgctgacgt	tctctagactgtccattttctccttc
cactcgctcagagcaggttattt	gactgggcagagaaaggaaatg	ttatttttagttatccagctccagag
gttcaatgatgatccagtaggttct	ggcaccccagcccca	aatttggattttgctcttctaattt
ggtttttctcttgctttgtgtttta	cgcgatgccccagcct	cccacgccctgtttctttg
cagtccgcaaacttttactatcataa	aacacagaccagcaccagcag	ctgtccacgggcctgtctc
cgttgtgatctccttctgaagtg	atggccagcgcaaccag	gctgcccgtctgggaatca
aaattttggatccagttgataacg	agccaaggcaatgagatagaca	cagaggtgtgaggatggtgcc
cattattctcatacaaaatactcgtgat	tcggcggcactgacagac	acaactgttcagacttctatcagaatg
aattttgaaccagatcaatagtgataa	tccagctgcccgtagttctt	cacgacctcttgagaatgcatg
cacatcattctgagtttttgagaag	tcccgggctggaaagatg	ttcctttcagtggctgattgg
aaataataagccacatcagctatgtc	tactcgctcataggatggtaggta	agttcctctgattgttaccattttaa
ggattcacctttaacatctttttca	atgggtgtggtaggtgggg	cagtatttttatttctaaaggtgttcttat
ggtccattttcttagaatgaaacaa	ggggcacatagcgccc	tttttcccctcagaggaagga
cagttgttccccatttactgtca	gggctacgatcggtactgctag	tgtttttgtattttaagttttttttg
aaagtttgaccaggatccagatc	attacctgcagaaaccttctcatag	tgggtcctcaataaataaatagaatgt
ggtgctttttcatcaacataataaatt	aagagaggctgctgccacc	ccggatctatttgcattttacca
ttagaccctgcattgagaattca	tgccactgctgggtttgtgt	tcataaaaatatgaatgcatttagaca
caataacagcaataacaccagctt	cctacaagttggcagaagtggc	gaaaaatatacaaagatatttacaaaacaa
aacaactgctatcaccacaacca	agcgggcgacgtgcc	
aaccagcacaacaattccagc	ctggctggccactcagctc	
gccattctcttctttctggaaat	cctgagtggagtggaatggca	
cctttatctcagccttctcatacttt		

Table 8.4: Target probe sequences for the detection of CTCs using CTCscope - N-Cadherin, Fibronectin, EGFR

N-Cadherin	Fibronectin	EGFR
acagacgcctgaagcaggg	acgaacatcggtgaagggg	tgagggcaatgaggacataacc
gcgatttcaccagaagcctct	tgcggttggtaaacagctgc	aaaggaattcgctccactgtgt
tcaggaaatccagtcttgcataat	gaggctgggggtgaggc	tctgatgatctgcaggttttcc
tcgataagactgcactgtaaacatct	tgacacagtggccatagggag	cataggaattttcgtagtacatatttcc
gctgtccttcatgcacatcct	acagagtagaccacaccactgtctg	gcatcatagttagataagactgctaagg
gttgctaaacttcacattgagaagag	cttcagccactgcatcccc	gctccttcagtccggttttattt
ttgtacttttcttttccattgca	aagcatttgcttatttccttgtgt	tcctgtaaatttctcatgggca
ctgcaggctcactgctctcata	tgcccaggcacgtgca	cggcgccatgcaggatt
gccatcttcatccaccttaaaat	ctcttggcagctgactccgt	gggttgttgctgaaccgca
gcttctcacggcatacaccat	ccgtaagtctgggttacagctgt	tccacgttgcacagggca
gcatgctcagaagagagtggaaa	ggctctccatttgagttgcca	tcccgccactggatgctc
cttgggcatatatcaggaacttg	cattgtaggtgaatggtaagacacat	gctgagaaagtcactgctgactatg
ccacttttcctgggtctctttgt	tgcaggagtagaacgtcctgc	tctggaagtccatcgacatgtt
ggctcaattttactgccacttg	tcctgtcgccctctgtgg	gcagctgcccaggtggt
tcctcagttaaggttggcttca	tgctgcaccaaagatgtccg	ggacagcttggatcacacttttg
acttctgctgactccttcactgac	gtcctgctcataattcgaagttg	ccccagcagctcccattg
tgtcttgggaacactatttcttca	ggtctgtgcagaaagagtatttctg	ggcagttctcctctcctgca
gtggccactgtgcttactgaat	tcgagtctgaaccaaaacagtgt	gcacagatgattttggtcagtttct
ccagtctctcttctgcctttgtag	gcaccattggaatttcctcc	gcccggagcactgctgg
gcaagttgattggagggatgac	ggaaggggaagtggcacaag	ggacttgccacggcagc
aaggtcccctggagttttctg	caatcagtgtaattgtggttgttgtata	tgtggcagcagtcactggg
gatcctgacaagctcttgaggaa	tgtctcttctgccctcagaagtg	cagcctgcagcacactggt
tgaaaggtttttatctctatcagacct	tggtcccacaccacttcatgt	tcccgggggcctgtg
gcccagttacactgtaccgcag	tggtcggcatcatagttctgtg	gcagaccaggcagtcgctc
ggaggctggtcagctcctg	tggggcagaacccaaacttc	cttcgtctcggaatttgcg
ggggttgataatgaagataccagtt	ttcctcgtgggcagcca	caggtgtccttgcacgtgg
ccgacagctgacccgagat	cccttcattggttgtgcagat	tgtagagcatgagtgggggg
cgatccaggggctttgtca	gatctccaatgcggtacatgac	catctggtacgtggtggggt
aaaccgggctatctgctcg	catgtcatgctgcttatcccact	gccctcggggttcacatc
tactgcatgtgccctcaaatg	cgtgcacctcatcatgtgacc	ggtggcaccaaagctgtattt
ttctccacttgatttccattaatatc	ccacgaccattcccaacaca	ggggacacttcttcacgca
attgatgacaatgtcaatgggg	aggcaatgcatgtccattcc	ccgtgatctgtcaccacataattac
ggtctgttgtcattcatgtcaataac	cactgatctcgaagctgcgagt	caggctcggacgcacgag
tccaaacctggtgtaagaactca	cattgtaagtgatgtcatcaacaatg	tcatagctgtcggcccca
atccctcaggaactgtcccat	gcttgtggaatgtgtcgttca	gacgccgtcttcctccatc
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cagcatcaattgctgttacggt	ctgaccgaagcatgtacagttca	ctttgcggcaaggccc
ttgagggcattgggatcgt	ttccacctgccccgacc	caccaatacctattccgttacaca
cgattctgtacctcaacatccca	attggtcgacgggatcacac	gcatttatggagagtgagtctttaaatt
ggtgcttggagcctgagaca	tcccagtctctgaatcctggc	gcagtttttgaagtgtttaatattcgta
gattgtaaacatgttgggtgaagg	catgaatctccaatttgataaaaacg	gagatcgccactgatggaggt
	tgacaccatgcacatacttctcc	gccaccggcaggatgtg
	gccatagcagtagcactggtatc	gtgtgaaggagtcacccctaaat
	ccactccccaatgccacg	ctgtggatccagaggaggagtat
	aggtctgtaaaggttggcaatg	ttacggttttcagaatatccagttc
	acaggaccacttgagcttggat	agcaaaaaccctgtgatttcct
		tttcaggccaagcctgaatc
		gcatggaggtccgtcctgt
		gcgtatgatttctaggttctcaaag
		catgttgcttggtcctgcc
		tgacgactgcaagagaaaactgac
		cccaaggatgttatgttcaggc
		ttatctccttgagggagcgtaat
		cctgaaattatcacatctccatcac
		tttgcatagcacaaatttttgttt
		ccaaacagtttttttccagtttattgta
		tggttttctgaccggaggtc
		gttttcacctctgttgcttataattt

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Comparison of bone marrow, disseminated tumour cells and blood-circulating tumour cells in breast cancer patients after primary treatment

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The purpose of this study was to determine whether primary breast cancer patients showed evidence of circulating tumour cells (CTCs) during follow-up as an alternative to monitoring disseminated bone marrow tumour cells (DTCs) by immunocytochemistry and reverse transcriptase (RT)–PCR for the detection of micrometastases. We planned to compare CTC and DTC frequency in low-risk and high-risk patients. We identified two cohorts of primary breast cancer patients who were at low (group II, T_1N_0, $n=18$) or high (group III, >3 nodes positive (with one exception, a patient with two positive nodes) $n=33$) risk of relapse who were being followed up after primary treatment. We tested each cohort for CTCs using the CellSearch system on 1–7 occasions and for DTCs by immunocytochemistry and RT–PCR on 1–2 occasions over a period of 2 years. We also examined patients with confirmed metastatic disease (group IV, $n=12$) and 21 control healthy volunteers for CTCs (group I). All group I samples were negative for CTCs. In contrast, 7 out of 18 (39%) group II primary patients and 23 out of 33 (70%) group III patients were positive for CTCs ($P=0.042$). If we count only samples with >1 cell as positive: 2 out of 18 (11%) group II patients were positive compared with 10 out of 33 (30%) in group III ($P=0.174$). In the case of DTCs, 1 out of 13 (8%) group II patients were positive compared with 19 out of 27 (70%) in group III ($P<0.001$). Only 10 out of 33 (30%) patients in group III showed no evidence of CTCs in all tests over the period of testing, compared with 11 out of 18 (61%) in group II ($P=0.033$). A significant proportion of poor prognosis primary breast cancer patients (group III) have evidence of CTCs on follow-up. Many also have evidence of DTCs, which are more often found in patients who were lymph node positive. As repeat sampling of peripheral blood is more acceptable to patients, the measurement of CTCs warrants further investigation because it enables blood samples to be taken more frequently, thus possibly enabling clinicians to have prior warning of impending overt metastatic disease.
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Breast cancer is the most common cancer among women in the United States and Europe. Although mammographic screening and the judicious use of adjuvant systemic therapy have improved the survival from this disease, many patients still relapse and develop metastatic disease. Metastases inevitably result in the death of the patient.

Over the past 10 years, long-term follow-up of patients in trials designed to evaluate adjuvant endocrine or cytotoxic chemotherapy has indicated that cure can be achieved in a proportion of patients by these treatments. For this reason, considerable efforts have been made to discover a means of monitoring these patients, in the hope of finding a test that would distinguish those who need further sequential adjuvant therapy from those for whom this treatment would not be necessary. This has become particularly important on account of our recent studies showing that 'interval' or 'switching' techniques improve disease-free (Coombes *et al*, 2004) and overall survival (Coombes *et al*, 2007).

Several studies have now shown that the presence of occult metastases in the bone marrow identifies a population of patients at high risk for recurrence (Redding *et al*, 1983; Cote *et al*, 1988; Diel *et al*, 1996; Mansi *et al*, 1999; Braun *et al*, 2000c; Wiedswang *et al*, 2003; Naume *et al*, 2004). In our original study (Neville *et al*, 1983), the presence of bone marrow occult metastases was correlated with tumour stage and vascular invasion, both of which are known predictors of poor prognosis. Other studies of note are Diel *et al* (1996) and Braun *et al* (2000b) who analysed bone

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marrows from 727 and 552 patients, respectively, using immunocytochemistry (ICC). Both these studies have shown that the occult bone marrow metastases were associated with larger tumour size, lymph mode involvement and high-grade tumour. The presence of micrometastases in bone marrow at surgery has been shown to be an independent prognostic factor in 817 breast cancer patients (Wiedswang *et al*, 2003). In a pooled analysis of 4703 patients with stages I, II or III breast cancer with 10-year follow-up (Braun *et al*, 2005), micrometastases at diagnosis were detected in 30.6% of patients. Those patients with micrometastases had larger tumours and higher histological grade and more frequent lymph node metastases and hormone receptor-negative tumours. This study shows that the presence of micrometastases in the bone marrow at the time of diagnosis of breast cancer was an independent predictor of a poor outcome and is associated with poor prognosis.

In studies from our laboratory and from others, it has been shown that it is possible to detect residual disseminated bone marrow tumour cells (DTCs) in the bone marrow, and circulating tumour cells (CTCs) in the peripheral blood during follow-up. However, the number of cells in the bone marrow is small even after using the best available techniques (i.e., using a pancytokeratin antibody (A45-B/B3), which is well characterized for DTC studies (Braun *et al*, 2000b)) for staining 2×10^6 cells and analysing automatically. On account of the difficulties inherent in subjecting patients to regular bone marrows, as well as the small number of cells detected in primary patients with no evidence of overt metastases, such tests have been difficult to apply in routine clinical practice. Furthermore, until recently, the peripheral blood has been shown to be generally negative for CTCs in all but a minority of patients, with most series showing 1–5% of patients found to be positive (Schoenfeld *et al*, 1997; Slade *et al*, 2005).

Many groups have attempted to develop a reproducible methodology for CTC detection to improve the detection rate. One of these is the CellSearch™ (Veridex LLC, Warren, NJ, USA) system with which Cristofanilli *et al* (2004) carried out a study of 177 patients with metastatic breast cancer. The results from this approach indicated that approximately 70% of patients with metastatic breast cancer have >1 CTC/7.5 ml of peripheral blood. They also found that patients with five or more CTCs per 7.5 ml of blood had a significantly worse progression-free and overall survival than patients with <5 cells per 7.5 ml of blood (progression-free survival was 2.1 *vs* 7.0 months and overall survival was 8.2 *vs* 18 months). It has subsequently been shown (Hayes *et al*, 2006) in this group of patients that elevated CTCs at any time during therapy are an accurate indicator of rapid disease progression and mortality.

We therefore decided to perform a pilot study to determine whether monitoring for CTCs using the CellSearch system was comparable with our current micrometastatic monitoring system using ICC and reverse transcriptase (RT)–PCR of bone marrow samples. This was performed in a group of patients on follow-up after a diagnosis of breast cancer, and none of them had evidence of overt metastases.

MATERIALS AND METHODS

Patients

The study was approved by each local ethics committee and conducted in accordance with the declaration of Helsinki and all patients gave written informed consent. Samples were blinded for analysis and patients understood that the results would not be made available to them. Patients diagnosed with early stage breast cancer, who were on routine follow-up, were invited to take part in the study. All women were attending local hospitals in the West London Cancer Network. All patients had previously histologically confirmed primary breast cancer and no evidence of distant metastases on chest radiology, bone scanning or liver ultrasound. It was not possible to compare the two sampling sites (i.e., blood and bone marrow), as the samples were not taken at identical time points for ethical reasons.

Breast cancer patients

To have a group of patients who were at very low risk of having recurrence in the future, we decided to take the subset of patients from our published study (Slade *et al*, 2005) to include only those patients with T_1N_0 (i.e., those who had a tumour $\leqslant 2$ cm)and who were node negative (group II) 4–13 years post-surgery. For the high-risk group (group III), we included patients who previously had >3 node-positive breast cancer (with one exception, a patient with two positive nodes) and were on follow-up and who had no sign or symptom of recurrence. We examined 3×7.5 ml samples of blood from all of these patients together with the same amount of blood from a cohort of women with no past history of breast cancer (negative control – group I). We repeated the sampling procedure 1–7 times over a 2-year period. As positive controls, we took 1×7.5 ml blood from a group of 12 patients with overt metastatic breast cancer (group IV).

Preparation of bone marrow samples

The skin was incised before the aspirates were taken to minimise the risk of epithelial contamination. Between 2 and 5 ml of bone marrow was aspirated into syringes primed with preservative-free heparin (Leo Labs, Risborough, UK). Bone marrow samples were prepared as described earlier (Slade *et al*, 1999; Smith *et al*, 2000). Ficoll (GE Healthcare, Chalfont St Giles, UK) was used to separate the mononucleocytes; these were counted and aliquoted for ICC on the basis of ideally 6×10^6 cells for each methodology but with a minimum of 3×10^6 cells for each. Those undertaking the ICC were blind to the clinical status and identity of the patients and their earlier assay results.

Immunocytochemistry

Cells were cytocentrifuged at a concentration of 5×10^5 per cytospin, air dried and stained as described earlier (Pantel *et al*, 1994; Slade *et al*, 1999; Smith *et al*, 2000). A total of six areas were stained, of which four were stained for the presence of cytokeratin-positive cells and two were isotype controls. The primary antibody (A45-B/B3 Micromet, Munich, Germany) directed to a common epitope of cytokeratin (Stigbrand *et al*, 1998) was used at $2\,\mu g\,ml^{-1}$. The rabbit anti-mouse antiserum (Z259; Dako, Hamburg, Germany) and the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (D651; Dako) were used as recommended by the manufacturer and the reaction developed with new fuchsin. An isotype IgG1 mouse myeloma antibody MOPC-21 (Sigma Aldrich, Poole, UK) served as negative control and the MCF-7 cell line as a positive control (Pantel *et al*, 1994; Slade *et al*, 1999; Smith *et al*, 2000). The cytospins were counterstained with haematoxylin and screened using the Automated Cellular Imaging System (ACIS) (Carl Zeiss Ltd, Welwyn Garden City, UK). Samples that were isotype positive were deemed uninterpretable and therefore excluded from the results.

CellSearch

Blood, 7.5 ml from the metastatic patients and 3×7.5 ml from the control and primary breast cancer patients, was collected in CellSave™ preservative tubes (Immunicon, Huntingdon, PA, USA) from patients in London, anonymised, and transported at room temperature to either the Institute of Tumor Biology in Hamburg or the Department of Oncology, Imperial College London for processing within 72 h of collection as recommended by the

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manufacturer. The CellSearch system was used for the isolation and enumeration of CTCs from each 7.5 ml of blood separately. The CellSearch Epithelial Cell Kit (Veridex) enriches the sample for cells expressing the epithelial-cell adhesion molecule (Ep-CAM) with antibody-coated magnetic beads, and labels the cells with the fluorescent nucleic acid dye 4,2-diamidino-2-phenylindole dihydrochloride (DAPI). Fluorescently-labelled monoclonal antibodies raised against leukocytes (CD45-allophycocyanin) and epithelial cells (cytokeratin 8, 18, 19-phycoerythrin) are used to distinguish epithelial cells from leukocytes. The identification and enumeration of CTCs were performed with the use of the CellTracks Analyzer (Immunicon), a semi-automated fluorescence-based microscopy system that permits computer-generated reconstruction of cellular images. Circulating tumour cells were defined as nucleated cells lacking CD45 and expressing cytokeratin in accordance with the criteria specified by Veridex.

Real-time QPCR

Real-time QPCR for CK-19 and ABL was performed using the LightCycler™ (Roche Diagnostics, Mahnheim, Germany). The assay used the LightCycler DNA FastStart SYBR Green 1 kit (Roche Diagnostics) using 2 μl of combined dNTPs, *Taq* DNA polymerase and SYBR Green plus 2.5 μl of cDNA and 0.625 μM of primers in a total volume of 20 μl.

Primers

CK-19S	5′-GCGGGCAACGAGAAGCTAA-3′
CK-19Do	5′-CTCATGCGCAGAGCCTGTT-3′
A2N	5′-CCCAACCTTTTCGTTGCACTGT-3′
A4-	5′-CGGCTCTCGGAGGAGACGTAGA-3′

The standard used for quantitation was the artificial construct described earlier (Slade *et al*, 1999), in the range $10^1 - 10^4$ for CK-19 and $10^3 - 10^6$ for ABL per 2.5 μl.

Statistical methods

Owing to the small numbers, exact two-sided non-parametric tests were used throughout at 5% level of significance. The exact Pearson χ^2-test was used for testing association of two categorical variables, the Mann–Whitney test for the comparison of continuous variables between the two groups, and the Spearman correlation for correlations of two continuous variables.

RESULTS

To perform this pilot study, we recruited 51 primary breast cancer patients (18 group II T_1N_0 'low risk' and 33 group III >3 nodes positive (with one exception, a patient with two positive nodes) 'high risk' patients) who were on routine follow-up, with no clinical evidence of disease, 4–13 years after diagnosis. No patient had any sign or symptom of metastatic breast cancer. Two control populations were also recruited, 21 non-cancer controls (group I) and 12 metastatic patients (group IV), to confirm earlier results with the CellSearch system (Cristofanilli *et al*, 2004, 2005; Riethdorf *et al*, 2007). Overall, 14 patients in group III and one patient in group II were taking adjuvant endocrine therapy. No patient changed treatment during the course of the monitoring period. Patients in group II had one bone marrow sample taken yearly. The higher risk patients in group III had two bone marrows taken 6–12 months apart. All patients had between 1–7 samples of peripheral blood taken (three tubes or 22.5 ml of blood at each time point) for immunocytochemical evaluation using the CellSearch system approximately 6–34 months apart. These patients also had an isotopic bone scan, liver ultrasonography and chest radiology, as well as blood evaluation for full haematological and biochemical screen to exclude overt metastatic disease. All but two patients had no evidence of metastatic disease. For ethical reasons, the non-cancer controls had two blood tests (15 ml) for the CellSearch system and only patient groups II and III had bone marrow aspirates taken. The number of CTCs and DTCs present in blood and bone marrow, respectively, were compared in the low- and high-risk primary breast cancer patient groups.

CTC results

In the patients with overt metastases, we found CTCs in seven patients; the mean number of cells detected was 51 with a range of 0–301. Fewer patients (7 out of 18 (39%)) in group II compared with group III (23 out of 33 (70%)) had at least one CTC ($P = 0.042$, exact χ^2-test). Results are shown for all patients in Tables 1 and 2. We then analysed our results taking >1 cell as positive: 2 out of 18 (11%) patients in group II *vs* 10 out of 33 (30%) in group III were positive using this cutoff point ($P = 0.174$, exact χ^2-test).

Groups II and III patients were tested for CTCs using the CellSearch system at 1–7 time points. There was no difference in the number of tests between the groups ($P = 0.812$), although the time from diagnosis to the start of testing and the time of intervals between tests were not associated with the results of the CTC tests within the groups (*P*-values 0.274 and 0.602). Neither oestrogen receptor (ER) or progesterone receptor (PR) status nor tumour size affected the proportion of the CTC-positive tests within each group ($P = 0.374$ and $P = 0.238$).

None of the non-cancer controls had any evidence of CTCs. Of the 18 group II patients five (28%) were positive at one time point, and two (11%) at two time points. By contrast, in group III, 13 (39%) patients were positive at one time point, six (18%) at two time points and four (12%) at three time points.

In all, only 10 out of 33 (30%) patients in group III showed no evidence of CTCs in all tests over the period of testing, compared with 11 out of 18 (61%) in group II ($P = 0.033$). The negative patients may reflect the potentially small number of patients who will not clinically relapse. Two patients developed evidence of overt metastases during the course of the study. Both of these patients had positive results on the CellSearch system, and in each patient, DTCs were detected by ICC in one of the two bone marrows and by QPCR in two of the bone marrows.

The chances of finding positive results did not seem affected by whether or not the patients were receiving endocrine therapy at the time of sampling ($P = 0.389$ and $P = 0.693$ for groups II and III, respectively).

Over this period of sampling for CTCs, we obtained 68 bone marrow samples for DTC analysis; 13 patients in group II and one patient in group III were tested once, and 27 patients in group III at two time points. Overall, 1 out of 13 patients (8%) in group II were positive for DTCs in contrast to the 19 out of 27 positive at least once when tested at two time points in group III ($P < 0.001$). Only 8 out of 27 (30%) patients in group III were negative at both time points, 16 out of 27 were positive once, and 3 out of 27 positive at two time points, but none had two or more cells at both time points (see Tables 1 and 2).

For bone marrow QPCR, none of the 13 patients in group II were positive in contrast to 16 of 27 patients tested at two time points in group III ($P < 0.001$); 16 were positive at both time points.

We also studied the relationship between the DTC results and the CTC results. Patients consistently negative for DTCs in bone marrow (measured by ICC) can have positive CTCs (5 out of 12 in group II and 4 out of 8 in group III), but among the patients with positive DTCs, there is a high proportion of patients with positive CTCs (16 out of 19 (84%) in group III). These numbers were too small to reach statistical significance ($P = 0.145$).

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Table 1 Patient characteristics, details of therapy and timing of bone marrow and peripheral blood sampling in group II patients

Patient no.	Date of diagnosis	Tumour type	Size (mm)	Grade	No. of LN	ER status	PR status	Therapy during study	Time from diagnosis BM1 (months)	QPCR	ICC	Time from diagnosis PB1 (months)	CTCs	Time from diagnosis PB2 (months)	CTCs	Time from diagnosis PB3 (months)	CTCs	Time from diagnosis PB4 (months)	CTCs	Time from diagnosis PB5 (months)	CTCs	Time from diagnosis PB6 (months)	CTCs	Time from diagnosis PB7 (months)	CTCs
1	03/03/1998	IDC	20	2	0/15	2+	3+	Nil	89	0.046	0	94	0	100	0	110	0	114	0	118	1	121	0		
2	23/03/1998	IDC	9	2	LN-	3+	2+	Nil	96	0	0	96	ND	102	0										
3	19/05/1998	IDC	10	2	0/26	2+	3+	Nil	90	0	0	92	0	98	0	109	0	112	0	117	0				
4	26/05/1998	IDC	7	1	LN-	3+	Neg	Nil								108	0	111	0	114	0	117	0	120	0
5	21/07/1998	2 foci and tubulo-lobular	10	1	0/22	3+	3+	Nil	87	0	0	90	0	96	0	106	1	109	0		ND	114	1	118	0
6	03/11/1998	IDC	10	2	0/20	3+	3+	Nil								101	0	104	0	107	0	110	0	114	0
7	03/11/1998	IDC	17	1	0/15	3+	3+	Nil	92	0	0	92	0		ND				ND	106	0				
8	25/05/1999	Multifocal ILC	10	2	0/34	Pos	Pos	Nil								100	1		ND	88	0	91	0	94	0
9	06/06/2000	IDC	15	3	0/27	Neg	Neg	Nil								81	0	84	0		ND	87	2		
10	24/10/2000	IDC	10	1	0/27	3+	3+	Nil	62	0.032	0		ND	68	0	78	0	81	0	82	0	85	1		
11	26/02/2001	ILC	18	2	0/29	3+	1+	Nil	52	0	0	60	0	66	0	76	0	79	0						
12	06/10/2003	IDC	1.5	3	0/4	Neg	—	Nil	17	0.002	0	29	0	35	0										
13	12/07/2002	IDC	12	3	0/17	Neg	Neg	Nil	33	0.023	0	43	0	49	0	59	0		ND	66	0	69	0		
14	10/12/2002	2 areas and IDC	20	1 and 2	0/28	3+	3+	Tam								54	0	57	0	60	0	63	0		
15	07/01/2003	IDC	19	3	0/31	3+	1+	Nil	29	0	1	36	0	42	0	53	1	56	2	59	0	63	0		
16	25/02/1994	IDC	17	3	LN-	Neg	Neg	Nil	135	0	0	135	ND	141	0	159	0	162	0		ND	169	1		
17	22/12/1997	IDC	17	3	0/16	2+	3+	Nil	90	0.006	0	96	0	102	0										
18	07/03/2000	IDC	10	2	0/23	3+	1+	Nil	60	0.018	0	68	0	74	0	88	0	91	0	94	0	97	0		

BM = bone marrow; CTCs = circulating tumour cells; DTCs = disseminated bone marrow tumour cells; ER = oestrogen receptor; ICC = immunocytochemistry; IDC = infiltrating ductal carcinoma; ILC = infiltrating lobular carcinoma; ND = not done; Neg = negative; PB = peripheral blood; Pos = positive; PR = progesterone receptor; QPCR = quantitative PCR; Tam = patient receiving tamoxifen at the time of analyses, all other patients were receiving no therapy. A CK-19 : ABL ratio of $\geqslant 0.1$ by RT–PCR is taken as positive. Results for CTCs and DTCs in 18 group II T_1N_0 primary breast cancer patients.

DISCUSSION

These data indicate that some patients who have had a diagnosis of breast cancer have evidence of micrometastatic breast cancer during follow-up, despite having no clinical evidence of metastases. Seven of 18 (39%) of the T_1N_0, group II patients were found to have CTCs compared with 23 of 33 (70%) of the patients with lymph-node-positive disease. Although the numbers of patients in this pilot study are small, the results suggest that the likelihood of finding CTCs and DTCs on follow-up is greater in the patients who are at higher risk of recurrence ($P = 0.042$ and $P < 0.001$, respectively) and that the numbers of CTCs are greater in the poor-risk patients.

We have previously published the fact that there is occasional evidence of DTCs on follow-up of patients over 4 years after treatment for primary breast cancer (Slade *et al*, 2005). Most patients show considerable variability. In this pilot study, we have intensively investigated a cohort of patients and found, on the basis of one or two separate tests over a 2-year follow-up period, that many patients show positive tests, sometimes confirmed by the CellSearch test for CTCs. It may be significant that a proportion of patients with bad prognosis on the basis of their tumour histology showed consistently negative results. None of these relapsed with overt disease during the course of the study, compared with two patients in the group with positive results.

There have been few studies in primary breast cancer patients, either at presentation or at follow-up. Wulfing *et al* (2006) discovered that 50% of patients had evidence of HER-2-positive cells in the blood and that this correlated with survival. It can be noted that some patients who were initially judged to have HER-2-negative tumours had HER-2-positive CTCs. It can be noted that some patients who were initially judged to have HER-2 negative tumours had HER-2 positive CTCs. A study of 456 primary patients showed that 28% had $\geqslant 1$ CTC in 3×7.5 ml of blood but the presence of CTCs did not correlate with any prognostic features of the primary tumour (Rack *et al*, 2006). Studies such as this are necessary in order to determine a cut-off for positivity in primary patients (both this study and ours used a cut-off of a single CTC) with clinical follow-up and also studies in patients with benign breast disease.

Discrepancies between the findings for DTCs and CTCs may be explained either by differences in the methodology of detection (RT–PCR for CK-19 only, as compared with ICC for CK8, 18 and 19 for the DTCs and Ep-CAM enrichment, followed by pan-cytokeratin staining for CTCs), or because DTCs and CTCs represent two different levels of risk. It has been shown that CTCs have a half-life of 1–2.4 h (Meng *et al*, 2004) and are non-replicating (Muller *et al*, 2005) and that these must be replenished by replicating cells from elsewhere. Potentially, this could be the bone marrow; however, the DTCs, when in the bone marrow, are also non-replicating in the majority of primary breast cancer patients (Pantel *et al*, 1993; Muller *et al*, 2005). In each case, we have used the best available methodologies for detecting the cells and further studies on a larger cohort of patients along with improvements in assays, for example, CellSearch applied to bone marrow aspirates, may improve the correlation between CTCs and DTCs. With regard to the CTCs and DTCs being biologically and genetically different cells or DTCs in bone marrow being a subset of CTCs circulating in blood, we are currently investigating this using single-cell PCR and microarray analysis.

The most commonly used methods for detection of tumour cells in breast cancer are immunocytochemistry and molecular methods in the form of RT–PCR. There has been extensive research into methods for detection of occult micrometastases in patients before clinical manifestation. To date, bone marrow has been the most common site investigated for micrometastatic organ involvement. This is probably because of easy accessibility and the physiological absence of epithelial cells in the bone marrow, and also because the bone marrow is a homing site and blood is a transition compartment, two different biological compartments

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Table 2 Patient characteristics, details of therapy and timing of bone marrow and peripheral blood sampling in group III patients

Patient no.	Date of diagnosis	Tumour type	Size (mm)	Grade	No. of LN	ER status	PR status	Therapy during study	Time from diagnosis BM1 (months)	QPCR	ICC	Time from diagnosis BM2 (months)	QPCR	ICC	Time from diagnosis PB1 (months)	CTCs	Time from diagnosis PB2 (months)	CTCs	Time from diagnosis PB3 (months)	CTCs	Time from diagnosis PB4 (months)	CTCs	Time from diagnosis PB5 (months)	CTCs	Time from diagnosis PB6 (months)	CTCs	Time from diagnosis PB7 (months)	CTCs
19	09/01/1999	IDC	30	3	4\13	3+	—	Arim	78	0.12	0	84	0.25	2	77	2		ND	99	0		ND	105	0	108	0	111	1
20	09/01/1995	IDC	30	3	12\17	1+	1+	Nil	126	0.18	1	132	0.11	1	125	0	132	0	149	0	152	1	156	2	159	0		
21	07/01/1994	ILC	>50	3	12\16	—	—	Nil	137	0.10	0	143	0.47	7	135	5	140	0	162	0	165	0	167	1	170	0		
22	05/01/1997	IDC	27	3	6\19	Neg	Neg	Nil	102	0.22	3	108	0.08	0	102	1	108	0	125	1	128	1		ND	134	0		
23	01/01/1996	IDC	33	3	4\20	—	—	Nil	117	0.03	0	123	0.00	0	119	0	125	0	138	0	141	1	144	0	147	0		
24	09/01/1996	ILC	55	2	5\29	3+	2+	Nil	129	0.02	1	135	0.10	1	119	0	124	0	138	0	142	0		ND	148	0		
25	23/12/1997	Tubular	11	-	2\13	Pos	Pos	Tam											113	0	117	0	121	0	123	0		
26	12/03/1997	IDC	16	2	16\36	1+	1+	Arim	92	0.14	0	104	0.00	0	98	0	100	0	122	0	126	0	129	0	132	0		
27	08/01/1999	IDC	10	2	5\15	3+	3+	Arim	68	0.22	1	74	0.04	0	76	0	82	0	100	0	104	0	107	0	112	0		
28	01/01/1997	IDC	20	3	8\21	Neg	Neg	Nil	107	0.20	3	113	0.20	0	106	0	112	2			Relapsed with metastatic disease 115 months after diagnosis							
29	11/01/1998	IDC	30	3	6\18	Neg	Neg	Nil	78	0.12	1	84	0.20	1	84	0	90	0										
30	02/01/2000	IDC	13	3	9\21	Pos	Neg	Arim	70	0.18	1	76	0.32	0		ND	76	0	89	0	92	0	94	1	97	0	100	0
31	05/01/1998	IDC	25	3	6\11	Pos	—	Nil	91	0.03	6	97	0.09	0	91	0		ND	113	0	116	0	119	0	121	0	124	1
32	03/01/1997	IDC	60	3	4\31	Neg	Neg	Nil	104	0.03	0	110	0.52	1	104	0	110	0	125	1	128	0	131	0	133	0		
33	27/09/1994	IDC	23	3	9\29	—	—	Nil											151	0	154	2	157	0	161	0	164	0
34	03/01/1995	Both IDC	2 × 16 and 22	3	9\24	1+	1+	Nil	126	0.06	0	132	0.13	3	132	8	136	0										
35	05/01/1997	IDC	50	3	4\22	Neg	Neg	Nil	95	0.02	0	101	0.00	0	95	0	101	0	125	0		ND		ND	132	0		
36	09/01/1997	T2 N1	25	2	11\20	2+	2+	Nil	90	0.06	0	96	0.06	1	96	0	109	0	124	0	127	0	130	1	133	1	136	0
37	10/01/1995	ILC	35	2	9\21	—	—	Nil	122	0.01	0	128	0.07	0	123	0	127	0	149	0	152	0		ND				
38	06/01/1999	IDC plus DCIS	25	3	4\29	2+	2+	Arim	78	0.01	0	84	0.05	0	78	0	84	0	100	1	103	1	107	23	112	0		
39	09/01/1997	IDC	40	3	10\21	1+	1+	Nil	93	0.02	1	99	0.00	0	99	0	103	0	126	1		ND	130	0	133	0		
40	02/01/1999	IDC	45	3	6\14	Pos	Pos	Arim	84	0	0	90	0.13	0	84	0	90	0	105	0	108	1	111	0				
41	03/01/1998	IDC	40	2	7\27	Pos	Pos	Arim	94	0.07	0	100	0.07	0	93	0	100	0	115	0	118	0	120	0				
42	08/12/1997	Multifocal IDC	>50	2	9\20	2+	3+	Arim											112	0	115	1	119	0	122	1	125	2
43	09/01/1997	TX N4	—	3	14\25	Neg	Neg	Nil	95	0.04	1	101	0.03	0	101	1	105	0										
44	04/01/1998	IDC	60	2	8\19	—	—	Tam	74	0.11	0	86	0.23	1		ND	96	7			Relapsed with metastatic disease 101 months after diagnosis							
45	05/09/1995	IDC	18	2	8\13	2+	Neg	Nil											144	0	147	0	151	0				
46	08/01/1997	IDC	20	2	7\19	3+	3+	Nil	85	0.12	1	97	0.26	0	101	0	106	0	126	0	129	1	132	0	135	0		
47	29/12/2003	IDC	15	3	45\45	Neg	Neg	CT (FEC)											41	0	44	0		ND	49	0	53	0
48	16/12/1998	Mucoid carcinoma	50	1	8\19	2+	3+	Arim	72	0.03	0	84	0.05	0	84	3	97	0	103	0	106	0	109	1	112	0		
49	08/01/1999	IDC	20	3	4\26	Neg	Neg	Nil	61	0.33	2	72	0.53	0	77	1	83	0	99	1		ND	105	0	108	0	111	0
50	02/01/2001	IDC	14	2	20\39	3+	3+	Arim	49	0.00	0		ND	ND	61	0	65	0	77	1	81	1	84	0	88	1		
51	01/01/2000	Multifocal lobular carcinoma	17	3	6\18	3+	3+	Arim	37	0.08	1	51	0.03	0	47	0	51	0	87	0	90	0	93	0	97	0	100	1

Arim = arimidex; BM = bone marrow; CT = chemotherapy; CTCs = circulating tumour cells; DCIS = ductal carcinoma *in situ*; DTCs = disseminated bone marrow tumour cells; ER = oestrogen receptor; FEC = fluorouracil, epirubicin and cyclophosphamide; ICC = immunocytochemistry; IDC = infiltrating ductal carcinoma; ILC = infiltrating lobular carcinoma; ND = not done; Neg = negative; PB = peripheral blood; Pos = positive; PR = progesterone receptor; QPCR = quantitative PCR; Tam = tamoxifen. High-risk patients therapy and monitoring details. Results for CTCs and DTCs in 33 group III high-risk primary breast cancer patients.

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in a metastatic cascade (Pantel and Brakenhoff, 2004; Pantel *et al*, 2008).

We have monitored patients with primary breast cancer using sequential bone marrow aspirates for several years. Although these results indicate that a proportion of patients have PCR or immunocytochemical evidence of micrometastases, the test is confounded by the problem of sampling errors and in disease variability. In our study (Slade *et al*, 2005), we followed up 131 primary breast cancer patients for 4 years after surgery with bone marrow aspirates; 73% showed a fall in the micrometastatic load as measured by PCR and 63% as determined by ICC during follow-up. Of 91 patients who had repeat samples assayed, 87 and 65% had positive results at some time using PCR and ICC, respectively.

Patients are reluctant to have bone marrow tests more often than every 6 months, and thus it is difficult to even repeat the test to see whether the result is consistent. The CellSearch system, requiring only a peripheral blood sample, can be used frequently during follow-up. Our results indicate that either CTC sampling or a combination of blood and bone marrow tests may provide a practical monitoring system for breast cancer patients on follow-up. In our experience, many patients find repeat bone marrow sampling difficult and painful; this study suggests that the number of CTCs found on blood sampling is similar to the number found in bone marrow samples. Thus, blood tests may, in the future, be used in place of bone marrow sampling. However, before this can be recommended, we believe that a prospective study should be done comparing both tests.

Others have shown that the persistence of DTCs after treatment indicates a poor prognosis. Braun *et al* (1999, 2000a) investigated the effect of 500 mg of Edrecolomab on bone marrow micrometastases in 10 primary breast cancer patients before and at days 5–7 after antibody treatment. They showed a reduction in the number of disseminated cells after therapy. We (Smith *et al*, 2000) analysed 145 blood samples obtained from 22 metastatic breast cancer patients, both by immunocytochemistry and PCR, over 13 months. Of the 25 assessable courses of treatment, PCR agreed with the clinical outcome in 17 cases (68%) and ICC in 12 cases (48%). When 356 disease-free patients (Wiedswang *et al*, 2003) were subsequently analysed with a second bone marrow aspirate after a 3-year follow-up, the presence of micrometastases at this stage in disease-free patients was shown to be an independent prognostic factor (Wiedswang *et al*, 2004). Stathopoulou *et al* (2003) used real-time RT–PCR to study 77 patients with primary breast cancer before and after adjuvant chemotherapy, and showed a marked reduction both in the number of positive patients (31.2–6.5%) and in the level of positivity. They also studied 47 patients with overt metastases before and after chemotherapy and found no differences either in the number of patients positive (40.4 and 42.6%) or in the levels of positivity. In a study of 228 patients followed up with a repeat bone marrow aspirate 21 months after diagnosis, Janni *et al* (2005) showed that recurrence-free survival in patients with no DTCs was 149.7 months compared with 86.5 months in patients positive for DTCs ($P = 0.0003$) and that overall survival was 162.1 months compared with 98.7 months ($P = 0.0008$), respectively.

Recently, the need for a monitoring system has been highlighted by several trials indicating that sequential treatment during the disease-free period may improve overall survival in breast cancer. Thus, the Inter-Group Exemestane Study (IES) and Arimidex-Noluadex (ARNO)/Austrian Breast Cancer Study Group (ABCSG) studies both indicate that this approach may be preferable, as does the concept of sequencing chemotherapy, such as indicated by the studies using anthracyclines followed by taxanes. We feel that further adjuvant therapy during the follow-up period may yield better results, providing that these patients are selected on the basis of residual DTCs or CTCs.

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Measurements of EGFR expression on circulating tumor cells are reproducible over time in metastatic breast cancer patients

Aims: Studies of EGFR expression in breast cancer have shown inconsistent results due in part to a large range of methods used. Anti-EGFR therapy trials have often not used patient selection because of this. We describe the use of the CellSearch™ system (Veridex LLC, NJ, USA) to enumerate and measure EGFR expression on the surface of circulating tumor cells (CTCs), derived from the peripheral blood of individuals with metastatic breast cancer over time. **Materials & methods:** The CellSearch system was used to quantify CTCs and EGFR measurement was performed on all samples. The specificity of EGFR phenotyping was further examined by spiking with cell lines with increased and low (or absent) levels of EGFR expression using the CellSearch system to enrich and phenotype the CTCs. **Results:** Serial samples were obtained from 33 individuals with metastatic breast cancer. CTCs derived from these individuals had consistent levels of EGFR expression at different time points, and none of the patients 'switched' from a positive to negative EGFR phenotype or vice versa. The specificity of EGFR phenotyping by the CellSearch system was verified by staining of EGFR only being present in a high EGFR expressing EGFR cell line (MDA-MB-468), as confirmed by Western blotting. **Conclusions:** Measurement of EGFR on the surface of CTCs, derived from individuals with metastatic breast cancer patients is possible using the CellSearch system and showed consistent positivity over time. The use of this system will now be validated in a prospective study aiming to identify patients for anti-EGFR therapy based on the expression profile of CTCs.

KEYWORDS: breast cancer, CellSearch™ system, circulating tumor cells, epidermal growth factor receptor

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The ErbB family of receptor tyrosine kinases are one of the most studied targets for cancer therapies [1,2]. They consist of four distinct receptors; the EGF receptor (EGFR), ErbB-2 (HER-2), ErbB-3 (HER-3) and ErbB-4 (HER-4). The overexpression of HER-2 is a well documented predictive factor in breast cancer [3,4]; the prognostic implications of this overexpression of EGFR are less well known. This is partly owing to the large range of methods available to detect it and the conflicting data produced. Studies have found a range of 14–91% positivity of EGFR in breast tumors, and most of these found only a weak association with progression free or overall survival [5]. Recent clinical trials of EGFR inhibitor therapies did not enroll patients on the basis of EGFR expression due to this lack of consensus regarding its measurement [6,7], while other studies have shown limited or variable responses, perhaps due to imbalances in patient selection [8,9].

The use of circulating tumor cells (CTCs) to monitor metastatic breast cancer patients has become readily available with the development of a standardized method called the CellSearch™ system (Veridex LLC, NJ, USA). This system potentially offers a real-time technique that can provide tumor material using a less invasive method, such as being derived from a single peripheral blood test. Once the phenotyping of CTCs has been validated as a surrogate marker for primary tumor expression, the potential exists to tailor therapies according to the expression patterns of biomarkers in CTCs. It is now known that expression levels of HER-2 on the surface of CTCs change over time: one study found that 9 out of 24 breast cancer patients whose primary tumor was HER-2 negative acquired *HER-2* gene amplification on the surface of their CTCs during disease progression [10]. In addition, a further recent investigation measuring the molecular phenotype of primary and relapsed breast cancer metastases found that 21% had changes in either the estrogen receptor/progresterone receptor (ER/PgR) or HER-2 status [11]. This implies the heterogeneous and potentially changeable nature of tumors and metastases, and importantly the need for constant monitoring.

Therefore, it is important to determine the natural fluctuations of EGFR expression in CTCs from patients over time to examine if it may be a relevant marker, and potentially enable patient selection with anti-EGFR therapies that

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Table 1. Clinical characteristics of patients and EGF receptor measurements of circulating tumor cells from all metastatic breast cancer patients included here.

Age (median/ range)	Histology		ER positive cases (n = 26)			PgR positive cases (n = 20)			HER-2 status by FISH			Median/range									
			+	++	+++	+	++	+++	+	++*	+++	CTCs time point 1 (pt = 33)	EGFR‡	CTCs time point 2 (pt = 19)	EGFR‡	CTCs time point 3 (pt = 10)	EGFR‡	CTCs time point 4 (pt = 7)	EGFR‡	CTCs time point 5 (pt = 5)	EGFR‡
	Ductal	*Lobular*																			
62 (40–81)	25	8	8	5	13	5	8	7	26	5	2	1 (0–835)	26 (0–100)	5 (0–1314)	44 (1–99)	2 (0–537)	73 (20–80)	6 (0–710)	19 (12–71)	2 (0–1940)	54 (0–60)

**Confirmed by FISH as positive in 0 cases. ‡Median as a percentage.*
CTC: Circulating tumor cells; EGFR: EGF receptor; ER: Estrogen receptor; FISH: fluorescence in situ *hybridization; PgR: Progesterone receptor; pt: Patients.*

target this cell surface signaling molecule. This may alleviate the need for repeat biopsies to establish the changing tumor phenotype. To study this further, we investigated the number of patients who had EGFR-positive CTCs, and the reproducibility and reliability of repeat measurements over time.

Materials & methods

A total of 33 individuals with metastatic breast cancer were recruited; an appropriate local ethics committee permission was obtained. All patients provided written informed consent in accordance with the declaration of Helsinki. Samples were blinded for analysis and patients understood that the results would not be made available to them. The CellSearch system was used to enrich and enumerate the CTCs as previously described [12]. A 7.5 ml blood sample was taken in a CellSave™ (Veridex LLC) preservative tube, kept at room temperature and processed within 72 h. The system enriched for epithelial cell adhesion molecule (EpCAM)-positive epithelial cells by incubating the sample with ferrofluid conjugated to anti-EpCAM antibodies. Cells were stained with the following fluorescent labeled mono clonal proprietary antibodies: EGFR-fluorescein isothiocyanate, CD45-allophycocyanin to distinguish the CTCs from leukocytes and pan-cytokeratin 8, 18 and 19 (cytokeratin-phycoerythrin) to stain epithelial cells. Nucleic acids were stained using 4,6-diamidino-2-phenylindole. Samples were then scanned on the CellTracks® analyzer II fluorescent microscope (Veridex LLC) for analysis.

HER-2, ER and PgR analysis were performed in accordance with the published UK guidelines [13]. HER-2 positivity was defined as more than 10% of cells staining 3+ by immunohistochemistry (IHC) or 2+ IHC with confirmation by fluorescence *in situ* hybridization (FISH). ER and PgR status was determined by standard IHC (ER – clone 6S11, PgR – clone PR88, Novacastra Laboratories, Newcastle, UK).

To investigate the utility of this system in detecting EGFR-positive cells, cell lines with high and low EGFR expression (MDA-MB-468 and MCF-7, respectively) were spiked into blood samples from healthy volunteers. Cells were collected and resuspended in phosphate-buffered saline and counted using a hemocytometer. A total of 100 cells from each cell line were spiked in a blood sample with the experiment being performed in duplicate. Samples were analyzed in the CellSearch system and the number of CTCs with EGFR expression were quantified.

To verify the EGFR expression in these cell lines, a Western blot was performed with total EGFR (Cell Signaling Technology, MA, USA) to examine the relative levels of EGFR protein present. Protein concentrations were measured with Bradford reagent (Pierce Biotechnology, IL, USA) and 20 µg/µl of each lysate was loaded onto a 7% SDS-TRIS polyacrylimide gel. Membranes were probed with total EGFR antibodies diluted in 5% bovine serum albumin (BSA) in 0.1% tris-buffered saline tween (TBST) overnight. This was followed by visualization using enhanced chemiluminescence reagents (Amersham Bioscience, Little Chalfont, UK).

Results

A total of 74 blood samples were taken from 33 individuals with metastatic breast cancer and all were analyzed for EGFR expression. Out of these, 19 patients had sequential samples taken (two to five samples) and 14 patients had one sample taken. Of the 74 samples taken, 51 (69%) had measurable CTCs and out of these, 44 (86%) were positive for EGFR.

Patient characteristics and the median EGFR measurements of their CTC samples are shown in Table 1. Figure 1A–C represent graphs of EGFR positivity in CTCs from three patients with three or more sequential samples in which CTCs were both present and positive for EGFR expression. Figure 1A & B demonstrates the number of CTCs increasing whilst the percentage of EGFR positive cells remains stable with an increase in the percentage of EGFR positives in the last sample taken. Figure 1C demonstrates that the number of CTCs decreases, whilst the number of EGFR-positive cells remains stable with minor fluctuations. Table 1 shows three further patients who had two sequential peripheral blood samples taken, with EGFR measurements taken on their CTCs. Patients 1 and 3 have a very low percentage of EGFR-positive CTCs with fluctuations of only 2% between repeat samples. (Table 2) The intensity of EGFR staining in patient CTCs was often weak (Figure 2, patient CTC samples 1–3).

When cell lines were used, EGFR expression was present in MDA-MB-468 cells but not in MCF-7 cells, demonstrating the specificity of this system for detecting EGFR (Figure 2). A Western blot of EGFR protein expression confirmed high-expression levels in the MDA-MB-468 cell line and undetectable expression levels in the MCF-7 cell line (Figure 3).

Discussion

In patients with metastatic breast cancer, we have demonstrated that the ratio of detected CTCs and EGFR expression from metastatic

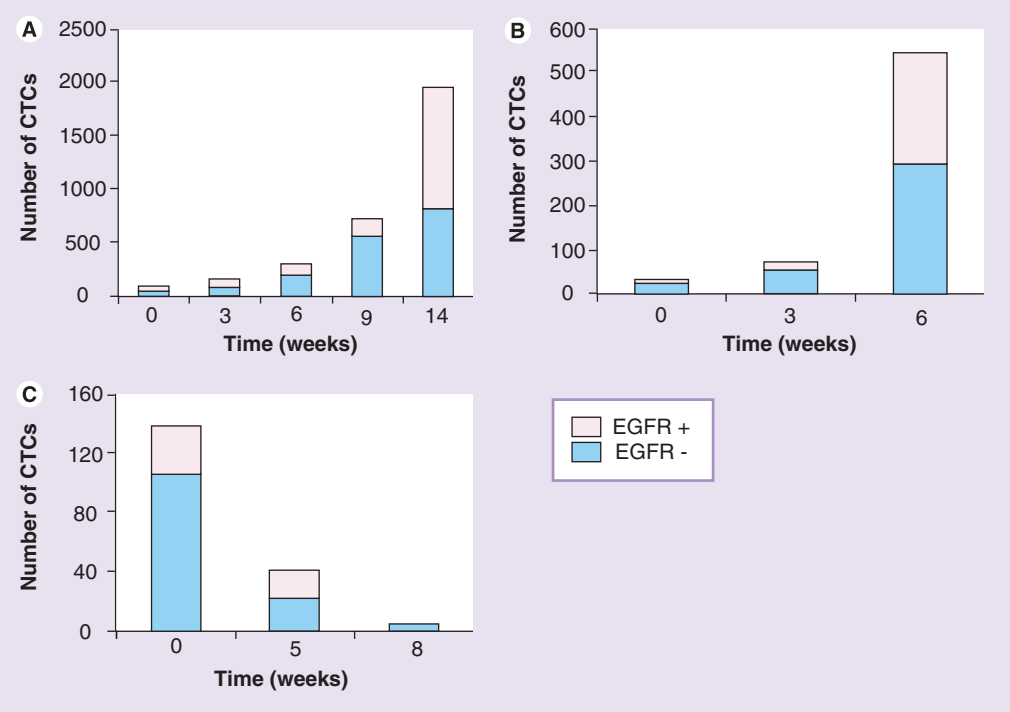


Figure 1. (A–C) Graphs showing the percentage of circulating tumor cells positive for EGF receptor expression in three individuals with metastatic breast cancer over time. These are different patients to those presented in Table 2.

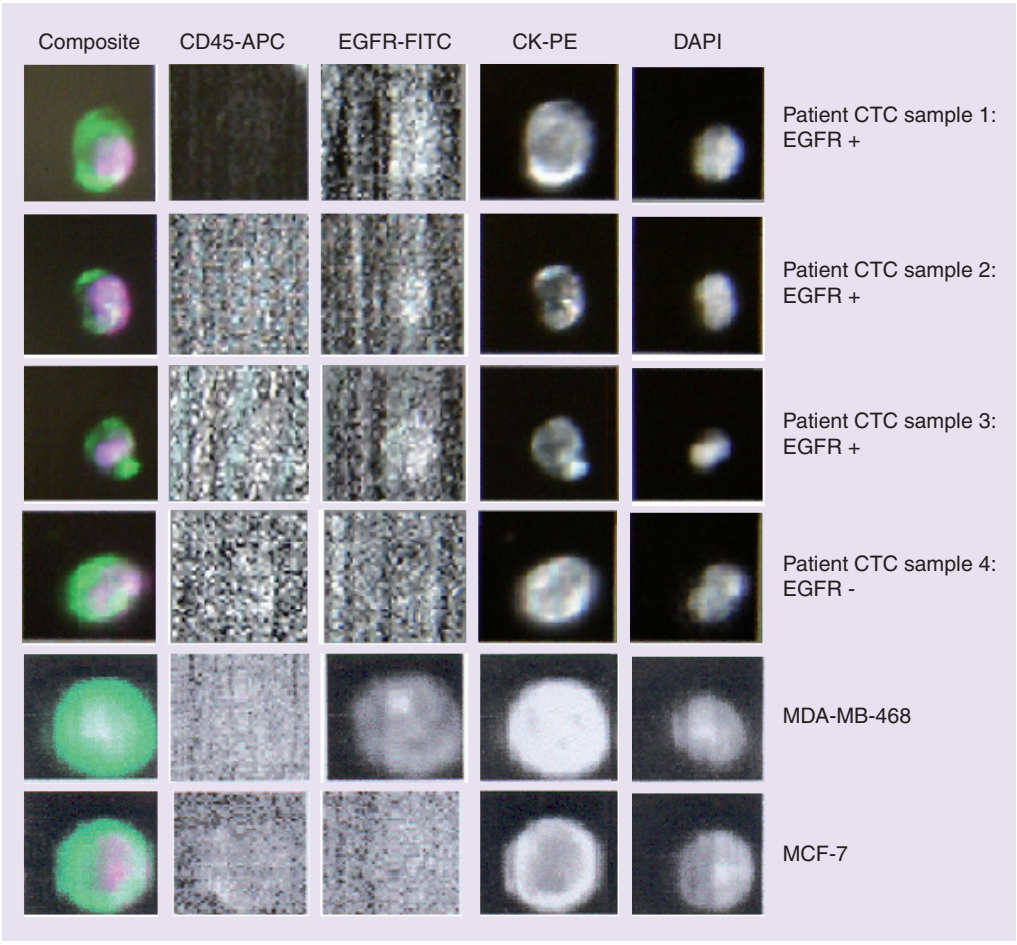


Figure 2. Gallery of images generated from the CellSearch™ system showing three representative EGFR positive patient CTCs and one EGFR negative patient CTC. Patient CTC samples often had weak EGFR staining and were determined only if a detectable edge to the cell was present with 'clear margins'. One representative MDA-MB-468 cell with EGFR staining and one representative MCF-7 cell is shown with absent EGFR staining.
CTC: Circulating tumor cells; DAPI: 4',6-diamidino-2-phenylindole; EGFR: EGF receptor.

breast cancer patients was consistent in some patients over multiple measurements in time using the CellSearch system. A total of six individuals who had multiple repeat CTC samples taken over a period of up to 14 weeks all showed a comparatively consistent percentage of EGFR-positive CTCs over time. All patients we studied had a wide range of EGFR-positivity in their CTCs from 0–100%; this was also the case in another study of circulating tumor cells in prostate cancer [14]. Their data suggested that the sampling of CTCs provided a better reflection of the biological heterogeneity of disease than a tumor biopsy that is taken from one site. A further study that used reverse transcriptase-PCR to detect EGFR in CTCs in a range of cancer patients found 59% had EGFR transcripts present [15]. This is in line with these data in which a greater proportion of patients was found to express EGFR on CTCs than observations based on primary breast tumors (our own and others' observations [5]).

One important observation in our study was that patient 2 (Table 1), who had stable disease, demonstrated a similar percentage of CTCs positive for EGFR at two time-points (48 CTCs with 25% EGFR positive at week 0 and 41 CTCs with 34% EGFR positive at week 6). Other patients who showed a small increase in CTC numbers over time also showed a consistent percentage of EGFR-positive CTCs with only minor fluctuations. These data implied that if a change did occur in either CTC number or expression of EGFR it occurred over weeks. Figure 1A represents CTC numbers and EGFR expression from a single patient with disease progression who demonstrated a rise in CTCs along with an increase in EGFR-positivity. The number of CTCs more than doubled from week 9 to week 14 whilst the

percentage of EGFR-positive also doubled. This could be a characteristic of disease progression with the most aggressive CTC subpopulations multiplying at a faster rate.

One limitation of the CellSearch system is the subjective nature of describing a CTC as being positives for a given marker. The EGFR staining was often weak in the patient samples, therefore, specific criteria were used to select positive cells. In particular, the staining had to be obvious with a clear distinguishable edge to the cell. Those cells that had 'slight' staining or that were hard to define were deemed negative. The EGFR staining was, however, validated by testing a breast cancer cell line known to be highly positive for EGFR expression (MDA-MB-468) and one that was known to have no or low EGFR-expression levels (MCF-7) [16]. These cell lines were spiked in healthy blood from volunteers and retrieved by the CellSearch system. Figure 3 shows only the highly expressing cell line was positive for EGFR staining. This was confirmed by an immunoblot showing only MDA-MB-468 cells had detectable EGFR protein present (Figure 2).

In patients with breast cancer, a number of biomarkers according to the ASCO 2007 guidelines [17], have been purported to have some applications for use in practice as follows: CA15–3, CA27.29, carcinoembryonic antigen (CEA), ER, PgR, HER-2, urokinase plasminogen activator (uPA), plasminogen activator inhibitor (PA-I) and certain multiparameter gene-expression assays. However despite years of research and hundreds of reports on tumor markers in oncology, the number of markers that have emerged as clinically useful is very small with initial promise replaced by inconsistent data. The development of guidelines for the reporting of tumor marker studies will encourage transparent and complete reporting so that relevant information will be broadly available to others and conclusions can be objectively ascertained [18]. The US FDA have now approved use of the CellSearch system for measurement of CTCs in patients with metastatic breast, colon or prostate cancer. Other new technologies for their measurement are becoming available, and these allow the molecular analysis of CTCs with the possibility of monitoring changes in epithelial tumor genotypes and phenotypes during the course of therapy [19,20]. Their routine prognostic and predictive use requires further validation, including comparison with standard tumor markers mentioned above. Whether CTCs can obviate the requirement for further biopsies on progression requires further investigation.

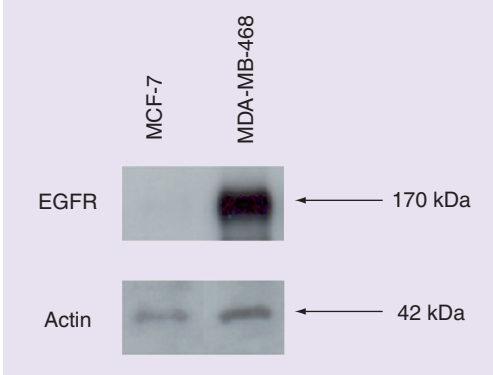


Figure 3. An immunoblot to show high EGFR protein levels in MDA-MB-468 cells and undetectable levels in MCF-7 cells.
EGFR: EGF receptor.

Conclusion

This preliminary study demonstrates that EGFR expression can be measured in patient samples over time in a reliable and relatively consistent manner. The drawback is the subjective nature of calling a cell positive due to the often weak levels of expression of EGFR in patient CTCs. The use of this methodology for the profiling of EGFR may only be validated once a prospective study of an anti-EGFR therapy finds it has predictive value for response in this setting. The use of patient selection using the staining characteristics of CTCs will be established now in a prospective study by our group, which intends to identify metastatic breast cancer patients for anti-EGFR therapy by determining those who have CTCs with 20% positive or more for EGFR expression. The cutoff figure of 20% EGFR staining has been

Table 2. The percentage of CTCs positive for EGFR over time (two time-points) in three individuals with metastatic breast cancer.

Patient	Time sample taken	No. of CTCs	No. EGFR positive (%)	Time sample taken	No. of CTCs	No. EGFR positive (%)
1	Week 0	529	9 (2%)	Week 15	309	3 (1%)
2	Week 0	48	12 (25%)	Week 6	41	14 (34%)
3	Week 0	99	5 (5%)	Week 6	536	19 (4%)

CTC: Circulating tumor cells; EGFR: EGF receptor.

Executive summary

- EGF receptor (EGFR) measurement in breast cancers is not standardized or shown to have significant prognostic value.
- This study demonstrates a potential method to phenotype EGFR on the circulating tumor cells (CTCs) of metastatic breast cancer patients using the CellSearch™ system (Veridex LLC, NJ, USA).
- In 33 metastatic breast cancer patients, CTC and EGFR measurements were performed. Of these 51 (69%) had CTCs present and 44 (86%) were positive for EGFR expression in a proportion of their CTCs.
- A total of 6 metastatic breast cancer patients with a proportion of their CTCs showing EGFR positivity had measurements taken over a period of 1–14 weeks and showed consistent positive results.
- Staining was often weak in patient samples.
- The specificity of the CellSearch machine for phenotyping EGFR was tested by spiking cells known to be high in EGFR expression and a cell line low or absent for EGFR expression (MDA-MB-468 and MCF-7 cells respectively) in blood and measuring CTCs and EGFR. The system only showed clear staining for EGFR in the highly-expressing cell line.
- The use of this system to select patients for anti-EGFR therapy will now be tested.

selected based on the mean value observed here. This study will also allow the additional analyses of the EGFR signaling pathway in CTCs, to determine superior markers of response to anti-EGFR therapy.

Future perspective

The aim of new targeted therapies is to enhance efficacy and minimize toxicity. In the 'pharmacogenomic era', we now have the potential to select patients for a particular treatment. The improvement in methodologies for both the enrichment and molecular profiling of CTCs will enable further insights into the mechanisms and process underlying invasion and metastasis, the potential to monitor response to therapy and the discovery of novel targets. The serial assessment of CTCs could provide important information for the effective management of metastatic breast cancer patients on therapy in the future, and may also enable CTC guided therapeutics to occur.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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- ▪▪ of considerable interest

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