Immune Responses in Primary and Metastatic Breast Cancer

A thesis submitted to the University of Glasgow by

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All the work described in this thesis was carried out by the candidate. Where technical assistance was required, this has been acknowledged. This thesis has not been presented to another institution nor has any other degree been conferred on account of it.

A number of publications and presentations have resulted from this thesis and these have been recorded in the appendices.

Paula M Loughlin

January 2009

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List of Abbreviations

ADCC	Antibody Dependent Cell Cytoxicity
AHCT	Allogenic hematopoietic cell transplantation
APC	Antigen Presenting Cell

APO 2 L	Apoptosis-inducing Ligand 2
BCL	B cell stimulatory factor
BMT	Bone Marrow Transplantation
Ca^{++}	Calcium
ci	Number of cells present in a calibration area (counted) in image i
Ci	Number of cells in image I (counted or estimated)
cm,cm',cm",	Number of labelled cells in calibration areas of first three of a set of 10 images ranked by the number of labelled pixels
CTL	Cytotoxic T Lymphocyte
DAB	Diaminobenzidine
DC	Dendritic Cell
ER	Oestrogen Receptor
FASL	Fatty Acid Synthase Ligand
FcγR	Immunoglobulin G Fc Receptor II
Fn	Calibration factor calculated from n images (n-1, 2,3 or 10)
GVT	Graft Versus Tumour
H+E	Haematoxylin & Eosin
HER2	Human Epidermal Growth Factor Receptor 2
IgG	Immunoglobulin G
IFN	Interferon
IHC	Immunohistochemistry
Lab	Colour space defined by luminance (L), green/red and yellow/blue colour axes
MBV	Mixed Bacterial Vaccine
MCA	Methylchloranthene
MCB	Medullary Carcinoma Of Breast
MHC	Major Histocompatibility Complex
NK	Natural Killer
Pi	Number of labelled pixels in image I ($1 \leq i \leq 10$)
pi	Number of labelled pixels in a calibration area in image i
pm,pm',pm",	Number of labelled pixels in calibration areas of first three of a set of 10 images ranked by the number of labelled pixels
RGB	Colour space defined by 8 bit red/green/blue colour axes
Sc	Suppressor Cell
TCR	T Cell Receptor
Th	T helper
Tr	T regulatory
TIL	Tumour Infiltrating Lymphocyte
TNF	Tumour Necrosis Factor
TRAIL	TNF- Related Apoptosis-Inducing Ligand
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
$\sum$Ci	Number of cells in set of 10 images (estimated)

$\sum Pi$ Number of labelled pixels in a set of 10 images

Abstract

Breast Cancer is a heterogeneous disease, which affects one in ten women in the UK today. Developments in recent years have led to earlier diagnosis and improved treatments and survival.

However, mortality is still high and metastatic disease remains incurable.

The role of the immune system in breast cancer has been questioned for over 100 years and more recently has led to major developments most notably in the form of Herceptin.

Current evidence suggests that the immune system is stimulated by tumours to manifest a response. Many breast cancers show evidence of this immune response in the form of tumour infiltrating lymphocytes. However contradictory opinions exist as to whether this response is favourable for the host or not. The significance of the findings of many of these studies is limited by several factors, including small patient numbers and the fact that qualitative rather than quantitative assessments of tumour infiltrating lymphocytes have been used.

The aims of this study were twofold:

Firstly, we set out to develop a practical and efficient method for quantifying immune responses in tissue specimens and secondly, the main aim was to establish the significance of this response, by quantifying the tumour infiltrating lymphocytes in a group of patients with breast cancer, using a well designed study.

Our patient group was derived from the Greater Glasgow Health Board database, which was established in 1995 to keep a record of all patients diagnosed with breast cancer in greater Glasgow area.

We designed a case-control study to include patients, who were matched on the basis of several factors, recognised as having prognostic significance in breast cancer.

The hypothesis to be tested was that metastatic relapse would be less likely in women with breast cancers in which a significant immune infiltrate was present, than in women with cancers in which there was no significant immune-cell infiltrate.

We established a reliable and efficient method for immune cell quantification, which will be of value in future studies looking at the immuno-phenotype of the cells that comprise the inflammatory cell infiltrate.

Additionally we found that most breast cancers show evidence of an immune cell infiltrate and that this response is likely to be protective.

To date there was conflicting evidence in the literature regarding the significance of the immune cell infiltrate in breast cancer. We have confirmed that this immune response is likely to favour the host, however we recognise that without knowing the phenotype of the cells contributing to this response, further therapeutic developments will not be possible.

We now have a well designed dataset of patients on whom to do this in addition to a practical and reliable method for cell identification and quantification.

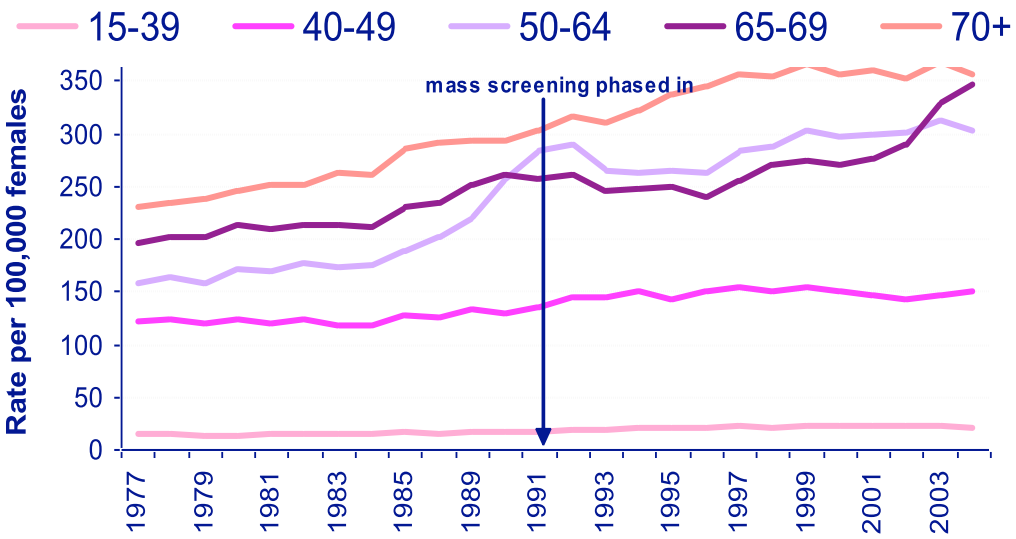
Chapter 1: Introduction

1.1 Background

Diagnosis and management of breast carcinoma has undergone many changes over the past few decades. Mammographic screening is detecting many more, early breast cancers. Despite this, and concurrent improvements in treatment, many women still die of breast cancer. It remains the commonest malignancy in women worldwide and after non-melanoma skin cancer is the commonest cancer in the UK. In the UK more than 44,000 cases of breast cancer are diagnosed each year (Cancer Research UK 2008). In developed countries the incidence of breast cancer has been increasing for many years. Over the twenty five year period 1980-2004 the incidence increased by 53%. The introduction of the National Breast Screening Programme to the UK in 1988 was responsible for a transient additional increase in the incidence of female breast cancer in

the 50-64 age. However, the underlying increase in incidence predates screening, and is still in evidence today, particularly in older women (Figure 1.1)

Figure 1.1 : Age specific incidence rates, female breast cancer,GB,1975-2004 (CRUK)

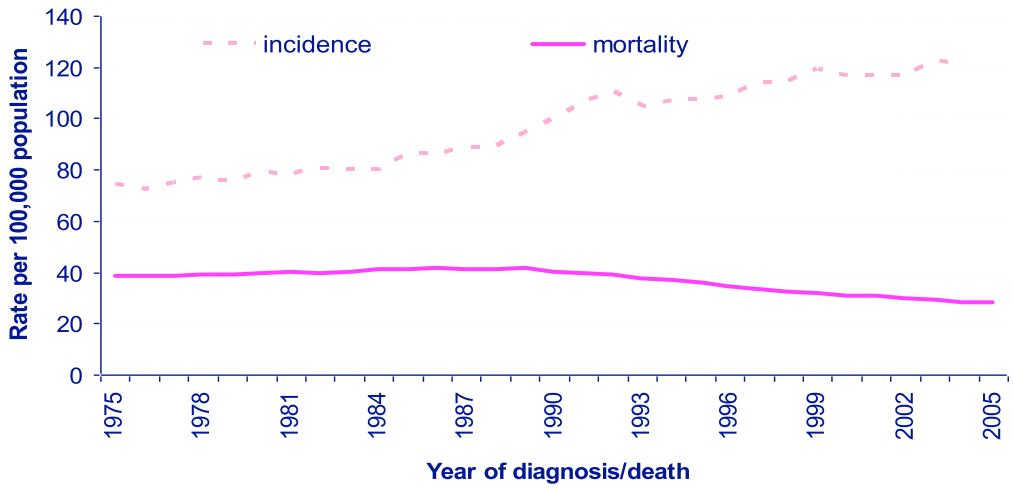


Breast cancer is a complex disease with pronounced morphological and biological heterogeneity, a tendency to become resistant to chemotherapy, and several different molecular pathways. Over the past few years, basic research developments have led to a better understanding of the molecular pathology and behaviour of breast cancer. The heterogeneity of the natural history and of the response to treatment, of breast cancer complicates patient management and influences survival. More recently, several biomarkers (steroid hormone receptor and HER2 status) have been added to classical pathological data which influence risk evaluation and therapeutic assessments. Evolving knowledge of molecular biology and newer techniques, such as genomics and proteomics, offer the potential to better define the biology of the disease, both for risk assessment and therapy choice. The International Breast Cancer Study Group (IBSG) was one of the first groups of investigators to analyze the concept of heterogeneity by evaluating the effects of adjuvant chemotherapy in different subgroups: oestrogen receptor (ER) rich, ER-intermediate, and ER-low. There is now substantial evidence of a greater benefit of chemotherapy in ER-low or ER-negative breast cancer (Henderson et al 2003, Early Breast Cancer Trialists Collaborative Group, 2005).

It is estimated that one in nine women in the UK will develop breast cancer at some point during their lifetime (Office for National Statistics 2007). While there has been an increase in the incidence of breast cancer during the 1990s the mortality rate in the UK has declined by 20% in the last ten years (ISD Online. Information and Statistics Division, NHS Scotland, 2007 and Office for National Statistics 2007) and latest

estimates suggest that 64% of those diagnosed with breast cancer today will be alive twenty years after diagnosis (CRUK 2007).

Figure 1.2: Age standardised (European) incidence and mortality rates, breast cancer, females, GB, 1975-2005 (www.cruk.com)



These statistics demonstrate the improved prognosis that advances in prevention, detection and treatment have yielded.

However in spite of earlier diagnosis and improved treatments, breast cancer remains a major cause of cancer morbidity and mortality. Length of disease-free survival in breast cancer is unpredictable, with relapse occurring up to ten years post treatment and even beyond. While some of this unpredictability is no doubt determined by tumour factors, it is also likely that some is related to host factors, including the immune response of the host to the presence of tumour cells.

1.2 Role of the immune system

1.2.1 Coley's Toxins

Dr. William B. Coley, an American orthopaedic surgeon, is considered by many today to be the father of present day tumour immunotherapy. In 1888, Coley was inspired by the unexpected complete recovery of a patient, terminally ill with a malignant bone tumour, following an attack of erysipelas (Cancer Research Institute 1976). He inferred from this event that the host response to a bacterial infection, in this case, with Streptococcus pyogenes, had also suppressed tumour progression. In 1891, he injected streptococci into a patient with an inoperable cancer, following which this tumour also underwent regression. Using a bacterial vaccine to treat primarily inoperable sarcoma, Coley achieved cure rates better than 10% (Coley 1893). He continued to use bacteria as a treatment in cancer patients for the next few decades, refining his method by mixing streptococcal toxins with those of Bacillus prodigiosus (now called Serratia marcesens).This increased tolerability for the patients. The reported results were best in bone and soft tissue tumours. This treatment came to be known as 'Coley's toxins'.

M.J. Shears, in 1943, discovered that the biologically active substance in Coley's toxins is lipo-polysaccharide (LPS), which is found in cell walls of gram-negative bacteria (Ward 1988). However, opinion within the medical community was not always supportive. James Ewing, who first described the bone tumour that was to be later named

after him, was an enthusiast for radiotherapy, and was especially opposed. Gradually Coley's treatment lost favour. Coley's toxins were produced commercially for many years with some reported successes but production ceased by the mid 1950s. Other studies analyzing their effects in humans and mice were on the whole favourable (Havas 1960 & 1990) and in the 70's various trials of mixed bacterial vaccines (MBV) – as Coley's toxins are now called - were carried out (Zhao 1992). The evidence from the clinical research that has examined this early form of cancer immunotherapy is limited by the small number of cases and the research methods used in the early 20th century. Some studies found that Coley's toxins did improved survival in certain forms of cancer, while others did not find significant benefit compared with more orthodox combined approaches (Chandler et al 1965). Modern forms of immunotherapy based on a better understanding of the effects of the immune system on cancer may ultimately prove more effective.

1.2.2 Paul Ehrlich.

The role of immunological function in the pathogenesis and progression of breast cancer has been under investigation for many years and remains an active field. The concept of an immunological mechanism directed against autologous cancer cells was first proposed by Ehrlich at the beginning of the last century (Ehrlich 1900). In 1909 Ehrlich suggested that the immune system could suppress tumour development, and proposed that the incidence of cancer would be much greater if not for the ability of the immune system to identify and eliminate nascent tumour cells (Silverstein 1999). Ehrlich also pre-empted, by 50 years, the theories of Jerne and Burnet on antibody formation. He postulated that

all physiologically active substances, antigens, function by attachment to preformed receptors or surface immunoglobulins on a cell, resulting in the release of the receptor and its regeneration by the cell. These receptors are released into the circulation as antibodies (Schwarz 2003).

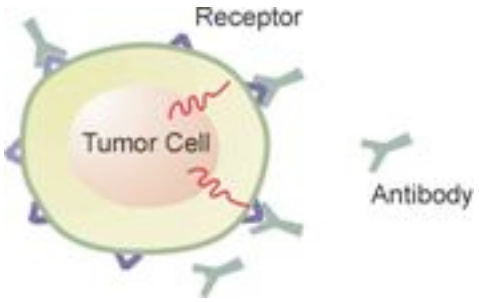


Figure 1.3: Ehrlich antigen receptor theory (https://savoypharmaceuticals.com/images/receptor.jpg)

However there was scepticism about his theory because of its implication that the immune system has the ability to generate unique receptors to an array of antigens before it is even exposed to them. Interest in this area was renewed in the middle of the twentieth century when studies in mice demonstrated an immune response capable of recognising and destroying transplanted tumour. However the underlying mechanism was thought to be one of allograft rejection, rather than being tumour specific.

1.2.3 Immune Surveillance.

In the 1950s the idea of 'immune surveillance' preventing the emergence of neoplasia was revived by Burnett and Thomas (Burnett 1957, Thomas 1959). Like Ehrlich, they believed the immune system had a role in control of carcinogenesis and that it could recognise and destroy nascent tumour cells. Central to their theory was the discovery of

tumour specific antigens. These were identified through studies showing that mice could be immunised against syngenic transplants of tumours induced by chemical carcinogens or viruses (Old et al 1964, Klein 1966). Burnet (1970) stated " …It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating potentially dangerous mutant cells and it is postulated that this is of immunological character". They postulated that lymphocytes were responsible for recognising incipient cancers (Burnett 1970). Studies seeking to challenge immune surveillance, by experimentally inducing immuno-suppression in animal models were inconclusive (Kaplan 1971, Stutman 1975), but following studies by Osias Stutman of athymic nude mice immune surveillance fell from favour. He demonstrated that these mice did not develop more chemically induced tumours when compared with their wild-type counterparts (Stutman 1973, 1979). It is, however, now known that nude mice do in fact possess some functional T cells and in particular also possess NK cells, so they are not completely immuno-compromised (Maleckar et al 1987) and these cells may well have a role in preventing tumour progression in nude mice.

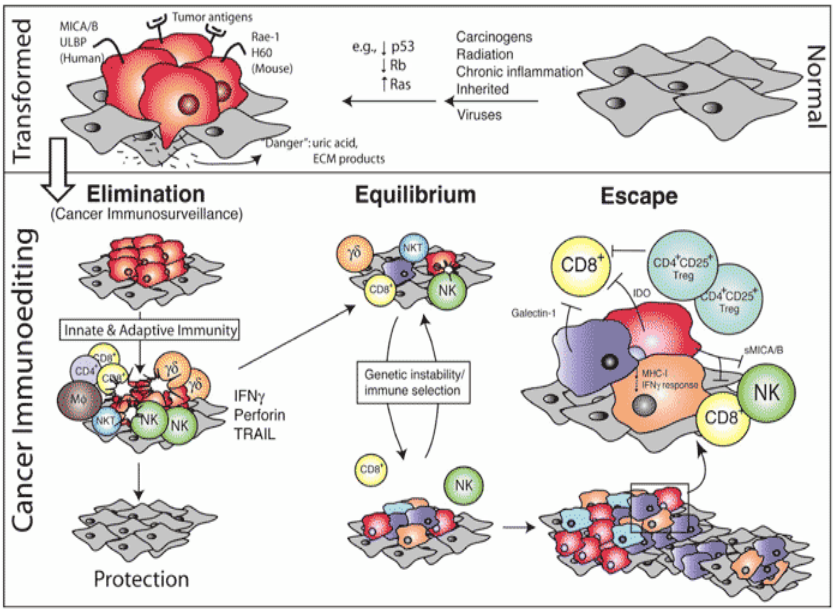
1.2.4 Recent Developments

It was not until the 1990s that further developments led to revived interest and renewed support for a significant role for the immune system in controlling tumour development. Both Interferon γ and Perforin, two important components of the immune system, were key to this. Endogenous interferon γ (IFN γ) was found to protect a host animal against transplanted, chemically induced and spontaneous tumours (Shankaran et al 2001). Perforin, a component of the cytolytic granules of cytotoxic T cells and natural killer

(NK) cells, was found to be an important mediator of lymphocyte-dependent of tumour cell killing (Russell et al 2002). Perforin deficient mice were more susceptible to chemically induced tumours than their wild-type counterparts. The development of mice with genetically defined mutations causing immunodeficiency allowed experiments conclusively demonstrating an increased risk of chemically induced and spontaneous epithelial tumours (Shankaran et al 2001). It is currently believed that T-cell mediated immunity evolved as a protection against infections (Zinkemagel et al 1979). The original theory of immune surveillance proposed the involvement of systemic, antigen-specific T-cell mediated immune responses of the type responsible for allograft rejection. Clinical studies have since confirmed that T-cell mediated immunity does indeed have a critical role in the development, progression and metastatic spread of cancer (Puisieux et al 1996, Topalian et al 1994). The tendency for immunosuppressed organ transplant recipients to develop malignancies supports this idea (Kliem et al 1997). It is significant that these are often tumours in which a viral aetiology is suspected or likely, such Epstein-Barr driven non-Hodgkin's Lymphoma (Penn 1999). However, an increased incidence of tumours of non-viral origin following organ transplantation has also been reported from several centres (Penn 1999, Birkeland et al 1995). Data from both clinical and studies in mice support the idea that immunosuppression, whether congenital or acquired is associated with malignancy (Gattie et al 1971, McClain 1997). Additionally there are several reports in the literature describing spontaneous regression of breast and other cancers. Some of these tumours were heavily infiltrated with lymphocytes (Van den Hove et al 1997). In these cases the mechanism of tumour destruction is presumed to be immunologic (Finke

et al 1994). Conversely, many tumours, even with heavy infiltration of lymphocytes do not spontaneously regress and in melanoma, histological evidence of regression is not associated with improved prognosis. The immune system can also potentially allow tolerance to develop or tumours may be able to escape from surveillance, so in spite of its known ability to destroy tumour cells, the immune system is not 100% successful as shown by tumour development in individuals who are immuno-competent. Recently the term 'immunoediting' has been coined to describe these paradoxical functions (Dunn et al 2002). Immunoediting is illustrated in Figure 4. This process is responsible for both eliminating tumours and 'sculpting' the immunogenic phenotypes of those tumours that do eventually form in immunocompetent hosts.

Figure 1.4: The three Es of cancer immunoediting: host protective versus tumour sculpting actions of immunity
(Dunn et al, Immunity, 2004)



A developing tumour is detected by the immune system, following cellular transformation and the failure of intrinsic tumour suppressor mechanisms. According to Dunn et al (2004), it faces several potential outcomes, depending on the host response. They proposed that one of the three things that could happen: 1) Elimination phase. Host immunity initiates its protective mode and eliminates the tumour. 2) Equilibrium phase: the tumour is maintained or permitted to persist in a dormant state. 3) Escape phase: the tumour evades the tumour suppressor actions of the immune response either by becoming non-immunogenic, or by elaboration of immunosuppressive molecules and cells.
Others have also shown that the immune system can influence the immunogenicity of a developing tumour. In recent years a number of studies in mice have shown that, in the absence of an intact immune system, developing tumours are more immunogenic than those arising in an immunocompetent host (Shankaran et al 2001). Numerous innate and adaptive immune effector cells and molecules participate in the recognition and destruction of cancer cells during cancer immunosurveillance. But cancer cells can avoid immunosurveillance through outgrowth of poorly immunogenic tumour-cell variants (immunoselection) and by subversion of the immune system (immunosubversion). During early stages of carcinogenesis, cell-intrinsic barriers to tumour development seem to be associated with stimulation of an active antitumour immune response, whereas overt tumour development seems to correlate with changes in the immunogenic properties of tumour cells. Immunogenic chemotherapy to re-establish anti-tumour immune responses could increase the chance of permanent success of treatments for cancer.

1.3 Tumour Infiltrating Lymphocytes

1.3.1 Background

Tumour infiltrating lymphocytes (TILs) are a manifestation of the immune response to tumours that have been increasingly researched in recent years. Tumour infiltrating lymphocytes are a common feature of many malignant neoplasms (Rosenberg et al 1986 & Marrogi 1997). Previously, their presence was taken as evidence of a host response against the developing tumour. More recent research suggests that tumours are recognised as self and lack strong foreign antigens. As Dunn (2004) suggested, it is thought that a tumour may be selected to manipulate the host immune system to prevent rejection. Their significance, however, remains controversial. Studies looking at TILs in melanoma and ovarian cancer have demonstrated that when present at high density, they are associated with an improved prognosis (Clark et al 1989, Curiel et al 2004)) but equally there are data correlating a high density of TILs with a poor prognosis (Coussens et al 2001& 2002). There are conflicting reports as to how exactly TIL density impacts on outcome in breast cancer, if at all (O'Sullivan et al 1994, Naukkarinen et al 1990). This idea that lymphocytes may actually facilitate cancer progression has been encouraged by the fact that some cancers are caused by infectious agents e.g. viruses that might cause chronic inflammation (Coussens et al 2002) or arise in the context of chronic inflammation. Approximately 50% of breast cancers have evidence of a lymphocytic infiltrate (Bilik et al 1989). Two studies in the last decade have demonstrated an association between a

lymphocytic infiltrate and improved outcome in certain subgroups of patients (Menard et al 1999, Pupa et al 1996). A dense lymphocytic infiltrate was associated with substantially improved survival in the short and long term. More recently a larger study by Lee et al (2006) demonstrated an association between a lymphocytic infiltrate and better prognosis on multivariate analysis. However, this improved outcome was seen only in patients under age 40 at the time of diagnosis. Medullary carcinoma of the breast (MCB), a morphologically and biologically distinct subtype of human breast cancer, has a diagnostic lymphoplasmacytic infiltrate. Despite cytologically anaplastic features, which would normally imply poor prognosis, there is some evidence for a better outcome than matched cases of infiltrating ductal carcinoma (Pederson et al 1991) and it has been proposed that its more favourable outcome may be a consequence of the prominent lymphoplasmacytic infiltrate. Several studies have shown that long term survival in MCB correlates with the intensity of tumour infiltration by these cells (Underwood 1974). However not all the evidence is in agreement. Rosen et al (1989) found an intense peritumoral lymphoplasmacytic infiltrate was associated with a poor prognosis. Other studies have shown no association at all (Aaltoma et al 1992).

Figure 1.5 illustrates the immune infiltration that may be seen in breast carcinoma (black dots) in comparison to the relative lack of it in normal breast tissue.

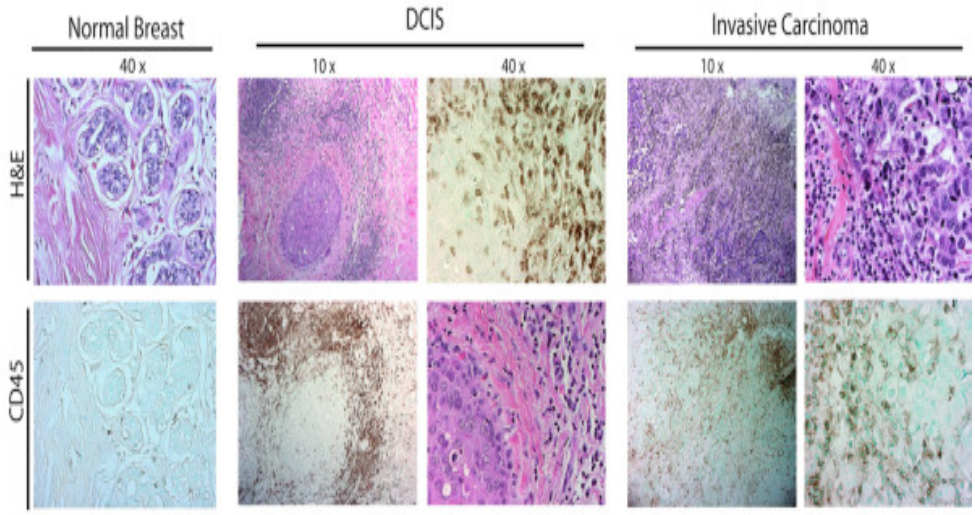


Figure 1.5: Development of human breast carcinoma is sometimes characterized by abundant infiltration of immune cells. Representative sections of normal, premalignant, and malignant human breast tissue stained with haematoxylin and eosin (H&E) (upper panels), and following immunodetection of CD45 (leukocyte common antigen, brown staining). (DCIS, ductal carcinoma in situ)
DeNardo and Coussens 2007

1.3.2 T Cells

More recently the focus has been on the type, rather than the quantity, of infiltrating cells. It is now thought the composition of the infiltrate may have a greater influence on prognosis. For example, T regulatory cells ($CD4^+$ $CD25^+$ T reg cells) may impair host defences against malignancy (Shimizu et al 1999, Jones et al 2002). T cells display wide diversity of phenotype, function and anatomical distribution. In terms of function they may be effector, regulatory or suppressor. Effector T cells are tightly controlled by various regulatory cells including dendritic cells, regulatory T cells and suppressor cells via secretion of inhibitory cytokines or contact mediated inhibition. (Kaplan 1971, Stutman 1975). Studies have suggested that suppressor T cells play a key role in the progression of cancer (North et al 1984, Awwad et al 1988). Many mice studies have

demonstrated T cells to be effectors in tumour immunity. Mice depleted of T cells are more susceptible to UV light induced tumours (Ward et al 1990) and there is some limited evidence from human studies of their importance. Recent clinical trials of adoptive transfer of in vitro expanded TILs in combination with chemotherapy have demonstrated a 50% positive response rate in selected patients with advanced aggressive tumours (Rosenberg 2001 & 2004). Most tumour infiltrating T cells are CD 4+ or CD 8+ (Marsigliante et al 1999, Chin et al). CD8+ T cells are cytotoxic lymphocytes (CTLs) which can induce tumour killing when presented with the tumour's MHC class 1 molecules. Most tumours are positive for MHC class 1 molecules. Cytotoxic T lymphocytes are one of the principal cells of the anti-tumour response in murine studies. In UV light induced tumours the CD8+ cells are required for tumour rejection (Ward et al 1990) and this has been confirmed in adoptive transfer studies, of in-vitro stimulated CD8+ lines, in humans (Rosenberg 2004). CD4+ T cells are also central to immune responses. They also can recognise tumour antigens and migrate to the site of a tumour in both murine and human cancers (Pardoll et al 1998). However, there is evidence that these cells can actually hinder activity of the CD8+ cells (Berendt et al 1980, Wang et al 2004). This effect is attributed to CD4+CD25+ T regulatory cells (Dieckmann et al 2001). CD 4+ cells can also eliminate tumour cells in the absence of CD8+ cells (Beatty et al 2001). However, they work more effectively together (Beatty et al 2000). This is partly because the absence of MHC class II molecules from a substantial number of tumour cells limits recognition by CD4+ T cells which are thought to function largely through activation of CD8+ T cells.

1.3.3 T Helper Cells

T helper (Th) cells are central to the amplification and regulation of cellular immune responses against infection and possibly malignancy. These are also CD4+ cells, which as mentioned recognise antigenic peptides presented with MHC class II molecules. These antigens are taken up by specialised antigen presenting cells (APC) and the resulting processed peptides exposed in MHC class II at the cell surface. This process is called cross presentation and is illustrated in figure 1.6

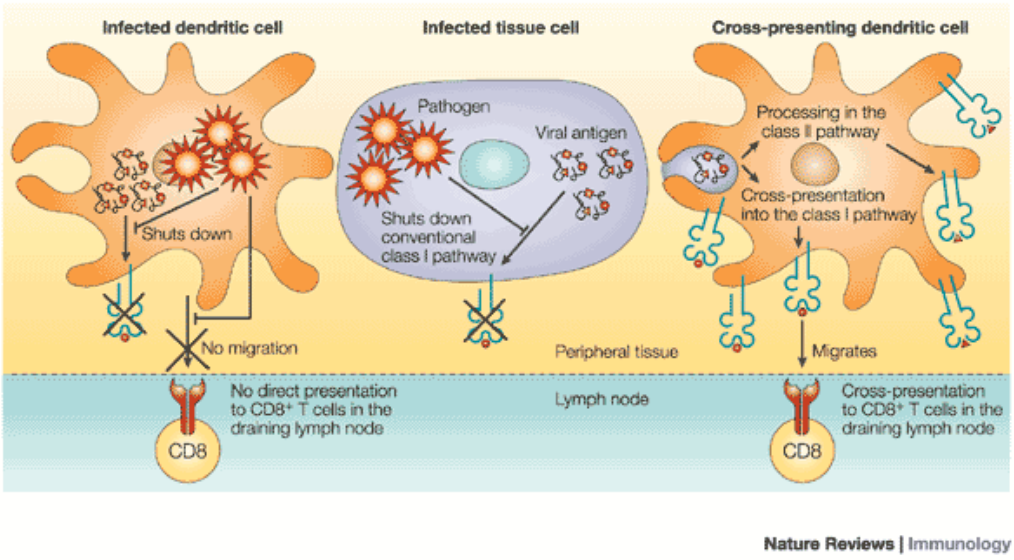


Figure 1.6: Viral subversion of dendritic cell function: cross priming is required to generate CTL immunity. Nature Reviews Immunology, November 2001

T helper cells become functional via the interaction between APC cells, such as dendritic cells and the CD4+ T cells. This combined with resulting release of cytokines contributes to the outcome of the cellular immune response. T helper cells can be divided into

different types according to their cytokine profile (Mosmann et al 1996). Type 1 cells are characterised by the production of IFNγ and interleukin-2 (IL-2) amongst others. Type 2 cells produce other cytokines including IL-4, -5 and -10, which can cross-regulate each other's function and development (O'Garra 1998). The cytokines play a role in the outcome of both cellular and humoral immune responses. The T helper cells activate antigen-specific effector cells including CTLs and B cells and can recruit other immune cells such as mast cells and macrophages (Kalams et al 1988, Ossendorp et al 2000). In 2002 Dudley et al evaluated the therapeutic efficacy of adoptive T cell therapy in malignant melanoma patients. There was evidence of tumour killing and that CD4+ T helper cells were central to this.

1.3.4 B Cells

Most studies in the literature to date have concentrated on T cell processes. B cells, while not so numerous as T cells in tumour lymphocytic infiltrates, are still potentially significant. One study, which examined a variety of tumours, found that anti- tumour antibodies were produced by tumour-infiltrating B cells in approximately 70% of cases (Punt et al 1994). Approximately 20% of invasive breast cancers contain significant numbers of B cells, with B cells constituting up to 60% of TIL in some cases (Coronella et al 2001, Grekou et al 1996). Several studies have found that when present CD 20+ TIL B cells occur in follicle-like aggregates. This is consistent with an in situ antigen-driven response generating anti-tumour antibodies. Nzula et al, 2003, confirmed this and have shown that it is accompanied by clonal proliferation and somatic hypermutation of

immunoglobulin V- genes, similar to the germinal centre response in sentinel lymph nodes. Preliminary data suggest that these B cells are responding to tumour antigen. Furthermore, recent work using a transgenic animal model of breast cancer has demonstrated that B cells and antibodies play an important role in the elimination of tumours . Immunisation of mice with three different types of tumour antigen preparation induced a protective immune response against development of transplanted or spontaneous mammary carcinomas (Renard et al 2003, Curcio et al 2003, Nanni et al 2004, Park et al 2005). This protective response was B cell, but not T cell, dependent.

1.3.5 Dendritic Cells

Another group of cells thought to be important for the immune response in tumours, are dendritic cells. These potent antigen-presenting cells play a major role in initiating anti-tumour immune response. While they are found in varying quantities in breast cancer, they are absent from normal breast tissue (Hillenbrand et al 1999). A similar situation exists for MHC class II molecules (Bartek et al 1987, Moller et al 1989). High tumour infiltrating dendritic cell (TIDC) densities are associated with a favourable prognosis in some tumour types (Wright-Browne et al 1997). A mouse study in 1997 found that active immunisation using dendritic cells mixed with tumour cells inhibited growth of primary breast cancer (Coveney et al 1997). Tumour antigens may either be presented by MHC class II expressing tumour cells or by actual antigen presenting cells attracted to the tumour site (Qi et al 2000). An intense inflammatory infiltrate may induce tumour MHC

II expression via release of cytokines. Dendritic cells may in this way favour the generation of tumour specific effector T cells and could therefore be associated with a more pronounced immune response. A study of 40 breast cancers showed a correlation between tumour MHC II expression and an associated inflammatory infiltrate containing CD1a+ dendritic cells (Hillenbrand et al 1999) and an association between p53 over-expression and the presence of immature dendritic cells.

1.4 Mechanisms of Tumour Killing: Humoral Immunity

1.4.1 Antibody Dependent Cellular Cytotoxicity (ADCC)

ADCC is an effector mechanism against tumour and virus-infected cells. They are destroyed by the combined action of specific antibodies of the IgG isotype directed against cell surface antigens, and effector cells, predominantly of the Natural Killer (NK) cell phentotype. ADCC requires three components: 1) target cells expressing tumour antigens on their surface; 2) IgG antibodies against the target antigen; and 3) effector cells bearing Fc gamma receptor (FcγR)(Clynes et al 2000). The antibodies, having recognised the antigen on the target cell, bind to it. Fc receptors on immunocompetent cells recognize the Fc portion of antibodies bound to tumour surface antigens (Figure 4). Most commonly the effector of ADCC is a natural killer (NK) cell. Following recognition and attachment via its Fc receptors, the NK cell can destroy the target cell through release of granules containing perforin and granzyme B and/or activation of the Fas/Fas ligand

apoptosis system in the target cell. Perforin molecules make holes or pores in the cell membrane, disrupting the osmotic barrier and killing the cell via osmotic lysis. The existence of ADCC has been verified in recent times by the introduction of a number of therapeutic unconjugated antibodies which are used to manipulate the host immune response to tumour. Most notable has been the advent of Herceptin.

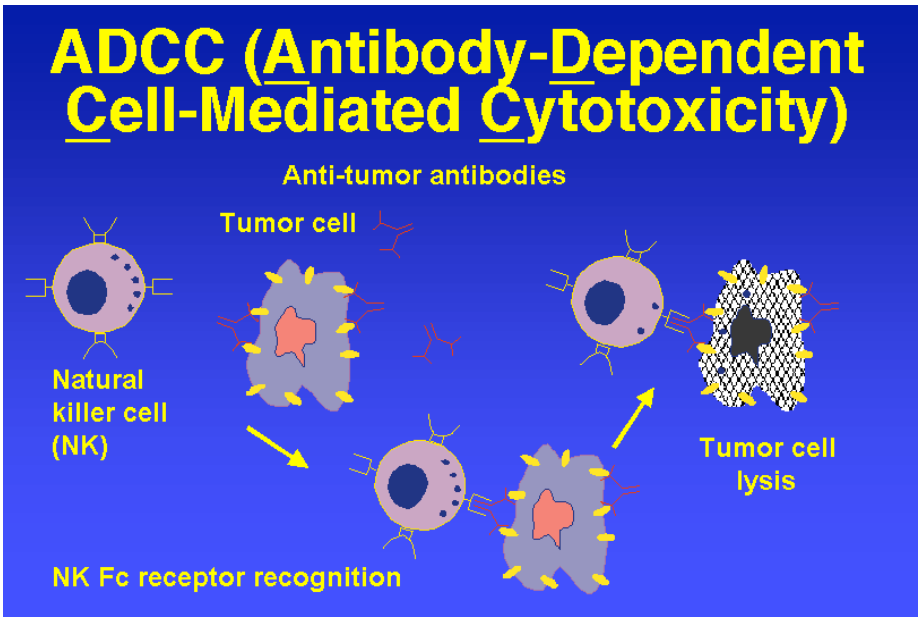


Figure 1.7: Antibody-Dependent Cell-Mediated Cytotoxicity. Naylor 2001

1.4.2 Herceptin (anti-HER2/neu antibody)

Herceptin is an antibody used to target the her2/neu gene. Her2/neu, a member of the human epidermal growth factor receptor family, is amplified in approximately 25% of invasive ductal carcinomas of breast. Amplification is associated with aggressive

behaviour and reduced survival (O'Sullivan et al 1994). Herceptin mediates ADCC in vitro and is postulated to do so in the clinical setting (Baselga et al 1996, Slamon et al 1998). Herceptin is directed against the extracellular domain of the her2/neu protein. Clinical trials have demonstrated that it prolongs the survival of patients with metastatic her2/neu over-expressing breast cancer in combination with chemotherapy and has recently been demonstrated to lead to dramatic improvements in disease-free survival in the adjuvant therapy setting, in combination with or following chemotherapy. Herceptin in combination with chemotherapy produces longer time to progression and improved survival (Piccart-Gebhart et al 2005)

1.5 Mechanisms of Tumour Killing: Cellular Immunity

1.5.1 Cellular Immunity

Cellular immunity comprises adaptive and innate components, both of which have a role in mediating tumour immunosurveillance (Rosenberg 1997, Banchereau 1998)). The adaptive component depends mainly on $CD4^+$ and $CD8^+$ T cells (Rosenberg 1997), which are primed by dendritic cells (DC)(Banchereau 1998) and recognize tumour antigens presented by major histocompatibility complex (MHC) class I molecules on tumour cells. Cytotoxic T lymphocytes (CTLs) are essential for host defences against both pathogenic micro-organisms and malignant cells (Russell et al 2002). The T cell receptor (TCR)-major histocompatibility (MHC)-peptide complexes are the cell surface molecules involved. When CTLs recognise target cells, the TCR on the CTL surface combines with MHC molecules. Specific cytotoxicity requires T cell receptor recognition of tumour-

associated antigens presented in the context of the MHC molecules and in addition a number of accessory molecules which mediate both target binding and delivery of additional regulatory signals. T cells are unresponsive or anergic when the TCR is occupied in the absence of a costimulatory signal (Schwartz et al 1996). However, according to Kuwano et al (1998), they are still capable of causing tumour cell death even in the absence of cytokines which anergic CTLs do not release. T cell-mediated toxicity results in unidirectional lysis of the target cell.

The innate component of cellular immunity to tumours has been attributed largely to natural killer (NK) cells, which can also lyse tumour cells (Banchereau et al 1998). NK cells achieve their lytic functions via perforin/granzyme, CD95 ligand (FasL) or tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) pathways, depending on the cytokines NK cells respond to, and on the expression patterns of NKG2D ligands on tumour cells (Smyth et al 2003). Recent studies have substantiated a pivotal role of NK cells, perforin, and IFN-γ in natural protection from primary tumour development induced by the chemical carcinogen methylcholanthrene (MCA) (Street et al 2001).

1.5.2 TNF- Related Apoptosis-Inducing Ligand (TRAIL Pathway)

TRAIL, also known as Apo2 ligand, is a type II transmembrane protein of the TNF super-family. At least five receptors for TRAIL have been identified in humans (only one, DR5 [TRAIL-R2] in mice) and two of them, DR4 (TRAIL-R1) and DR5, are capable of transducing an apoptotic signal (Degli-Esposti 1999, Ashkenazi 2002). Controversy

exists over the intracellular signalling pathways responsible for TRAIL receptor-induced apoptosis. Most recent studies suggest DR5 signals through FADD- and caspase-8-dependent pathways (Bodmer et al 2000). A clear role for TRAIL, in the T cell-mediated immune defence against tumour, has been demonstrated in recent years (Schmaltz et al 2002).

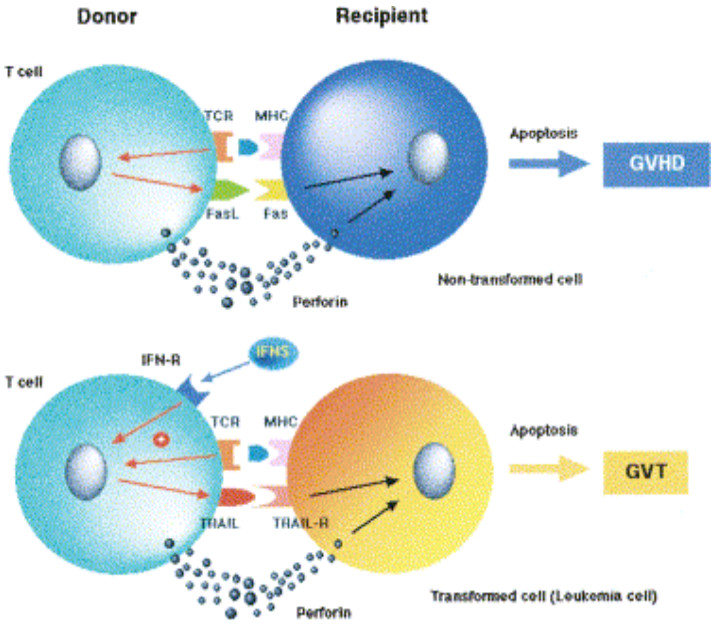


Figure 1.8: TRAIL pathway (Smyth et al 2003)

Allogeneic hematopoietic cell transplantation (AHCT) is an important therapy for a variety of malignant diseases. The anti-tumour activity of allogeneic donor T cells (graft versus tumour, GVT) provides evidence for T cell mediated anti-tumour activity with clinical relevance and is at present the most potent immunotherapy of cancer available. GVT activity is triggered by the recognition of tumour-specific antigens expressed on malignant cells. Several mouse bone marrow transplantation (BMT) models have shown the TRAIL pathway to be a prerequisite for optimal GVT activity by donor T cells

(Schmaltz et al 2002). It has also been shown that the TRAIL pathway can selectively kill transformed cells preferentially over normal cells. Although the only NK cell subset to express TRAIL constitutively is restricted to the liver, many NK cells in the lungs, liver, and spleen can be induced to express TRAIL by interleukins-2 (IL-2) or -15 and interferons (IFNs), and then kill tumour cells in vivo through a TRAIL-dependent pathway (Smyth et al 2003, Kayagaki et al 1999).

1.5.3 Perforin

Perforin is a pore-forming toxic protein synthesized and stored in cytoplasmic vesicles of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. It is secreted when these effector lymphocytes encounter virus-infected or neoplastic cells (Trapani et al 2007) and has a role in tumour cell killing. This has been demonstrated by several authors eg Smyth et al (2000) who demonstrated that perforin-deficient mice were more prone to spontaneous NK cell tumours. Thus it can be deduced that perforin is an important immune effector molecule (Jaatela et al 1995). Like perforins, granzymes (Baselga et al 1996) are lytic cytoplasmic granules found in CTLs and NK cells. They stimulate formation of pores in target cell memebranes which not only cause osmotic lysis but may also initiate the apoptotic process (Henkart 1985). These granules are thought to be released following receptor-mediated binding of target cells by NK cells or CTLs which stimulate a Ca++- dependent degranulation by the effector cell. The released perforin/ granzymes then lead to target cell lysis. Most studies have concentrated on demonstrating the presence of perforin and granzymes in activated lymphocytes and correlating their presence with cytotoxicity (Darmon et al 1995, Froelich et al 1996). There is evidence

that perforin is a marker for cytolytic activity but that does not necessarily mean it is directly involved in target cell killing. Until recent years there was also a lack of evidence for the role of granzymes in cell killing. Granzymes and perforin may work together to produce cell death: it has been suggested that granzymes released along with perforin travel via perforin-induced pores to the target cell cytoplasm where they induce apoptosis (Lieberman et al 2003) and some investigators believe that granzyme activity requires perforin. Several groups (Shivers et al 1992, Nakajima et al 1995) have found that transfection of cells with granzymes alone does not lead to target cell death. However when the same cells are transfected with perforin, cell death was induced.

Recently, studies using gene-targeted mice have demonstrated an important role for perforin in tumour immunity. Perforin was initially shown to play a broad role in protecting the host from experimental tumour challenge and from tumours initiated by some carcinogens (Van den Broek et al 1996) and it has since been shown that perforin is involved in inducing the anti-metastatic activities of NK cells against a variety of non-lymphoid tumours, including breast carcinomas (Smyth et al 1999, 2000). It has been suggested that aggressive tumour variants may escape from cytotoxic T cell (CTL) attack by down-regulating Fas, rather than becoming perforin-insensitive (Kagi et al 1994).

1.5.4 Fas/Fas Ligand.

Fas is a transmembrane receptor that belongs to the tumour necrosis factor (TNF) family. It can induce apoptosis after cross-linking with either agonistic antibodies or with Fas Ligand (Fas L). Fas ligand is expressed on the membranes of activated B and T cells, in

addition to several organs and tumour cells (Locksley et al 2001). Much of the literature suggests that CD4+ cells, which frequently lack perforin-containing cytoplasmic granules, kill target cells through a Ca++ independent Fas-mediated mechanism (Lancki et al 1991). About a decade ago, the death factor Fas L was identified as the natural trigger of Fas/CD95-dependent apoptosis and as an inducer of Fas-dependent activation-induced cell death. It is now known that this molecule not only contributes to target cell lysis in the immune system but also to the establishment of immune privilege and tumour survival (Igney et al 2005).

There is increasing evidence that Fas/Fas L have an important role in tumour development. Not only are they involved in promoting apoptosis but there is evidence to suggest they can activate numerous non-apoptotic signalling pathways, leading to increased tumourigenicity and metastasis (O'Brien et al 2005). It has also been demonstrated that apoptotic killing may result in reduced tumour immunogenicity compared with lytic mechanisms which induce heat shock protein and produce inflammation (Melcher et al 1998). This is because apoptotic bodies are efficiently removed by phagocytosis without a significant inflammatory response. Therefore, mechanisms of tumor lysis may influence outcomes of immune responses to tumours and impact on how such responses might be manipulated. Rouvier et al (1993) demonstrated that murine cells transfected with Fas became susceptible to CTL-induced apoptosis when previously they had been resistant. In contrast to the calcium-dependent perforin pathway, Fas requires target cells to be in a susceptible state for apoptosis (Alderson et al 1993). Jaatela et al (1995) demonstrated that overexpression of bcl-2 or bcl-x can block

Fas- mediated cytotoxicity. This may be a mechanism by which tumours can overcome natural cytyotoxic pathways. The BCL2 genes regulates cell death by affording protection against apoptotic signals. On the other hand, CTL and NK cells are heterogeneous, and may have more than one method of destroying target cells.

1.5.5 Cytokines

Cytokines are inducible chemical messengers produced by a variety of cells throughout the body. They are low-molecular weight glycoproteins involved in inflammatory and immune responses (Dinarello 2000). They are secreted by healthy and diseased cells and act on many different target cells. Cytokines regulate cell survival, growth, differentiation, and effector functions (Heinrich et al 1998). Unsurprisingly tumour growth in vivo is influenced by cytokines. They may act as tumour promoters or inhibitors and can be pro- or anti-inflammatory. Cytokines, as mediators of the effector response from innate and acquired cellular immunities (Abbas et al 1994), are probably involved in tumour cell evasion of immunosurveillance.

1.5.6 Interleukins

Interleukins (IL) and tumour necrosis factor are familiar cytokines. The interleukin (IL) 1 family of cytokines (IL-lα, IL-1β), is frequently expressed in breast cancer cell lines, in human breast cancer tissue, and in the tumour microenvironment (Miller et al 2000, Pantschenko et al 2003, Singer et al 2003). Similarly, high concentrations of IL-6 have also been found (Liu et al 2002, Honma et al 2002, and Kurebayashi et al 2000). Several cell types including lymphocytes (mainly Th2 cells) are thought to be an important

source of IL-6. IL-6 promotes tumour growth by up-regulating anti-apoptotic and angiogenic proteins in tumour cells (Heinrich et al 1998, Knupfer et al 2004, Sabbioni et al 2000).

IL-2 has been one of the most commonly studied cytokines in clinical trials. It has been shown that higher serum values of soluble IL-2 receptors are associated with favourable prognostic factors, including ER positivity (Zhang et al 1999). In several studies of metastatic breast cancer, multivariate analysis identified high serum IL-6 levels as an independent adverse prognostic variable for disease-free and overall survival (Bachelot et al 2003, Colombo et al 2002). In vitro many cytokines have a therapeutic anti-tumour effect. To date, however, IL-2 and IFNα are the only cytokines approved for oncological use (Gresser et al 1969). IL-2 has been used in clinical trials of patients with advanced breast cancer both with IFNα and in conjunction with Herceptin (Meehan et al 1999, Kimmick et al 2004, Flemming et al 2002). While it was well tolerated, the benefits have not been impressive. Median overall survival was improved in only one study (Nicolini et al 2005).

1.5.7 Tumour Necrosis Factor

Members of the tumour necrosis factor (TNF) family of cytokines are expressed by effector lymphocytes and are important mediators of apoptosis that both shape and regulate the immune system.

Tumour necrosis factor alpha (TNFα) is a cytokine that acts as an important mediator of the apoptotic process that also demonstrates selective cytotoxicity against breast cancer cells.(Park et al 2002,Fujiki et al 2002,Basu et al 2001).

It is mainly produced by macrophages and T lymphocytes. Various studies have examined its anti – tumour potential and role in recruiting the immune system to defend against malignancy (Bower et al 2002, See et al 2002).

Higher TNF – alpha secretion however has been found in solid tumours and appears to be closely linked to the development of metastasis.(Ardizzoia et al 1992).

Recently its been demonstrated that co-cultivation of breast cancer cells with macrophages leads to increased invasiveness of the malignant cells due to TNF – alpha- dependent up regulation of metalloproteases (Hagemann et al 2004).

1.5.8 Interferons

Interferon (IFN) was originally identified by Isaacs and Lindenmann in 1957, when they discovered that it was released following the incubation of heated virus with membranes, and demonstrated its ability to interfere with viral replication. In 1969 Gresser et al demonstrated that treatment with IFN can inhibit tumour growth in animals. IFNs are now recognized as central regulatory mediators of the immune response. Their functions of include anti-tumour and immuno-regulatory activities. Interferons α and β are type I IFNs with antitumor activity (Abbas et al 1994). They down-regulate oncogene expression and induce tumour suppressor genes which result in anti-proliferative activity. They also increase expression of MHC class I molecules in tumour cells, which can enhance immune recognition (Belardelli et al 2002). Recent studies have identified additional immunological effects of IFNs. They can stimulate proliferation and prolonged

survival of human cytotoxic lymphocytes. Dendritic cell function is also promoted via their activity (Belardelli et al 2002). Moreover, in clinical trials on melanoma and renal cell carcinoma, IFNα increases both NK cell and T helper lymphocyte activity, as well as in-vitro T-cell responses and tumour infiltrating lymphocytes numbers (Belardelli et al 2002).

1.6 Tumour Infiltrating Lymphocytes and Prognosis

There have been numerous studies over the last few decades analysing the lymphocytic infiltrate found within breast and other cancers. Many of these studies have examined the relationship between intensity of this infiltrate and the eventual outcome of the tumour (Marsigliante et al 1999, Bassler et al 1981, Ogmundsdottir et al 1995). The results have been conflicting. Some studies suggest that an intense infiltrate represents an active immune response against the tumour, and is therefore a favourable prognostic sign (Rilke et al 1991). Conversely, others have suggested that an immune response of this nature is associated with a poor prognosis (Parl et al 1982) while others again suggest there is no link between inflammation and outcome (Roses et al 1982, Alderson et al 1971). However, there have been limitations with many of these studies. Many have been observational studies only of relatively small case numbers. There have also been many studies looking at phenotypes of various tumour-infiltrating cells but their value has often been limited by purely qualitative assessment. In the absence of conclusive findings investigations of the search for the clinical significance of tumour infiltrating lymphocytes continue.

1.7 Immunotherapy

1.7.1 Background

Cancer immunotherapy has developed as an additional treatment modality in the management of breast cancer. Two major strategies have been explored. Firstly, the application of monoclonal antibodies, such as Herceptin, directed against tumor-associated antigens; and secondly, cancer vaccines targeting breast cancer antigens through the patient's own immune system.

1.7.2 Tumour Antigens

Ultimately, the main practical aim of examining tumour immune responses is to identify means of manipulating such responses to facilitate tumour killing. In order to mediate tumour rejection, an optimal combination of antigen, adjuvant and administration is required. However, conditions required for successful immunotherapy are poorly understood. Malignant cells frequently express antigens which can be recognised by the host immune system, but we know this response is often ineffective. Many tumour antigens have been identified and are capable of inducing a cytotoxic response. P53, Her2 and MUC-1 are examples in breast cancer. In addition, many breast cancers express tumour-associated antigens which are potential therapeutic targets.

Dendritic cells are the most potent antigen presenting cells; they play a role in initiating anti-tumour immune responses. Qi et al (2000) showed that tumour antigens may be presented either by class II-positive tumour cells or by specialised antigen-presenting

cells attracted to the tumour site. As discussed previously, high densities of tumour infiltrating dendritic cells (TIDC) are associated with a favourable prognosis in some tumour types (Wright-Browne et al 1997).

P53 is a transcription factor that regulates diverse genes involved in DNA repair and apoptosis (Zhao et al 2000). It is over-expressed in 57% of breast cancers and over-expression is associated with a poor prognosis. Immunity to P53 peptides has been observed in breast cancer and P53 has been suggested as a target for immunotherapy. MUC1, an antigen significantly up regulated in breast cancer, is also a focus of ongoing research. In a rat study, 60-80% of animals immunised with vaccinia virus–MUC1 survived challenge with MUC1+ tumour cells (Hareuveni et al 1990). Murine models of breast cancer and in vitro systems have also provided insights for the study of breast cancer in humans. One successful approach in these murine tumours involved cytokine gene transfection of mammary cancer cells (Lebowski et al 1997). Although the antigens are not known, these genetically modified tumour cells invoke a dense lymphocytic infiltrate and in some cases the subjects were then resistant to further challenge with the parent tumour-cell line (Cavallo et al 1992).

1.7.3 Vaccines

Cancer vaccines aim to stimulate anti-tumor immune responses mediated by immunological effector cells such as CD4+ and CD8+ T lymphocytes and NK cells. Vaccination strategies used over the past years to immunize breast cancer patients (Ko et al 2003) have included use of irradiated or transfected tumour cells, dendritic cells pulsed with tumour cell lysate or peptides, and dendritic cells transfected to express tumour antigens. All of these clinical trials have been phase I or phase II. There were many limitations on these studies and their main significance has been to demonstrate that vaccines can activate antigen-specific cellular and humoral immune responses, which could potentially impact positively on outcome (Jager et al 2005).

In summary, immune responses have the potential to influence outcome in breast cancer. With the development of Herceptin, some progress has been made towards the goal of manipulating immune responses for therapeutic effect. However, if immune mechanisms are to be harnessed effectively for breast cancer treatment, better understanding of the contributions of B and T cell mediated processes, and the role of other inflammatory cell types, is required in breast cancer.

1.8 Summary & Hypothesis

A problem in many studies of the immune response in cancer is the use of qualitative or at best semi-quantitative measures of, for instance, density of tumour-infiltrating lymphocyte and other inflammatory cell populations. We wanted to attempt to quantify intra-tumoral immune responses in a group of breast cancer patients in a way that would improve the likelihood of obtaining meaningful results, and to investigate relationships between such measurements and clinical outcome. The experimental design chosen was a retrospective case – control study, which was felt to be the most effective way to control for known prognostically significant factors including grade, stage and steroid receptor status.

A major objective, therefore, was to develop an efficient method for quantifying the densities of the various immune cells contributing to the intra-tumoral immune response. To succeed in this objective would pave the way for further research on the contribution of different populations of infiltrating inflammatory cells in breast cancer, as identified by their distinctive immuno-phenotypes. Since breast cancer treatment is now standardised in the UK, and has been for about the last ten to fifteen years, we were able to take a group of patients in whom management had been planned according to standardised protocols in a uniform regional framework, and match 'cases' (women with breast cancer in whom metastatic relapse of breast cancer did occur) with 'controls' (women with breast cancer in whom metastatic relapse of breast cancer did not occur in the follow up period). Matching was to be based on several known, classical prognostic factors.

The hypothesis to be tested was that metastatic relapse would be less likely in women with breast cancers in which a significant immune infiltrate was present than in women with cancers in there was no significant immune-cell infiltrate.

Chapter 2: Quantifying Tumour-Infiltrating Lymphocyte subsets on Immuno-histochemistry (IHC)

2.1 Tumour Infiltrating Lymphocytes and Imunohistochemistry

Breast cancers evolve over varying periods of time from intraepithelial neoplasia to distant metastases. We know that it is likely that TILS can influence this process, through the mechanisms discussed in chapter 1 including cytotoxic T cells and ADCC. As metastatic progression is responsible for most cancer deaths, immunological killing of cancer cells shed into the blood or lymph could be important. The extent to which such mechanisms do actually control growth and dissemination of spontaneous human cancer remains unclear, but is the object of intense research, in diverse tumours including breast cancer(Liyanage et al 2002 , Hussein & Hassan 2006), melanoma (Hussein et al 2006) and others (Mufson 2006, Willimsky 2005).

It is plausible that not only the intensity, but also the composition of the host response, in the form of an inflammatory infiltrate, could indicate the potential of immune surveillance to prevent progression to metastatic cancer.

In order to test this hypothesis we must measure the tumour-infiltrating lymphocyte populations. An efficient method for doing this that is not prohibitively laborious is essential. In doing this we also wanted to use a quantitative method rather than the

widely used qualitative and semi-quantitative methods, which are poorly reproducible. Hussein and Hassan in 2006 performed this manually, but in an endeavour to expedite the process, and to make it more efficient, we sought to design an automated method which could be easily employed in studies, even with large numbers of cases.

2.2 Cell types in Breast Cancer

In developing a method to facilitate quantification of the individual TIL types in breast cancer, we elected to quantify those cells types that others have shown to be present frequently, although in varying quantities, in breast cancers.

T cells display extensive diversity of phenotype, function and distribution. Many studies have confirmed the heterogeneity of TILs in breast tumours(Leong et al 2005, Georgiannos et al 2003).Several authors have found a predominance of CD4+ T cells in breast carcinomas(Marrogi et al 1997,Wong et al 1998).Others have however found that CD8+ T cells were present in greater numbers (Leong et al 2005).

Georgiannos et al (2003), in a study of 60 breast carcinomas, showed that CD3+ T cells were present in all of the tumours, in addition to CD4+ and CD8+ T cells.

Although there has been less in interest in quantification of the B cell component of the immune cell infiltrate, B cells are often present in large numbers (Coronella et al 2001) and CD20+ B cells have been found in breast carcinomas (Baxevanis et al 1994). CD35 is found inter alia on follicular dendritic cells and is of interest as a potential

marker of ectopic germinal-centre like structures in neoplasia, predominantly on B lymphocytes. We elected to examine for CD 3+, 4+ and 8+ T cells and CD20+ B cells and 35+ cells, in this pilot study of our quantification method, as a precursor to future quantification in a larger study group.

2.3 Cell Quantification

The best counting method for a particular study depends on many variables including the level of accuracy and precision required, the nature of the objects to be counted and the available equipment and labour. There is no single described quantitative method that is absolutely reliable and without bias (Guillery and August 2002). However making a quantitative assessment, even with some weaknesses, should be superior to a purely qualitative analysis, which the majority of studies to date, examining TIL's in breast cancer, have been(Pupa et al 1996,Georgiannos et al 2003).

In this particular study we wished to quantify individual cells. As cells are discrete, countable entities then an obvious unit of measurement would be cells per mm^3. However, this measurement requires 3D stereological tools such as the 'optical dissector' or 'unbiased brick' which may not be practicable for a large project and may indeed have their own biases (Guillery and August 2002, Von Bartheld 2002).

As histological sections are quasi-two dimensional, a more accessible measure might be cells per mm^2. Other possibilities include the volume fraction V_V occupied by the immune cells, which, by the principle of Delesse (Royet, 1991),

could be estimated from the area fraction A_a of an infinitesimally thin section (approximated in reality by a histological section of conventional thickness).
Point counting estimates the area fraction and also therefore, the volume fraction, but if the volume fraction is small, the number of points which must be counted to estimate it with a specified degree of precision is relatively large. A subjective element may also influence the decision as to whether a sampling point falls on the object to be measured.
Binary thresholding of a digital image closely resembles 'point counting'. Every pixel is treated as a sampling point, and its colour properties determine whether it is to be counted. Theoretically this seems straightforward, however, determining the appropriate threshold is not. For an immuno-peroxidase signal visualised with diamino-benzidine and counterstained with haematoxylin, the problem is deciding which pixels are 'brown' enough to represent signal to be counted and which are either blue(counterstained nuclei) or unstained background.

A variety of approaches have been proposed, from simple thresholding of colour channels to complex image deconvolutions (Brey et al., 2003, Matkowskyj et al., 2000 & Ruifrok and Johnston, 2001).
The approach we adopted was based on the idea that relevant pixels resembling each other in colour, would therefore be co-located in an appropriate 3D colour space (Poynton, 1995). In 24-bit RGB colour space 3 separate 8-bit (one byte) numbers plotted on mutually perpendicular red, green and blue colour axes define a

colour cube composed of 256 × 256 × 256 individual elements specified by each possible RGB number triple. In Lab colour space, luminance (L) is plotted against mutually perpendicular chromaticity axes (a= green/red, b= yellow/blue). Proximity of points within a colour space designed to be perceptually uniform (Sangwine ,1998) such as Lab colour space implies similarity of colour, and it appeared therefore that this proximity could be used to identify pixels representing the signal to be counted. We also wanted to use readily available software, so Adobe photo-shop 7 tools were employed.

2.4 Materials and Methods

2.4.1 Cases and Immunostaining

Sixteen invasive breast carcinomas of no special histological type were chosen at random from archives at Glasgow Royal Infirmary. For each case four micron sections of one representative block were immunostained as a single batch using a standard automated immunoperoxidase methodology and DAKO primary antibodies against CD3, CD4, CD8, CD20 and CD35. For each immunostained slide ten consecutive adjacent digital images of fields located at the infiltrative edge of the carcinoma were acquired, starting at a random point, using a Fuji HC300Z digital camera and a Nikon Eclipse E600 microscope with a x40 apochromatic objective, and saved as uncompressed 24-bit RGB TIFF files. Field size (measured by stage graticule) was 218°—170 microns=0.03706 mm2 =1/26.98 mm2 ≈1/27 mm2, so cell counts multiplied by 27 equal cell counts per mm2.

2.4.2 Measuring the immunostaining signal

Adobe Photoshop (version 7) allows the colour of any individual pixel to be sampled and set as the 'foreground' colour. The 'Select/Colour Range' tools allow all pixels in the image having a colour similar to the foreground colour to be selected, which can then be counted using the 'Histogram' tool. How closely colours in the image must resemble the foreground colour is determined by setting a property known as 'fuzziness': a low value ensures that only pixels closely similar in colour will be selected, while a higher value broadens the selected colour range, making it possible to count the number of image pixels of a particular colour or colour range, narrowly or broadly defined. Even if the open image file is in RGB mode, the Photoshop 'Colour Range' tool operates in Lab colour space. This can be demonstrated using the 'LabMeter' colour measurement software tool, available as a free download (www.curvemeister.com). This provides a square image representing all values on the green–red (a) and yellow–blue (b) chromaticity axes of Lab colour space (i.e. the a,b colour plane), at a user-specified luminance value. Setting the foreground colour in Photoshop to RGB 160, 67, 23 (Lab 41, 38, 44) and 'fuzziness'=100, the 'Select/Colour Range' command chooses a square portion of the Lab Meter image for which a=38±15 and b=44±15, inclusive. Varying the luminance (L value) of the test image or a gradient image showed a slightly wider selection range on the luminance axis, with L=41±19 inclusive being chosen. Lab 41, 38, 44 corresponded to a brown equivalent to DAB staining of moderate intensity in our sections. Using fuzziness=100 includes

weaker and stronger staining in the selected area. This value and range were chosen to select approximately 90% of the area of the section in which the DAB signal could be identified visually. One hundred percent selection was not sought, to avoid identifying areas of the section lacking specific staining. Figure 2.1 illustrates an example of pixel selection in Photoshop with these parameters for an image of a immunostained section containing many labeled cells, and one containing none. In all cases there was visual control of the selected area. It is also instructive to examine a 3D histogram of the pixel distributions of these images in Lab colour space (Figure 2.2).

The stained section includes pixels corresponding to the DAB signal while the image without labeled cells shows pixels corresponding to the background and nuclei only. In essence, we are counting a representative subset of these pixels.

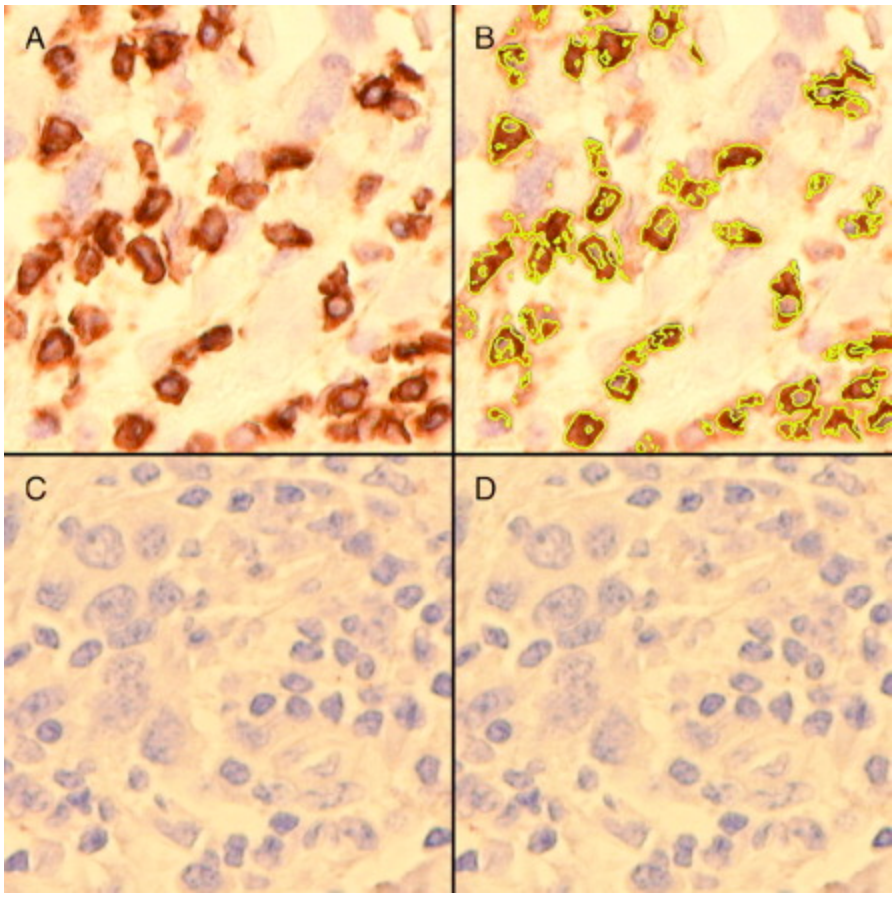


Figure 2.1 : A: Immunoperoxidase staining shows many CD3+tumour-infiltrating lymphocytes in this breast carcinoma.

B: outlined areas selected in Photoshop using 'Select/Colour Range' as described in materials and methods. C and D: No CD35+cells are present in this field (C), and no pixels are selected by Photoshop (D).

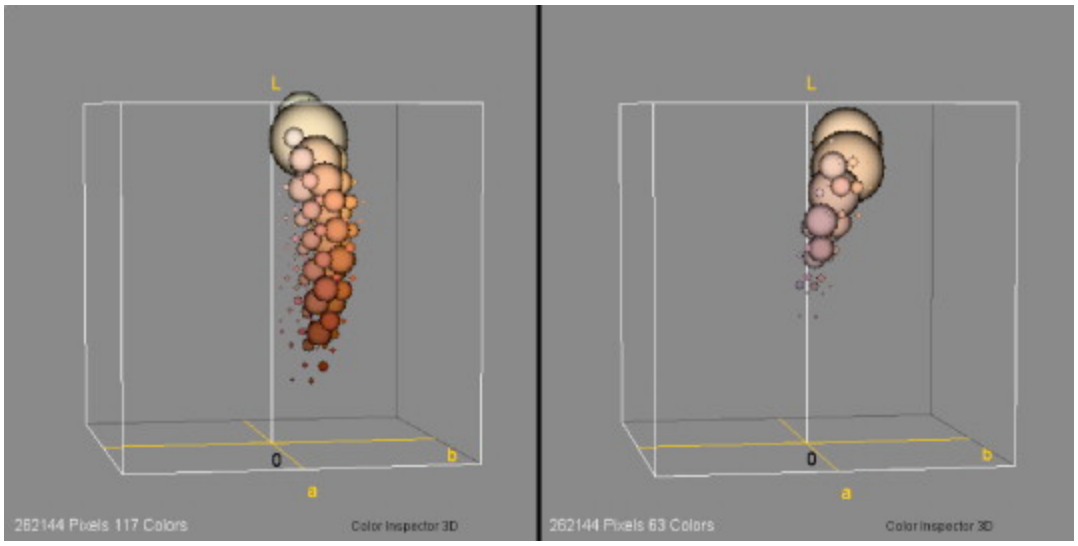


Figure 2.2 : These images are 3D histograms of those seen in figure 2.1.

On the left is CD3 and on the right is CD35. The difference in the colour distribution of coloured pixels between the two images as a consequence of the numerous CD3+cells and the absence of CD35+cells is obvious. These 3D histograms were generated using the '3D Color Inspector/Color Histogram' plug-in [Kai Uwe Barthel] for the public-domain image processing software Image (available at: http://rsb.info.nih.gov/ij/). Luminance is on the vertical axis and the chromaticity axes a and b are indicated on the base of the Lab colour cube.

2.4.3. Associating the measured signal with cell counts

The next step was to examine the relationship between the number of cells present in an image and signal strength measured by the number of pixels falling into a particular Lab colour range. A total of 16 cases°—5 antibodies°—10 images=800 images were collected on the same microscope, camera, and light setting, of slides

stained in a single batch on an automated staining machine. These precautions were observed to maximize consistency of analysis without extraordinary measures. CD35 positive cells were present in significant numbers in very few of these images, and images of CD35 staining were excluded from subsequent analysis.

Each image was opened in Adobe Photoshop 7 with foreground colour set to R=160, G=67, B=23 and 'fuzziness' set to 100. The 'Select/Colour Range' tool was used to select the labeled pixels; these are highlighted on screen so that labeled cells can be identified. The number of selected pixels was recorded from the Image/Histogram dialogue. The file was then closed and the process repeated file by file until all files had been processed. If the number of labeled cells in an image was not too great, all were counted to calibrate for that particular image the relationship (labeled pixels per cell) between total signal and cell number. If there were too many cells to be counted easily, the 'Rectangular Marquee' or 'Lasso' tools were used to define a representative sub-region of the image within which all labeled cells could be counted and within which colour selection and pixel counting allowed a calibration (labeled pixels per cell) value to be calculated for that particular image. A complete field or field subset cell count with the corresponding pixel count was made for every image. To be certain that labeled cells were neither missed nor counted twice, the Eraser tool was used to place a spot of colour on each labeled cell as it was counted. This was quick and efficient.

The expectation was that the number of labeled pixels, for a particular combination of case and antibody, would be proportional to the number of cells present in a

field; and that once the system had been calibrated, it would not be necessary to count individual cells, but only the pixels using the semi-automated methodology described above. Particular interest was attached to variations in calibration from field to field, case to case and antibody to antibody. Batch-to-batch variation was not addressed, because if case-to-case variation in the relationship between pixel counts and cell number is significant, then calibration will have to be undertaken in every case, which will control for batch-to-batch variation also.

For any individual field i the data available are the number, Pi, of labeled pixels in the whole image; the number, pi, of labeled pixels and the number, ci, of cells present in the calibration area.

The estimated number of cells in a field is $Ci=Pi / (pi / ci)$;

in fields containing few enough cells to count them all, the formula is $Ci=Pi / (Pi / Ci)$, which cancels to $Ci=Ci$, as expected.

The best estimate of the number of cells, ΣCi, in all 10 fields is:

$$\Sigma C_1 = P_1 (p_1 / c_1) + \ldots. P_i (p_i / c_i) + \ldots. + P_{10} (p_{10} / c_{10})$$

and from this figure an estimate of the average calibration factor, F10, weighted in proportion to the number of cells present in individual fields can be derived as

$$F_{10} = \Sigma P_i / \Sigma C_i$$

We expected that it would be necessary to calibrate each case and antibody combination individually, on account of differences in fixation and processing field between cases, and differing epitope robustness and antibody binding affinity. We

wondered ,however, whether calibration for every field was necessary, given that all ten fields were adjacent to each other on the same section, and had been exposed to identical handling, dissection, fixation, processing, storage and staining. We sought, therefore, to find the minimum number of fields which would have to be calibrated by cell counting to allow an acceptable estimate of the number of cells present, in comparison with the number of cells estimated by calibrating every field.

Our data allow us to examine variations in the pixels/cell calibration factor on a field-to field, case-by case and antibody-by antibody basis. Plotting all individual pi / ci measurements against Pi for all cases, antibodies and fields (CD3, CD4, CD8, CD20) allows us to see relationships between the calibration factors and the total labeling (Figure 2.3).

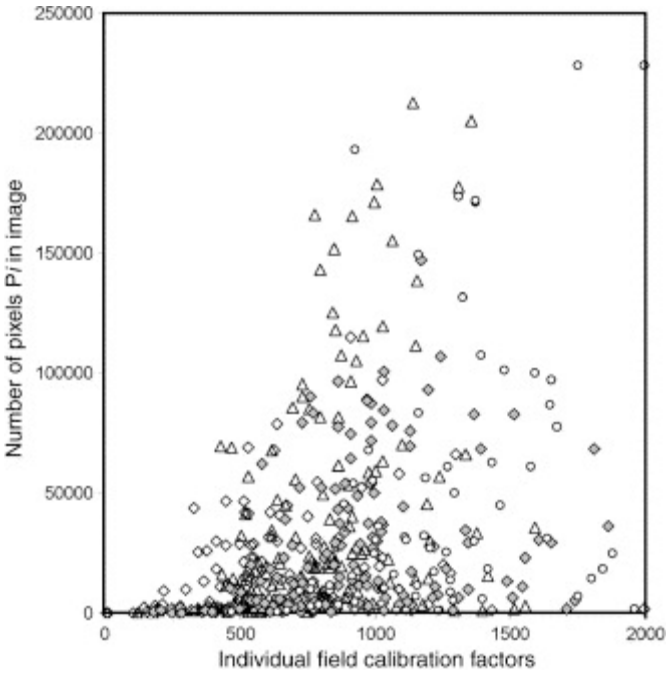


Figure 2.3 : Scatter plot of calibration factors pi/ci for all evaluable fields.

The calibration factor is plotted on the x axis against the number of labeled pixels Pi in the image which yielded that calibration factor for CD3 (triangle), CD4 (diamond), CD8 (grey diamond) and CD20 (circle)

Where few labeled pixels are present in a field, there is wide variation in the calibration, but the estimates of the calibration factor derived from fields in which more labeled pixels are present fall within a narrower range. It appeared appropriate therefore to base the calibration on the fields containing the largest number of labeled pixels, to reduce noise associated with smaller cell and pixel counts.

Accordingly, three different estimates were made for each case/antibody combination: the calibration factor (over all 10 fields) was calculated as described above (F_{10}); and estimated using calibration factors calculated from the field *m* containing the greatest number of labeled pixels as $F_1=(p_m / c_m)$; from the sum of that field and the field *m′* with the next largest number of labeled pixels as $F_2=(p_m +p_{m'}) / (c_m+ c_{m'})$ and from the sum of the three fields with the largest, second and third largest number of pixels as $F_3=(p_m+p_{m'}+ p_{m''} /(c_m+c_{m'} +c_{m''})$.

In expressing actual cell counts, the number of cells per field has been multiplied by 27 to give the results in cells per mm^2.

2.5. Results

2.5.1 Comparison of F_{10} against F_1, F_2 and F_3

A scatter plot (Figure 2.4) shows a greater degree of scatter for F_1 against F_{10} than for F_2 or F_3 against F_{10}, as might have been expected. We can look more closely at the degree of agreement (following Bland and Altman1986) by computing $100(F_1-F_{10}) / 0.5(F_1+F_{10})$, $100 (F_2-F_{10}) / 0.5(F_2+F_{10})$ and $100(F_3-F_{10}) / 0.5(F_3+F_{10})$ to express the degree of agreement between the different calibration factors as a percentage of their means. The mean difference and its standard deviation are: F_1 v F_{10}, 8.3% (17.4%); F_2 v F_{10}, 5.3% (10.2%) and F_3 v F_{10}, 4.4% (7.8%). Clearly, F_3 agrees best with F_{10}, but F_2 is nearly as good and is less work to derive, requiring only two calibration measurements.

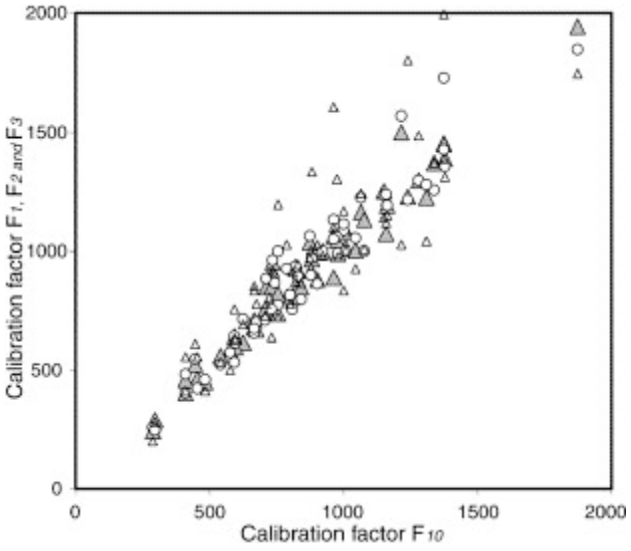


Figure 2.4: Scatter plot of calibration factors (pixels per cell) derived from all evaluable fields. The calibration factor F_{10} is plotted on the x axis against the three calibration factors derived from available measurements for the single field with the most labeled pixels (F_1; small Δ), the two fields with the most and second most labeled pixels (F_2; o), and the three fields with the three most labeled pixels (F3; larger grey Δ). One-field calibration shows considerably more scatter than two- or three-field calibration

The differences are normally distributed (judged by normal probability plots and Shapiro–Wilk W test), so these figures tell us that we can be 95% confident that a cell count using F_1 will not be more than 42.5% greater and not more than 25.9% less than a cell count derived using F_{10}. The 95% confidence limits for F_2 are +25.3% and −14.7% and for F_3 are +19.6% and −10.9%. For many purposes these will offer adequate

accuracy and precision.

2.5.2. Differences in calibration between antibodies and between cases

This section looks at whether it is necessary to calibrate cell counting for different cases or different antibodies. Figure 2.5 plots the raw calibration data for CD3, CD4, CD8 and CD20.

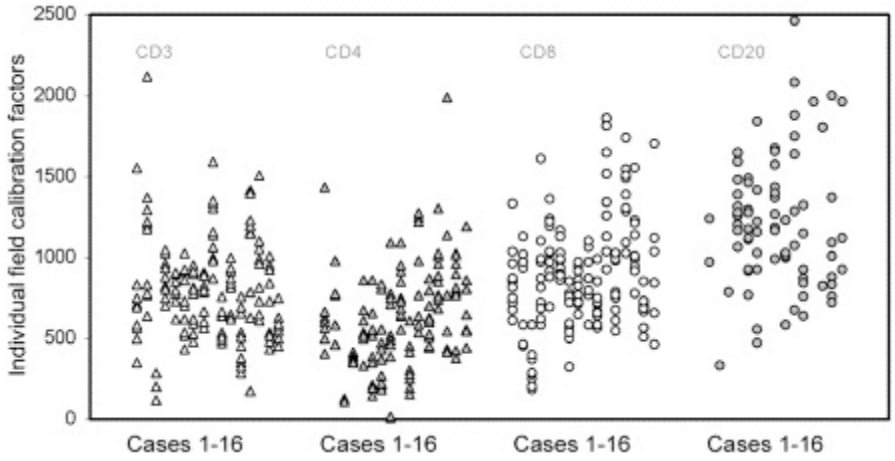


Figure 2.5 Scatter plot of calibration factors (pixels per cell) derived from all evaluable fields arranged by case and by antibody. Each vertical column of data points represents one case from 1 to 16 and the order of cases is the same for each antibody. Calibration factors are comparable for CD3 and CD8; those for CD4 tend to be lower and for CD 20, higher.

Another way of looking at this is to take mean F_{10} values for all cases for each antibody. For CD3 the mean F_{10} is 770 pixels/cell±SEM 61; for CD4 it is 603±53; for CD8 it is 896±67; and for CD20,1161±103. Were one to take the mean of these values (857.5 pixels/cell) to represent them all, the number of CD20+B cells and CD8+T cells would be systematically overestimated by 35% and 4.5% and CD3 and

CD4+T cells would be underestimated by 10.2% and 29.3%. These represent non-trivial biases which for many purposes would not be acceptable.

Figure 2.6 looks at case to case variation. It plots normalized factors F10 for CD3, CD4, CD8 and CD20 and shows that that generally the calibration factors F10 lie in a range between about 75% and 140% of the average for the series. One case is clearly an outlier and may have been subjected to unusually lengthy fixation. Again, the differences in the relationship between the pixel counts and the cell counts which they imply suggest that this variation must be taken into account.

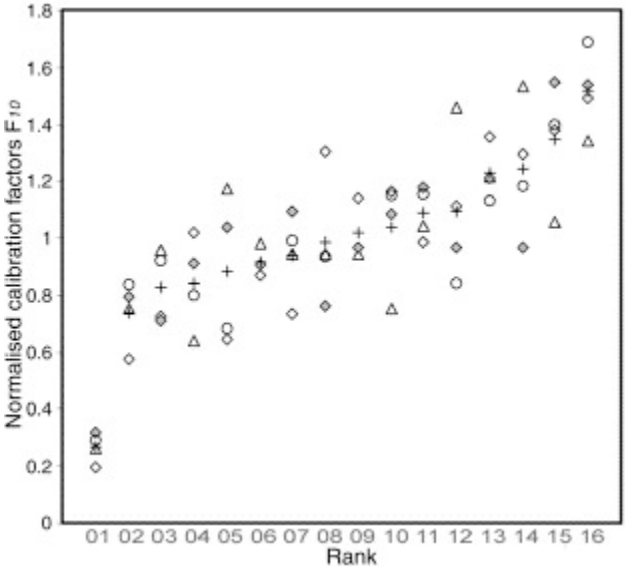


Figure 2.6 : Scatter plot of calibration factors F_{10} for CD3, CD4, CD8 and CD20: CD3 (triangle), CD4 (diamond), CD8 (grey diamond) and CD20 (circle). Data are normalised against mean F10 and ranked by mean normalized F10 value. This plot indicates that for the most part calibration factors F10 lie in a range between about 75% and 140% of the average. The first case is clearly an outlier and may have been subjected to unusually lengthy fixation. '+' indicates mean normalized F_{10} values

Finally, it is worth taking a preliminary look at the range of densities of different TIL populations in breast cancer. This gives some indication of the kind of precision which must be achieved to detect biologically significant differences; if the range is very wide then greater precision may not be required in comparison to the situation which would obtain if the range of observed densities was small.

Figure 2.7 and Table 1 present this data in units of cells per mm2.

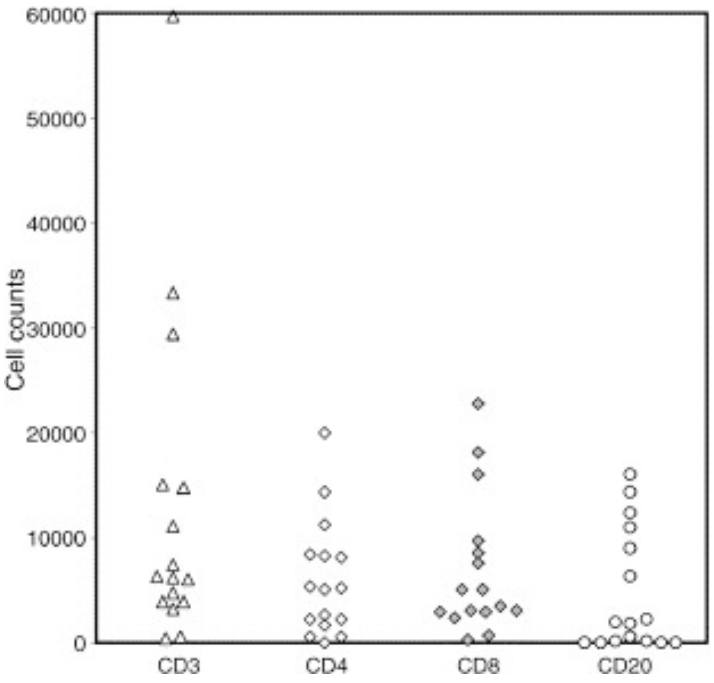


Figure 2.7 : Scatter plot of cell counts for CD3, CD4, CD8 and CD20: CD3 (triangle), CD4 (diamond), CD8 (grey diamond) and CD20 (circle). There is a wide range of values for each lymphocyte sub-population

Table 2.1 :

The range of TIL densities is very wide; there are major differences between different cases in the intensity of the TIL response evoked, in keeping with subjective impressions of the situation.

	CD3	CD4	CD8	CD20
Mean	12,900	6016	7010	4750
SD	15,700	5570	6600	5850
Median	6280	5160	4280	1860
Total Range	490–59,700	54–20,000	270–22,800	0–16,092
IQ range	3969–14,158	1958–8330	2997–9126	54–10,031

This is a pre-condition for significant differences in tumour behaviour to be related to TIL density, and makes the task of analysis easier. IQ range = interquartile range.

2.6. Discussion

Immunostaining pixel counts made using a widely available tool (Adobe Photoshop) can be converted into cell counts per mm2 for tumour infiltrating lymphocyte subtypes. Likely errors associated with this conversion are moderate in comparison with the range of TIL densities in breast carcinomas in this preliminary study, although one must not be unrealistic about the accuracy likely to be achieved. While heterogeneity of staining between samples is reasonably well compensated for by the calibration process, compensation for heterogeneity within samples will depend on the number of fields in which calibration is carried out.

A purpose of this study was to develop a methodology employable in larger-scale studies. Many studies published in the pathology literature are statistically underpowered. Our ongoing case/control study of outcomes in breast cancer is designed to examine 111 breast cancer patients (‘cases’) in which metastatic relapse occurred and 222 cancer patients (‘controls’) without relapse.

Carcinomas are matched for size, grade, estrogen-receptor and lymph node status, and the women are matched by age as a surrogate for menopausal status.

Examining a core set of TIL subtypes (CD3, CD4, CD8 and CD20) will require measurement of 333°—4°—10=13,320 digital images. High-throughput approaches to the analysis of≈40 gigabytes of image data are required.

Manual processing of all images in Photoshop is a non-starter. We view this enabling, preliminary study as a stepping stone to a more streamlined approach.

Calibration of the conversion from pixel counts to cell counts is required for each case/antibody combination.

For a set of histological sections immunostained as a batch on automated staining equipment, identical colour selection parameters can be used on all sections in that batch; visual inspection showed satisfactory selection of areas identified visually as immunostained with DAB from field to field, case to case and antibody to antibody, all in the same batch.

We studied 640 images in this pilot study. Total labeled pixel counts were made manually for all these images. We have made good progress towards automation of this step and a program has been written, by Dr Alison Gray, University of Strathclyde, for batch processing of image files.

This program is written in C, for which compilers are readily available for different computer platforms (Windows, Apple, Unix/Linux etc.), and calls the public domain image processing software 'ImageMagick' (http://www.imagemagick.org) to convert different image file types into a format suitable for image arithmetic to count the number of image pixels which meet the user-specified criteria (i.e. L,a,b ranges) for the signal.

These criteria may be established within Photoshop. Photoshop does not offer a tool revealing Lab ranges implied by specific 'Colour Range' and 'Fuzziness' settings; indeed there is no published algorithm detailing precisely how Photoshop performs the image arithmetic behind these tools. However, these parameters can be derived using the LabMeter tool (free from www.Curvemeister.com) to measure the a,b range and a very useful array of 100 small images of the Lab colours with luminance vales

from 1 to 100, created by Gernot Hoffman (see www.fho\-emden.de/~hoffmann/ hungams17042004.pdf).

Even in its present form the method could be used for studies in a small to medium scale. We do not think it will be possible to avoid the need to calibrate images manually, although 'histogram specification' may be worth exploring to see how it performs in this context.

This transforms each colour plane in a new image to have the same colour histogram as a reference image. There are no a priori reasons to think the method will not work with cytoplasmic or nuclear antigens, although these were not included in our studies. Validation studies would be prudent before applying the method to such antigens.

Although we did not address this issue, the number of fields which need to be measured before a stable estimate of TIL density is obtained could be established from running means.

Another issue we did not address is that of quantifying TILs in different compartments within a carcinoma, e.g. stromal, epithelial, or perivascular (Hussein and Hassan, 2006). Our method could be adapted to this by using the Photoshop 'Lassoo' tool to define separate compartments within which TILs are to be quantified.

For some purposes, calibration based on a single image from a set may define a conversion factor with adequate precision in a particular application. For the application we have described two fields appear sufficient. This represents an 80% reduction in the labour of counting, compared to 10-field calibration. This brings the method within the range of what is practicable and should facilitate the use of

objective histological cell counting in clinical and experimental tumour immunology.

Chapter 3: **Quantification of Tumour-Infiltrating lymphocytes on H& E stained tumour sections: A Case-Control Study**

3.1 Study Design

3.1.1 Background to Study Design

As stated in the hypothesis, our aim was to examine the lymphocytic infiltrate in a cohort of breast cancers and examine the association between this infiltrate and prognosis . In designing a prognostic study it is essential to control for already recognised prognostic factors. A defined minimum length of follow up of is required, due to the variable time to progression in breast cancer, which may be prolonged.

Prognosis in operable breast cancer is influenced by a number of clinical and pathological factors. A prognostic factor is any measurement available at the time of surgery that predicts disease-free or overall survival in the absence of systemic adjuvant therapy. In contrast, a predictive factor is any measurement allowing a degree of prediction of response to a given therapy.

Some factors, such as hormone receptor expression and HER2/neu over expression, are both prognostic and predictive

3.1.2 Nottingham Prognostic Index (NPI)

Many prognostic factors in breast cancer have been described but only some of these retain independent significance in multivariate analysis, for example the numbers of nodes involved by tumour.

The importance and utility of such independently significant factors is recognised in the well established and widely used Nottingham Prognostic Index (Haybittle et al 1982). This is used to predict survival in operable breast cancer and was first described in 1982.

NPI is compiled from the size, grade and lymph node status of the primary tumour. It has both intra- and inter- centre validation (Todd et al 1987, Sundquist et al 2002). The ability of this index to separate patients into groups with significantly differing probable survival, to achieve wide separation between these groups and the fact that its applicable to all operable breast cancers, be they symptomatic or screened (Lawrence et al 2003) means it is a satisfactory discriminatory tool when predicting survival.

NPI is calculated in the following manner:

NPI = 0.2 × tumour size (cm) + lymph node stage + grade (1-3) and prognosis and 5 year survival rates are detailed below

NPI Score	Prognosis	5 year survival
2.0-2.4	Excellent	93%
2.4-3.4	Good	85%
3.4-5.4	Moderate	70%
> 5.4	Poor	50%

Table 3.1: NPI & Survival

3.1.3 Nodes

It has been known for many years that lymph node involvement by metastasis is the single most important prognostic factor, following surgery for primary breast cancer (Smith et al, 1977), and there is a direct relationship between the number of involved axillary nodes and the risk of distant recurrence (Saez et al 1989).

Lymph node involvement contributes scores of 1-3 to the NPI as shown below:

Score	Number of nodes involved
1	0 Nodes
2	1-3 Nodes
3	≥ 4 Nodes

Table 3.2: Nodal Groups for NPI

3.1.4 Carcinoma Size

The size of a carcinoma does correlate with the number of involved axillary lymph nodes, but is also an independent prognostic factor in breast cancer. The larger the tumour, the greater the risk of developing distant metastases

(Carter et al 1989). Metastases from larger cancers, do occur more quickly but the increased risk does persist over long term follow up (Rosen et al 1993).

In those cancer patients who have no axillary nodal involvement, the size of the tumour becomes a proportionately more important prognostic factor. Tumour size influences decisions regarding adjuvant therapy and in general the larger the tumour the more likely one is going to need some form of such treatment.

3.1.5 Carcinoma Grade

Pathologic characteristics of a carcinoma, including its grade and histological type, have prognostic significance. Certain subtypes such as tubular, mucinous and possibly medullary have a more favourable prognosis (Carstens et al 1985).

Multiple grading systems have been proposed, with the most widely accepted being the Scarff-Bloom-Richardson (SBR) classification (Bloom et al 1957), which became the basis of the Nottingham grade (Elston & Ellis 1991). This refinement of the SBR grade addresses issues of reproducibility by defining appropriate grading criteria. Tumour grade particularly influences treatment decisions for lymph node-negative patients and those with borderline tumour sizes.

NPI scores tumour grade in the following way:

Score	Grade
1	Well differentiated
2	Moderately differentiated
3	Poorly differentiated

Table 3.3: NPI; Tumour Grade

3.1.6 Oestrogen receptor status

Oestrogen receptors (ER) are nuclear steroid hormone receptors. Their activation is linked with the regulation of certain genes, particularly those controlling cell growth. Oestrogen receptor (ER) assays in breast cancer has been a routine test for approximately 30 years. Early biochemical assays have been superseded by more reliable immuno-histochemical assays. ER status predicts response to hormonal treatment (Jensen et al 1985), and in particular it is used to predict the clinical response to anti-oestrogen treatments, for example tamoxifen and more recently aromatase inhibitors. It was later recognised that determination of ER status could help in the prediction of overall prognosis(Hahnel et al 1979).

Several studies have demonstrated that the breast cancer specific survival of ER positive cancers is better than ER negative cancers (Hahnel et al 1979, Hahnel et al 2004).

However the early survival benefit of ER positivity is not maintained over time and Hahnel et al (2004) found a trend towards increased mortality after five years, with a tumour that is ER positive.

The combination of ER and PR status may further refine the prediction of

the response to endocrine treatment. Overall, ER is perhaps best considered as a "predictive" rather than prognostic factor.

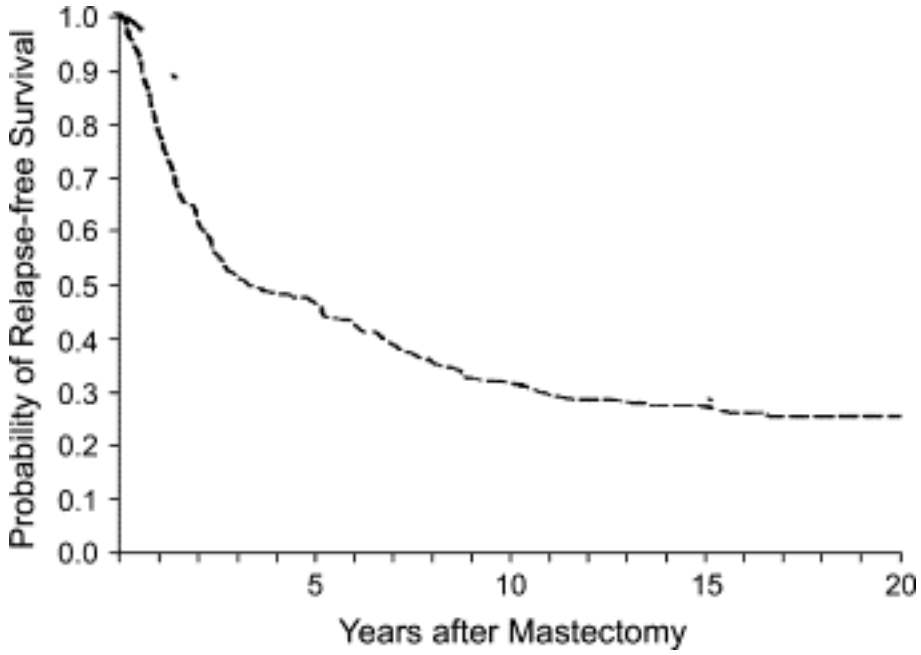
3.1.7 Age/Menopausal status

Age is an important predictor of response to both hormonal and chemotherapies, and has prognostic implications. In women under the age of 35 there is a greater incidence of high grade tumours and an association with reduced survival (Nixon et al 1994). Many studies evaluating the influence of age on outcome in breast cancer have been small, with conflicting results (Fowble et al 1994 & Mueller et al 1978). Two relatively large trials did, however, demonstrate a worse prognosis for patients younger than 35 years of age, even after adjustment for other prognostic factors (Nixon et al 1994 & Albain et al 1994). However, age is generally used as an adjunct to other prognostic factors that are better validated such as tumour size, when determining treatment and expected outcome.

3.1.8 Follow up and Recurrence

Breast cancer is a heterogeneous disease. Unlike for example, rectal cancer, which can be considered 'cured' if a patient is disease free five years after primary surgery (Moore et al 2005), breast cancer can recur up to fifteen and twenty years after initial diagnosis.

Figure 3.1: Illustrates the probability of relapse free survival over a 20 year period (Retsky et al 2005)



In this cohort of patients, approximately 75% of all recurrences had occurred within five years. This indicates that a minimum follow up of five years allows for meaningful comment on disease free survival, whereas a shorter follow up period would not.

Age standardised relative survival (%) at one, five, ten and twenty years since diag female breast cancer, England and Wales, 1971-2003 (CRUK)

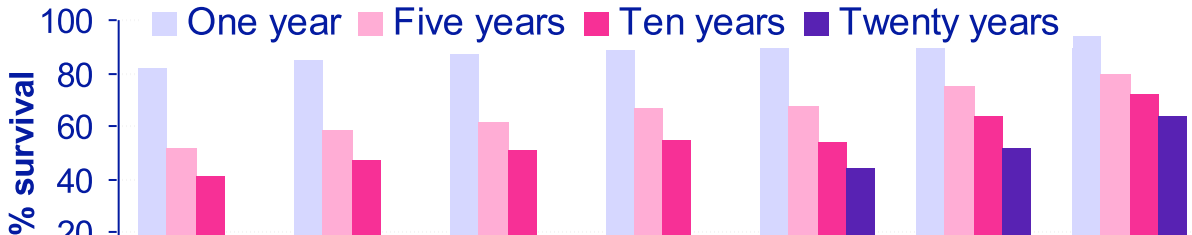


Figure 3.2: Illustrates that while overall survival is increasing, mortality from breast cancer can still occur up to twenty years later. However, most recurrences still occur within the first five years.

3.1.9 Patient Source

We selected our patients from the Greater Glasgow Health Board (GGHB) database. This is a database established in 1995 with the intention of keeping a prospective record of all patients diagnosed with breast cancer in the Greater Glasgow area. It records patient demographics in addition to clinical and pathological data, and subsequent treatments. The intention is that the database should be followed up over the long term to allow outcomes to be recorded. I participated in gathering a significant proportion of this data through case note review.

Information regarding death, which was not recorded in case notes, was obtained from the Scottish cancer death registry.

The GGHB database documents that between October 2005 and December 2000, 3204 patients have been diagnosed with primary operable breast cancer.

Overall there were 684 deaths from this period, however only 392 were directly

attributable to breast cancer. The database does not have a record of overall numbers diagnosed with metastatic disease, however it is accepted that if one dies as a result of breast cancer one has to have distant metastatic disease. All of the cases used in this study had documented evidence of systemic metastases.

3.2 Ethics

Ethics approval was sought from the North Glasgow University Hospitals NHS Trust Ethics committee and was approved on 21ST June 2004.

Ethics Reference no: 04/S0705/7

3.3 Type of study & Power Calculation

In order to investigate the influence of lymphocytic infiltration in breast cancer on patient survival, we decided to implement a case-control study .

It was felt that with access to such a large database that this was the method that would provide results of most significance. In particular, since so many different factors can influence the outcome in breast cancer , we felt that this would be the most appropriate way to control for these factors.

At the time of commencing the study, we had planned to include only those patients diagnosed between 1995 and 2000, assuming in 2005, that we would not have completed five year survival data beyond this.

However we were subsequently able to extend the study period to include 2001, as we had completed follow up data by the time of completion of the study.

The power calculation, was based on the number of patients diagnosed with breast cancer between 1995 and 2000 (i.e. 3204 patients) and the probability that approximately 50% of them would have evidence of an inflammatory response (Billik et al 1989).With the aid of the statistics department at the University of Glasgow, it was

estimated that we would require 111 cases and 222 controls. It was calculated that these numbers would give the study a power of 80% to detect a two-fold relative risk of relapse between groups, with 95% confidence. This calculation was performed using the statistical programme Epi-info version 6.

3.4 Patient Selection

3.4.1 Background

Having elected to perform this study as a case – control series we determined that our cases would be those individuals who had developed distant metastatic disease .
In choosing our controls we felt it would be necessary to account for the aforementioned prognostic data. Therefore we matched our cases and controls based on the following five characteristics :

1. Menopausal status – we chose 50 as the cut off for being assigned to either the pre- or postmenopausal groups as this is usual practice in the clinical setting when determining adjuvant therapies.

2. Tumour size – categories were as per those used in NPI;
   - < 2cm
   - 2-5cm
   - > 5cm
3. Tumour grade – as per NPI

4. Number of positive axillary lymph nodes - again as per NPI

5. Oestrogen receptor status – positive / negative

We did not control for treatment as this has been standardised to the extent that it is reasonable to assume that patients with similar disease and patient characteristics would have had similar treatments.

3.4.2 Inclusion Criteria

A. Cases

All those on the database who were diagnosed with primary breast cancer and who subsequently developed distant metastatic disease or whose death was attributable to their breast cancer.

B. Controls

Two individuals were chosen to control for each case. Any individual on the database who matched a case, based on the previously mentioned five characteristics and had at least five years of completed follow up.

3.4.3 Exclusion Criteria

A. Cases

1) Any case who did not have surgery.

2) Any case for which two matched controls could not be identified.

3) Any case for which a haematoxylin and eosin stained tissue section could not be found.

B. Controls

1) Those who did not have at least five full years of follow up

2) A patient who had developed distant metastases at any time during the follow up period was not included as a control and would automatically become a 'case'.

3) Any individual whose death was attributable to their breast cancer.

4) Anyone who did not have surgery.

5) Anyone for whom a haematoxylin and eosin stained tissue section could not be found.

3.4.4 Final patient group

The study was carried out over the period 2005-2007. Therefore in order to ensure

that our controls had a minimum five year follow up, we did not use patients who had a primary diagnosis after 2001.

Between 1995 and 2000 the GGHB database has recorded a total of 3204 patients diagnosed with primary operable breast cancer. Of these, 392 died from breast cancer and 292 of another cause.

In total we had 127 cases for which we could identify two matched controls. There were obviously many more 'cases' on the database, however, we had some difficulty matching all of them with suitable controls. The majority of those that could not be matched were the largest tumours, i.e. those over 5cm and or the ones with large numbers of positive lymph nodes. The reason for this, unsurprisingly, was that it was difficult to find patients in these categories who had not developed distant metastases.

However our final number in the study was reduced because haematoxylin and eosin stained sections, from resected cancer specimens, could not be found for 57 patients. This number includes both 'cases' and 'controls'.

Our final numbers for inclusion are:

A) 90 cases

B) 180 controls.

3.5 Materials and Methods for counting on Haematoxylin & Eosin (H&E) stained sections

3.5.1 Background

Many previous studies of lymphocytes in breast cancer have used semi-quantitative (Scholl et al 1996) or qualitative methods (Lee et al 2006).

We wished to use a strictly quantitative approach, which would however not be prohibitively laborious.

We therefore used a validated method, established by Going in 1994, to count the inflammatory cells. This method is based on 1) random sampling (Simpson et al 1992) and 2) the unbiased counting rule (Gunderson 1978).

The usual approach to counting cells in a microscope is to use an eyepiece graticule in the form of a 10 x 10 square grid and to count all the cells in this area ,using the grid as a guide to avoid counting the same cells more than once. Going established that by counting cells in a random subset of small squares, for example 10, and then multiplying this number by 10 would give an unbiased estimate of the number of cells in a grid of 100 small squares. By repeating the procedure over ten fields the degree of total error was reduced to an acceptable level, ± 5% of the fully counted total, and significantly the observer only needed to count 10% of all cells present.

3.5.2 Imaging the Sections for counting

Tumour infiltrating lymphocytes may be found within the stroma of the tumour but

also often at the edge of the tumour (Lee et al 1996).We counted the inflammatory cells on H+E stained sections of breast cancer in ten consecutive fields along the edge of the tumour. One reason for this choice was to avoid inflammatory responses to areas of necrosis.

Using a computerised camera microscope system (Fuji HC300Z digital camera and a Nikon Eclipse T600) at x40 Apo chromatic objective, a photograph was taken of 10 consecutive fields starting wherever tumour edge was found closest to the top left hand corner of each section. This random starting point was consistently used for each case. A total of ten images was taken for each of 366 cancers (a mixture of both cases and controls). However only 270 of these could be used in the final study as the remainder were either cases for which two control slides could not be found or vice versa.

The photographs were saved as uncompressed 24-bit RGB TIFF files, for counting at a later date.

3.5.3 Counting the Inflammatory cells

We used Image J (http://rsbweb.nih.gov/ij/), an image processing tool kit, available in the public domain, to facilitate the counting of inflammatory cells on the images we photographed.

The ‘process’ application in Image J was used to superimpose one of four different pre-constructed counting grids, via the ‘AND’ mode, on each the ten images of the H&E stained section for each case.

Each counting grid had within it 10 highlighted rectangles (Figure 3.3).The same arrangement of rectangles was highlighted in grids (A-D), but rotated by 90^0 between A-D, B-C, C-D and D-A.

One of the four grids was selected at random for counting each case.

Using the unbiased counting rule (Gunderson 1978) inflammatory cells were only counted if they either fell within the highlighted squares on the grid or along the ‘northern’ or ‘western’ border of these highlighted squares , as illustrated in figures 3.4. and 3.5 . As per Going’s counting method, the total number of cells over the counted 10 squares was then multiplied x 10 to give an estimate of the cell count for the whole image. . Field size (measured by stage graticule) was 218°—170 microns=0.03706 mm^2 =1/26.98 mm^2 ≈1/27 mm^2, so cell counts multiplied by 27 equal cell counts per mm^2.

A record was kept of which grid was used with each case.

For the first twenty tumours imaged, cell counts were performed by two observers trained in the counting method.

The counts were largely comparable.

Figure 3.3 : Grid A

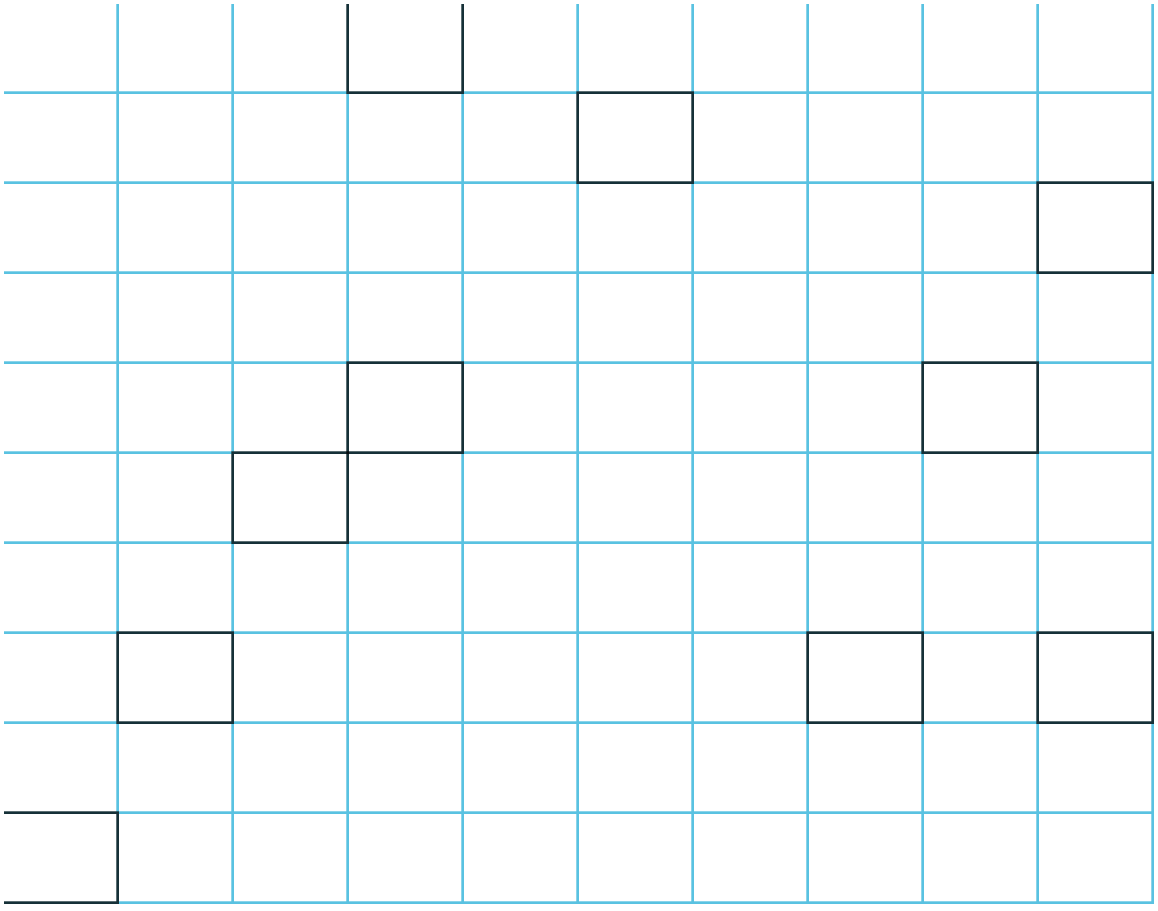
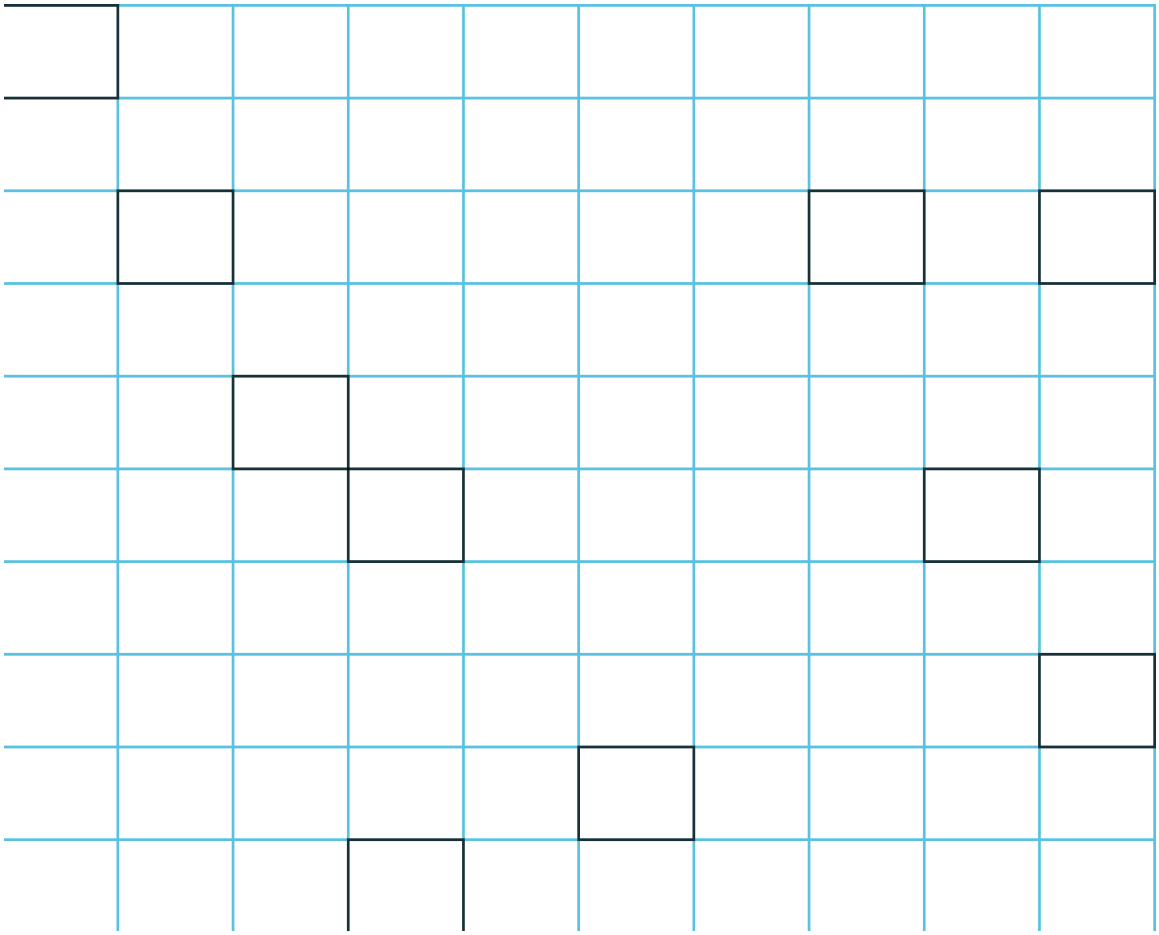


Figure 3.4 : Grid D



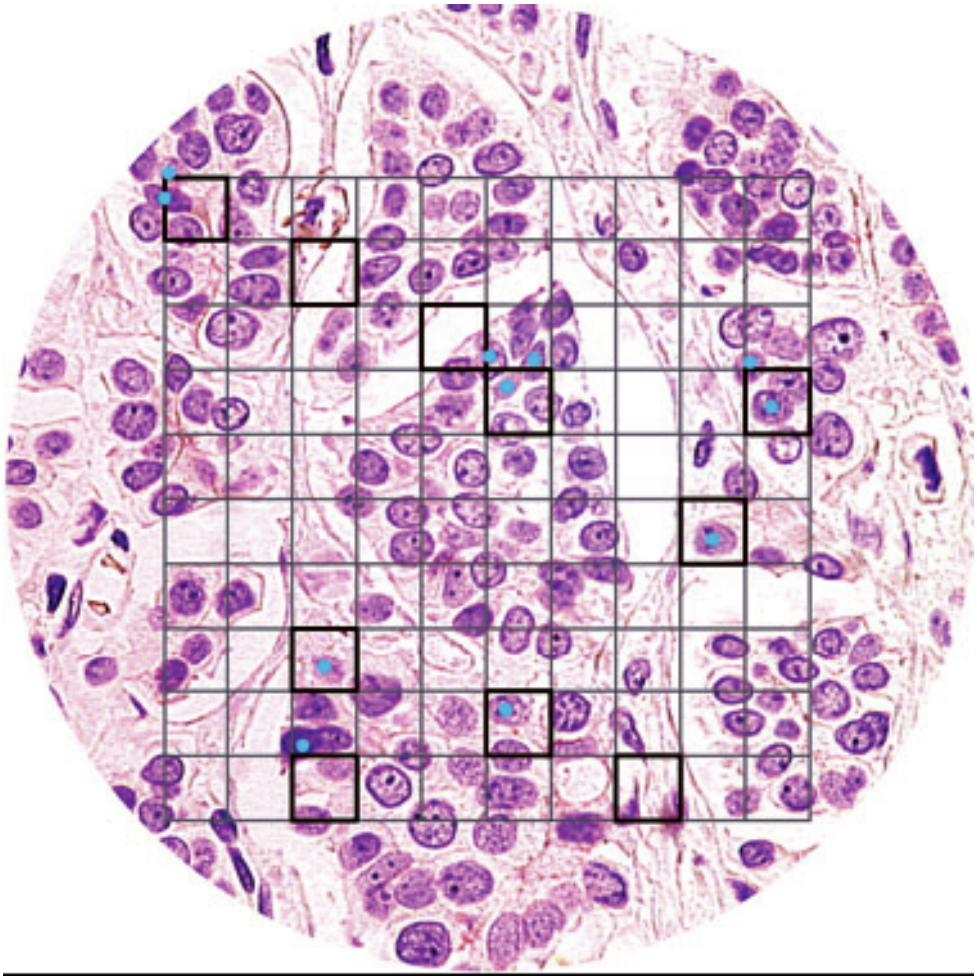


Figure 3.5: Example of Counting Grid superimposed on an image of a H&E stained breast cancer section. The cells to be counted are highlighted in blue (Going, 2006)

Figure 3.5 illustrates the counting process. The large square is subdivided into 100 small squares, 10 of which are picked out by their heavier border. Eleven cells (marked with blue spots) to be counted are identified by the unbiased counting rule using upper and left inclusion edges, giving an estimate of 11 x 10 = 110 cells in the large square. Actual cell count is 124.

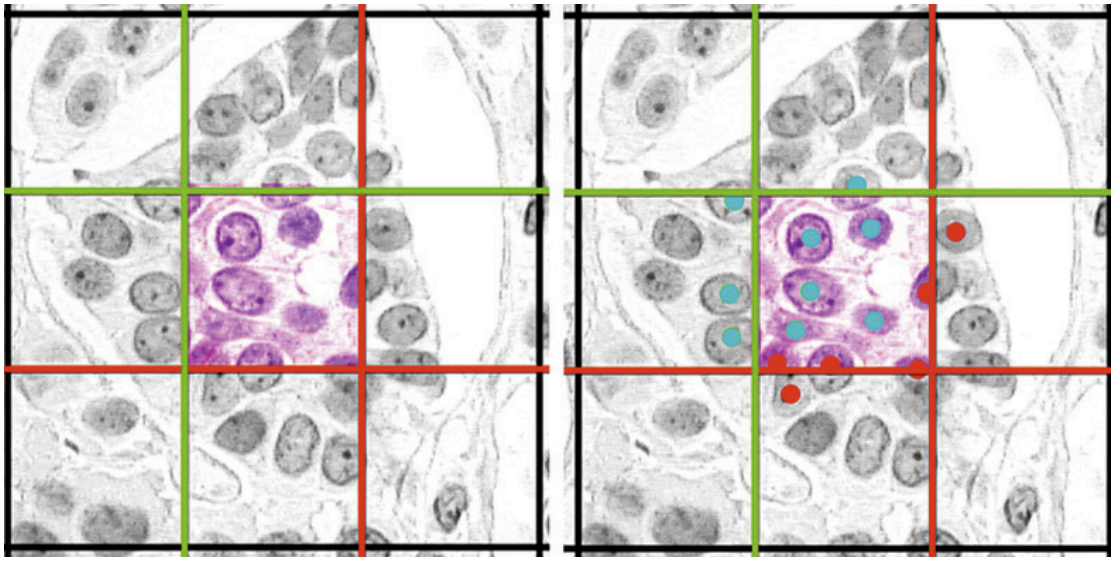


Figure 3.6 : Illustration of the unbiased counting rule (Going 2006)

Figure 3.6 illustrates the unbiased counting rule. Consider the sampling square in the centre of this 3 x 3 array. Imagine two inclusion edges (green) above and to the left and two exclusion edges (red) below and to the right of the sampling square. Cells to be included in the count (blue dots) touch or lie below or to the right of both inclusion edges, but do not touch or lie below or to the right of either exclusion edge Excluded cells are identified with red dots. This rule is easy to apply in practice once learned. In expressing actual cell counts, the number of cells per field has been multiplied by 27 to give the results in cells per mm^2.

3.5.4 Statistics Employed

Analyses were performed using a combination of Microsoft Excel for the descriptive data and programme R , a web based statistics package , for the analysis.

A combination of paired and unpaired t –tests were used

3.6 Results

3.6.1 Descriptive Statistics

90 ‘Cases’ had cell counts calculated on H&E stained tumour sections.

Range: 0-1770

Median count: 583.8

This demonstrates the wide variation in counts between all the cases.

180 Controls had cell counts calculated on H&E stained tumour sections

Range: 0- 4790

Median Count: 627.8

Again a wide variation is seen in the range of cell counts for all 180 controls.

Figures 3.7 and 3.8 are examples of ‘cases’ with a low IID (overall cell count for this ‘case’ was 93 per Square mm) and a high IID (2241 per Square mm) respectively.

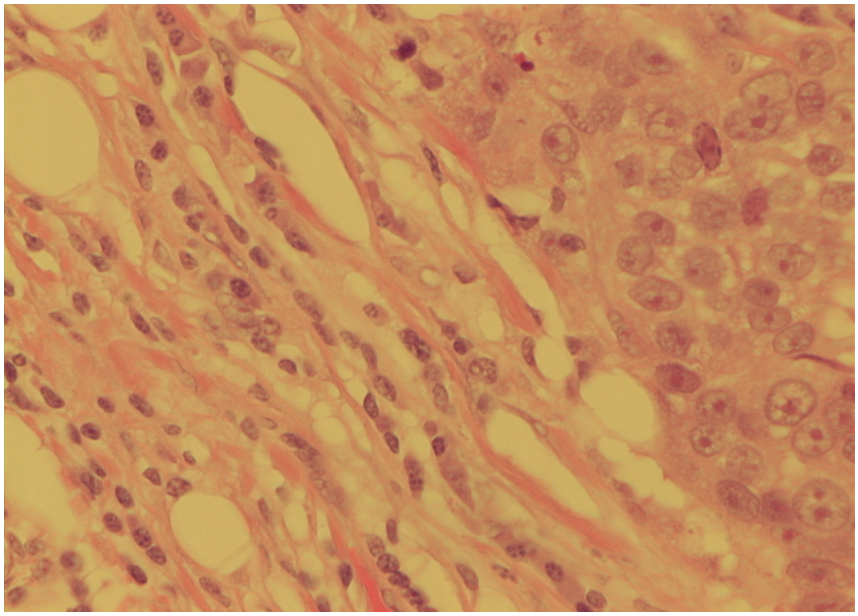


Figure 3.7: ‘Case’ 432

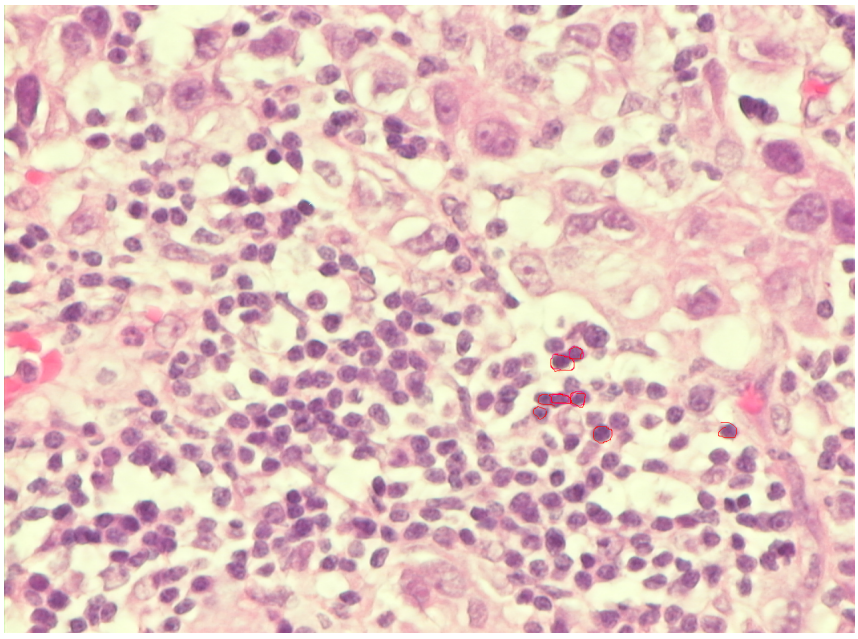


Figure 3.8: ‘Case’ 1257 (some of TILs circled in red)

Figures 3.9 and 3.10 are examples of ‘controls’ with a low IID (overall cell count =108 per Square mm) and a high IID (1426 per Square mm) respectively.

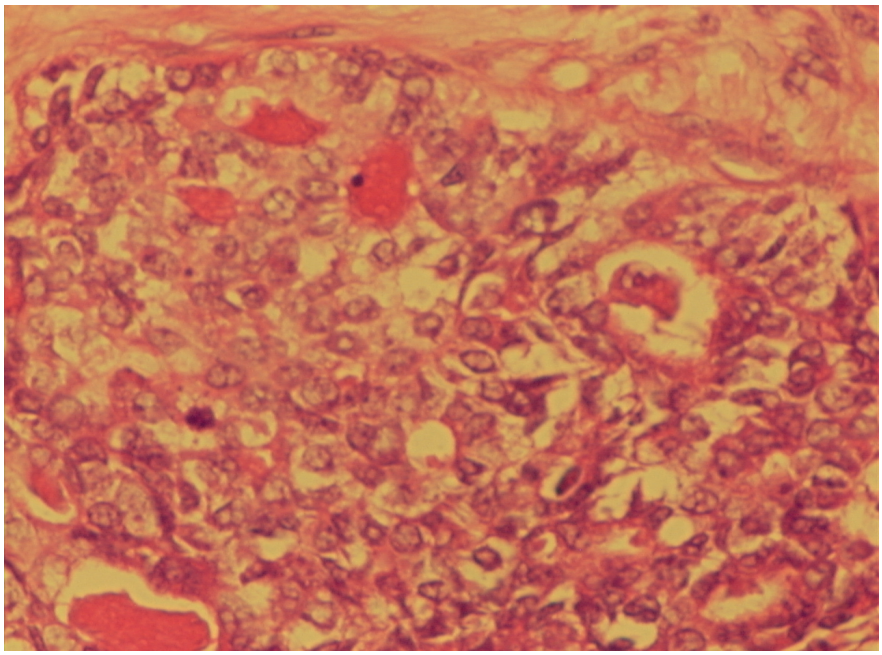


Figure 3.9: ‘Control’ 1437

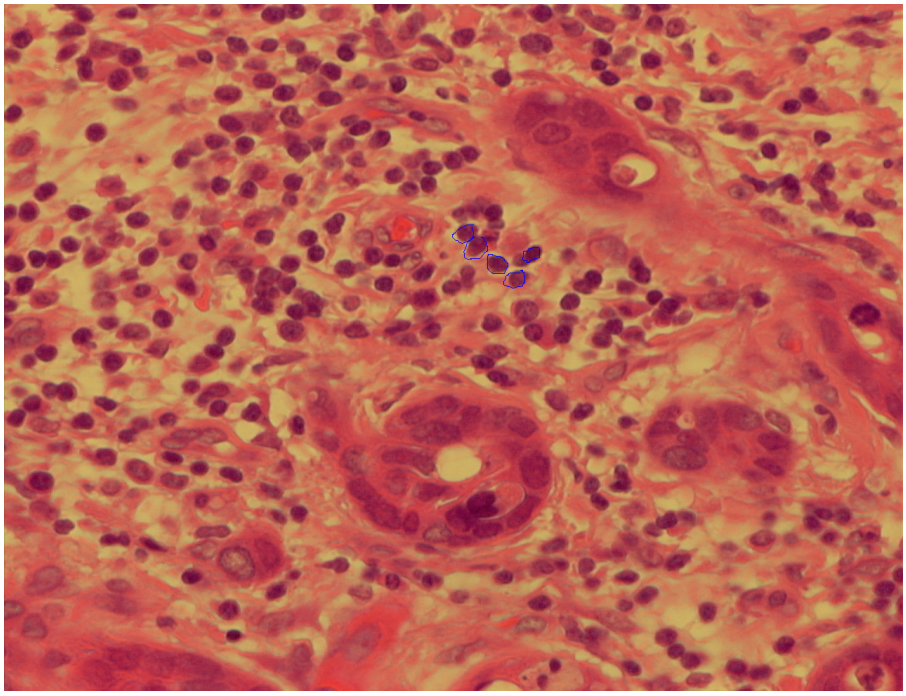


Figure 3.10: 'Control' 542 (some of TILs circled in blue)

Menopausal Status

Overall , out of the 270 patients 183 of them were presumed to be post-menopausal, as determined by age >/= 50 . 87 of them were pre-menopausal. This is in keeping with the expected distribution of age given that the incidence of breast cancer increases with age.

Table 3.4: Distribution of patients according to menopausal status

Pre-menopausal	87 (32.2 %)
Post-menopausal	183 (67.7 %)

Size

The range of size was 4-60mm for the cases and 5 – 55mm for the controls. Overall the median size was 20mm.This is not unexpected. Since the introduction of breast cancer screening, more tumours are being diagnosed earlier and are therefore likely to be smaller (Hofvind et al 2008).

Tumour grade

As illustrated in table 3.3 the majority of the tumours were either grade 2 or 3. Only 12 out of 270 patients had a grade 1 tumour. The median tumour grade was 2.

Table 3.5: Distribution of patients by tumour grade

Grade	Total Number (%)
1	12 (4.4 %)
2	132 (48.9 %)
3	126 (46.7 %)

Nodal Status

The range of positive i.e. tumour involved axillary lymph nodes was

0-17 for the 'Cases'

0-19 for the controls

The median number of positive nodes was 1.

Oestrogen Receptor Status

Table 3.6 shows the distribution of ER status amongst all tumours.

Table 3.6:

ER Positive	168 (62.2%)
ER Negative	102 (37.8%)

These figures are in concordance with the literature. Approximately 60% of those with breast cancer will have functioning oestrogen receptors.

3.6.2 Data Analysis

3.6.2.1 Data distribution and Transformation

For the initial analysis, data from all patients (n=270), i.e. both 'case' and controls, were grouped together. This was done to analyse the data in the whole group, without regard to pairing. We firstly wanted to establish if the inflammatory infiltrate densities (IID) were

normally distributed, or not.

To do this a quantile-quantile plot (Q-Q plot) is used. It is clear from figure 3.11 that the line is strongly curved indicating that the data is highly non-normal.

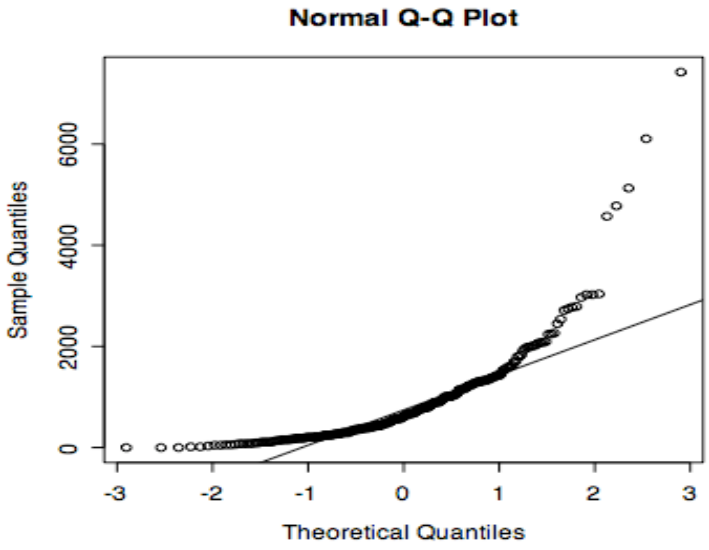


Figure 3.11 : Q-Q plot of untransformed Inflammatory Infiltrate Density data (per mm^2)

A histogram of the same data seen in figure 3.12 again confirms the data is strongly skewed, and non-normal.

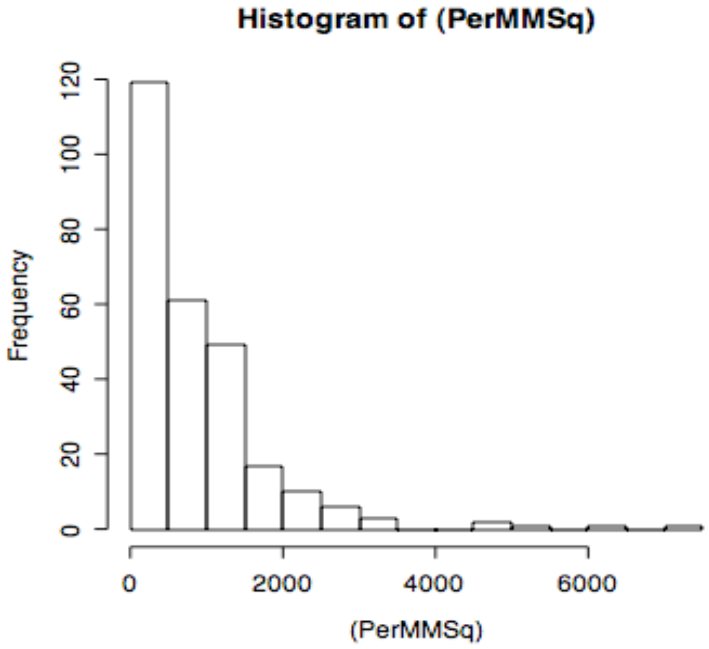


Figure 3.12: Histogram of IID per mm^2

On the advice of Dr. J Paul, statistician at the University of Glasgow, we then went on to 'transform' the data into a form in which it was normally distributed. We looked at both square and cube root transformations, of which the latter was felt to be optimal. These statistical transformations are perfectly legitimate because they are applied equally to all of the data.

Figures 3.13 and 3.14 illustrate the Q-Q plots of the square root and cube root

transformed data respectively.

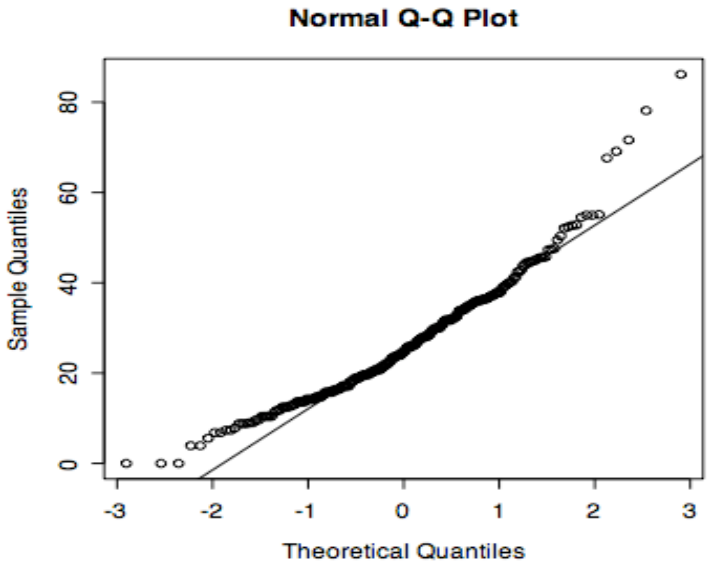


Figure 3.13: Q-Q plot of the square root transformed IID data

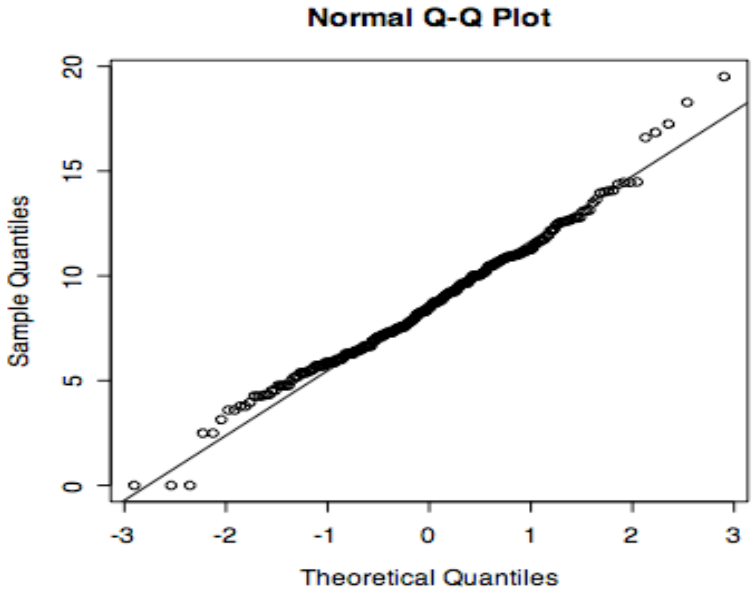


Figure 3.14: Q-Q plot of the cube root transformed IID data

Clearly the cube root transformation is most linear and represents the data in a normally

distributed manner. This was the transformation therefore chosen for statistical testing.

3.6.2.2 Comparison of IID between 'Cases' and Controls

The range of cell counts for both groups of patients were widely distributed. This is illustrated in the histograms in figures 3.15 and 3.16

Figure 3.15: Histogram – Range of cell counts for all 'Cases' n =90

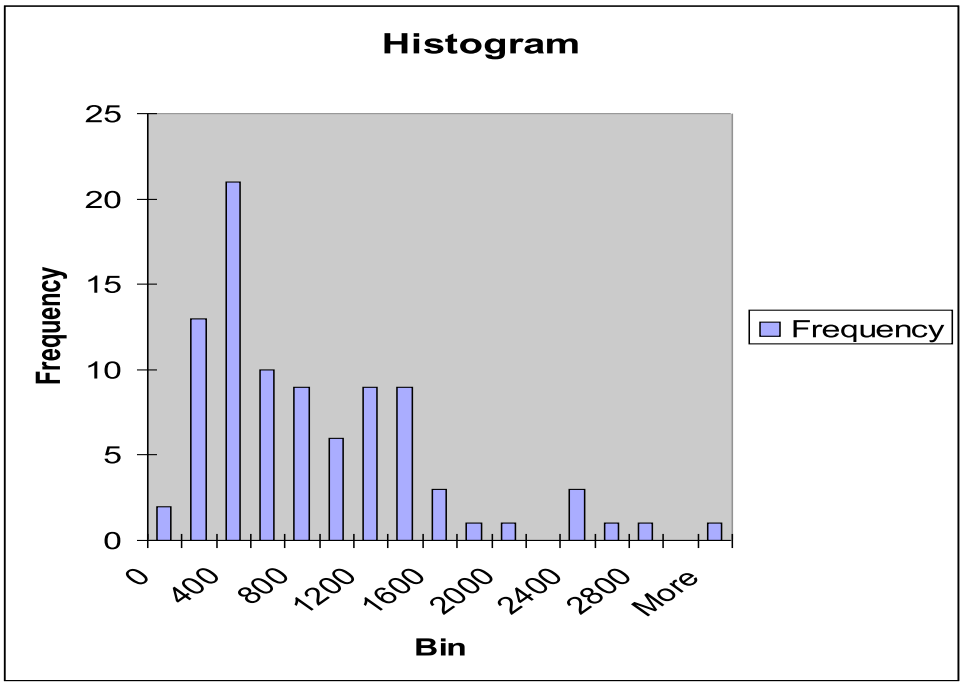
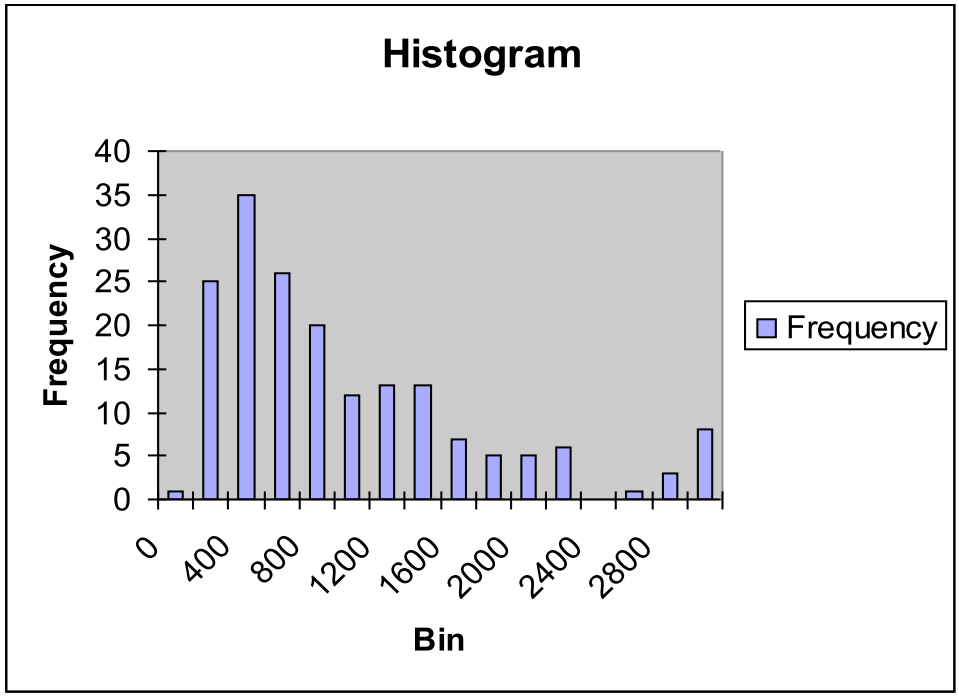


Figure 3.16: Histogram – Range of cell counts for all Controls n= 180



Next we examined the relationship between the IID and status of the patient as a case or a control. To do this parallel boxplots were constructed, figure 3.17

It would appear that there is no obvious difference in the distribution of the IID values between 'cases' and controls.

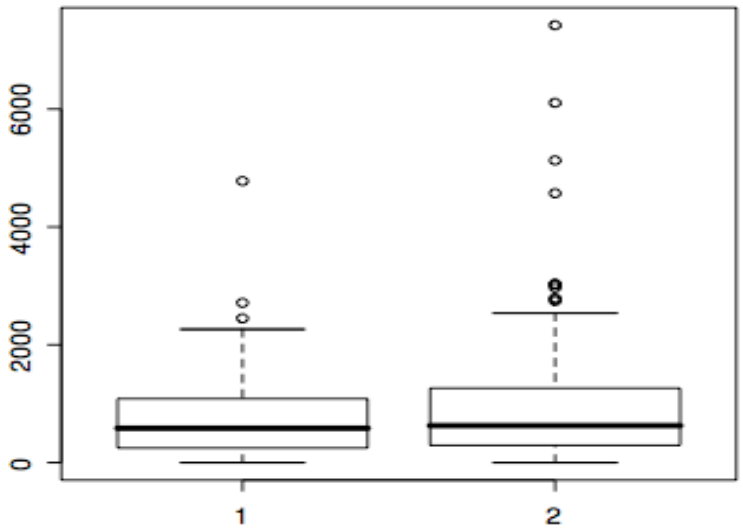


Figure 3.17: Boxplot comparing IID per mm^2 Cases (1) and Controls (2)

We used a two sample t-test to compare the difference. Since this assumes normality of the data it was applied to the cube root transformed data. It also assumes the variances of the data are the same in the two groups.

The result was non-significant, p = 0.2319, 95% confidence interval.

Whilst this is a useful test it does not take into account that our data was made up of 'cases' paired with two controls.

In order to compare 'cases' directly with 'controls' the mean value of the two cell counts permm^2 , for each of the two controls was calculated. It was then possible to perform a paired t-test which did show a significant difference between the two groups,

p = 0.04478, 95% confidence interval.

This demonstrated that the IID is significantly greater in the controls than in the ‘cases’. Although the unpaired data did not allow this conclusion, it was not the most appropriate analysis.

However, the average difference in IID between ‘cases’ and controls is small. A boxplot, seen in figure 3.18 of the untransformed data illustrates this.

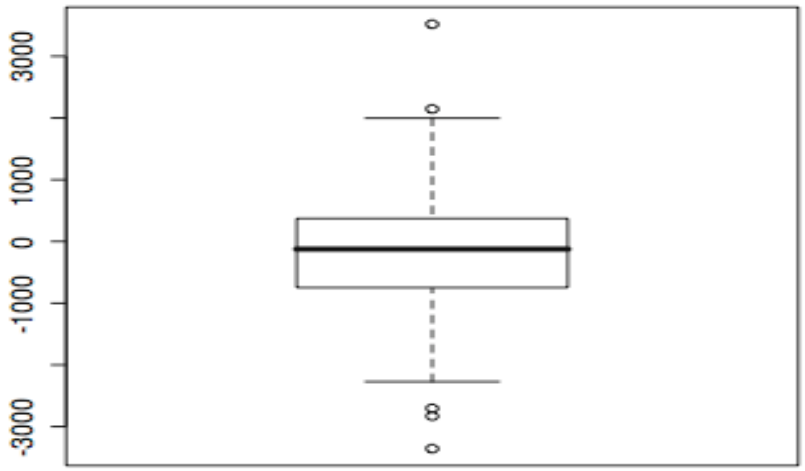


Figure 3.18: Boxplot of the average difference between ‘cases’ and controls

The average difference, although statistically significant, is much less than the ‘noise’ in an individual case and therefore would have no prognostic value in an individual case.

.3 Association between IID and Prognostic Factors

3.6.2.3.1 IID and Menopausal Status

Table 3.7 illustrates the IID in patients when classified according to menopausal status. The maximum cell count 7424.5 for the post-menopausal group is much greater than that of the pre-menopausal patients, 4557. There is, however no significant difference between mean cell count per mm^2 in the two groups,($p = 0.66$).

Table 3.7 :Differences in cell count data between pre- and post-menopausal groups

Postmenopausal	Count mm^2	Premenopausal	Count mm^2
Mean	865	Mean	929.2
Standard Error	71.8	Standard Error	93.9
Median	567	Median	798.2
Range	7424.5	Range	4557
Minimum	0	Minimum	15.5
Maximum	7424.5	Maximum	4572.5

3.6.2.3.2 IID and Tumour Size

Table 3.8: Cell count data for all tumours in relation to size

Size <20mm	Count mm^2	Size 20-50mm	Count mm^2	Size >50mm	Count mm^2
Mean	828	Mean	872.1	Mean	735.2
Standard Error	70.2	Standard Error	65	Standard Error	267.7

Median	573.5	Median	604.5	Median	592.7
Range	6107	Range	7424.5	Range	1491.1
Minimum	0	Minimum	0	Minimum	46.5
Maximum	6107	Maximum	7424.5	Maximum	1537.6

The degree of inflammatory infiltration does not appear to be related to tumour size based on this data. When put into groups based on tumour size being ≤ 20mm or > 20mm there was no difference in the mean IID, P = 0.1695

3.6.2.3.3 IID and Tumour Grade

Table 3.9 : Illustrates the IID data for tumours according to grade

Grade 1	Count mm ^{2}	Grade 2	Count mm ^{2}	Grade 3	Count mm ^{2}
Mean	1068	Mean	712	Mean	1049.9
Standard Error	469.6	Standard Error	66.2	Standard Error	89.6
Median	570.2	Median	433	Median	879.5
Range	5998.5	Range	5130.5	Range	7424.5
Minimum	108.5	Minimum	0	Minimum	0
Maximum	6107	Maximum	5130.5	Maximum	7424.5

The difference between the mean IID for grade 1 and the means of the other two were not significant. However there was a significant difference between the grade 2 and grade 3 tumours (p = 0.002). The grade two tumours had a significantly lower IID. We then classified the tumours into two groups, low grade (includes grades 1+2) and high grade (3).Again the difference was highly significant, p = 0.00045. The high grade

tumours had a significantly denser IID.

3.6.2.3.4 IID and Nodal Status

Table 3.10 : Cell counts based on nodal status

0 Nodes Positive (Group1)	Count mm ^{2}	1-3 Nodes Positive (Group 2)	Count mm ^{2}	≥ 4 Nodes Positive (Group 3)	Count mm ^{2}
Mean	929.6	Mean	916.7	Mean	712.3
Standard Error	88.6	Standard Error	100.9	Standard Error	88.3
Median	720.7	Median	570.2	Median	565.7
Range	6107	Range	7424.5	Range	2241
Minimum	0	Minimum	0	Minimum	0
Maximum	6107	Maximum	7424.5	Maximum	2241

When the means were compared between the groups 1, 2 and 3 as individual pairs there was no significant difference in cell counts between any of the pairs.

We also performed a separate analysis by grouping the tumours into either node negative or node positive groups. Again there was no significant difference ($p= 0.32$).

3.6.2.3.5 IID and ER status

Table 3.11: Cell count data based on ER status

ER Negative	Count mm ^{2}	ER Positive	Count mm ^{2}
Mean	1127.7	Mean	715.7
Standard Error	108.3	Standard Error	62.3
Median	899	Median	480.5
Range	7393.5	Range	6107
Minimum	31	Minimum	0
Maximum	7424.5	Maximum	6107

The mean and median cell counts per square mm were significantly greater in the ER negative patients. Er negative tumours had a greater IID ($p < 0.0002$).

3.7: Discussion

We recall that our hypothesis was that metastatic relapse would be less likely in women with breast cancers in which a significant immune infiltrate was present than in women with cancers in there was no significant immune-cell infiltrate.

In keeping with the literature (Bilik et al 1989, Lee et al 2006) we found that the majority of the tumours studied had some evidence of an immune cell infiltrate. The range was wide, as illustrated in figures 3.15 an 3.16. However only 3 of the tumours in our data set of 270 showed absolutely no evidence of tumour infiltrating lymphocytes. This supports previous investigations concluding that breast cancer is an immunogenic tumour and interacts with the host immune system, provoking a response.

In agreement with our hypothesis, we did find a statistically significant different between our ‘cases’ and controls. However, this difference was on average too small to be considered biologically significant. Nevertheless, it does support the idea that host immune responses are in some way mounting a successful response to the tumour.

There have been many studies over the last twenty years looking at the relationship between lymphocytic infiltrates in breast cancer and prognosis. The results have been conflicting. The most recent large study by Lee et al in 2006, aimed to establish an association between tumour lymphocytic infiltration and better prognosis. They examined the tumours of 745 patients and looked at the degree of lymphocytic infiltration. They established, on multivariate analysis, that overall prognosis was better when there was evidence of diffuse inflammation, in tumours containing necrosis.

Prior to this there have been several studies that have concluded that there was no association between the inflammatory response in the the tumour and prognosis (Haybittle 1982, Aaltoma 1992). Carlomagno in 1995 and Scholl in 1996 both found that it was associated with a worse prognosis. However many of these studies only looked at inflammation as one variable amongst many and details regarding assessment of inflammation was limited.

Our study was a well designed case-control study which controlled for the several factors with prognostic significance in breast cancer. The power calculation had estimated that we needed a total of 333 patients, i.e. 111 ‘case’ and 222 controls. Unfortunately as we explained in the methodology section it was not possible to achieve this, however we still did achieve more than 80% of the total suggested.

As shown in the results we went on to look at the relationship between the factors we controlled for and the inflammatory infiltrate density.

Similarly to previous work (Menard et al 1997) we found no association between lymphocytic infiltration and age / menopausal status, although Menards group did

establish that the presence of a lymphocytic infiltrate was associated with improved survival in those under the age of 40. Although several authors (Macchetti et al 2006, Wernicke et al 2003) have found an association between the presence of CD 4+ T cells within the tumour and lymph node metastases, we found no such association between IID and this most important of prognostic factors.

The only significant findings, in our study, in relation to prognostic factors, were of a greater IID in tumours of higher grade and also in those tumours that were ER negative. Lee et al in 2006 found that there was an increasing intensity of diffuse inflammation within the tumour, in those of higher histological grade. They also found that diffuse inflammation was a marker of better prognosis in those with grade 3 tumours. This would suggest that higher grade, or more aggressive, tumours are stimulating a more intense host inflammatory response, perhaps in an attempt to eradicate it.

Recently a small Brazilian study by Macchetti et al (2006) found an association between the presence of CD 4 + T cells within the tumour and lymph node metastases. This was in keeping with findings of other authors (Wernicke et al 2003).

We have confirmed that breast tumours are clearly interacting with the host immune response in some way as is evidenced by the very presence of tumour infiltrating lymphocytes. We have also shown that this is a positive response, which can impact on outcome.

Further studies quantifying the lymphocytic infiltrate without phenotypic evaluation of these cells are unlikely to be of significant value. We now need to identify what cells are

contributing to this response and the mechanisms of how they are defending the host. We now have a well designed dataset on which to do this and this will be where future work will be focused.

Chapter 4 The Future

4.1 Overview

Increasingly, it is recognised that the immune system has a significant role to play in tumour development, and subsequently outcome. Research, particularly, during the last decade indicates that the host immune response to a tumour is influenced by the type of response initiated. This is illustrated in figure 4.1 which documents the contrasting roles of different types of lymphocytes.

On the one hand Th 1 CD4 and CD 8 T cells, during the acute inflammatory response, may initiate tumour rejection via pathways referred to in chapter

1. Conversely in chronic inflammation, Th2 CD4 T cells in conjunction with T- reg cells can suppress the cytolytic ability of the CD8 T cells.

It has become apparent that the extent of the total lymphocyte density in breast cancer, in itself, is perhaps not of significance without the knowledge of the phenotype of these cells.

In our study examining the intensity of the lymphocytic infiltrate, in multiple breast cancers, we have helped to confirm this by demonstrating there is a greater lymphocytic infiltrate in those tumours that do not progress to metastatic disease.

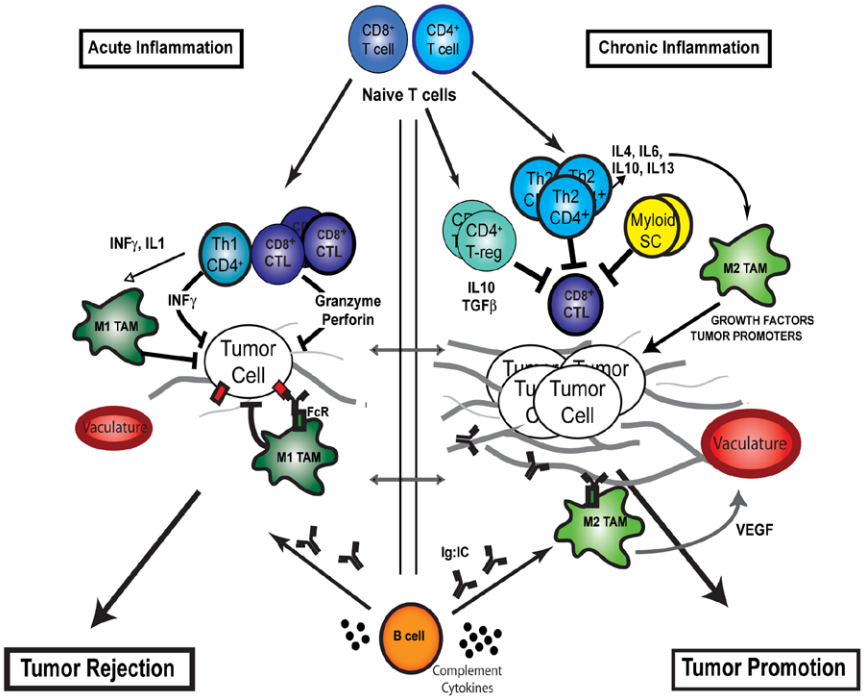


Figure 4.1 Contrasting roles of adaptive lymphocytes during tumour development. DeNardo and Coussens Breast Cancer Research, 2007

We see in figure 4.1 the significance of knowing the types of inflammatory cells taking part in the host response to the presence of a tumour. Now that we know that the inflammatory response in breast cancer is significant in predicting survival, we can focus on eliciting more information about it.

If we are to be able to manipulate the response that is occurring we need to identify the individual cells and demonstrate the extent to which they can influence outcome.

The role of the immune system in cancer development is complex. We have clearly shown in our study, that the majority of breast cancers exhibit evidence of an immune response as reflected by the lymphocytic infiltrate, however variable the extent of this is. It is accepted that chronic infiltration of human tissue by some innate immune cell types, such as monocytes and macrophages, is associated with the development of epithelial cell cancers (Coussens et al 2002).

So where does this leave us in trying to develop better, specifically immune based treatments for breast cancer? We must remember that in spite of improved prognosis, the overall mortality from breast cancer is still significant. Metastatic disease remains incurable.

There have been improvements over recent years, not least from the recent introduction of Herceptin, the recombinant humanized anti-HER2 monoclonal antibody trastuzumab, to the physician's armament. Preclinical models demonstrated that this

antibody has significant anti-tumor activity as a single agent and has synergy with certain chemotherapeutic drugs. Phase II and III clinical trials performed in women with metastatic breast cancer that overexpress HER2 have shown that trastuzumab has clinical activity when used as first-, second- or third-line monotherapy, and improves survival when used as first-line therapy in combination with chemotherapy(Slamon et al 2001).The anticancer effects of these antibodies, is mediated as we previously discussed via antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity (Naylor et al 2001). Monoclonal antibodies have emerged as a class of novel oncology therapeutics. Many are being developed and as many as three different solid tumours, in addition to breast cancer, have antibodies licensed for use in clinical treatment. They include Bevacizumab for both colorectal and lung cancer (Yan 2008).

We recall that the concept of using antibodies as “magic bullets” to specifically attack malignant tumor cells was originally proposed by Ehrlich 1 at the beginning of the 20th century. Almost a century later, in spite of varying success along the way we are again focusing on this theory.

Active immunotherapy is still an investigational approach to the treatment of breast cancer. In general, active immunization of those with cancer, using tumour specific antigen vaccine does not induce regression or cure. It is possible that success will be from using cancer vaccines as a preventative measure, of either primary disease or recurrence. Active immunization of cancer patients with HER-2/neu helper peptide vaccines can boost antigen-specific T cell frequencies in vivo (Knutson et al 2001). Because of the heterogeneous nature of solid tumours, vaccines that can act against

several different cancer antigens may be necessary. We know that tumours escape immune surveillance via a number of mechanisms including loss of cancer antigens and MHC- 1 expression (Jager et al 2002 &1997). Future studies will need to focus on vaccine use in the adjuvant setting where tumour heterogenicity and immune escape may not yet have developed (Jager et al 2005). Combining vaccines with monoclonal antibodies in addition to standard adjuvant therapy may be necessary. Immunomonitoring is an essential component of the development of immunotherapy.

Another area of focus in recent years has been the systemic inflammatory response. Al Murri et al (2006) found that the systemic inflammatory response, as evidenced by elevated circulating concentrations of C-reactive protein(CRP) and hypo-albuminaemia, was independently associated with decreased survival in breast cancer patients with advanced disease. There is also some evidence that these acute-phase proteins have independent prognostic value in primary breast cancer (Lis et al 2006, Al Murri et al 2007). So is there any relationship between this systemic response and the local one? A recent study by Al Murri et al(2008) found a positive association between an elevated CRP and the presence of CD4+ T cells in a group of patients with primary operable breast cancer. However no association between CRP, or tumour infiltration by T lymphocytes, and survival was found. Their findings would suggest that there is that there is a connection between the local and systemic responses. This may be important when considering new therapies. For example it is well recognized in colon cancer that inflammatory diseases of the colon increase the risk of colorectal cancer (Rhodes and

Campbell 2002) and there is some evidence that the use of nonsteroidal anti-inflammatory drugs may protect against colon cancer (Gupta and Dubois 2001). As is the case with breast cancer an elevated CRP is associated with decreased survival in those with colorectal cancer.

It may be possible for us to look at this in the future on our dataset of patients and correlate it with the local response.

4.2 Conclusion

In conclusion it would seem that the inflammatory response in breast cancer does have a role to play in the development of the disease and that those tumours which do not metastasise are more likely to have evidence of this response. We also have established a way to accurately and practically measure this on immunohistochemistry .

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Appendix A.

Algorithm for counting TILs

1. Immunostain sections in one batch using automation if available.

2. Capture ten ×40 digital images of fields for counting, using the same microscope, camera, and light intensity. Save as RGB tif files. Do not use lossy file compression.

3. Open a representative image in Photoshop. Use 'Colour Picker' tool to choose a Foreground Colour equal to mid-range DAB staining. Note RGB and Lab values corresponding to this colour, so you can reset it later if need be.

4. Open 'Colour Range' tool. Set 'Fuzziness' to 100. Verify that these settings choose pixels corresponding to the DAB staining. Adjust Foreground Colour and Fuzziness until satisfied with selection.

5. With these settings (check), open each file in turn; apply the Colour Range tool; confirm that the selection looks right; and record the number of pixels selected from the Image/Histogram dialogue in an spreadsheet.

6. For each set of 10 images, open again the two images with the most labelled pixels.

7. If these images do not include too many DAB labelled cells, count the total number of cells. If there are too many to count them all, select an area containing a countable number of cells. Count them. Then count the number of signal pixels in this area by applying the Colour Range tool to this area only.

8. Calculate the average number of labelled pixels per cell over these two images.

9. Use this calibration to calculate the number of labelled cells in all 10 images.

10. Convert to cells per mm^2 (using image size measurements).

Appendix B

ID	MENO	SIZE	GRADE	NODES	ER	COUNT	PerMMSq
16	POST	60	2	4	POS	30	46.5
234	PRE	55	3	2	NEG	50	135
252	POST	40	2	0	POS	190	294.5
334	POST	30	2	12	POS	360	558
339	POST	12	2	2	POS	100	270
349	POST	11	2	0	NEG	640	992

352	POST	25	2	1POS	430	666.5
359	POST	20	2	5POS	130	201.5
365	PRE	17	3	0POS	140	217
399	POST	12	2	1POS	20	54
431	POST	24	3	1NEG	650	1007.5
432	PRE	20	3	1NEG	60	93
460	POST	20	2	0POS	170	263.5
473	PRE	12	3	2NEG	860	1333
474	POST	25	2	0POS	390	604.5
617	POST	30	2	7POS	430	666.5
622	POST	20	3	11NEG	670	1038.5
651	PRE	19	3	1POS	370	573.5
673	PRE	17	2	13POS	1160	1798
684	PRE	17	3	0NEG	270	418.5
690	POST	25	1	17POS	370	573.5
696	POST	17	2	3POS	250	387.5
702	PRE	15	3	0POS	450	697.5
729	POST	40	2	0NEG	580	899
730	POST	17	3	0POS	70	108.5
755	POST	16	3	0NEG	560	868
757	POST	22	3	5POS	0	0
781	PRE	14	3	0NEG	510	1377
782	POST	20	3	0POS	130	201.5
806	POST	21	3	4NEG	770	1193.5
819	PRE	21	2	0NEG	830	1286.5
820	POST	35	2	1POS	190	294.5
853	PRE	30	3	2NEG	370	573.5
863	POST	20	3	2POS	210	325.5
867	PRE	17	2	1POS	120	186
869	PRE	20	2	1POS	440	682
880	POST	13	2	3POS	260	403
1018	POST	20	2	2POS	240	372
1019	POST	12	3	2POS	660	1023
1034	POST	24	2	1POS	140	217
1042	POST	29	3	2NEG	110	170.5
1054	POST	32	3	0NEG	650	1007.5
1061	PRE	30	3	2POS	160	248
1076	POST	8	2	4POS	140	217
1105	POST	35	2	0POS	160	248
1151	POST	11	2	0NEG	700	1085
1257	POST	17	3	5POS	830	2241
1315	POST	30	3	1NEG	1770	4779
1318	PRE	15	2	0POS	350	542.5
1420	POST	32	2	2POS	160	248
1431	POST	25	2	0POS	470	728.5
1439	POST	17	1	0POS	210	567
1440	PRE	17	3	0NEG	330	891
1444	POST	25	1	17POS	300	810
1446	POST	20	2	0POS	160	432
1483	POST	20	3	1NEG	470	1269

1505	PRE	25	3	2	NEG	460	1242
1513	POST	26	2	2	POS	210	325.5
1516	PRE	20	2	2	NEG	840	1302
1563	PRE	20	3	0	NEG	130	201.5
1670	PRE	20	3	0	NEG	250	387.5
1709	POST	4	3	3	POS	1290	1999.5
1721	POST	5	3	0	NEG	1750	2712.5
1785	POST	30	2	9	POS	430	666.5
1794	POST	35	2	2	POS	70	108.5
1797	PRE	15	3	2	NEG	230	356.5
1801	PRE	20	3	0	NEG	540	1458
1803	PRE	40	3	0	POS	390	1053
1805	PRE	36	2	5	NEG	140	378
1806	POST	20	2	1	POS	100	155
1834	POST	45	2	0	POS	490	1323
1899	POST	15	2	1	NEG	840	1302
1927	POST	22	3	0	NEG	650	1007.5
1981	POST	25	3	0	POS	1580	2449
2006	POST	15	3	2	NEG	500	775
2012	POST	30	3	1	NEG	510	790.5
2034	PRE	12	1	1	POS	920	1426
2314	POST	19	2	0	POS	30	46.5
2341	PRE	35	2	2	POS	1030	1596.5
2358	POST	40	2	16	POS	120	186
2443	PRE	13	2	1	POS	120	186
2980	POST	35	2	0	POS	0	0
2981	POST	35	2	0	POS	20	54
3333	PRE	20	3	1	NEG	1450	2247.5
3458	POST	14	2	0	POS	1460	2263
4454	POST	20	3	0	POS	790	1224.5
4480	POST	28	3	4	POS	230	356.5
4496	PRE	20	3	2	POS	220	594
4499	POST	35	2	0	NEG	740	1147
A	POST	20	3	0	NEG	580	899

ID	MENO	SIZE	GRADE	NODES	ER	COUNT	PerMMSq
3193	POST	50	2	19	POS	1260	1953
3357	PRE	55	3	1	NEG	770	2079
549	POST	25	2	0	POS	740	1147
1522	POST	25	2	12	POS	160	248
871	POST	10	2	2	POS	150	232.5
1332	POST	6	2	0	NEG	50	77.5
788	POST	15	2	1	POS	310	480.5
1346	POST	25	2	5	POS	60	93
1831	PRE	8	3	0	POS	610	945.5
260	POST	5	2	1	POS	130	201.5
718	POST	25	3	2	NEG	160	248
775	PRE	20	3	1	NEG	1960	3038
563	POST	25	2	0	POS	160	248

3514	PRE	12	3	2	NEG	430	1161
1339	POST	25	2	0	POS	860	1333
1101	POST	35	2	5	POS	180	279
1348	POST	20	3	13	NEG	1002	1553.1
799	PRE	19	3	1	POS	610	945.5
§	PRE	22	2	6	POS	230	356.5
351	PRE	12	3	0	NEG	520	806
1011	POST	15	1	10	POS	650	1007.5
1082	POST	8	2	1	POS	560	868
1022	PRE	18	3	0	POS	500	1350
384	POST	30	2	0	NEG	30	81
1605	POST	18	3	0	POS	740	1147
774	POST	8	3	0	NEG	820	1271
X	POST	30	3	7	POS	810	1255.5
1424	PRE	10	3	0	NEG	570	1539
2004	POST	20	3	0	POS	310	480.5
792	POST	23	3	6	NEG	410	635.5
293	PRE	21	2	0	NEG	20	31
196	POST	35	2	1	POS	510	790.5
343	PRE	28	3	2	NEG	80	216
1562	POST	40	3	1	POS	300	465
1157	PRE	8	2	2	POS	140	217
1844	PRE	20	2	1	POS	1100	2970
64	POST	16	2	1	POS	650	1007.5
1610	POST	17	2	1	POS	120	186
1006	POST	18	3	1	POS	190	294.5
1114	POST	25	2	1	POS	3310	5130.5
546	POST	20	3	1	NEG	1090	1689.5
1783	POST	20	3	0	NEG	50	77.5
791	PRE	20	3	2	POS	750	1162.5
1669	POST	18	2	15	POS	320	496
4473	POST	42	2	0	POS	290	449.5
1461	POST	11	2	0	NEG	100	270
1253	POST	18	3	6	POS	60	162
2745	POST	20	3	1	NEG	1050	1627.5
1781	PRE	18	2	0	POS	250	387.5
1087	POST	20	2	1	POS	480	744
1099	POST	35	2	0	POS	100	155
2124	POST	11	1	0	POS	280	434
1662	PRE	18	3	0	NEG	1280	1984
1013	POST	20	1	8	POS	330	511.5
672	POST	20	2	0	POS	130	201.5
828	POST	25	3	1	NEG	880	1364
1939	PRE	20	3	3	NEG	50	77.5
1880	POST	22	2	1	POS	0	0
1128	PRE	30	2	1	NEG	100	155
542	PRE	20	3	0	NEG	920	1426
789	PRE	35	3	0	NEG	680	1054
1841	POST	16	3	1	POS	710	1917
1142	POST	7	3	0	NEG	420	651

110	POST	40	2	10	POS	500	775
1552	POST	40	2	3	POS	480	1296
437	PRE	12	3	2	NEG	220	341
941	PRE	28	3	0	NEG	1350	2092.5
719	PRE	25	3	0	POS	240	372
2769	PRE	30	2	4	NEG	430	666.5
4474	POST	25	2	2	POS	430	666.5
1631	POST	22	2	0	POS	330	891
1164	POST	9	2	3	NEG	1330	2061.5
1423	POST	38	3	0	NEG	900	1395
23	POST	24	3	0	POS	1160	1798
2748	POST	10	3	2	NEG	400	620
660	POST	40	3	1	NEG	260	403
1538	PRE	12	1	1	POS	70	108.5
112	POST	19	2	0	POS	260	403
797	PRE	20	2	2	POS	920	1426
2951	POST	30	2	16	POS	680	1836
813	PRE	15	2	2	POS	770	1193.5
617	POST	30	2	0	POS	350	542.5
1438	POST	21	2	0	POS	940	2538
3004	PRE	5	3	1	NEG	630	976.5
368	POST	12	2	0	POS	280	434
671	POST	20	3	0	POS	650	1007.5
2364	POST	35	3	10	POS	80	124
243	PRE	42	3	3	POS	210	567
367	POST	25	2	0	NEG	1770	2743.5
1459	POST	30	3	0	NEG	370	573.5

ID	MENO	SIZE	GRADE	NODES	ER	COUNT	PerMMSq
2335	POST	50	2	9	POS	430	1161
1116	PRE	50	3	2	NEG	490	759.5
475	POST	25	2	0	POS	110	170.5
556	POST	25	2	13	POS	250	387.5
798	POST	10	2	1	POS	100	270
680	POST	10	2	0	NEG	230	356.5
937	POST	20	2	1	POS	90	139.5
579	POST	35	2	6	POS	490	1323
1851	PRE	10	3	0	POS	890	1379.5
1639	POST	10	2	1	POS	170	263.5
26	POST	20	3	1	NEG	4790	7424.5
1092	PRE	38	3	1	NEG	790	1224.5
1480	POST	25	2	0	POS	100	155
2142	PRE	16	3	2	NEG	1040	1612
1091	POST	22	2	0	POS	910	1410.5
1174	POST	24	2	6	POS	60	162
355	POST	30	3	10	NEG	300	465
1073	PRE	15	3	1	POS	280	434
1116	PRE	50	3	2	NEG	490	759.5
1260	PRE	10	3	0	NEG	2950	4572.5
623	POST	18	1	7	POS	450	697.5

1324	POST	15	2	1	POS	160	248
1007	PRE	16	3	0	POS	240	372
1025	POST	20	2	0	NEG	130	201.5
340	POST	15	3	0	POS	460	713
1460	POST	15	3	0	NEG	1950	3022.5
Y	POST	37	3	6	POS	850	1317.5
1023	PRE	10	3	0	NEG	980	1519
194	POST	20	3	0	POS	310	837
1141	POST	23	3	4	NEG	1340	2077
1378	PRE	22	2	0	NEG	510	790.5
1712	POST	28	2	1	POS	40	62
2262	PRE	22	3	1	NEG	840	1302
2548	POST	23	3	1	POS	250	387.5
382	PRE	15	2	1	POS	580	899
268	PRE	30	2	3	POS	440	682
1033	POST	15	2	2	POS	1100	1705
341	POST	18	2	1	POS	130	201.5
662	POST	12	3	3	POS	230	356.5
459	POST	20	2	1	POS	1300	2015
796	POST	40	3	3	NEG	190	513
1790	POST	25	3	0	NEG	400	620
1913	PRE	35	3	1	POS	650	1007.5
1043	POST	15	2	5	POS	510	790.5
4497	POST	42	2	0	POS	250	387.5
1606	POST	15	2	0	NEG	180	279
732	POST	18	3	9	POS	210	325.5
2929	POST	25	3	3	NEG	330	891
488	PRE	7	2	0	POS	10	15.5
61	POST	35	2	1	POS	670	1038.5
762	POST	22	2	0	POS	190	294.5
804	POST	6	1	0	POS	3940	6107
1662	PRE	18	3	0	NEG	1280	1984
1325	POST	20	1	4	POS	90	139.5
1711	POST	20	2	0	POS	170	459
354	POST	26	3	1	NEG	320	496
1536	PRE	35	3	1	NEG	120	186
697	POST	40	2	3	POS	360	558
786	PRE	25	2	3	NEG	150	232.5
140	PRE	12	3	0	NEG	580	899
1014	PRE	25	3	0	NEG	190	294.5
1961	POST	15	3	2	POS	540	837
1323	POST	14	3	0	NEG	310	480.5
1070	POST	23	2	8	POS	190	294.5
1326	POST	20	2	1	POS	270	418.5
1010	PRE	19	3	2	NEG	270	418.5
1437	PRE	25	3	0	NEG	70	108.5
1473	PRE	45	3	0	POS	720	1116
1158	PRE	50	2	8	NEG	350	542.5
821	POST	20	2	1	POS	1950	3022.5
1012	POST	30	2	0	POS	290	783

560	POST	10	2	2	NEG	170	263.5
536	POST	28	3	0	NEG	270	418.5
1321	POST	20	3	0	POS	280	434
3725	POST	18	3	2	NEG	190	294.5
2108	POST	25	3	2	NEG	750	2025
736	PRE	8	1	1	POS	280	434
614	POST	15	2	0	POS	480	744
812	PRE	20	2	2	POS	10	15.5
2138	POST	23	2	4	POS	440	682
1074	PRE	12	2	1	POS	220	341
472	POST	40	2	0	POS	390	604.5
1173	POST	30	2	0	POS	450	1215
3320	PRE	15	3	1	NEG	1790	2774.5
69	POST	12	2	0	POS	590	914.5
949	POST	30	3	0	POS	30	81
3332	POST	36	3	5	POS	120	186
480	PRE	37	3	2	POS	40	108
855	POST	25	2	0	NEG	1800	2790
357	POST	20	3	0	NEG	1020	1581

Appendix C

Publications & Presentations

Peer Reviewed Paper

Quantification of tumour-infiltrating lymphocyte subsets :a practical immunohistochemical method
Paula M. Loughlin, Timothy G. Cooke, W. David George, Alison J. Gray, David I. Stott and James J. Going.
Journal of Immunological Methods
Volume 321, Issues 1-2, 10 April 2007, Pages 32-40

Abstracts-Presentations

Quantifying tumour-infiltrating lymphocyte subsets by colour image histogramming

Presented. Presented at the 4th Joint meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain and Ireland.July 2007
Published in The Journal of Pathology.213 S1,101;2007
PM Loughlin,TG Cooke,WD George,DI Stott,JJ Going

Prognostic Significance of tumour-infiltrating lypmphocyte density in breast cancer H&E sections. Presented at the 4th Joint meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain and Ireland.July 2007
Presented in The Journal of Pathology.213 S1,59;2007
PM Loughlin,TG Cooke,WD George,DI Stott,JJ Going

Quantifying lymphoid infiltrates in breast cancer. Presented at the British Association of Surgical Oncologists meeting November 2005.
Published in the European Journal of Surgical Oncology,31,9;1092;Nov 2005
P Loughlin , T Cooke , W George , D Stott , J Going.