

THE UNIVERSITY OF HULL

**Significance of Interleukin-10 and Interleukin-12 levels in breast cancer patients and
their possible role in tumour immunology**

being a Thesis submitted for the Degree of

MD

in the University of Hull

by

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Synopsis

Patients with cancer are thought to have significant impairment of the immune system. One of the manifestations is believed to be skewing of the cytokine profile in favour of the immunosuppressive Th2 cytokine family as opposed to the immunofacilitatory Th1 cytokine family. In this MD thesis, a prospective study was undertaken to determine whether this occurred in breast cancer patients.

IL-10 (representing the Th2 cytokine family) and IL-12 (representing the Th1 cytokine family) levels in the serum, IL-12 production capability of peripheral blood mononuclear cells (PBMC) along with the levels of IL-10 and IL-12 in the tumour microenvironment as detected by immunohistochemistry were determined in breast cancer patients and compared with levels in healthy volunteers. No difference in serum IL-10 and IL-12 levels or IL-12 production capability of PBMC was noted between breast cancer patients and controls. Among the known prognostic factors (tumour size, grade, lymph node involvement and oestrogen receptor status), grade was found to be inversely related to serum IL-12 levels (by univariate and multivariate analysis; $P=0.045$). No significant association between these prognostic factors and IL-10 levels were noted. Immunohistochemistry revealed mild to intense staining for IL-10 in the tumour vicinity, but in the case of IL-12, the staining was inconclusive. The effect of therapy on the levels of IL-10 and IL-12 was also investigated. Following surgical excision, serum IL-10 levels and IL-12 production capability of PBMC did not show significant change. However, serum IL-12 levels were significantly elevated after surgery raising the possibility of a partial skewing of the immune response in favour of the Th1 cytokine profile (Wilcoxon signed ranks test, $P=0.001$). After completion of adjuvant therapy (chemotherapy and/or radiotherapy), serum IL-10 levels again did not show any significant change, but serum IL-12 levels showed a downward trend which may be due to the immunosuppressive effect of the adjuvant therapy (Wilcoxon signed ranks test, $P=0.06$).

In conclusion, this study did not find any evidence of Th2 bias as a manifestation of immune suppression in breast cancer patients. The change in serum IL-12 levels following surgery and adjuvant therapy is interesting and may reflect a change in immune status following the therapeutic interventions. The significance of this must be elucidated by studies with long term follow-up before definite conclusions can be made.

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- Sir Isaac Newton

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List of abbreviations

CI	Confidence interval
HRT	Hormone replacement therapy
DCIS	Ductal carcinoma <i>in situ</i>
LCIS	Lobular carcinoma <i>in situ</i>
NK	Natural killer cells
TGF	Transforming growth factor
TNF	Tumour necrosis factor
MHC	Major histocompatibility complex
TIL	Tumour infiltrating lymphocytes
IL	Interleukin
IFN	Interferon
DC	Dendritic cell
LAK	Lymphokine activated killer cell
CTL	Cytotoxic T lymphocyte
TAA	Tumour associated antigen
kDa	Kilo Dalton
LPS	Lipopolysaccharide
HLA	Human leukocyte antigen
Th1	T helper 1 cell
Th2	T helper 2 cell
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
ELISA	Enzyme linked immunosorbent assay
IQR	Interquartile range

Declaration

I declare that I composed this thesis and undertook all the laboratory work, analysis and interpretation of results involved in this thesis, with acknowledged help from the laboratory assistants, guidance and advice from the supervisors as appropriate.

This thesis has not been submitted for any other degree or professional qualifications to any other institute.

Publications and presentations in support of the thesis

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- Changes in serum IL-10 & IL-12 levels pre- and post-operatively in Breast cancer patients. Oral poster presentation at the 50th Annual Meeting of the Society of Academic and Research Surgery, Newcastle, 12th-14th January, 2005.

1.1 Epidemiology

Breast cancer is the most common cancer among women worldwide and is the leading cause of cancer death in women. More than one million women were diagnosed as having the disease world wide in 2000, accounting for 18-25 per cent of all female malignancies and 373,000 women died constituting 14 percent of cancer related deaths in women (Althuis et al, 2005). Breast cancer was recognised as early as 1600 B.C. by the ancient Egyptians, however, it has only become a major health problem over the last 50 years causing immense anxiety, suffering and loss to affected patients and their families (Fentiman, 2002).

Although the burden of breast cancer is increasing in both the developed and developing countries, there is a four-fold variation in incidence by geographical location between countries with the highest and the lowest rates (Butler and Cunningham, 2001). Overall, North American and North European countries have the highest incidence, followed by intermediate levels reported in Western Europe, Scandinavia and Israel with the lowest levels being in Eastern Europe, South and Latin America and Asia. Incidence rates ranged from 97 per 100,000 among white American women to 27 per 100,000 in Asian countries. Studies have shown that migrants from Japan to Hawaii assume the rate in the host country within one or two generations, indicating that environmental factors are of greater importance than genetic factors.

Breast cancer is the most common cancer in women in England. In 2003, 36,500 new cases were diagnosed accounting for 32 per cent of all cancers in women giving an overall incidence of 120 cases per 100,000 of the female population. Incidence increases with age, with a peak in distribution of new cases in women aged 50-54 years due to screening. Breast cancer accounts for 17 per cent of female deaths from cancer in U.K. with around 12,509 women dying of the disease in 2005 (www.statistics.gov.uk).

1.1.1 Risk factors

Various risk factors, some of which can be modified, have been attributed to the increased incidence of breast cancer in the developed world. These include:

i) Age: This is the strongest risk factor for breast cancer. Incidence increases with age and almost doubles every decade until menopause. The risk escalates from 1 in 15,000 up to age 25, 1 in 50 by age 50, 1 in 15 by age 70 and to 1 in 10 by age 85. The lifetime risk of developing breast cancer up to the age of 85 years is estimated to be 11 percent, with an overall lifetime risk of 1 in 9 (www.statistics.gov.uk).

ii) Reproductive history: The following factors are thought to increase the risk of breast cancer in women in the developed world when compared to those in the developing countries:

- **Age at menarche:** Early age at menarche is found to be associated with increased risk of breast cancer. This is particularly seen in developed countries where the average age at menarche is 12-13 years when compared to 16-17 years in low risk countries such as China. Nutrition in early life is thought to be a key factor influencing age at menarche (Tanner, 1973).
- **Age at first childbirth:** The risk of breast cancer bears an inverse relationship to the age of the woman at first childbirth. The relative risk of developing breast cancer increases by 3% for each year of delay (Collaborative group on hormonal factors in breast cancer, 2002). The risk in women who have their first child after the age of 30 is about twice that of women who have their first child before the age of 20. Those who have their first child after the age of 35 have the highest risk; these women appear to be at even higher risk than nulliparous women.

- **Parity:** The effect of parity on the risk of breast cancer has been recognised as early as the 18th century. One meta analysis has shown nulliparity to be associated with a 30% increase in risk compared to parous women (Ewertz et al., 1990).
- **Breastfeeding:** Women who breastfeed have a lower risk of developing breast cancer when compared to women who do not breastfeed their children. The protective effect is directly proportional to the duration of breastfeeding; it is estimated that there is a 4.3% reduction in risk for each year of breastfeeding (Collaborative group on hormonal factors in breast cancer, 2002).
- **Age at menopause:** Delayed menopause increases the risk of breast cancer. There is an approximate 3% increase in risk of breast cancer for every year of delay in attaining menopause (Collaborative Group on Hormonal Factors in Breast Cancer, 1997).

iii) **Endogenous hormones:** High circulating levels of sex hormones are thought to increase the risk of breast cancer. A pooled analysis of nine prospective cohort studies reported a significant increase in breast cancer risk in post menopausal women with high levels of sex hormones (Endogenous Hormones and Breast Cancer Collaborative Group, 2002). Women with oestradiol levels within the top quintile were more than twice likely to develop breast cancer than women with levels in the bottom quintile. However, evidence for pre menopausal women is inconclusive.

iv) **Exogenous hormones:**

- **Oral contraceptives:** The use of oral contraceptives is associated with a small increase in breast cancer risk in current and recent users (relative risk: 1.24; 95 per cent confidence interval (95% CI): 1.15-1.33), however this tends to disappear within ten years of cessation of use (Collaborative Group on Hormonal Factors in Breast Cancer, 1996).

- **Hormone replacement therapy:** The use of hormone replacement therapy (HRT) is associated with an increase in risk of breast cancer. Follow-up of over 1 million women in the Million Women Study confirmed findings from other recent studies that women currently using HRT are more likely to develop breast cancer than those who are not using HRT. This effect is substantially greater for combined (oestrogen-progestagen) HRT than for oestrogen-only HRT; and that the effects were similar for all specific types and doses of oestrogen and progestagen, for oral, transdermal and implanted HRT, and for continuous and sequential patterns of use. The risk increases with duration of use and is estimated to be 66% higher in users than in non-users. It is also reported to reduce the sensitivity of mammography with obvious implications for screening and early detection. The estimated risk for current users of oestrogen-progestogen combinations for 10 or more years was 2.31 (95% CI: 2.08-2.56) compared to 1.74 (95% CI: 1.60-1.89) for the first four years of use. However, this risk decreases with cessation of use (Beral et al, 2002). In the last decade (1993-2003), it is estimated that over 20,000 extra cases of breast cancer have occurred among women aged 50-64 as a result of HRT in the U.K (Banks, 2001; Dixon, 2001; Chlebowski et al, 2003; Beral, 2003).

v) **Body weight:** Obesity moderately increases the risk of post menopausal breast cancer and is one of the few modifiable risk factors (van den Brandt et al, 2000). It is estimated that about 8% of breast cancer cases in the U.K. are attributable to obesity (Bergstrom et al, 2001). After menopause, adipose tissue becomes the primary source of endogenous oestrogen as ovarian production ceases. This, coupled with low levels of sex hormone binding globulin seen in obese women, leads to increased bioavailability of oestradiol and subsequent exposure to high levels of oestrogen (Endogenous Hormones Breast Cancer Collaborative Group, 2003). In pre menopausal women, on the contrary, some studies have shown a slightly reduced risk of breast cancer with a high body mass index. This is thought to be due to reduced exposure to endogenous oestrogens as a result of increased incidence of anovulatory cycles in these women.

vi) **Height:** Tall women are observed to have an increased risk of breast cancer (Hunter, 1993). A pooled analysis estimated that the relative risk for women 1.75 metres or taller compared with women shorter than 1.6 metres was 1.22 for all women and 1.28 for post menopausal women (van den Brandt et al, 2000). There was an approximate increase in

relative risk of 7% for each additional 5 centimetres in height for post menopausal women and 2% for pre menopausal women. The underlying mechanism for this association between height and breast cancer risk is not clear, and it is likely that height may be a marker for other exposures that influence breast cancer risk (Lawlor et al, 2003).

vii) Physical activity: Moderate levels of physical activity are reported to have a preventive effect on breast cancer (Key et al, 2001). This is believed to be as a result of direct effects on hormonal and growth factor levels and indirect effects on lowering the body mass index and preventing obesity. Studies have reported a 30-40% reduction in the risk of breast cancer in women participating in a few hours per week of vigorous activity versus none.

viii) Alcohol intake: A significant association between alcohol intake and breast cancer has been reported, with an increase in risk of 7% for each alcoholic drink consumed on a daily basis (Hamajima et al, 2002). It is estimated that around 4% of breast cancers in women in developed countries are attributable to excessive alcohol consumption.

ix) Diet: A diet rich in fat has been positively associated with breast cancer in various correlation studies (Rose et al, 1986), animal studies (Welsch, 1992) and case control studies (Howe et al, 1990). However, pooled analyses of cohort studies found no significant association between fat intake and breast cancer risk (Hunter et al, 1996; Smith-Warner et al, 2001) while a meta-analysis found a modest positive relation with both total and saturated fat intake (Boyd et al, 2003). Overall, the evidence suggests that fat intake may cause a small increased risk of breast cancer but probably is not a major factor as was once thought.

x) Socio-economic status: In contrast to other cancers, breast cancer is more common in the higher socio-economic strata and this may be due to influence of other factors such as reproductive history and nutrition. The age standardised incidence rate is 97.3 per 100,000 population in the most deprived quintile when compared to 115.1 in the least deprived quintile (www.show.scot.nhs.uk).

xi) Ionising radiation: Exposure to ionising radiation is a well known risk factor for breast cancer. The effect is related to the age at exposure and is maximal if received at a young age. This is usually encountered in patients who receive radiotherapy as

treatment for other cancers such as Hodgkin's or Non Hodgkin's lymphoma (Bhatia et al, 1996).

xii) Benign breast disease: Certain benign breast diseases are associated with an increased risk of breast cancer. Women in the third and fourth decades who suffer from fibrocystic disease are at slightly increased risk of breast cancer. Women with proliferative breast disease without atypia have a two fold increased risk and those with atypical hyperplasia have two to five fold increased risk (Byrne et al, 2000)

xiii) Personal history of breast cancer: Women with previous history of breast cancer have two to six times the risk of developing a second primary breast cancer than the general population (Chen et al, 1999).

xiv) Family history: It is estimated that hereditary factors account for a quarter of differences in individual susceptibility to breast cancer (Key et al, 2001). A woman with one affected first degree relative (mother or sister) has approximately double the risk of developing breast cancer compared to a woman with no family history of the disease. Like-wise, if two (or more) relatives are affected, her risk increases further (Collaborative Group on Hormonal Factors in Breast Cancer, 2001).

A small proportion of women (about 2-5%) have a particularly strong family history of breast cancer and hence are at a very high risk (over 50%) of developing the disease (Claus et al, 1994). This is due to an abnormal gene generally inherited as an autosomal dominant trait with limited penetrance. The BRCA 1 gene is located on the long arm of chromosome 17 (17q) and the gene frequency in the population is approximately 0.0006. In certain populations, it occurs with greater frequency such as in Ashkenazi Jews, in whom this is a more common mutation. The BRCA 2 gene is located on chromosome 13 q and this is associated with male breast cancer (Mc Intosh et al, 2004). Women who are thought to be gene carriers may be offered breast screening, genetic counselling and mutation analysis. Those who are proven to be gene positive have a 30-50% risk of developing pre menopausal breast cancer. They may be offered risk reduction mastectomy which reduce the risk considerably (Sainsbury, 2004) .

Rare familial cancer syndromes associated with increased risk of breast cancer are summarised in Table 1 (Ford et al, 1998; Emery et al, 2001; Wooster et al, 1995).

However, over 85% of women who have a close relative with breast cancer will never develop the disease, and more than 85% of women with breast cancer have no family history of it . Only if there are several family members with early onset breast cancer is there a likelihood of a significant inherited predisposition to the disease (Collaborative Group on Hormonal Factors in Breast Cancer, 2001).

Gene	Cancer syndrome	Associated tumours	References
BRCA 1	Breast/ovarian predisposition	Breast, ovary, bowel, prostate	McIntosh et al, 2004
BRCA 2	Breast/ovarian predisposition	Breast, ovary, pancreas, prostate	McIntosh et al, 2004
p53	Li Fraumeni syndrome	Childhood sarcoma, brain, leukaemia, adrenal cortex, early onset breast cancer	Emery et al, 2001; McIntosh et al, 2004
PTEN	Cowden's syndrome	Breast, gastrointestinal, benign and malignant thyroid	Emery et al, 2001
ATM	Ataxia telangiectasia	Non Hodgkin's lymphoma, ovary, breast (heterozygous)	McIntosh et al, 2004

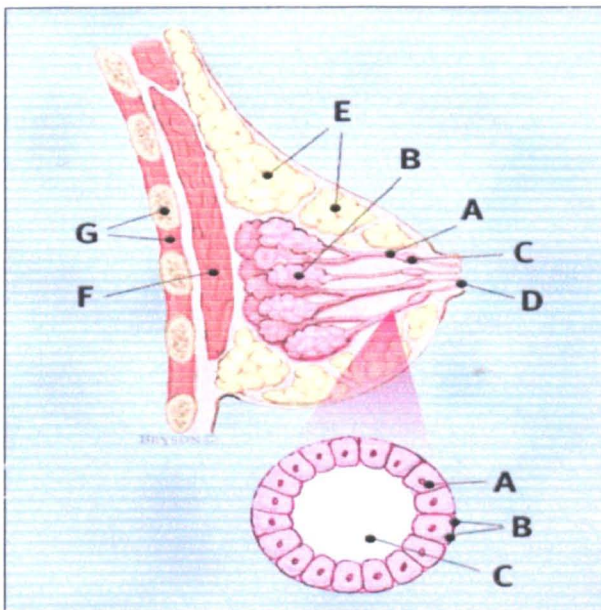
Table 1. Rare familial cancer syndromes associated with breast cancer.

1.2 Pathology

1.2.1 Applied anatomy

The breast lies between the second and sixth ribs. Breast tissue may be found within a pentagonal area that extends from the clavicle to the infra mammary fold, and from the lateral edge of the sternum to the latissimus dorsi muscle. An oblong projection called the tail of Spence extends towards the axilla.

The adult female breast is made up of two major components: the epithelial elements, which consist of the ducts and lobules (glandular components) and the stromal elements, which consist of fibrous tissue and fat. The glandular components of the breast are grouped into 12-15 lobes. Each lobe is subdivided into 20-40 lobules, composed of alveoli. Ducts from each lobe converge toward the apex of the breast, enlarging into lactiferous sinuses just under the nipple (Fig 1.21). Cooper's suspensory ligaments link the skin of the breast to the anterior fascia of the pectoralis major muscle, providing support. The principal blood supply to the breast is from the internal mammary and lateral thoracic arteries. Lymphatic drainage is about 97% to the axillary nodes, and 3% to the internal mammary chain. About 1/3 of the breast in younger women consists of adipose tissue; the other 2/3 is glandular tissue. The proportion of fat increases with age, making the breast more radiolucent and easier to evaluate mammographically (Sainsbury, 2004).



Breast profile:

- A Ducts
- B Lobules
- C Dilated section of duct to hold milk
- D Nipple
- E Fat
- F Pectoralis major muscle
- G Chest wall/rib cage

Enlargement

- A Normal duct cells
- B Basement membrane
- C Lumen (centre of duct)

Fig 1.21 Normal breast with an enlarged cross-section of normal duct (http://www.breastcancer.org/pathology_intro.html)

1.2.2 Pathology

The term "breast carcinoma" encompasses a diverse group of lesions which differ in microscopic appearance and biological behaviour, although these disorders are often discussed as a single disease (Schnitt and Guidi, 2000).

The epithelial lining of the lactiferous duct and ductule is of two-three cell thickness. The superficial layer is composed of cuboidal epithelium in the lobule and columnar epithelium in the extralobular system and the deep cell layer is made up of mesoepithelial cells.

A range of growth from normal to abnormal cells is encountered in various disease processes afflicting the breast (Schnitt and Guidi, 2000). These include:

- Too many cells (ductal hyperplasia)
- Too many cells that appear to be abnormal (atypical ductal hyperplasia or ADH)
- Too many cells that are growing like a cancer but are still confined to the inside of the duct (Ductal carcinoma *in situ*; DCIS)
- Uncontrolled growth of duct cells that have broken through the basement membrane (invasive ductal cancer)

Malignant tumours may arise from any of the breast structures. The most common are ductal adenocarcinomas which arise from the epithelium lining of the lactiferous ducts and ductules, followed by lobular carcinomas and malignancies arising from other connective tissues.

As in other body tissues, breast tumours grow by cell division, which can take on average anything from 85 days to 15 months. Consequently, in the majority of cases, a tumour may have been in the body for several years before it is detected. A breast tumour can be successfully detected by palpation when it reaches a diameter of approximately 1cm and a weight of 1 gram. By this time it will have gone through 20–30 doublings and it is highly possible that metastasis has already started. Most primary breast cancers have invaded into the stroma of the breast at the time of diagnosis. The great majority of these present as breast lumps, although, a small proportion erodes through the skin of the breast by the time they are first seen, presenting as fungating tumours. Lesser degrees of skin involvement lead to dimpling or tethering of the skin

and peau d'orange – where skin infiltration leads to local lymphatic obstruction (Schnitt and Guidi, 2000).

1.2.3 Classification: The wide variability in histological appearance has led to several attempts to classify these tumours according to their microscopic characteristics (Table 1.21; Iglehart and Kaelin, 2001). Descriptive terms are often used, but they are generally unhelpful since they have little bearing on patient prognosis.

Breast carcinomas can be divided into two major groups:

- *In situ* (non invasive) carcinoma — The tumour cells remain confined to the ducts or lobules and show no evidence of invasion into the surrounding stroma or beyond the breast.
- Invasive (infiltrating) carcinoma — The tumour cells invade the breast stroma; they have the potential to metastasize and spread to the other parts of the body through the blood or lymphatic system (Fig 1.22).

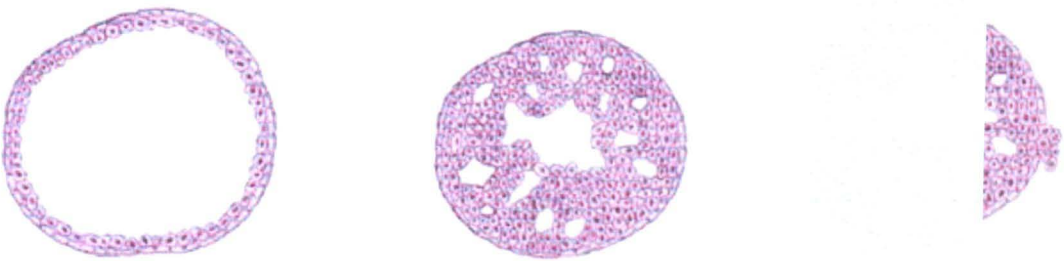


Fig 1.22 Normal cells

In situ carcinoma

Invasive carcinoma

(http://www.breastcancer.org/is_cancer_invasive_html)

Table 1.21 Classification of malignant tumours of the breast (WHO classification)

Malignant tumours

1. Non invasive
 - a. Ductal carcinoma *in situ*
 - b. Lobular carcinoma *in situ*
2. Invasive
 - a. Invasive ductal carcinoma
 - b. Invasive ductal carcinoma with a predominant intraductal component
 - c. Invasive lobular carcinoma
 - d. Mucinous carcinoma
 - e. Medullary carcinoma
 - f. Papillary carcinoma
 - g. Tubular carcinoma
 - h. Adenoid cystic carcinoma
 - i. Secretory (juvenile) carcinoma
 - j. Apocrine carcinoma
 - k. Carcinoma with metaplasia
 - i. Squamous type
 - ii. Spindle cell type
 - iii. Cartilaginous and osseous type
 - iv. Mixed types
1. Others
3. Paget's disease of the nipple

Non invasive carcinoma: *In situ* or non invasive carcinomas of the breast are increasingly recognised as early lesions with the advent of screening programmes. These are not usually detectable by clinical examination and are picked up by mammography based mass screening programmes. These include:

- **Ductal carcinoma *in situ* (DCIS):** This is the most common variant accounting for 85-90% of non invasive carcinomas. It arises as a heterogeneous group of lesions, characterized by proliferation of malignant appearing cells within the ductal system that do not cross the basal membrane (Morrow et al, 2000). DCIS is often multifocal, appearing in more than one duct, but usually in only one breast (Allred et al, 1997).
- **Lobular carcinoma *in situ* (LCIS):** This is less common than DCIS accounting for 10-15% of non invasive carcinomas. These are found only on microscopic examination, rather than clinically or on mammograms. It is therefore usually reported as an incidental finding and occurs more frequently in pre-menopausal women. LCIS is characterized by solid proliferation of small cells with uniform, nuclei. Unlike DCIS, LCIS tends to be multi centric, appearing in several separate areas in the breast, and often in the other breast, placing both breasts at equal risk (Morrow and Schnitt, 2000).

Invasive carcinoma: Unlike *in situ* lesions (LCIS and DCIS), invasive cancers do cross the wall of the duct or lobule and invade adjacent tissues. Invasive cancers account for the vast majority of breast neoplasms. These include:

- **Invasive (infiltrating) ductal carcinoma:** This is the most common type of invasive breast cancer, accounting for 70 to 80 percent of invasive lesions (Fig 1.24). It is also termed infiltrating carcinoma of no special type, or infiltrating carcinoma not otherwise specified (NOS). The tumours occur throughout the age range of breast carcinoma, being most common in women in their middle to late fifties. It is characterised by its solid core, which is usually hard on palpation and an associated ductal carcinoma *in situ* is frequently present. Invasive ductal carcinoma commonly spreads to the regional lymph nodes and this along with nuclear and histological grade is shown to be effective predictors of prognosis (Berg and Hutter, 1995).

- **Invasive lobular carcinoma:** This is relatively uncommon, comprising only 5–10% of breast tumours (Fig 1.25). Invasive lobular carcinomas are characterised by greater proportion of multicentricity in the same or the opposite breast. The lesions tend to have ill-defined margins, and occasionally the only evidence is subtle thickening or induration. Patients with infiltrating lobular carcinoma are especially prone to have bilateral carcinoma (20%) (Iglehart and Kaelin, 2001).

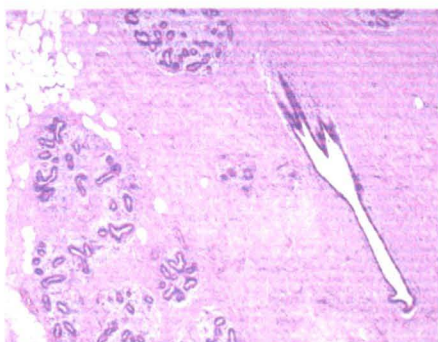


Fig 1.23 Normal breast tissue

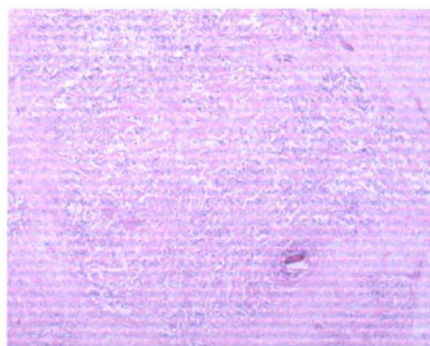


Fig 1.24 Invasive ductal carcinoma

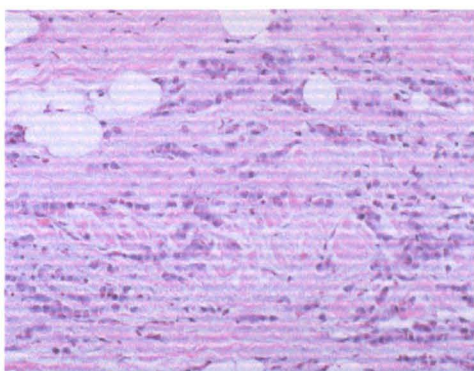


Fig 1.25 Invasive lobular carcinoma

(<http://www-medlib.med.utah.edu/WebPath/BRESHTML/BREST020.html>; [BREST008.html](http://www-medlib.med.utah.edu/WebPath/BRESHTML/BREST008.html); [BREST011.html](http://www-medlib.med.utah.edu/WebPath/BRESHTML/BREST011.html))

TNM Classification and Staging: The stage of a breast cancer lesion is based upon its size and degree of spread. Carcinomas have a propensity to spread via lymphatics at an early stage. When breast cancers metastasise, they often go first to the axillary lymph nodes into which most lymphatics from the breast drain. More distant metastases are

also possible and these include supraclavicular lymph nodes and other organs such as lung, bone, brain and liver (Sainsbury, 2004).

The most widely used system for staging primary breast cancer has evolved from classifications proposed by the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC). The TNM classification system is based on the description of the primary tumour (T), the status of regional lymph nodes (N) and the presence of distant metastasis (M) (Appendix 1).

The designations of the tumour (T), lymph node (N) and metastasis (M) in TNM staging can be combined to give a summary staging category as shown in Table 1.22):

Stage I	Tumour 2 cm or less in greatest diameter and without evidence of regional (nodal) or distant spread	T1 N0 M0
Stage II	Tumour more than 2 cm but not more than 5 cm in greatest dimension with regional lymph node involvement, but without distant metastasis OR tumour more than 5 cm in diameter without regional (nodal) or distant spread	T0 N1 M0 T1 N1 M0 T2 N0 M0 T2 N1 M0 T3 N0 M0
Stage IIIA	Tumour less than 5 cm in size with fixed axillary lymph nodes OR tumour more than 5 cm in size with axillary lymph node involvement	T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1 M0 T3 N2 M0
Stage IIIB	Tumour of any size with possible skin involvement, pectoral and chest wall fixation, fixed axillary lymph nodes and involved internal mammary nodes but with no distant metastasis	T4 Any N M0 Any T N3 M0
Stage IV	Tumour of any size with or without regional lymph node involvement but with evidence of distant metastasis	Any T Any N M1

Table 1.22 Staging of breast cancer

This system helps to determine stage based on clinical examination and radiological investigations and pathological stage after histological evaluation of the tumour after surgery. Thus it aids in deciding treatment strategies and seeks to provide information about the prognosis of patients after treatment and to allow comparison of results within and between clinical settings (Iglehart and Kaelin, 2001).

1.3 Diagnosis and treatment of breast cancer

1.3.1 Clinical presentation

In the early stages, a cancerous lesion seldom gives rise to symptoms. But as growth progresses, it gives rise to various manifestations. These include:

- A lump or thickening in or near the breast or in the armpit
- A change in size or shape of the breast
- Abnormal nipple discharge (usually bloody, or clear/yellow/green/purulent fluid)
- Change in colour or feel of the skin of the breast, nipple or areola:
 - Dimpled, puckered or scaly
 - “Orange peel” appearance
 - Redness
 - Attenuated veins on the breast surface
- Change in appearance of the nipple (retraction, destruction, itching)
- Breast pain, enlargement or discomfort
- Symptoms of advanced disease such as weight loss, bone pain, swelling of one arm and skin ulceration.

On clinical examination, the usual characteristics of a cancerous lump include lack of tenderness, firm to hard in consistency, restricted mobility and irregular margins. If the draining lymph nodes in the armpit are involved, they too may be palpable. Rarely advanced lesions may manifest as ulcerating fungating lesions fixed to the chest wall (Sainsbury, 2004).

1.3.2 Investigations

Evaluation of a breast lump is done by triple assessment. This entails clinical assessment, imaging to determine the nature of the lesion and cytological/histopathological examination of a tissue sample of the lesion to confirm the

Various modalities include:

- **Mammogram** is an X-ray examination of the breast and is used to evaluate a breast complaint or abnormality detected by the clinician. It is a valuable tool used in both the screening and diagnosis of breast cancer. It detects the lesion much earlier than it is clinically palpable and thereby picks up the lesion at an early stage. This helps in instituting breast conserving therapy avoiding the need for mastectomy and improves survival. A combination of data from eight randomised controlled trials reported a 27% reduction in breast cancer deaths in favour of the screened populations compared with non screened women over the age of 50 (Iglehart and Kaelin, 2001). In the UK, breast screening by mammography is offered to women between 50 and 70 years of age once every 3 years (Thompson and Dewar, 2002).

Mammographic features of a malignant lesion include density abnormalities such as masses, asymmetries and architectural distortions and microcalcifications (Fig 1.31) (Iglehart and Kaelin, 2001).

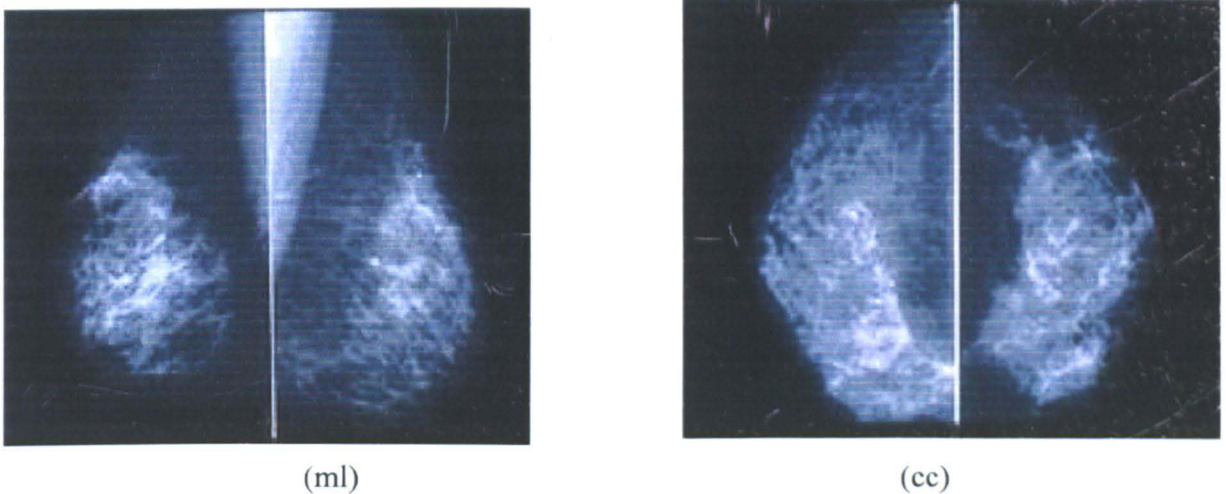


Fig 1.31 Mammogram showing a region of irregular increased density in both side to side (medial-lateral; ml) and top to bottom (cranio-caudal; cc) images (http://www.breastcancer.org/testing_images.html)

- **Ultrasound** is used to evaluate whether a lump is solid (such as cancer) or fluid filled (such as a benign cyst) (Fig 1.32). It is also used to precisely guide biopsy needles to suspicious areas that are impalpable. Ultrasound is useful in young women with dense breasts for whom mammograms are difficult to interpret (Sainsbury, 2004).



Fig 1.32 Ultrasound showing a dark irregular mass in the breast representing a cancerous lesion (http://www.breastcancer.org/testing_images.html)

- **Magnetic resonance imaging (MRI)** is better in evaluating lesions in women with dense breasts, implants and after breast conservation therapy. However, it does not show calcifications which may represent *in situ* disease and is associated with a high false positive rate (Sainsbury, 2004).

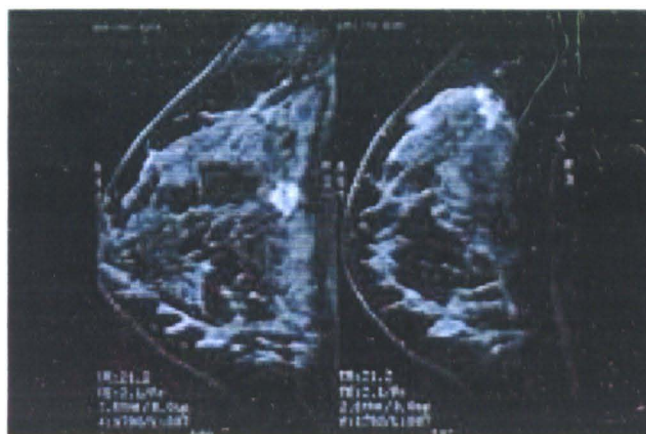


Fig 1.33 MRI scan of the breast showing two discrete areas of abnormality representing cancerous lesions (http://www.breastcancer.org/testing_images.html).

- **Core biopsy** is essential to get histological confirmation of a diagnosis. A fine needle such as a trucut or corecut biopsy device is passed through the lump if it is palpable or under ultrasound guidance if impalpable and the specimen is analysed in the laboratory (Sainsbury, 2004). A series of tests are done which help guide treatment decisions and these include:
 - **Tumour characteristics:** whether invasive or *in situ*; ductal or lobular; well, moderate or poorly differentiated and whether there is vascular or lymphatic invasion.
 - **Oestrogen/progesterone receptor status:** Tumours positive for these receptors respond to adjuvant hormone therapy.
 - **Her-2/neu status:** Her-2 is a protein that is over expressed in about 25% of breast cancers. If positive, they can be treated with a drug called trastuzumab or Herceptin which blocks the receptors.

1.3.3 Treatment

There have been tremendous advances in the treatment of breast cancer over the past few years resulting in significant improvement in survival rates. The two basic principles of treatment are to reduce the chance of local recurrence and the risk of metastatic spread, thereby improving quality of life (Sainsbury, 2004). The treatment offered largely depends upon the clinical stage of the disease at presentation. For stage I, II, or III cancers, the main considerations are to adequately treat the cancer and prevent a recurrence either at the place of the original tumor (local) or elsewhere in the body (metastatic). For stage IV cancer, the goal is to improve symptoms and prolong survival.

Treatment of early breast cancer will usually involve surgery with or without radiotherapy. Systemic therapy such as chemotherapy or hormone therapy is added if there are adverse prognostic factors such as lymph node invasion indicating a high likelihood of relapse. Locally advanced or metastatic disease is usually treated by systemic therapy to palliate symptoms with surgery playing a minor role. Thus

depending upon the stage of the disease, patients get a combination of treatments as part of the multidisciplinary team approach in most specialist centres (Thompson and Dewar, 2002).

Various modalities of treatment include:

- **Surgery** plays a key role in the management of breast cancer. It usually involves removal of the cancerous lesion from the breast and assessing the lymph node involvement in the axilla. Recently, there has been a shift from radical procedures such as mastectomy to more conservative surgery such as wide local excision followed by radiotherapy. This has been validated by clinical trials which have shown equivalence in survival between mastectomy and wide local excision followed by radiotherapy for early stage breast cancer (Sainsbury, 2004). Various surgical options include:
 - **Mastectomy** involves removal of the whole breast. This is now limited to large tumours (in relation to the size of the breast), central tumours beneath or involving the nipple, multifocal disease, local recurrence or patient preference.
 - **Wide local excision** entails removal of the tumour along with a rim of at least 1cm of surrounding normal breast tissue. This is suitable for small localised breast tumours and helps in avoiding the need for mastectomy thereby alleviating the significant associated psychological morbidity. This is usually supplemented with radiotherapy to the remnant breast tissue to ensure that any residual cancer cells are destroyed and thereby prevent the chance of recurrence.
 - **Axillary surgery** is used to assess the axillary lymph nodes as presence of metastasis in these nodes is still the best marker for prognosis and determines the need for adjuvant systemic therapy. Various options

include sampling (level I – removal of 4-5 nodes mostly from the anterior group close to the axillary tail), clearance (level I,II &III – removal of all nodes from the axilla) or sentinel node biopsy which entails removal of node/nodes draining the tumour after localisation by patent blue dye and radio isotope. If the axillary lymph nodes are involved due to tumour spread, then adjuvant therapy in the form of radiotherapy or chemotherapy is considered after taking into account other prognostic factors.

- **Radiotherapy** is usually used in the adjuvant setting after breast conserving surgery. The standard regimen involves doses of 40-50 Gray (Gy) delivered in 15-25 fractions over 3-5 weeks (+ tumour bed boost of 10-20 Gy) (Thompson and Dewar, 2002). It is given after wide local excision for high grade, large, node positive tumours with lympho vascular invasion. It is thought to destroy any cancer cells that may linger and thereby prevents recurrence. Targeted radiotherapy is administered to the remaining breast tissue after breast conserving surgery, to the chest wall after mastectomy in cases of chest wall infiltration and to the axilla and supraclavicular fossa in node positive disease following sampling. An overview of all trials of post-operative radiotherapy (including post-mastectomy and breast conservation trials) showed an absolute benefit in reduction in breast cancer mortality of 2.1% at 10 years and 1.2% at 20 years (Early Breast Cancer Trialist's Collaborative Group, 2000).
- **Hormone therapy** is used as adjuvant systemic treatment for oestrogen receptor positive tumours. About 60% of breast cancers are oestrogen receptor positive and hence the goal of hormone therapy (anti-oestrogen therapy) is to deprive the cancer cells of oestrogen that triggers their growth and multiplication. It reduces the risk of recurrence in patients with early stage disease and shrinks the tumour in patients with advanced or metastatic disease. Various modalities of hormone therapy include:

- **Blocking the receptors:** These agents act by blocking the hormone receptors so that growth signals cannot get through to the cancer cells. These include the selective oestrogen receptor modulators such as tamoxifen which is given for five years after surgical resection of the tumour. This is also shown to reduce the risk of tumours in the contralateral breast by up to 50%. The Early Breast Cancer Trialist's Collaborative Group (EBCTCG) overview confirms that it reduces the odds of recurrence by about 25% and of death by about 17% which translates to an absolute survival benefit of about 6% at 10 years (Early Breast Cancer Trialist's Collaborative Group, 1998a).

- **Reducing oestrogen production:** These agents act by reducing the level of circulating oestrogen and thereby the growth signals to the hormone receptors that facilitate tumour growth. These include aromatase inhibitors such as anastrozole, letrozole and exemestane. These agents are effective in post-menopausal women as they inhibit oestrogen production from sources other than the ovary. In premenopausal women, agents such as goserilin and leuprolide inhibit oestrogen production by the ovary. Other options in premenopausal women include surgical removal of the ovaries and radiation induced menopause (Thompson and Dewar, 2002).

- **Eliminating receptors:** These agents reduce the availability of receptors to receive the growth signals from oestrogen. These include oestrogen receptor downregulators such as fulvestrant which attach to the receptors and destroy them. These agents are the subject of on going trials.

- **Chemotherapy** is a mode of systemic treatment that can be used in a neoadjuvant or an adjuvant setting. Before surgery, it is used to shrink large tumours so that they are amenable to breast conserving surgery. After surgical excision,

chemotherapy is offered with the intention of killing cancer cells that might have spread to other parts of the body away from the site of origin

Chemotherapy is now offered to both pre- and post-menopausal women with tumours having poor prognosis. These include large tumours that are node positive, oestrogen receptor negative and of high nuclear grade. A combination of 2-3 agents is used to deal with the heterogeneity of cancer cells making up the tumour. It is reported that a 6 monthly cycle of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) will achieve a 25% reduction in the risk of relapse over a 10 – 15 year period (Sainsbury, 2004). The EBCTCG overview confirms that 6 months of anthracycline-based polychemotherapy (eg, with FAC or FEC) reduces the annual breast cancer death rate by about 38% (SE 5) for women younger than 50 years of age when diagnosed and by about 20% (SE 4) for those of age 50–69 years when diagnosed, largely irrespective of the use of tamoxifen and of oestrogen receptor (ER) status, nodal status, or other tumour characteristics. Such regimens are significantly ($2p=0.0001$ for recurrence, $2p<0.00001$ for breast cancer mortality) more effective than CMF chemotherapy (Early Breast Cancer Trialist's Collaborative Group, 2005). Chemotherapy is associated with significant side effects as apart from cancer cells, it affects rapidly dividing cells in other parts of the body such as bone marrow, intestines, hair and oral cavity. Considering these possible side effects is an important part of the careful process of weighing benefits versus risks.

- **Targeted therapy** in the form of herceptin is found to be very effective in a sub-group of patients with advanced breast tumours that are Her-2 positive. About 1 in 5 breast cancers are Her-2 positive and these tend to be more aggressive than tumours that do not express the Her-2 gene (Slamon and Clarke, 1988). Herceptin acts by binding to the Her-2 receptors on the surface of cancer cells and blocks them from receiving growth signals. This effectively stops the growth of cancer cells and also alerts the immune system to destroy the cancer cells that it attaches to. A multi-centre clinical trial looking at therapy for patients with metastatic disease that expressed Her-2 receptors, reported a three fold increase in disease free interval and increase in survival time by 4 to 6 months following addition of herceptin to paclitaxel (Shak, 1999). Thus it is a promising mode of targeted immune therapy which is thought to reduce the chance of disease recurrence in women with Her-2 positive tumours.

1.4 Immunology

Breast cancer, in common with other malignancies, is a complex disease with multifactorial aetiology. The period of development can span several decades and the clinical course is highly variable. Breast cancer is the most common malignancy in women, accounting for approximately one third of all female cancers (www.cancerresearchuk.org).

The immune system is thought to play an important role in various phases of tumour growth – immunosurveillance, initiation, promotion, progression and regression. Both the innate and acquired arms of the immune system are believed to play crucial roles in the anti-tumour response and the interaction between host immune system and tumour cells has been the subject of intense research over the past decades (Dranoff, 2004; Ben-Baruch, 2003).

Innate response The innate responses occur early, are not antigen specific and depend largely on the proper functioning of natural killer (NK) cells and phagocytic cells, such as monocytes, macrophages and neutrophils which do not need prior sensitisation to respond to an immunogen. Cytokines including transforming growth factor – beta (TGF- β), tumour necrosis factor (TNF), IL-1, IL-6, IL-10, IL-12, IL-18 and interferons play an important role in the innate response. Macrophages can present tumour antigens to T cells and may exert direct anti tumour activity through secretion of tumour necrosis factor or oxyradicals such as nitric oxide. Macrophages and neutrophils can phagocytose and destroy antibody coated tumour cells. NK cells can efficiently lyse tumour cells made susceptible by loss of major histocompatibility complex (MHC) alleles (Roda-Navarro et al, 2000).

Acquired response In contrast, the acquired response develops late after antigen processing and clonal expansion of T and B cell subsets and is antigen specific. The functions of the acquired or adaptive immune system are mediated by effector cells with specific receptors that recognize tumour antigens. Tumour specific antigens that are unique or shared by other tumours have been discovered that are recognized by antibodies or T cells. The T-cell receptor molecule is designed to bind to molecules of

the MHC; CD8+ T cells bind to MHC class I molecules, which are expressed on all nucleated cells; whereas CD4+ T cells generally bind to MHC class II molecules, which are expressed predominantly on cells of the immune system. As most tumour cells do not express MHC class II, CD4+ T cells are dependent on presentation of tumour antigen by professional antigen presenting cells, such as dendritic cells and macrophages (Qin et al, 1998). T-cell activation is a two signal process. In addition to the T-cell receptor binding to the MHC peptide complex, a number of costimulatory signals are required. These signals are provided through interaction of certain ligands on the peptide presenting cell and their receptors expressed on the T cell. When properly activated, T cells undergo cell division (clonal expansion) and become functionally active. Expansion of T cells is also dependent on the availability of growth factors, such as IL-2, partly secreted by CD4+ Th1 cells, highlighting the requirement for both cytotoxic and helper cells to be activated in a co-ordinated and precise manner. T cells can directly lyse tumour cells and this is thought to be largely responsible for 'spontaneous regression' of solid tumours. Thus T-cell mediated lysis is dependent on recognition of tumour antigens by the T-cell receptor on the T cell upon which the lytic machinery of T cells is activated (Davis and Bjorkman, 1988). NK cells, macrophages and neutrophils also help in tumour cell lysis by antibody dependent cellular cytotoxicity.

Tumour escape mechanisms The sequence of events which occur in the innate and adaptive arms of the immune system and the subsequent elimination of tumour cells by T cells often does not occur due to tumour escape mechanisms. These include factors related to the tumour, host or both.

Tumour related factors These refer to certain properties of the tumour which enable it to evade the host immune system. The tumour may not be immunosensitive as it may lack antigens that induce a T cell response. There may be no or low expression of major histocompatibility complex alleles. Tumour cells may not be able to process or present tumour antigens and the antigens may be masked or modulated. Studies have shown that human breast cancer cell lines fail to present alloantigen (Gimmi et al, 1996). Tumour cells may be resistant to tumoricidal effector mechanisms such as induction of apoptosis. The tumour may not be immunogenic in that they may express tumour antigens, but still not induce an immune response because the tumour cells lack expression of costimulatory molecules. Monocytes derived from patients with early

breast cancer have been demonstrated to have a defect in expression of costimulatory molecules and induction of T-cell proliferation. It is hypothesized that these defects might contribute to tolerance of the immune system towards the presence of malignant cells in these patients (Wolfram et al, 2000). It has been shown that lack of expression of intracellular adhesion molecule (ICAM-1) or leucocyte function-associated antigen (LFA-3) confers resistance to T cell mediated lysis. Tumour cells may produce immunosuppressive factors that inhibit T cell function or induce signalling defects in T cells. Tumours may shed antigen (eg:- CA 125 in ovarian cancer; CEA in colon cancer) or it may induce T cell tolerance. Tumours may induce apoptosis in activated T cells by expressing the apoptosis inducing molecule, Fas ligand, on the cell surface that can bind to the Fas receptor expressed on activated T cells (O'Connell et al, 1999).

Host related factors Certain host related factors have been implicated in tumour escape from immune surveillance. These include inherited or acquired immunodeficiency, immunosuppression induced by radiotherapy or chemotherapy, tumour progression that may outpace tumour regression and the immune system, deficient presentation of tumour antigens by antigen presenting cells, lack of access of effector cells to the tumour (stromal barrier) or expression of immunodominant antigens on parental tumour that prevents stimulation with other tumour antigens (Goedegebuure and Eberlein , 2001).

It has been known for a number of years that there is significant impairment of the immune system in breast cancer patients (Stewart, 1996). The lack of an effective immune response to tumour genesis, in spite of the presence of tumour infiltrating lymphocytes (TIL) *in vivo*, is believed to be due in some way to production by inflammatory cell infiltrates, of direct or indirect modulators of breast cell growth, e.g. cytokines, peptide or steroid hormones, enzymes involved in steroid metabolism, as well as auto antibodies to growth factors or their receptors. Data from various studies suggest that instead of being a host defence reaction, the immune response may actually facilitate cancer development in some instances. These include studies in breast cancer patients which show a correlation between the intensity of lymphocytic infiltration into the tumour mass with poor prognosis, studies that demonstrate a similar correlation between delayed hypersensitivity reactivity or *in vitro* assays of immune reactivity to tumour cell membranes or non-specific antigens and poor prognosis and analyses of cancer incidence in chronically immunosuppressed, kidney transplant recipients who

develop an unexpectedly low incidence of breast cancer. These are backed up by observations in mouse mammary tumour models that also demonstrate immune enhancement of breast cell proliferation *in vitro* and of breast cancer development *in vivo* (Stewart and Heppner, 1997). Also, breast cancer has long been considered to be weakly immunogenic and hence is only poorly recognized by the immune system, meaning this type of tumour is generally thought to be a poor candidate for immune therapy (Allan et al, 2004). Hence, these immune facilitatory mechanisms must first be understood if immune-based therapies are to be applied successfully in breast cancer.

1.5 Role of cytokines

Cytokines are known to play a key role in the innate and adaptive arms of the immune response. They are glycoproteins secreted by cells such as lymphocytes or macrophages or as components of a tissue such as the intestinal epithelium. They alter the function of target cells in a paracrine or autocrine manner and less commonly in an endocrine fashion. Cytokines have been categorised on the basis of structure as Type I cytokines, which include, interleukin (IL) – 2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, IL-15 and granulocyte colony stimulating factor (G-CSF) and Type II cytokines which include interferon (IFN) – alpha, IFN-beta, IFN – gamma and IL-10 . On the basis of the nature of their function, cytokines are also classified as the pro-inflammatory Th1 cytokines which include IL-2, IFN-gamma and IL-12 and the anti-inflammatory Th2 cytokines which include IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Fink, 2001).

Cytokines have several common structural and functional characteristics:

- They tend to be glycosylated polypeptides
- They are biologically active at very low concentrations (nano to picograms/ml)
- They exert their effects by binding to receptors on the target cell surface
- Their effects are target cell-specific
- They are pleiotropic, i.e. a cytokine has multiple biological activities, depending on the target cell types involved and the presence or absence of other modulating factors
- They are redundant, i.e. multiple different cytokines mediate similar effects
- Their effects are additive, synergistic or antagonistic
- They interact on various target cells forming a complex network.

Thus it is the integration of these functions that determine the overall outcome.

It is recognised that there is significant impairment of the immune system in breast cancer patients (Stewart, 1996). One of the manifestations is reduction in the level of immunostimulatory cytokines and increase in levels of inhibitory cytokines. The lack of an effective immune response to tumourigenesis, in spite of the presence of TIL *in vivo*, is believed to be due in some way to the action of “inhibitory” cytokines in the tumour microenvironment. Cytokines have the potential to stimulate breast cancer growth, increase angiogenesis and decrease cell adhesion. In addition to their effects on the immune system, cytokines have also been reported to influence aromatase activity and oestrogen synthesis directly in the tumour vicinity and hence promote tumour growth and aggressiveness (Purohit et al, 2002). Certain cytokines are found to be elevated in patients with advanced disease, metastasis and during relapse.

In contrast to many of the *in vivo* observations, however, certain cytokines have been demonstrated to promote generation and/or efficacy of anti-tumour effectors, including dendritic cells (DC) and lymphokine activated killer (LAK) cells, and cause inhibition of tumour growth or, in certain instances, regression (Allan et al, 2004). Most of this work has been *in vitro* although some *in vivo* data do exist. Adjuvant therapy including chemotherapy and hormone therapy has been demonstrated to improve the efficacy of the host immune response by enhancing the levels of immunostimulatory cytokines. With rapid advances in viral-based delivery systems and nanotechnology, local targeted delivery of cytokines at a tumour site with a view to boost anti-tumour response may become a feasible treatment option to modulate therapeutically one or more components of the immune system.

Thus cytokines seem to have an important role in the host immune response towards breast cancer. On one hand they seem to facilitate breast tumourigenesis and on the other hand, certain cytokines seem to aid in control of tumour growth and regression. They have a potential role as prognostic markers to assess disease stage, biological behaviour, tumour aggressiveness, disease progression and possible use in the evolving immune therapy regimens. The role of Type 1 and Type 2 cytokines in breast cancer has been extensively investigated and are reviewed here to elucidate their roles in breast cancer.

1.51 Interleukin – 10

IL-10 and IL-10 receptor Interleukin-10, originally called cytokine synthesis inhibitory factor, is a 18 kDa non-glycosylated protein expressed by a variety of immune cells such as CD4⁺ T cells, natural killer cells, macrophages and dendritic cells. Human IL-10 is encoded by five exons on chromosome 1. IL-10 binds to its receptor (IL-10R) with high affinity (Moore et al, 2001). The IL-10 receptor (IL-10R) consists of two subunits that are members of the interferon receptor family. The ligand binding subunit (IL-10 R1 or IL-10R α) binds IL-10 with high affinity and is expressed by most haemopoietic cells (Ho et al, 1993). The accessory signalling subunit (IL10 R2 or IL-10R β) has little affinity to IL-10, but appears to play a crucial role in signal transduction pathway by recruiting two protein kinases of the Jak family (Finbloom and Winestock, 1995). IL-10 is a pleiotropic cytokine that can exert either immunosuppressive or immunostimulatory effects on a variety of cell types, depending on its relative concentration in the microenvironment and the presence of other modulatory factors.

Effects on immune cells IL-10 promotes survival of normal B cells and is a potent cofactor for B cell proliferation promoted by IL-2 (Rousset et al, 1992). IL-10 inhibits the production of IL-2 and IFN- γ by Th1 cells (Del Perte et al, 1993; de Waal Mallefyt et al, 1993) as well as their proliferation and chemotaxis (Taga et al, 1993; Jinquan et al, 2000). As IL-10 has been shown to have anti-inflammatory/immunosuppressive properties and B cell stimulatory function *in vitro*, it is widely regarded as a Th2 cytokine which terminates T cell mediated immune response and initiates humoral immune response. IL-10 impairs the antigen presenting functions of DC by down regulating their expression of HLA class II molecules (Koch et al, 1996), intercellular adhesion molecules (eg:- ICAM-1) (Chang et al, 1994) and costimulatory molecules (CD80/B7-1 and CD86/B7-2) (Buelens et al, 1995). Thus IL-10 inhibits primary alloantigen-specific T cell responses as T cell activity is dependent on its interaction with DC (Caux et al, 1994). IL-10 inhibits monocyte/macrophage dependent, antigen stimulated cytokine synthesis by human peripheral blood mononuclear cells and NK cells. IL-10 acts to down regulate the inflammatory response by inhibiting the

production of other cytokines such as IL-1, TNF- α , IL-6, IL-8, IL-12 and GM-CSF by monocytes and macrophages.

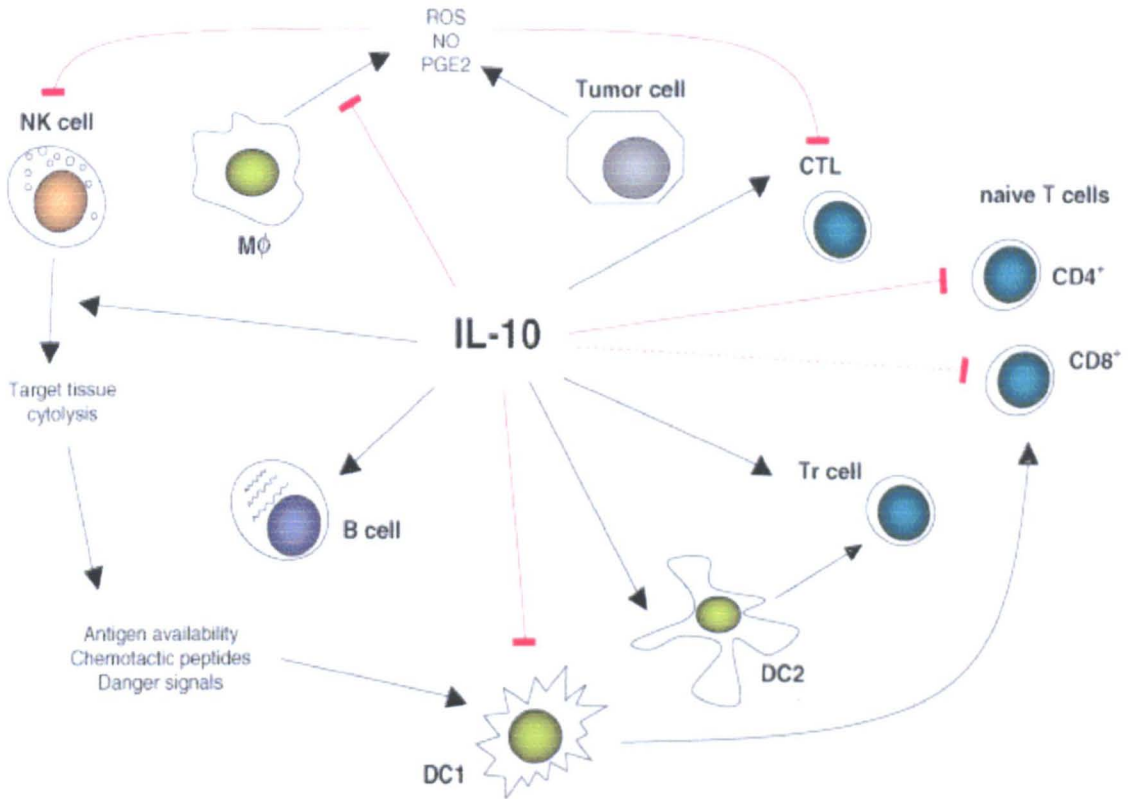


Fig 1.51 The major effects of IL-10 on the innate and adaptive immune response cell mediators (Mocellin et al, 2004). Stimulatory effect (black lines), inhibitory effect (red lines), uncertain effect (dotted lines). ROS: reactive oxygen species; NO: nitric oxide; PGE2 : prostaglandin E2; NK cell: natural killer cell; M ϕ : macrophage; DC1: dendritic cell activating naïve T cells; DC2: tolerogenic dendritic cell; Tr cell: T regulatory cell; Th1/Th2 cell: T helper/T helper 2 cell; CTL: antigen-experienced cytotoxic T lymphocyte.

However, more recent studies have reported apparently contradictory findings that IL-10 has stimulatory and facilitatory functions on monocytes and macrophages and enhances antibody-dependent cellular cytotoxicity. Immunostimulatory properties of IL-10 have been demonstrated in several *in vitro* and *in vivo* models. Leukocyte recruitment is shown to be enhanced by IL-10 via chemotaxis and induction of

endothelial cell adhesion molecule expression (Vora et al, 1996; Jinquan et al, 1993). IL-10 has been demonstrated to increase CD8+T cell migration (Jinquan et al, 2000) and enhance NK cell cytotoxicity both in *in vitro* and in pre-clinical models (Shibata et al, 1998). As the data on IL-10 function accumulates, its biological activity appears to be more complex and there is a growing body of evidence to suggest that IL-10 can have both an immunosuppressive and an immunostimulatory effect on immune cell mediators (Fig 1.51).

Venetsanakos and colleagues investigating the expression of cytokine mRNA in human breast tumour sections by reverse transcriptase polymerase chain reaction (RT-PCR) reported detection of significant levels of IL-10 mRNA in tumour tissues with little or no expression in normal breast tissue (Venetsanakos et al, 1997). They postulated that the reduced function of TIL is related to IL-10 concentration. IL-10 is reported to inhibit tumour associated antigen presentation by DC, thereby preventing the TIL from mounting an effective immune response against malignant cells (Marincola et al, 2000). This is supported by *in vitro* tumour models where CD8+ T cells are seen to be anergized towards melanoma associated antigens when stimulated with IL-10 conditioned DC (Steinbrink et al, 1999). Furthermore, IL-10 production by basal and squamous cell carcinoma cell lines derived from biopsy specimens have been shown to prevent *in vitro* lysis of autologous tumour cells by TIL (Kim et al, 1995). IL-10 secretion by T regulatory cells has been shown to induce anergy of TAA-specific CTL in a mouse lymphoma model (Rohrer and Coggin Jr, 1995).

However, IL-10 has also been demonstrated to have potent anti-tumour activity. Dorsey *et al* demonstrated that systemic administration of recombinant human IL-10 to animals bearing established, highly malignant, mammary tumours led to significant growth inhibition which was associated with increased numbers of CD4+ve cells and production of Mig (monokine induced by IFN- γ) and IP-10 (inducible protein 10) (Dorsey et al, 2002). Mig and IP-10 are chemotactic for activated T and NK cells and are potent inhibitors of angiogenesis. Furthermore, IL-10 is reported to have potential anti-metastatic activity which is believed to be dependent on NK function (Kundu and Fulton, 1997). These workers showed that IL-10 expression downregulates MHC class I expression on tumour cells, leading to enhanced NK cell lysis. This dual role for IL-10 is now thought to be due to its concentration in the tumour microenvironment with

low concentrations (picograms) facilitating tumour growth but higher concentrations (nanograms) having considerable anti-tumour activity. It is suggested that IL-10 might act as an immunological adjuvant maintaining the number of antigen experienced CTL during vaccine induced tumour rejection. This is based on the following observations: i) IL-10 exerts a positive effect on antigen exposed CTL; ii) a continuous supply of tumour associated antigens (TAA) to secondary lymphatic organs is necessary to maintain an effective adaptive immune response and iii) IL-10 can facilitate TAA availability by stimulating NK cell mediated tumour cytolysis (Ochsenbein, 2002; Peterson et al, 1998; Kundu et al, 1996; Cai et al, 1999; Terres and Coffman, 1998). Hence it is hypothesised that IL-10 may have a permissive effect on vaccine induced adaptive immunity by increasing NK cell anti-tumour function .

Data on the *in vivo* immunostimulatory effects of IL-10 is scarce. Intravenous administration of human recombinant IL-10 in healthy volunteers given 1 hour after induction of endotoxaemia has been shown to generate pro-inflammatory response by enhancing the release of IFN- γ , IP-10 and other monokines induced by IFN- γ (Lauw et al, 2000). These volunteers also had increased plasma levels of granzyme-B reflecting activation of cytotoxic T lymphocytes and natural killer cells. Similarly, patients with acute myelogenous leukaemia showed an elevation in plasma levels of TNF- α and IL-1 upon administration of IL-10 (Tao et al, 2001). Thus the potency of IL-10 has been demonstrated, however, it may prove a difficult reagent to use therapeutically because of the obvious potential for unwanted, deleterious, immunosuppression.

1.52 Interleukin-12

IL-12 and IL-12 receptor Interleukin-12, also known as natural killer cell stimulatory factor or cytotoxic lymphocyte maturation factor, is a pleiotropic cytokine principally produced by phagocytic cells such as monocytes and macrophages, B cells and dendritic cells . It is a 75 kDa glycoprotein heterodimer composed of two genetically unrelated subunits (p35 and p40) linked by a disulphide bond. Both subunits are required for biological activity (Germann and Rude, 1995). IL-12 acts on the IL-12 receptors (IL-12R) which are primarily expressed on activated T cells and NK cells. IL-12 R has two sub units and are designated as IL-12R β 1 and IL-12R β 2. They are type I transmembrane glycoproteins and expression of both subunits is required for the generation of high affinity IL-12 binding sites. The IL-12R β 2 subunit appears to function as the signal transducing component of the high-affinity receptor complex (Gately et al, 1998).

Effects on immune cells IL-12 is a pivotal factor for the initiation of cellular immunity and has been shown to have multiple effects on T cells and NK cells (Schmitt et al, 1994). It plays an essential role in the interaction between the innate and adaptive arms of the immune response. IL-12 promotes the polarization of Th1-like T cell development, increases the differentiation and activation of cytolytic T cells and, in concert with TNF- α , IL-1, IL-2 and IL-18, induces IFN- γ production by T cells and NK cells (Germann and Rude, 1995; Trinchieri, 1994). IL-12 activated NK cells have enhanced cytotoxic activity that may induce tumour cell death or affect the integrity of the tumour vascular endothelium. IFN- γ , TNF- α and other pro inflammatory cytokines induced by IL-12 also induce the production of chemokines such as IP-10 and MIG which are powerful inhibitors of tumour angiogenesis (Fig 1.52).

The activities of IL-12 are antagonized primarily by IL-10 which is associated with the development of naïve T helper cells into Th2-like cells and mediation of the humoral immune response. IL-10 inhibits IFN- γ production by suppressing the transcription of the IL-12 gene (Wang et al, 1994). Like IL-1, IL-12 is not essential for the generation

of CTL, however, its addition (either exogenously or endogenously) or inhibition markedly improves or inhibits CTL development respectively (Bhardwaj et al, 1996).

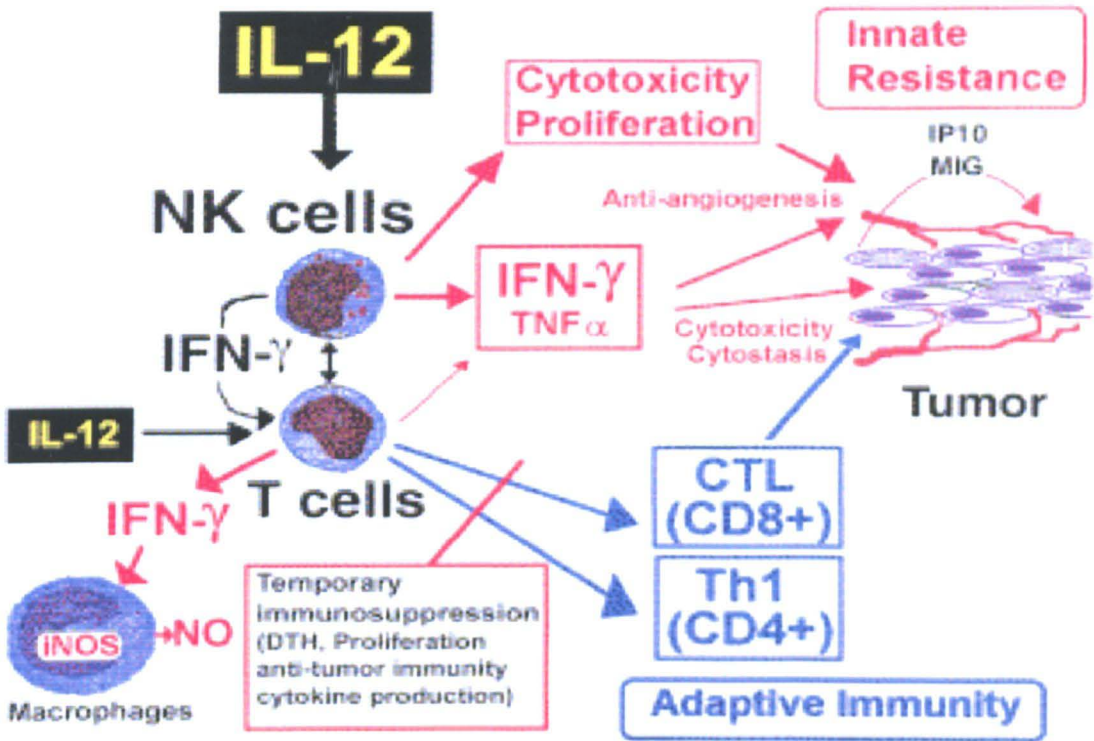


Fig 1.52 Mechanisms of IL-12 mediated anti-tumour activity (Colombo and Trinchieri, 2002). NK cells: Natural killer cells; IFN- γ : Interferon-gamma; TNF- α : Tumour necrosis factor-alpha; CT : Cytotoxic T lymphocytes; IP10 : Inducible protein 10 (a chemokine); MIG : Monokine induced by gamma (a chemokine); NO : Nitric oxide.

IL-12 and IL-10 generation are most likely to be co-regulated by a complex monocyte and macrophage-derived cytokine network and an impairment of this can be observed in neoplastic diseases including breast cancer. Mononuclear cells of patients affected by breast cancer have been demonstrated to have a defective IL-12 production capability while generating higher amounts of IL-10 ($n=15;p<0.05$; Merendino et al, 1999). A similar pattern has been reported in patients with advanced colorectal cancer ($n=60$), who had low IL-12 production by PBMC ($p<0.001$) and higher serum levels of IL-10

($p=0.004$) when compared to controls ($n=30$), suggesting an impaired Th1-cell mediated anti-tumour response (O'Hara et al, 1998).

The anti-tumour effects of local and systemic recombinant murine interleukin-12 (rIL-12) have been demonstrated in mice harbouring spontaneously metastasising mammary adenocarcinomas (Cavallo et al, 1999). Leukocyte infiltration of the tumour site and production of mRNA for secondary cytokines (mainly IFN- γ) and chemokines all occurred more quickly and were more effective in the mice receiving systemic as compared with local rIL-12. Neutrophils were demonstrated to take part in the IL-12 mediated effect against tumour vessels leading to micro haemorrhages and thrombosis resulting in tumour necrosis. IL-12 mediated production of IFN- γ resulted in generation of IP-10 and MIG and this inhibited sprouting of new vessels in tumour neo-angiogenesis (Di Carlo et al, 2001). Sabel and colleagues more recently demonstrated that intra-tumoural administration of poly-lactic-acid-encapsulated microspheres (PLAM) containing IL-12, TNF- α and GM-CSF, in a murine model of breast cancer, enhanced CD8+ve T-cell infiltration resulting in tumour regression (Sabel et al, 2004). Importantly, it also induced tumour specific T-cells in the lymph nodes and spleen, resulting in a memory immune response. Thus IL-12 utilises effector mechanisms of both innate resistance and adaptive immunity to mediate anti tumour response.

IL-12 is one of the many co-stimulatory molecules expressed by professional antigen presenting cells such as DC and over the last few years, attempts to harness this property in a number of therapeutic vaccination strategies involving DC have yielded encouraging results. Human DC co-activated *in vitro* by CD40L and IL-1 β produced high levels of IL-12 and subsequently IFN- γ and thus became potent T cell-stimulators (Luft et al, 2002). The dual CD40L/IL-1 signal enables the production of pro-inflammatory cytokines including IL-1, IL-12, IL-18 and IFN- γ by DC, and enhances the differentiation of naïve T cells into effectors of a Th1 cellular immune response. Hence, it is predicted that such DC will function as effective vaccines for the induction of cellular immunity *in vivo*. Once again, the challenge now is to translate the findings from murine models to human subjects. It has been shown that DC generated *in vitro* from breast cancer patients have similar antigen-specific reactivity against infectious agents to DC from normal donors but have significantly decreased allostimulatory ability, suggesting that further manipulation of these cells is required prior to use *in vivo*

(Pedersen et al, 2005). A recent review has noted, however, that caution is needed in interpreting data on DC vaccination strategies and has highlighted the need for clinical response to be the main means of assessment of efficacy (Rosenberg et al, 2004).

Although results from pre-clinical experimental models of immunotherapy utilising IL-12 as an anti tumour agent are tantalisingly encouraging, clinical trials of IL-12 have shown only modest clinical efficacy with considerable toxicity. Phase I & II trials in patients with advanced malignancies resulted in cessation of disease progression and in some cases, regression. But this was dampened by severe toxicities including fever/chills, fatigue, nausea, vomiting, headache, anaemia, lymphopenia, neutropenia, hyperglycemia, thrombocytopenia, hypoalbuminemia, oral stomatitis, abnormalities in liver function tests and capillary leak syndrome (Atkins et al, 1997; Gollob et al, 2000; Hurteau et al, 2001). These adverse effects seem to be less evident when IL-12 is administered subcutaneously rather than intravenously. A small trial in patients with cutaneous T cell lymphoma yielded encouraging results with complete regression in some patients (overall response rate of 56%; Rook et al, 1999). Preliminary results of trials exploring the use of IL-12 in gene therapy and as adjuvants in cancer vaccines have shown some promising results (Kang et al, 2001; Lee et al, 2001). Therapeutic trials involving IL-12 have not been reported in breast cancer patients to date. Clinical protocols that minimise toxicity without affecting the anti-tumour effect of IL-12 need to be envisaged. It is hoped that with the advent of nanotechnology and micro drug delivery systems, it may be possible to deliver IL-12 locally into the tumour microenvironment and thereby avoid the manifestations of systemic toxicity.

1.53 Other relevant cytokines

These include cytokines demonstrated to have an important role in the host immune response against breast cancer. They seem to have the potential to function as prognostic markers for assessing disease stage, tumour aggressiveness and disease progression and a possible role in novel immune therapy regimens (Table 1.53.1 & 1.53.2). They include:

Transforming growth factor – beta (TGF- β) TGF- β is a pleiotropic cytokine produced by numerous tumour types. Three TGF- β isoforms (TGF- β 1, TGF- β 2 & TGF- β 3) have been isolated in mammalian cells, each encoded by a unique gene (Derynck et al, 1985). They are made as large precursors of 390 amino acids (TGF- β 1) or 412 amino acids (TGF- β 2 and TGF- β 3). Each precursor contains an N-terminal signal peptide, a long prosegment and a 112 amino acid C-terminal polypeptide that constitutes the mature TGF- β monomer. The mature form of TGF- β has a homodimeric structure with a molecular weight of 27 kDa and has two chains of 112 amino acids, each containing nine cysteine residues (Roberts and Sporn, 1985). TGF- β binds to a unique cell surface receptor and is known to have dual effects on cell growth, either stimulatory or inhibitory. It is known to induce angiogenesis, impair DC functions *in vitro*, block T-cell activation, mediate production of extracellular matrix (ECM) and facilitate the production of cytokines by fibroblasts and endothelial cells.

TGF- β is known to have anti-proliferative effects on a range of epithelial cells and hence a potential tumour suppressor with clinical significance. Early stage of breast carcinogenesis is associated with a growth inhibitory response to TGF- β which is lost with tumour progression. Hence it is postulated that boosting of endogenous TGF- β in early disease stage and anti-TGF- β strategies at later disease stage could yield therapeutic gains (Benson, 2004).

There are two major studies of interest with respect to TGF- β 1 and breast cancer prognosis. Firstly, Ivanovic *et al* investigated the association between plasma TGF- β 1 level and disease progression in advanced breast cancer patients (n = 44). They found that plasma TGF- β 1 values were significantly elevated (P < 0.05) in stage IIIB/IV

patients compared with healthy donors, suggesting that TGF- β 1 may have potential as a prognostic marker for breast cancer patients with advanced disease and is worthy of further investigation (Ivanovic et al, 2003). Secondly, Kobie *et al*, observing the lack of effectiveness of dendritic cell (DC)-based vaccines in treating established tumours, demonstrated that TGF- β exposure inhibits the ability of DC to present antigen and stimulate a specific T cell response. Furthermore, blocking of TGF- β with the neutralizing monoclonal antibody 2G7 enhanced the ability of DC vaccines to inhibit the growth of established 4T1 murine mammary tumours. Treatment of 4T1 tumours transfected with TGF- β with a combination of DC and 2G7 monoclonal antibody inhibited tumour growth and resulted in complete regression of tumours in 40% of mice in an *in vivo* model (Kobie et al, 2003). These results clearly suggest that neutralization of TGF- β is an important factor to be considered when designing DC vaccines for clinical use in humans as it is seen to enhance the efficacy of DC-based vaccines in murine models.

Tumour necrosis factor-alpha (TNF- α) TNF- α is principally produced by lymphocytes and NK cells. TNF- α is initially synthesized as a 26 kDa cell surface associated molecule that is anchored by an N-terminal hydrophobic domain. This biologically active membrane bound form of TNF- α is cleaved by a specific TNF-converting enzyme to release soluble 17 kDa form of TNF- α that binds to a family of TNF receptors (Kriegler et al, 1988). TNF- α exhibits various anti-tumoural effects including the induction of apoptosis and necrosis, activation of cytolytic effector cells and upregulation of expression of intercellular adhesion molecule-1 (ICAM-1) on tumour cells, an important event in the interaction with lymphokine activated killer (LAK) cells (Wang et al, 2003).

Spontaneous production of TNF- α by polymorphonuclear cells (PMN) in breast cancer patients was reported to be lower than that found in healthy controls (Jablonska, 1998). This lower level of TNF- α production by cancer patients was also seen following *in vitro* stimulation with Zymosan or lipopolysaccharide (LPS). However, there were no significant differences in circulating serum levels of TNF- α between breast cancer patients and controls. More recently, impaired production of TNF- α by monocytes derived from patients with breast cancer as well as in healthy carriers of BRCA1 mutations has been reported (Zielinski et al, 2003). Whether these changes are caused

by the tumour or are the result of the anti-tumour response now needs to be elucidated as this knowledge could facilitate new therapeutic approaches.

Tsuge and colleagues found that infiltration of breast cancer lesions by DC, an important subset of antigen-presenting cells characterized by their potent capacity to activate naïve T cells, requires expression of TNF- α , along with granulocyte macrophage colony stimulating factor (GM-CSF) and IL-1, on cancer cells (Tsuge et al, 2000). Candido *et al* investigated whether intra-tumoural delivery of DC alone would have any anti-tumour effect on a malignancy with a relatively high apoptotic index. Their studies revealed intense mononuclear cell infiltration during and after DC injections and significant apoptosis of the tumour cells. They also reported that the level of tumour apoptosis could be increased substantially by co-administration of TNF- α with DC (Candido et al, 2001). It has been reported that human peripheral blood DC activated by pro-inflammatory stimuli produce TNF- α , which subsequently has a potent direct anti-tumour effect against breast cancer cell lines *in vitro*, leading to growth inhibition and apoptosis (Manna and Mohankumar, 2002). Based on the above observations, it is plausible that TNF- α could be included as part of an immunotherapeutic regime, however, potential side effects of any such strategies, including possible induction of an autoimmune response due to generalised T cell activation, would need to be carefully considered.

Interferon-gamma (IFN- γ) IFN- γ is a pro-inflammatory mediator produced principally by T cells (CD4+ve and CD8+ve) and NK cells. It is composed of 143 amino acids with a molecular weight ranging from 34-50 kDa and exists as a non covalent homodimer. Key functions include activating cells of the monocyte/macrophage lineage, promoting differentiation of naïve CD4+ve T cells into Th-1 like cells and inhibiting differentiation of CD4+ve cells into Th-2 like cells (Schreiber and Schreiber, 2003).

IFN- γ has been demonstrated to be a critical component in regulating an innate phagocytic response against metastatic breast cancer (Pulaski et al, 2002). Furthermore, Kamamura and colleagues incubated peripheral blood mononuclear cells (PBMC) from breast cancer patients with IFN- γ and found that this increased LAK activity (Kamamura et al, 1998). The use of intravesical instillation of recombinant IFN- γ has been demonstrated to be effective against tumour recurrence in patients with early stage

superficial transitional cell bladder cancer (Giannopoulos et al, 2003). For this to be feasible in breast cancer new delivery systems allowing targeted cytokine release are required.

Interleukin-2 (IL-2) IL-2 was originally identified as a growth factor for T lymphocytes. It is a 15 kDa protein produced mainly by CD4⁺ Th1-like cells that promotes proliferation of T cells and activated B cells, stimulates secretion of cytokines by T cells and increases the cytotoxicity of NK cells. There are two distinct, membrane-associated IL-2 binding components in the high-affinity IL-2 receptor: a ligand binding alpha subunit and a signal transducing beta subunit, which associate in a non-covalent manner (Gaffen and Liu, 2004).

Reduced IL-2 production has been described as one of the most frequent immune dysfunctions observed at relapse in patients with disseminated solid neoplasm. Patients with breast cancer are observed to have a much lower rate of relapse (4.7%) if plasma IL-2 levels were normal compared with those patients with decreased amounts (33.3%) one year post-operatively (Arduino et al, 1996). Not only have systemic levels been shown to vary in cancer patients but also IL-2 production by lymphocytes in the tumour vicinity is reported to be impaired and it is suggested that this decrease will limit the cytotoxic potency of CTL (Blidaru et al, 1998).

It is well-known that hypoxia is a common event within a tumour mass and it has been postulated that this can affect the ability of tumour cells to be recognized by the immune system. IL-2-transfected tumours showed decreased hypoxia and increased vascularisation compared with parental tumours and these changes were associated with a pronounced infiltration of activated T cells (Lee et al, 1998). Thus, expression of IL-2 at the tumour site may enhance immunity not only by inducing the generation of reactive CTL but also by allowing increased infiltration of these activated cells into the tumour mass.

A number of positive clinical results have been observed after IL-2 treatment in a variety of solid tumours, e.g. melanoma, pulmonary metastasis of renal carcinoma, unresectable pancreatic head carcinoma and malignant mesothelioma. In contrast, IL-2 therapy for breast cancer has not been generally effective although, recently, peri-tumoural administration of IL-2 was reported to significantly improve survival in mice with advanced, relatively aggressive, tumours (Moiseeva et al, 2003). Furthermore, in a

Phase I trial with HER2-overexpressing metastatic breast cancer, a combination of Trastuzumab and IL-12 yielded encouraging results (Repka et al, 2003). In this pilot study, ten women with metastatic breast cancer received multiple cycles of IL-2 and Trastuzumab and no significant toxicities were seen. Clinical responses were: one partial response, five cases of stable disease, and four cases of progressive disease. Immune assays *in vitro* showed NK cell expansion and Trastuzumab-mediated increased NK killing of breast cancer targets, although this immune parameter did not correlate positively with clinical response. This study suggests that IL-2 can be safely administered without inducing significant toxic side effects and that further work is required to ascertain the mechanism of action.

Interleukin-4 (IL-4) IL-4 originally described as a B cell growth factor, is a 15-20 kDa glycoprotein produced by Th2-like cells, mast cells, basophils and eosinophils. It effects down regulation of pro-inflammatory and cell-mediated immune responses and up regulation of humoral immune responses. IL-4 induces differentiation of naïve CD4+ T cells into Th2-like cells, inhibits production of TNF- α , IL-1, IL-8 and PGE2 by stimulated monocytes/macrophages and downregulates endothelial cell activation induced by TNF- α . It acts as a co-mitogen for B cells and promotes expression of the class II major histocompatibility complex on these cells (Nagai and Toi, 2000).

IL-4 is known to provide potent anti-tumour activity against various tumours, including breast cancer. These effects are thought to be mediated directly by IL-4 through initiation of apoptosis, and indirectly by regulation of enzymes involve in oestrogen synthesis including 17beta-HSD and 3beta-HSD. A number of human breast carcinoma cell lines have been shown to express high affinity interleukin-4 receptors (IL-4R) that on ligation with IL-4 undergo apoptosis. These findings imply that IL-4 is a key factor not only for Th2 type immune reactions but also for tumour cell growth itself in human breast cancer (Leland et al, 2000).

Interleukin-6 (IL-6) IL-6 is a pluripotent cytokine produced by monocytes, macrophages, lymphocytes, endothelial cells and intestinal epithelial cells and its expression is known to be induced by IL-1, TNF- α and Platelet Activating Factor. It is a glycoprotein with a molecular mass ranging from 21-28 kDa and consists of 212 amino acids including a 28 amino acid hydrophobic signal sequence (Hirano et al,

1986). IL-6 promotes B-cell maturation and differentiation, stimulates T-cell proliferation and differentiation, stimulates the hypothalamo-pituitary-adrenal axis and induces production of acute phase reactants (Heinrich et al, 2003).

Serum IL-6 concentration was found to be significantly higher in patients with breast cancer compared with healthy controls (n=26; p<0.0001) (Benoy et al, 2002). Median IL-6 serum levels were nearly 10 times higher in patients with metastatic breast cancer (n=73) as compared to those with locoregional disease (n=31) (6.0 pg/ml versus 0.7 pg/ml respectively). A small preliminary study reported that continuously elevated serum IL-6 levels correlate with poor survival in patients with hormone-refractory metastatic breast cancer (n=12). They also reported that pre-treatment IL-6 and IL-8 levels are predictive indicators of response to therapy and prognosis of patients with recurrent breast cancer (Yokoe et al, 2000). This was confirmed more recently by another study involving 87 patients with hormone refractory metastatic breast cancer (Bachelot et al, 2003). Thirty nine percent of patients had detectable serum levels of IL-6 and high levels were significantly correlated to a shorter survival. In a multivariate analysis together with clinical prognostic parameters, serum IL-6 was identified as an independent adverse prognostic variable for overall survival (p<0.001). Similar associations between IL-6 levels and disease progression and survival have also been reported in patients with non-small cell lung cancer and prostate cancer.

Interleukin-8 (IL-8) IL-8 is secreted by monocytes, macrophages and endothelial cells. It is expressed as a 99 amino acid precursor and is secreted after cleavage of a 22 amino acid leader sequence. It has a molecular weight of 11 kDa (Xie, 2001). Also classified as a chemokine, it stimulates chemotaxis and triggering of neutrophil degranulation, increased expression of surface adhesion molecules and production of reactive oxygen metabolites (Schmid and Weissmann, 1987).

Elevated expression of IL-8 in human breast cancer cell lines was observed to be associated with breast cancer invasiveness and angiogenesis (Lin et al, 2004). Furthermore, IL-8 levels are inversely related to oestrogen receptor (ER) status with ER positive cells generally expressing low levels and ER negative cells expressing higher levels of IL-8. Bendre *et al* demonstrated a direct effect of IL-8 on osteoclast

differentiation and activity, implying a possible role in osteolysis, an event frequently associated with metastatic breast cancer (Bendre et al, 2003). IL-8 levels were found to be significantly increased in breast carcinoma patients and were correlated with stage of disease. It has been suggested previously that IL-8 measurement may be useful in estimation of disease progression in women with breast carcinoma (Yokoe et al, 2000).

Interleukin-18 (IL-18) IL-18, a recently described member of the IL-1 cytokine superfamily, is a 22 kDa protein structurally related to IL-1 β that is now recognized as an important regulator of innate and acquired immune responses. Formerly called interferon-gamma-inducing factor (IGIF), IL-18 is a cytokine that plays an important role in the Th1 response, primarily by its ability to induce IFN- γ production by T and NK cells. IL-18 is produced early during the acute immune response by macrophages and immature DC. In addition to IFN- γ , IL-18 also induces GM-CSF, TNF- α and IL-1 expression, and acts in collaboration with IL-12 (Lebel-Binay et al, 2000).

Coughlin *et al* studied the anti-tumour mechanisms activated by murine IL-12 and IL-18, cytokines that induce IFN- γ production, using a transfected murine mammary carcinoma model (Coughlin et al, 1998). Cells expressing mIL-12 or mIL-18 were less tumorigenic and formed tumours more slowly than non-transfected control cells. Antibody neutralization studies revealed that the anti-tumour effects of secreted mIL-12 and mIL-18 were mediated via IFN- γ .

IL-18 also appears to be a potent stimulator of DC function, enhancing *in vitro* tumour vaccination strategies by increasing recruitment of Th1 cytokine-producing cells and thus increasing numbers of antigen-specific CD8⁺ T cells, when transfected into DC (Ju et al, 2001). A study investigating the effects of IL-18 on bone metastasis of human breast cancer cells, MDA-231 cells, in a nude mouse model found that IL-18 inhibited osteolytic growth at bone metastatic sites and suppressed an early onset of bone metastasis (Nakata et al, 1999). Furthermore, systemic daily administration of mIL-18 significantly inhibited the number and the total area of osteolytic bone metastasis by the RWGT2 human lung cancer cells in nude mice. Thus, IL-18 may be useful clinically for suppression of osteolytic bone metastasis in patients with advanced breast cancers, however, the problems of unwanted systemic effects of IL-18 still remain to be overcome (Iwasaki et al, 2002).

Serum IL-18 levels were found to be significantly increased in breast cancer patients (n=56; p<0.05) when compared with controls (n=14). This change was found to be associated with established clinically-used prognostic factors such as tumour size, axillary lymph node involvement and disease stage (Gunel et al, 2003). A second study from this group also showed that IL-18 levels were significantly higher in patients with metastatic spread compared with those without (38 metastatic vs. 26 non metastatic patients; p<0.001). Despite the relatively small cohort size, the findings of these studies suggest that serum IL-18 levels may be useful as a prognostic marker in monitoring breast cancer patients after treatment and/or in tumour surveillance to detect bony metastasis (Gunel et al, 2002). The results from the human studies appear to contradict the data from the murine models and therefore further work is required to clarify the role of this cytokine in carcinogenesis.

Interleukin 24 (IL-24) IL-24 exhibits homology to IL-10 and is found within the IL-10 family gene cluster. It is secreted by activated monocytes and T cells and is generally considered to have tumour suppressive activity. IL-24 was initially described as melanoma differentiation-associated gene 7 (mda-7) (Caudell et al, 2002).

Overexpression of IL-24 is growth inhibitory for a range of human tumour cells. Su *et al*, using a nude mouse model, showed that breast tumour cells transfected with IL-24 underwent apoptosis, whereas the same cell line established tumour masses when IL-24 was given exogenously (Su et al, 1998). It appears that IL-24 mediates its anti-tumour effects in a p53-independent manner. The endogenous mode of action of IL-24 has been shown *in vivo* through infection via adenoviral vectors. McKenzie and colleagues evaluated the impact of combination therapy of a recombinant adenovirus vector encoding for human IL-24 and Herceptin on Her-2/neu-overexpressing breast cancer cell line MCF-7-Her-18 *in vitro* and in a nude mouse model *in vivo*. The combination therapy resulted in growth inhibition *in vitro* and a marked reduction in tumour size *in vivo* (McKenzie et al, 2004). IL-24 has generated considerable interest as a novel tool for cancer gene therapy and is currently undergoing phase II clinical trials to determine its efficacy in patients.

Cytokine	Reported changes in breast cancer patients	References
TGF- β	Raised levels in patients with advanced disease (n=44; p<0.05)	Ivanovic V, Todorovic-Rakovic N <i>et al</i> , 2003
IL-2	Decreased levels associated with relapse	Arduino S, Tessarolo M <i>et al</i> , 1996
IL-6	Raised levels in patients with metastases (n=73; p<0.0001). Levels correlate to survival and response to therapy (n=87; p<0.001)	Benoy I, Salgado R <i>et al</i> , 2002; Yokoe T, Iino Y <i>et al</i> , 2000; Bachelot T, Ray-Coquard I <i>et al</i> , 2003
IL-8	Levels increased in advanced disease and increase with disease progression	Zakrzewska I, Kozlowski L <i>et al</i> , 2002
IL-18	Raised levels in breast cancer patients (n=56; p<0.05). Levels higher in patients with metastases (n=38; p<0.001)	Gunel N, Coskun U <i>et al</i> , 2002, 2003

Table 1.53.1 Summary of cytokines with potential role as prognostic markers in breast cancer.

Cytokine	Proposed mode of action	References
TNF- α	Enhances anti-tumour effect of DC-based vaccines. Inhibits growth and promotes apoptosis of tumour cells in breast cancer cell lines <i>in vitro</i> .	Candido KA, Shimizu K <i>et al</i> , 2001; Manna PP and Mohanakumar T, 2002
IFN- γ	Enhances LAK activity.	Kamamura Y, Takahashi K <i>et al</i> , 1998
IL-2	Enhances tumour infiltration by CTL. When used with Trastuzumab in phase I trials, enhances ADCC and NK cell lysis of breast cancer cells.	Lee J, Fenton BM <i>et al</i> , 1998; Repka T, Chiorean EG <i>et al</i> , 2003
IL-10	Inhibits tumour growth in murine models of breast cancer by elaboration of chemokines, inhibition of angiogenesis and stimulation of NO production. Has potent anti-metastatic activity by inducing NK lysis of tumour cells.	Dorsey R, Kundu N <i>et al</i> , 2002; Kundu N and Fulton AM, 1997; Sun H, Gutierrez P <i>et al</i> , 2000
IL-12	Demonstrated to induce tumour destruction in murine models of breast cancer by inhibiting angiogenesis and activating CTL.	Cavallo F, Di Carlo E <i>et al</i> , 1999; Sabel MS, Skitzki J <i>et al</i> , 2004
IL-18	Facilitates tumour rejection by augmenting cytotoxic effects of NK cells and T cells and inhibiting angiogenesis. Enhances anti-tumour effect of DC-based vaccines.	Coughlin CM, Salhany KE <i>et al</i> , 1998; Ju DW, Tao Q <i>et al</i> , 2001; Nakata A, Tsujimura T <i>et al</i> , 1999
IL-24	Induces selective apoptosis of tumour cells in murine model of breast cancer while sparing normal mammary epithelial cells.	Su ZZ, Madireddi MT <i>et al</i> , 1998; McKenzie T, Liu Y <i>et al</i> , 2004

Table 1.53.2. Summary of cytokines with potential role in immune therapy of breast cancer.

1.6 Aims

1.6.1 Background

Cytokines are known to have a plethora of diverse functions including lymphocyte recruitment and activation, cell proliferation and stimulation of antibody dependent cellular cytotoxicity. It appears that IL-12 and IL-10, which are key representatives of the Th1 and Th2 cytokine profile respectively, are two of the most important cytokines in determining the anti-tumour response.

It appears that IL-10 and IL-12 generation is regulated by a complex monocyte/macrophage, T cell and tumour cell derived cytokine network which remains poorly understood. The only previous study looking specifically at the levels of IL-10 and IL-12 in breast cancer identified a group of patients who displayed a defective IL-12 production capability while generating higher amounts of IL-10 (Merendino et al, 1999). Although supportive of the hypothesis that the balance of IL-10 and IL-12 is of great importance, this study was based on only 15 patients with early stage (Stage I & II) disease.

Data from various studies reported in literature suggest that there is an impaired immune anti-tumour response in cancer patients and one of the manifestations of this impairment is an imbalance of the Th1/Th2 cytokine profile. Measurement of levels of these cytokines may also be utilized to monitor disease progression and hence as prognostic markers. Here, it is hypothesised that the tumour in patients with breast cancer is causing some degree of immune impairment manifested by the skewing of the cytokine profile in favour of Th2 cytokines as against the Th1 cytokine profile. Hence, it was decided to investigate whether the changes in IL-10 and IL-12 levels reflective of Th1 or Th2 bias occur in breast cancer patients.

1.6.2 Aims of the study

- i) To determine the serum IL-10 and IL-12 levels and IL-12 production by peripheral blood mononuclear cells (PBMC) in a cohort of breast cancer patients and to investigate the relationship of these cytokine levels with parameters such as tumour size, grade, pathological type (*in situ* versus invasive), lymph node involvement and oestrogen receptor status

- ii) To determine the effect of surgery and post-operative adjuvant therapy (radiotherapy and chemotherapy) on the serum levels of IL-10 and IL-12 and IL-12 production by PBMC.

- iii) To determine the profile of IL-10 and IL-12 expression in breast tumour specimens using immunohistochemistry and to correlate this with serum levels, IL-12 production capability of PBMC and clinico pathological factors detailed in (i).

2. Materials and methods

2.1 Study design

The project was a prospective study involving patients diagnosed with primary breast cancer referred to the breast care unit, Castle Hill Hospital, Cottingham, East Yorkshire. This breast care unit is a tertiary referral centre for the Yorkshire region with an annual intake of 250-300 new breast cancer patients. It was proposed to recruit at least 100 newly diagnosed primary breast cancer patients and at least 30 age matched healthy volunteers to act as controls.

Ethical approval for this study was granted by the Hull and East Riding Local Research Ethics Committee (Ref No. LREC/10/03/213).

2.2 Study population

It was proposed to include eligible patients diagnosed with primary breast cancer at the breast care unit at Castle Hill Hospital under the following criteria:

Inclusion criteria:

- All patients with confirmed diagnosis (with pre-operative core biopsy) of primary breast cancer
- All patients who agree to participate in the study and give written consent for the same.

Exclusion criteria:

- Patients with recurrent disease
- Patients with coexisting infectious disease
- Patients with autoimmune disease
- Patients with other coexisting neoplastic disease
- Patients on immunosuppressive therapy.

Patients in the above mentioned groups in the exclusion criteria were excluded from the study to negate the influence if any, of the above mentioned disorders on the inflammatory response and hence the cytokine levels. Patients with *in situ* carcinoma were also included in the study as it was thought that it might be interesting to compare this group (considered to have pre-invasive disease) with patients having invasive carcinoma.

A cohort of healthy women of similar age with no known malignancy was recruited to act as control subjects.

2.3 Methodology

All eligible patients with confirmed diagnosis of primary breast cancer were seen at the pre-assessment clinic one week before surgery. They were given the patient information sheet which detailed the background and the outline of the study. If they agreed to be included in the study, written informed consent was obtained. Relevant clinical details were recorded in the clinical record form in an anonymised manner. Letters were sent to the patient's general practitioner informing them about the patient's participation in the study.

Healthy volunteers were also given an information sheet. Once they agreed to participate in the study, written informed consent was obtained.

After obtaining the consent, venous blood (approximately 25 ml) was obtained by venepuncture from a peripheral vein. About 15 ml was collected into heparinised tubes (x 3; BD Vacutainer™ containing 102 IU of lithium heparin, BD Biosciences, Plymouth, UK) for separation of PBMC and 10 ml was collected into gel tubes (x 2) for separation of serum. After collection, the blood samples were transported to the laboratory for further processing and analysis.

Further blood samples were collected after surgery in the clinic at follow up. This was usually at 10-14 days after surgery in an attempt to negate any non-specific influence of surgery on the cytokine levels. If the patients received post operative adjuvant therapy, then a further blood sample was taken 4-6 weeks after completion of the adjuvant treatment. Thus the number of blood samples was either 2 (if patient had only surgery and no adjuvant treatment) or 3 (in patients who had surgery followed by adjuvant treatment). Only a single blood sample was collected from healthy volunteers.

Histopathology slides of patients were cut from the diagnostic tumour specimens by colleagues in the Department of Pathology for immunohistochemical staining to

determine the level of the cytokines in a semi-quantitative manner. Analysis of slides after staining was undertaken by Dr. Read and Dr. Long, consultant histopathologists.

2.4 Separation of serum

About 10 ml of blood collected into two gel tubes (BD Vacutainer[®], BD Biosciences, Plymouth, UK) was centrifuged at 750 g for 10 minutes. The separated serum was collected and stored in 1ml aliquots in 1.5ml polypropylene tubes (Sarstedt, Leicester, UK) at -80°C until analysis. Pre-operative samples were labelled as the serial number followed by A, post-operative samples were labelled as B and post adjuvant samples were labelled as C. Freeze-thawing of samples was avoided.

2.5 Preparation of peripheral blood mononuclear cells

This was done in a sterile setting in a class II cabinet (sterile environment maintained by Ultra violet light). Heparinised blood (15ml) was diluted 1:1 with phosphate buffer saline (PBS). Then the peripheral blood mononuclear cells were isolated by the Ficoll-Hypaque density gradient purification technique (Blanchard and Djeu, 1988) as follows:

- 15 ml of Histopaque (Histopaque-1077; Sigma-Aldrich Co. Ltd., Irvine, United Kingdom) was aliquotted into 2 tubes.
- 15 ml of heparinised blood diluted 1:1 with PBS was gently layered on top of the histopaque in each tube.
- The tubes were centrifuged at 400 g for 30 minutes (no brake). At the end of this step, the blood-PBS mixture is separated into 4 distinct layers (from top to bottom: buffer, buffy coat, histopaque, red blood cells, Fig 2.5).
- The middle layer (buffy coat) was extracted into a tube containing an equal volume of PBS.
- This was again centrifuged at 400 g for 10 minutes (brake on) to remove traces of histopaque.
- The supernatant was discarded and the pellets were resuspended in 10 ml of PBS.
- This was again centrifuged at 400 g for 10 minutes (brake on).

- The samples were labelled in a similar fashion as the serum samples and stored at - 80°C for 24 hours and subsequently stored in liquid nitrogen until use.

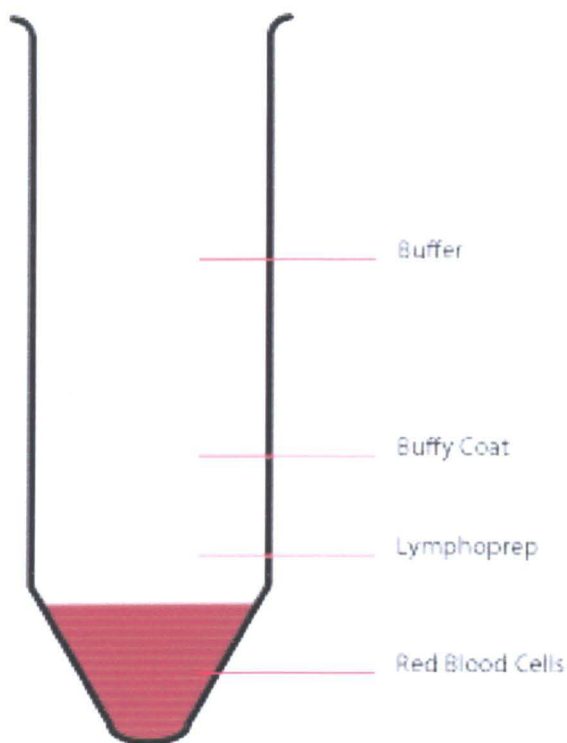


Fig 2.5 Various layers after centrifugation of heparinised blood diluted 1:1 with PBS

2.6 Cell culture and stimulation

In order to determine the IL-12 production capacity of PBMC, cell culture and stimulation was done as follows:

- After retrieving the samples from liquid nitrogen, the PBMC suspended in FCS+DMSO was thawed gradually to room temperature in a sterile class II cabinet.
- The pellets were resuspended in 10 ml of media [RPMI 1640(Life Technologies Ltd, Paisley, UK) supplemented with 1% v/v fetal calf serum, penicillin (100units/ml), streptomycin (100µg/ml) and glutamine (2mM)].
- This suspension was centrifuged at 400 g for 3 minutes (brake on).
- The pellets were resuspended in 1 ml of media.
- 10µl of this cell suspension was mixed with 10µl of 0.2% w/v Trypan Blue in a 0.5 ml polypropylene tube. This stain helps in identifying non viable cells

- The pellets were resuspended in 1 ml of media.
- 10 μ l of this cell suspension was mixed with 10 μ l of 0.2% w/v Trypan Blue in a 0.5 ml polypropylene tube. This stain helps in identifying non viable cells (stained blue) from viable cells (colourless and refractile) and makes cell counting easy.
- 10 μ l of this suspension was instilled into the haemocytometer (Improved Neubauer) on each side and a count of viable cells was done in the square area defined (0.1mm² under x40 magnification). An average of the two counts was taken.
- The concentration of cell suspension was calculated to give a final concentration of 1 x 10⁶ cells/ml. (No. of cells x 2 x 10⁴ = no. of cells/ml; No. of ml of cell suspension = no. of cells/1 x 10⁶ cells).
- Stimulation of IL-12 production was performed with fixed SAC [Staphylococcus aureus Cowens Strain I (Pansorbin, Calbiochem-Novabiochem Corporation, La Jolla, CA), 0.0075% w/v added to the cells in a 24 well tissue culture plate and incubated for 24 hours at 37°C (D'Andrea et al, 1992) . At least 2 wells in each experiment had cell suspensions which were not stimulated and thus act as negative controls.
- After 24 hours of incubation, the supernatants were collected taking care not to disturb the cells stuck to the bottom of the wells into 1.5 ml polypropylene tubes.
- These were centrifuged at full speed (13000 g) for 5 minutes in a micro centrifuge.
- The supernatants were collected and stored in 1.5ml polypropylene tubes at - 80°C until analysis.

2.7 Measurement of IL-10 & IL-12 levels

IL-10 levels in serum were measured in duplicate with a solid phase sandwich enzyme linked immuno sorbent assay (ELISA; Human IL-10 Ultrasensitive; Biosource International Inc, USA). IL-12 levels in serum and cell culture supernatant after stimulation were measured in duplicate with a solid phase sandwich enzyme linked immuno sorbent assay (ELISA; Human IL-12 Ultrasensitive; Biosource International Inc, USA). In a few samples, IL-12 levels in cell culture supernatants were measured

using the Quantikine HS IL-12 ELISA kit (Quantikine HS Human IL-12 Immunoassay, R&D Systems Inc, USA). Unlike the biosource kit, this measures only the biologically active heterodimer (p70) and excludes the β -chain (p40 subunit). This was done due to persistent lack of difference in IL-12 levels in supernatants obtained from stimulated wells and unstimulated wells (negative controls).

All reagents were thawed to room temperature before use. 12 x 8 well strips were used in each assay. The standard diluent buffer (100 μ l) was added to the zero wells. The standards (100 μ l) were added to the appropriate microtitre wells. Then the standard diluent buffer (50 μ l) was added to each well followed by 50 μ l of serum sample. For IL-12 estimation in PBMC supernatants, 100 μ l of sample was added to each well.

Various steps in IL-10 ELISA (Fig 2.7.1) are:

- After addition of the standards, serum sample and the standard diluent buffer into the appropriate microtitre wells, the wells are incubated at 37°C for 2 hours. A monoclonal antibody specific for human IL-10 has been pre-coated onto the microtitre wells and this binds to any IL-10 antigen present in the sample on a specific site. The standards used are in the range of 0 to 50pg/ml.
- Solution from the wells is aspirated and the wells are washed 4 times with the freshly diluted wash buffer.
- 100 μ l of biotinylated anti-IL-10 (Biotin conjugate) solution is added into each well and incubated for 1 hour at room temperature. This biotinylated monoclonal antibody is specific for human IL-10 and this binds to the immobilised human IL-10 captured during the first incubation.
- Solution from the wells is aspirated and the wells are washed 4 times with the wash buffer to remove the excess second antibody.
- 100 μ l of Streptavidin-HRP Working Solution is added to each well and incubated for 30 minutes at room temperature. Streptavidin-Peroxidase is an enzyme which binds to the biotinylated antibody to complete the four member sandwich.
- Solution from the wells is aspirated and the wells are washed 4 times with the wash buffer to remove all the unbound enzyme.
- 100 μ l of stabilized chromogen is added to each well and incubated for 30 minutes at room temperature. The liquid in the wells begin to turn blue as the

- The absorbance of each well is read at 450 nm and the standard curve is plotted. Values obtained for the samples are multiplied by 2 to correct for the 1:2 dilution.

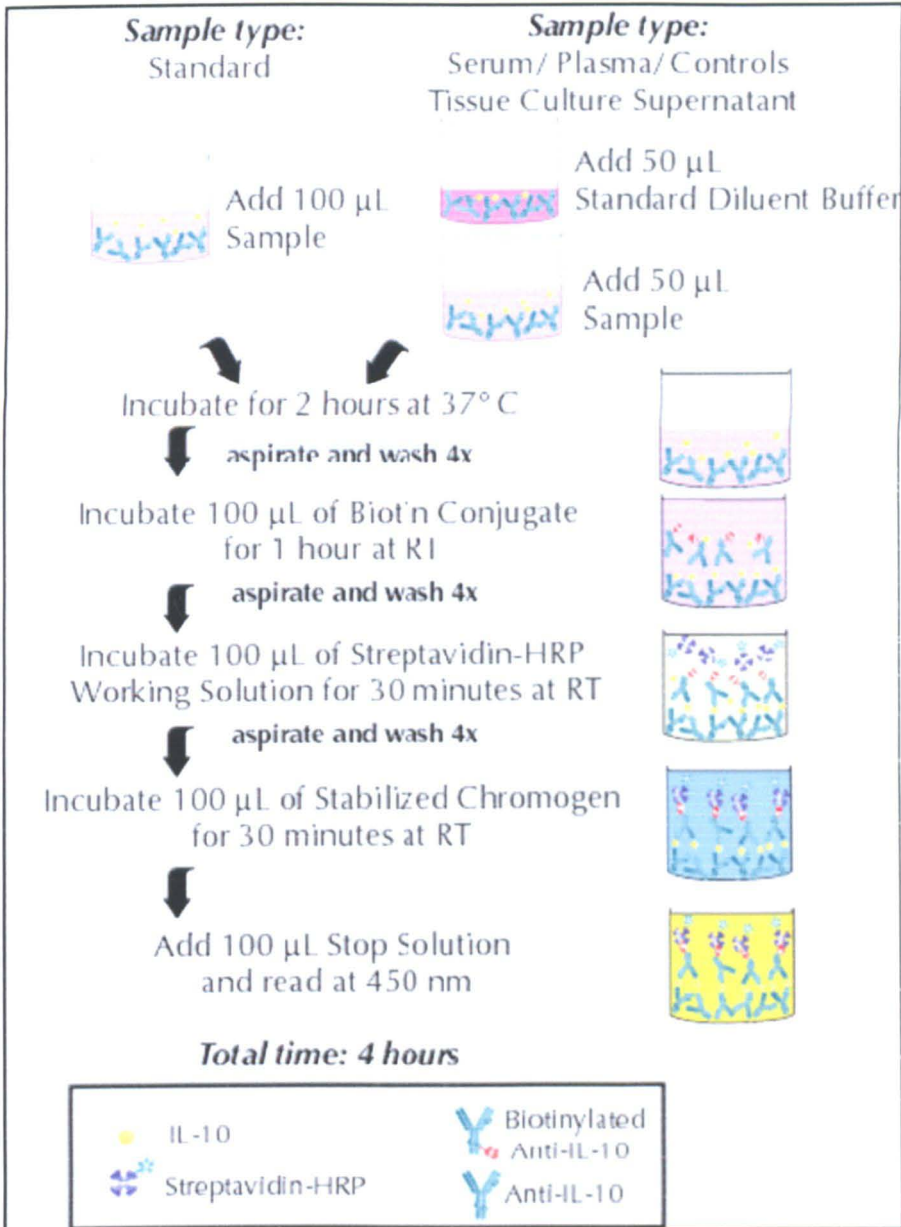


Fig 2.7.1 Human IL-10 US Assay summary.

(www.biossource.com/content/catalogContent/tds3/moreinfo/KHC0104.pdf)

Various steps in IL-12 ELISA (Fig 2.7.2) are:

- After addition of the standards, serum samples or PBMC supernatant samples and the standard diluent buffer into the appropriate microtitre wells, the wells

Various steps in IL-12 ELISA (Fig 2.7.2) are:

- After addition of the standards, serum samples or PBMC supernatant samples and the standard diluent buffer into the appropriate microtitre wells, the wells are incubated for 3 hours at room temperature. A monoclonal antibody specific for human IL-12 has been pre coated onto the microtitre wells and this binds to any IL-12 antigen present in the sample on a specific site. The standards used are in the range of 0 to 100pg/ml (Biosource kit) and 0 to 40pg/ml (R&D kit).
- Solution from the wells is aspirated and the wells are washed 6 times with the freshly diluted wash buffer.
- 100 µl of biotinylated anti-IL-12 (Biotin conjugate) solution is added into each well and incubated for 45 minutes at room temperature (200 µl of IL-12 conjugate; 2 hours of incubation for the R&D kit). This biotinylated monoclonal antibody is specific for human IL-12 and this binds to the immobilised human IL-12 captured during the first incubation.
- Solution from the wells is aspirated and the wells are washed 6 times with the wash buffer to remove the excess second antibody.
- 100 µl of Streptavidin-HRP Working Solution is added to each well and incubated for 30 minutes at room temperature (50 µl of substrate solution; 1 hour of incubation for the R&D kit). Streptavidin-Peroxidase is an enzyme which binds to the biotinylated antibody to complete the four member sandwich.
- Solution from the wells is aspirated and the wells are washed 6 times with the wash buffer to remove all the unbound enzyme (This step is omitted in the case of the R&D kit).
- 100 µl of stabilized chromogen is added to each well and incubated for 25 minutes at room temperature (50µl of amplifier solution; 30 minutes of incubation for the R&D kit). The liquid in the wells begin to turn blue as the chromogen is acted upon by the bound enzyme. The intensity of the colour change is directly proportional to the amount of conjugated secondary antibody that is dependent on the concentration of human IL-12 present in the sample.
- 100 µl of stop solution (1N Sulphuric acid aqueous) is added to each well and this changes the colour of the solution in the wells from blue to yellow (50µl of stop solution in the case of the R&D kit).

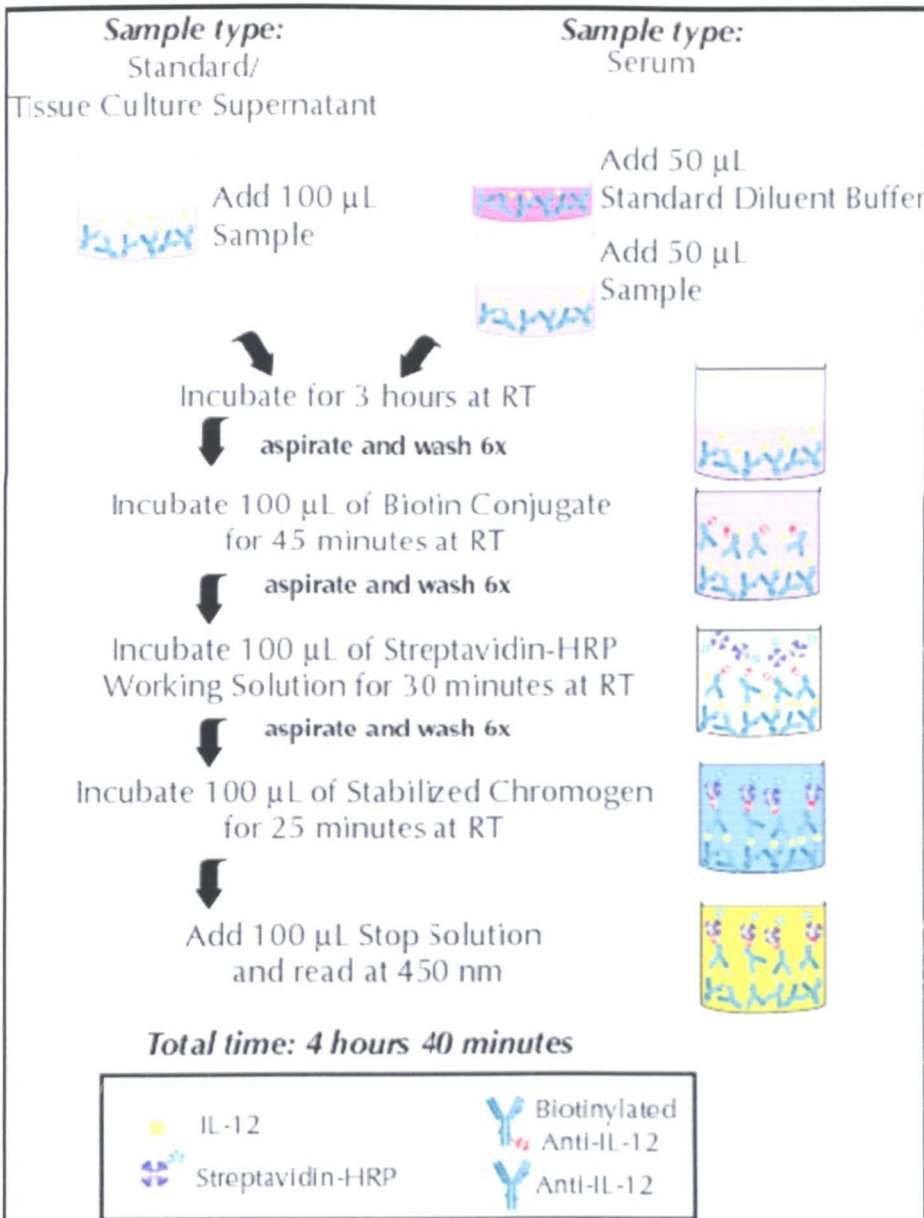


Fig 2.7.2 Human IL-12 US Assay summary

(www.biossource.com/content/catalogContent/tds3/moreinfo/KHC0123.pdf)

2.8 Immunohistochemistry

Immunocytochemical staining of deparaffinized fixed slides was performed after antigen retrieval by boiling in pressure cooker with vector antigen unmasking solution of halogen power 10 for 3 minutes (1500ml distilled water with 15ml of vector antigen unmasking solution). The slides were incubated with avidin solution (for 15 minutes to block the endogenous biotin) followed by biotin solution (for 15 minutes to block the endogenous avidin; Vectastain ABC Kit; Vector Laboratories, Berlingame, CA, USA). The antibody was added to the samples and the positive controls and casein to negative

of halogen power 10 for 3 minutes (1500ml distilled water with 15ml of vector antigen unmasking solution). The slides were incubated with avidin solution (for 15 minutes to block the endogenous biotin) followed by biotin solution (for 15 minutes to block the endogenous avidin; Vectastain ABC Kit; Vector Laboratories, Berlingame, CA, USA). The antibody was added to the samples and the positive controls and casein to negative controls and the slides were incubated for 2 hours at room temperature (Mouse anti human IL-10; Serotec Ltd., United Kingdom; MCA926 and monoclonal anti human IL-12 antibody; (R&D systems, Inc; MAB219). In order to determine the optimum antibody concentration, repeated runs of immunohistochemical staining using both tumour specimens and tonsil slides as controls with differing concentrations of antibody (IL-10: 1,5,10 & 20 µg/ml; IL-12: 5,10,20 &25 µg/ml) was done. The best results were obtained when IL-10 antibody was used at a concentration of 20µg/ml and the IL-12 antibody was used at a concentration of 10µg/ml and hence this was adopted as the test concentration. The peroxidase was developed by DAB (3,3'- Diamino benzidine tetra hydrochloride 97%; Sigma Aldrich Inc, Germany) and counter stained with Harris haematoxylin. The positive control antibody was cytokeratin (1:50). Tonsil slides were used as controls as IL-10 and IL-12 have been detected by immunohistochemistry in paraffin embedded human tonsil tissue sections using these antibodies. After the staining, the slides were reviewed independently by two histopathologists in a blinded manner to assess the intensity of staining for IL-10 & IL-12 in the tumour.

Due to paucity of staining for IL-12, antigen retrieval was also attempted by a different technique utilising microwave heating with citrate buffer (Poindexter et al, 2004). In this technique, the antigen retrieval was done by boiling the deparaffinized slides in a microwave oven for 20 minutes in high setting (650 ml citric acid 10Mm in water set to pH 6 with 2M sodium hydroxide). Various concentrations of IL-12 antibody were tried out to get the optimum immunohistochemical staining for IL-12.

2.9 Statistical methods

Statistical analysis was done using SPSS[®] version 13 (SPSS, Chicago, Illinois, USA). A power calculation to determine sample size was not plausible due to the fact that this was the first study of its kind to be undertaken. Hence what constituted a significant difference in IL-10 and IL-12 levels (between patients and healthy controls) was not

known in order to facilitate a power calculation. Cytokine distributions were tested for normality. If normality was found to be invalid, data was analysed by non parametric methods (Wilcoxon signed rank test for paired samples and Mann-Whitney or Kruskal-Wallis test for independent samples). Otherwise parametric methods such as T tests or one way analysis of variance were used. For assessing the impact of pathological factors such as grade, size, lymph node involvement and oestrogen receptor status on the cytokine levels, univariate followed by multivariate analysis was used. Results were considered to be statistically significant when P was <0.05 . The statistical analysis was validated by Dr. Eric Gardiner, statistics consultant, University of Hull.

3. Results

3.1 Overview

One hundred and four patients with primary breast cancer were recruited between 01/01/2004 to 28/02/2005. The median age of this cohort of breast cancer patients was 58 years (range: 31-81) with 88 patients being post menopausal. Approximately a third of these patients (31) had a family history of breast cancer and 39 patients were on HRT (Median 72 months; range 1-300). Thirty one healthy female volunteers were recruited as controls.

The majority of the patients (58) presented with symptomatic breast lumps with 2 patients having bilateral disease and the remainder were diagnosed at routine mammograms as part of the national breast screening programme. Three patients had neo adjuvant therapy; 1 was commenced on hormone therapy, 1 had neo adjuvant chemotherapy and 1 patient received hormone therapy and radiotherapy. One hundred and one patients had surgery as the primary modality of treatment with 54 patients having breast conserving surgery (wide local excision with or without axillary surgery (Fig 3.1.).

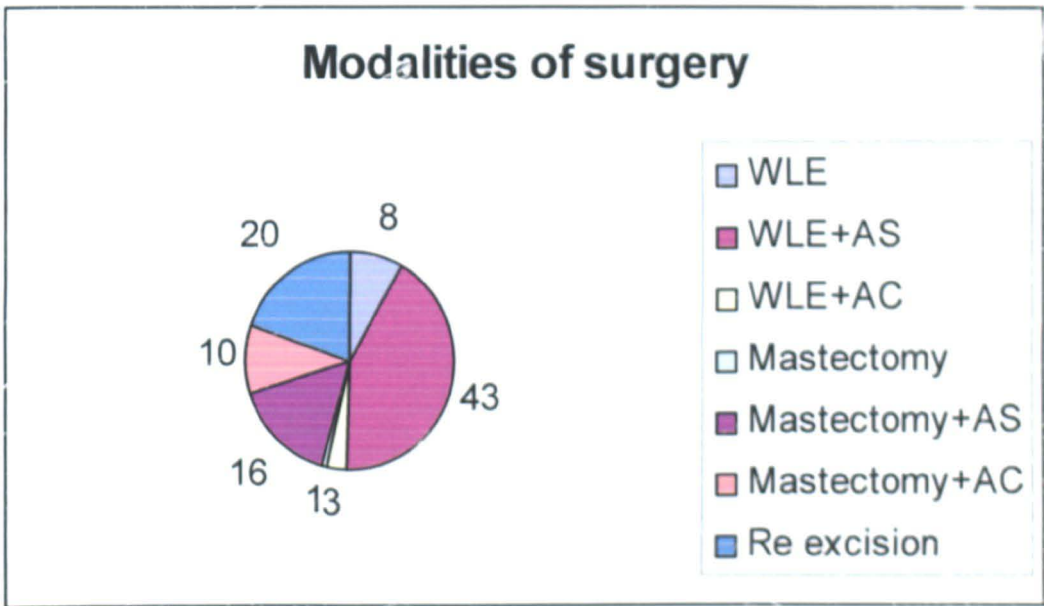


Fig 3.1 Modalities of surgical treatment. WLE: Wide local excision; WLE+AS: Wide local excision and axillary sampling; WLE+AC: Wide local excision and axillary clearance; AS : axillary sampling; AC : axillary clearance

Histopathology reports indicated that 11 patients had *in situ* disease, 34 patients had invasive disease and 58 patients had invasive and *in situ* disease. The pathological characteristics of the tumour in the study cohort in terms of tumour size, grade, lymph node involvement and oestrogen receptor status are summarised in Table 3.1. The discrepancy in the patient numbers in various subgroups is due to different surgical modalities of treatment and is based on the final histopathology report. In total, 93 patients received adjuvant therapy in the form of radiotherapy or chemotherapy or both and endocrine treatment depending on the oestrogen receptor status.

Parameter	No. of patients
Pathological type	
<i>In situ</i>	11
Invasive	34
<i>In situ</i> + Invasive	58
Tumour size	
<20 mm	52
20 – 50 mm	37
>50 mm	1
Tumour grade	
Grade 1	14
Grade 2	38
Grade 3	36
Lymph node status	
Positive	20
Negative	68
Oestrogen receptor status	
Positive	68
Negative	21

Table 3.1 Pathological characteristics of the tumour in the study cohort.

3.2 Serum levels

Serum levels of IL-10 and IL-12 were measured by ELISA as described in Section 2.7. The patients were split into two groups: those with invasive disease and those with *in situ* disease. Due to paucity in patient numbers in the *in situ* disease group, statistical analysis of this group of patients was not done. The results were:

Pre-op levels: The serum levels of IL-10 and IL-12 were measured in 91 patients with invasive disease and 11 patients with *in situ* disease and 31 healthy controls. Serum samples could not be obtained from 2 patients due to difficulty in venesection.

Pre-op IL-10 levels in breast cancer patients: IL-10 levels in a substantial number of patients were below the sensitivity of the ELISA kit. Since the levels of IL-10 obtained on ELISA were essentially comparative rather than absolute values, the negative values were deemed 0 pg/ml for the purpose of statistical analysis. The minimum detectable level of human IL-10 by the Biosource kit is <0.2 pg/ml. Hence for statistical analysis, the patient groups were sub-divided into two sub groups: those with readings greater than 0 and those with readings considered 0. Recordable levels of IL-10 were detected in 61.5% of patients with invasive disease (56/91), 27.3% of patients with *in situ* disease (3/11) and 45.2% of healthy controls (14/31). The levels of IL-10 expressed in terms of median (IQR) were: 1.32 pg/ml (1.02-1.83) in patients with invasive disease, 1.83 pg/ml in patients with *in situ* disease and 1.45 pg/ml (0.93-3.08) in healthy controls.

This grouping was tested for association with the disease status of patients and controls by a chi square test. The result was of borderline significance (chi square=6.198; df=2; $P=0.045$) with the invasive group most likely to record a positive value. The second stage of the analysis was to compare the levels of IL-10 between the patient groups and controls when positive readings were recorded. This was done by Kruskal-Wallis test. This gave (chi square approximation) to Kruskal-Wallis statistic =0.087; df=2 and $P=0.957$. No significant difference in IL-10 levels between screening (n=46) and symptomatic patients (n=58) was detectable ($P=0.700$). Hence there appears to be no evidence of higher IL-10 levels in one group than in another assuming a positive value has been recorded.

Pre-op IL-12 levels in breast cancer patients: Only one patient had IL-12 levels below the sensitivity of the ELISA kit (negative values were deemed 0pg/ml). The minimum detectable level of human IL-12 by the Biosource kit is <0.8 pg/ml. The levels of IL-12 expressed in terms of median (IQR) were: 44.48 pg/ml (25.98-73.40) in patients with invasive disease, 38.73 pg/ml (28.51-55.09) in patients with *in situ* disease and 52.9 pg/ml (29.12-91.06) in healthy controls. Hence comparison of the IL-12 levels between the patient groups and controls was done by Kruskal-Wallis test without needing to split the patient group as was done in the case of IL-10. This gave (chi square approximation) to Kruskal-Wallis statistic = 1.385; df =2 and $P=0.5$. As in the case of IL-10, no significant difference in IL-12 levels were noted between screening and symptomatic patients ($P=0.612$). Thus there appears to be no significant difference in levels of IL-12 between the patient groups and healthy controls.

The levels of IL-10 and IL-12 in patients with *in situ* disease and invasive disease and controls are summarised in table 3.2.1.

	IL-10 level (pg/ml) Median (IQR)	IL-12 level (pg/ml) Median (IQR)
Control (n=31)	1.45 (0.93-3.08)	52.9 (29.12-91.06)
<i>In situ</i> disease (n=11)	1.83	38.73 (28.51-55.09)
Invasive disease (n=91)	1.32 (1.02-1.83)	44.48 (25.98-73.4)

Table 3.2.1 Serum IL-10 & IL-12 levels in patients and controls

Correlation of key clinico-pathological factors such as size, grade, lymph node involvement and oestrogen receptor status with serum levels of IL-10 and IL-12 were assessed. Patients were subdivided on the basis of tumour size into three groups: 0-20mm, 21-50mm and 50mm and above; on the basis of grade into three groups: grade I, grade II and grade III, on the basis of lymph node involvement into two groups: positive

and negative and on the basis of oestrogen receptor status into two groups: positive and negative (in line with the TNM classification).

Correlation of IL-10 levels with known prognostic factors: Since a number of patients had undetectable levels of IL-10 (deemed 0 pg/ml), the analysis was split into two stages as above. In the first instance, a logistic regression of the two groups (those with readings = 0 and those with >0 pg/ml) was performed. The pathological factors listed above were entered into univariate logistic regression models and also a multivariate logistic regression model. None of these factors were found to be statistically significant in any of these models (Table 3.2.2). Hence there was no evidence of a correlation between these pathological factors and the likelihood of a positive reading for serum IL-10 levels.

Factors	No. of patients <i>n</i>	<i>P</i> value	
		Univariate	Multivariate
Size (0-20,21-50,>50)	49, 33, 1	0.215	0.298
Grade (grade I, II& III)	14, 35, 34	0.376	0.537
Lymph node involvement (Positive & Negative)	17, 66	0.45	0.566
Oestrogen receptor status (Positive & Negative)	65, 18	0.346	0.592

Table 3.2.2 Univariate and multivariate logistic regression analysis of pathological factors in relation to serum IL-10 levels (including 0 and positive readings)

In the second stage of the analysis, the subset of patients with positive readings of IL-10 was analysed. The influence of individual factors on the levels of IL-10 was analysed using univariate analysis. This was followed by analysis of the joint effects of these factors on the IL-10 levels using a multivariate analysis. None of these factors were found to be statistically significant in either univariate or multivariate analysis. However since the positive readings of IL-10 levels were skewed when tested for normal distribution, the analysis was repeated on log transformation of the positive IL-10 readings and this did not alter the findings (Table 3.2.3). Hence there was no evidence to suggest that there was any significant correlation between various pathological factors and serum IL-10 levels assuming a positive reading is recorded in the first instance.

Factors	No. of patients <i>n</i>	<i>P</i> value	
		Univariate	Multivariate
Size (0-20,21-50,>50)	28, 23, 1	0.107(0.223)	0.27(0.35)
Grade (grade I, II & III)	9, 19, 24	0.533(0.307)	0.537(0.212)
Lymph node involvement (Positive & Negative)	12, 40	0.2(0.178)	0.363(0.282)
Oestrogen receptor status (Positive & Negative)	39, 13	0.246(0.552)	0.417(0.683)

Table 3.2.3 Univariate and multivariate analysis of pathological factors in relation to serum IL-10 levels (including only the positive values). *P* value for the log transformed IL-10 values is given in parenthesis.

Correlation of IL-12 levels with known prognostic factors: Since there was no need of splitting the patient group on the basis of undetectable levels as in case of IL-10, the correlation between individual clinico-pathological factors with the serum levels of IL-

IL-12 was analysed using univariate analysis. This was followed by analysis of the joint effects of these factors on the IL-12 levels using a multivariate analysis. Apart from grade, none of the factors were close to being statistically significant in either univariate or multivariate analysis (Table 3.2.4). Hence there was no evidence to suggest that factors such as size, lymph node involvement and oestrogen receptor status had any effect on serum IL-12 levels in breast cancer patients.

The grade of the tumour was of borderline significance ($p=0.061$ in univariate analysis and $p=0.045$ in multivariate analysis) for IL-12 levels in breast cancer patients. The plot shows that mean levels of IL-12 decrease with increasing grade of the tumour (Fig 3.2). Hence there is some evidence to suggest a degree of correlation between grade and serum IL-12 levels.

Factors	No. of patients <i>n</i>	<i>P</i> value	
		Univariate	Multivariate
Size (0-20,21-50,>50)	49, 33, 1	0.734	0.399
Grade (grade I, II & III)	14, 35, 34	0.061	0.045
Lymph node involvement (Positive & Negative)	17, 66	0.424	0.453
Oestrogen receptor status (Positive & Negative)	65, 18	0.557	0.681

Table 3.2.4 Univariate and multivariate analysis of pathological factors in relation to serum IL-12 levels.

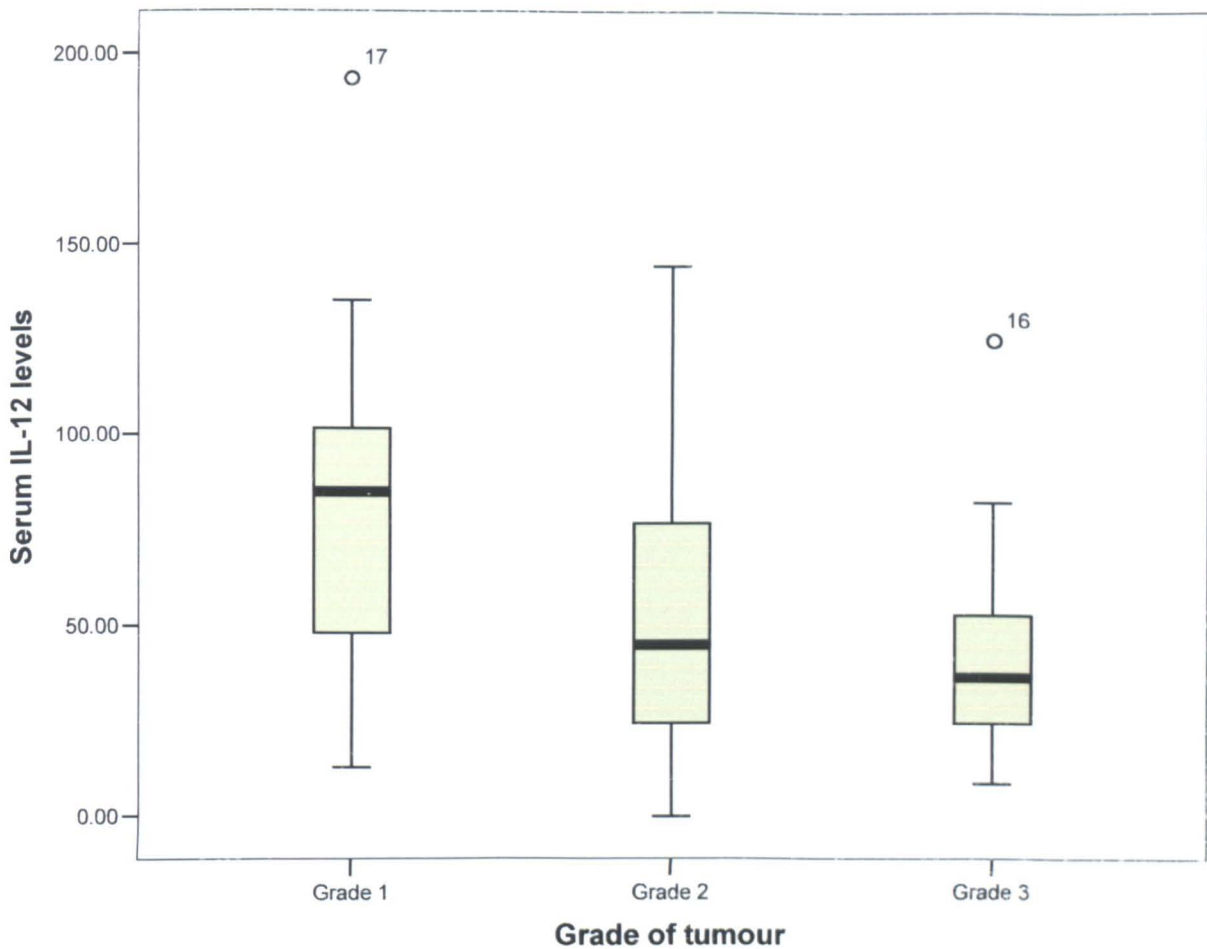


Fig 3.2 Box plot demonstrating decreasing serum levels of IL-12 in relation to grade of tumour

Comparison of pre-op with post-op levels: Levels of IL-10 and IL-12 were also measured in serum samples obtained post operatively before the commencement of adjuvant therapy and these were compared with pre-operative levels. The post-operative samples were collected at a median interval of 21 days after surgery (IQR: 13-30) in an effort to negate the effect of the inflammatory response to surgery, if any, on the levels of IL-10 and IL-12. Patients were again split into two groups: those with invasive disease (n=51) and those with *in situ* disease (n=6).

The median levels of serum IL-10 after surgery compared to pre-operative period were: 1.26 pg/ml compared to 1.13 pg/ml in the invasive group and 0.62 pg/ml compared to 0

compared with pre-operative period were: 62.25 pg/ml versus 50.45 pg/ml in the invasive group and 50.32 pg/ml versus 33.62 pg/ml in the *in situ* group (Table 3.2.5).

	IL-10 levels (pg/ml)		IL-12 levels (pg/ml)	
	Median (IQR)		Median (IQR)	
	Pre-op	Post-op	Pre-op	Post-op
<i>In situ</i> disease (n=6)	0 (0-0.72)	0.62 (0-0.91)	33.62 (16.73-48.76)	50.32 (18.96-93.64)
Invasive disease (n=51)	1.13 (0.55-1.61)	1.26 (0.70-1.86)	50.45 (26.71-81.99)	65.25 (40.02-89.12)

Table 3.2.5 Pre-op and post-op serum IL-10 & IL-12 levels in patients with *in situ* disease and invasive disease.

There was no statistically significant difference in pre- and post-op levels of IL-10 and IL-12 in patients with *in situ* disease. However, this observation should be tempered by the fact that the patient numbers in the *in situ* group are small (n=6) and a study with larger numbers is needed before definite conclusions can be made. In patients with invasive disease, although there was no difference in IL-10 levels, there was a significant rise in IL-12 levels after surgical excision of the tumour ($P = 0.001$; Wilcoxon signed ranks test). The change in IL-12 levels with time since surgery was analysed using a scatter plot and this showed that the IL-12 levels remain high even 20-70 days after surgery (Fig 3.2.2).

In order to compare the change in IL-10 and IL-12 levels after surgery between the invasive and non invasive groups, Mann-Whitney test on these changes were performed. No significant change in IL-10 ($P=0.958$) or IL-12 levels ($P=0.640$) were shown.

The post-operative blood samples could be collected only from 57 patients (51 invasive and 6 *in situ*). This was due to logistical problems of seeing these patients when they come for follow up and also due to patient refusal in some cases.

	IL-10 levels (pg/ml)		IL-12 levels (pg/ml)	
	Median (IQR)		Median (IQR)	
	Pre-op	Post-adjuvant	Pre-op	Post-adjuvant
<i>In situ</i> disease (n=5)	0 (0-0.95)	0	53.6 (21.6-78.87)	48.76 (28.85-66.33)
Invasive disease (n=17)	1.05 (0-1.52)	0.43 (0-1.50)	57.07 (31.94-76.87)	35.0 1(22.94-68.03)

Table 3.2.6 Pre-op and post-adjuvant levels of serum IL-10 & IL-12 in patients with *in situ* disease and invasive disease.

There was no significant change in serum levels of IL-10 and IL-12 after completion of adjuvant therapy in patients with *in situ* disease. Although there was no discernible change in IL-10 levels in patients with invasive disease, there was a trend towards a fall in IL-12 levels after adjuvant treatment in this group ($P=0.06$; Wilcoxon signed ranks test).

Serum samples could be collected only from 22 patients after completion of adjuvant therapy. Again, this was due to logistic problems, patient refusal and difficult venesection.

3.3 IL-12 production by peripheral blood mononuclear cells (PBMC)

PBMC were stimulated to produce IL-12 using fixed SAC. During the process of stimulation, a few wells containing PBMC were not subjected to SAC stimulation and were used as negative controls in the tissue culture plate to confirm that the SAC stimulation was effective and to demonstrate the difference in IL-12 production following the SAC stimulation. Initially the IL-12 level of the supernatants harvested from the cell cultures following stimulation and from the negative controls (unstimulated PBMC) was measured using the Biosource kit (h IL-12 US ELISA kit; Biosource). The Biosource kit measures both the free p40 sub unit and the biologically active heterodimer (p70). The IL-12 levels in the supernatants from the negative controls were found to be higher than those from the stimulated wells in samples from both the patients and healthy controls. Since no plausible explanation to this strange

phenomenon could be given, the experiment was repeated under supervision on a number of samples and also by another member of staff independently to rule out faulty technique. This again yielded the same results. The experiment was repeated using fresh reagents to rule out contamination and this resulted in little change to the outcome.

Due to the persistent lack of difference between stimulated and unstimulated samples, estimation of IL-12 in a few samples was assessed using the quantitative sandwich enzyme immunoassay technique (h IL-12 Quantikine HS ELISA kit; R&D Systems). This kit, unlike the Biosource kit, measures only the biologically active heterodimer (p70) and excludes the β -chain (p40 subunit). This kit has been used to determine IL-12 levels in supernatants following SAC stimulation of PBMC in previous studies. But again, the IL-12 levels in the supernatants from unstimulated wells were found to be higher than in the stimulated wells.

In order to rule out the possibility of cryopreservation of samples being the factor responsible for this discrepancy in IL-12 levels, the SAC stimulation was done on fresh samples. At this stage, since the recruitment of breast cancer patients was completed, the fixed SAC stimulation of PBMC was repeated on fresh samples immediately after PBMC separation from whole blood on a cohort of head and neck squamous cell carcinoma patients. The IL-12 levels in the supernatants from unstimulated wells were found to be in the same range as that in supernatants from stimulated wells.

A group from the University of Colorado discovered that lymphocyte proliferation assay responses of PBMC to various stimulants (cytomegalovirus, varicella-zoster virus, candida and tetanus toxoid antigens and phytohemagglutinin) were significantly high if performed on fresh samples compared with samples subjected to overnight transportation and cryopreservation (Weinberg et al, 1998). In the present study, PBMC after separation from whole blood were stored in liquid nitrogen at -160°C for varying periods of time ranging from 6-14 months before fixed SAC stimulation. Hence this period of storage may be a factor responsible for the unusual levels of IL-12 even in unstimulated negative controls. Since there was no opportunity to repeat the experiment on fresh samples from breast cancer patients, the findings obtained on the cryopreserved samples are reported in this study. Only patients with invasive disease were included in the study. The levels were:

Pre-op levels : IL-12 production by PBMC after fixed SAC stimulation was measured in 43 patients with invasive disease and 5 healthy controls using the Biosource kit and 30 patients with invasive disease and 5 healthy controls using the R&D kit .

The IL-12 levels detected by the Biosource kit and the R&D kit expressed in median (IQR) is illustrated in Table 3.3. The minimum detectable level of human IL-12 by the R&D kit is <0.5pg/ml.

	IL-12 production by PBMC (pg/ml)	
	Biosource kit	R&D kit
Control	1.04 (0.43-28.24) (n=5)	1.08(0.12-12.21) (n=5)
Invasive disease	5.86(0-33.45) (n=43)	0.7(0.24-2.53) (n=30)

Table 3.3 IL-12 production by PBMC in patients and controls estimated by Biosource and R&D kits.

There was no statistically significant difference in IL-12 production by PBMC detectable either with the Biosource kit or the R&D kit between patients with invasive disease and healthy controls ($P=0.668$; $P=0.962$; Mann-Whitney test).

Comparison of pre-op with post-op levels: IL-12 production by PBMC was measured in samples obtained post operatively before the commencement of adjuvant therapy and compared with pre-operative levels. The post-operative samples were collected at a median interval of 26 days after surgery (IQR: 21-35) in an effort to negate the effect of the inflammatory response to surgery, if any, on the PBMC production of IL-12. Due to paucity of numbers (n=33) and the fact that there appeared to be no difference between the Biosource kit and the R&D kit as demonstrated in the pre-op data, only the Biosource kit was used.

IL-12 production by PBMC after surgical removal of the tumour compared to pre-operative levels expressed in median (IQR) were: 6.16 pg/ml (2.41-19.43) compared to 5.86 pg/ml (0-41.68). This was not found to be statistically significant ($P=0.71$; Wilcoxon signed ranks test).

No comparison between pre-operative and post adjuvant IL-12 production by PBMC could be done due to lack of patient numbers and samples.

3.4 Expression of IL-10 and IL-12 in tumour tissue detected by immunohistochemistry

Immunohistochemical staining for IL-10 & IL-12 was done on paraffin sections of breast cancer specimens of 21 patients. Cytokeratin staining was used as a positive control and tonsil slides were also used apart from tumour sections. The slides were analysed by two histopathologists independently and assessed for intensity of staining of tumour cells on a scale of 1-3. This was then correlated to various pathological factors such as size, grade, lymph node involvement and oestrogen receptor status.

The tumour sections showed appreciable staining for IL-10. Cytokeratin staining was also positive for both tumour and tonsil sections. Of the 21 sections, 13 were reported to show mild staining for IL-10 (Scale 1), 6 were reported to show moderate staining for IL-10 (Scale 2) and 2 were reported to show strong staining for IL-10 (Scale 3). However, no correlation to known pathological factors was demonstrable, perhaps due to the small sample size (Table 3.4).

Scale	Size		Grade			Lymph node		Estrogen receptor	
	0-20	21-50	I	II	III	Positive	Negative	Positive	Negative
1 (mild)	4	9	2	6	5	4	8	11	2
2 (moderate)	3	3	1	3	2	2	5	3	3
3 (strong)	2	0	0	1	1	0	2	1	1
Total	9	12	3	10	8	6	15	15	6

Table 3.4 Intensity of staining for IL-10 compared to various pathological factors.

Unfortunately, the intensity of staining for IL-12 could not be reported with confidence and though it appeared that the staining was weak or negative, concerns were raised regarding the quality of staining. Different techniques of antigen retrieval as detailed in section 2.8 were employed with little improvement in intensity of staining. The staining was repeated with different concentrations of antibody and again, this did not make significant difference to the intensity of staining.

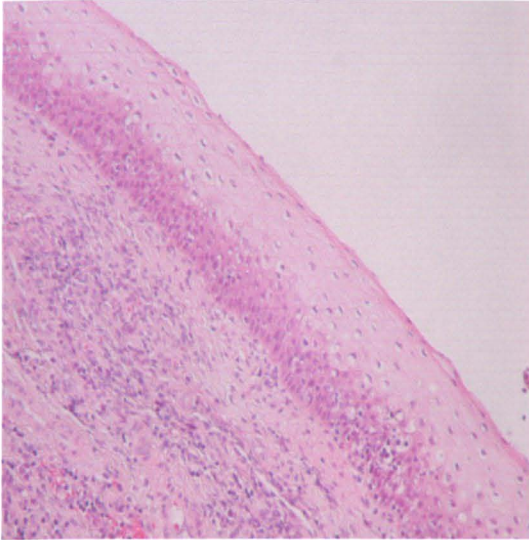


Fig 3.4.1 Histology slide showing section of tonsil (H&E) x10

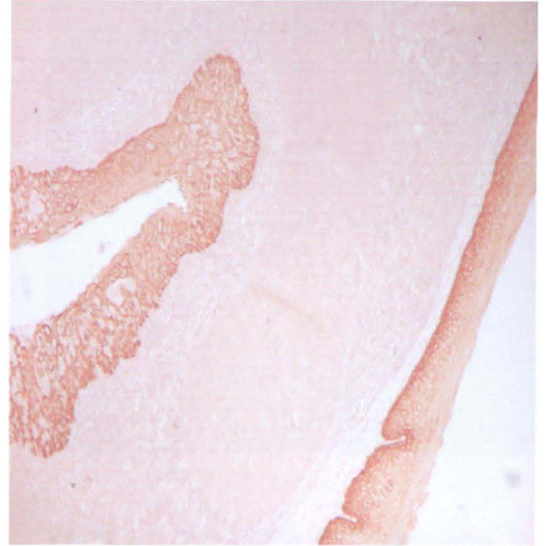


Fig 3.4.2 Histology slide showing section of tonsil (immunohistochemical staining with cytokeratin) x 10

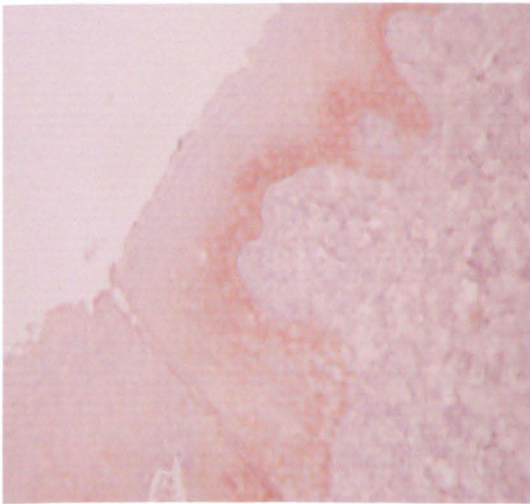


Fig 3.4.3 Histology slide showing section of tonsil (immunohistochemical staining with IL-10) x 10

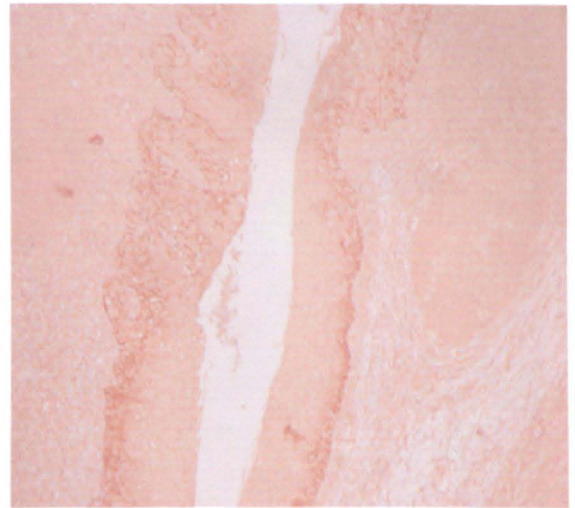


Fig 3.4.4 Histology slide showing section of tonsil (immunohistochemical staining with IL-12) x 10

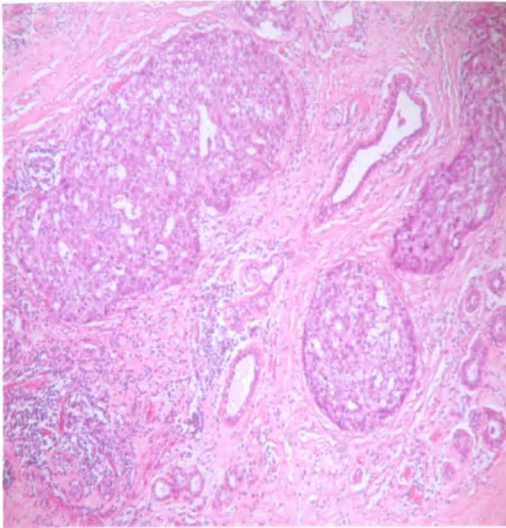


Fig 3.4.5 Histology slide showing section of ductal carcinoma *in situ* (H&E) x 10

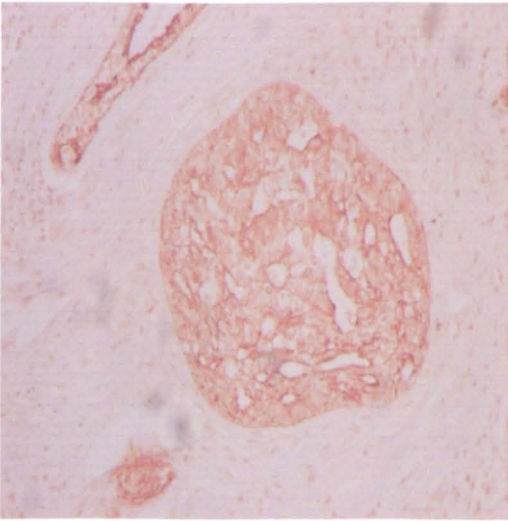


Fig 3.4.6 Histology slide showing section of ductal carcinoma *in situ* (immunohistochemical staining with cytokeratin) x 10

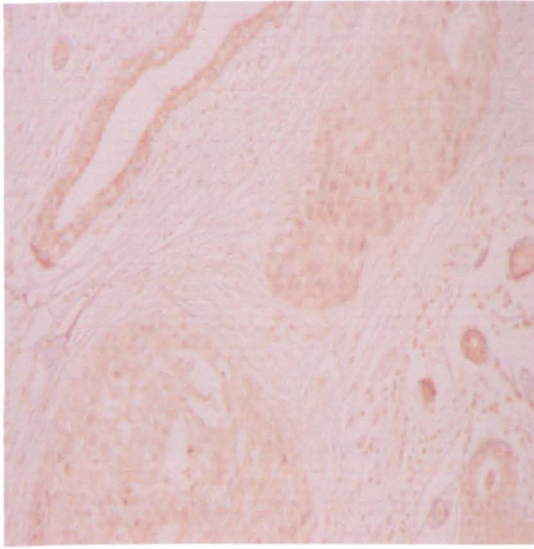


Fig 3.4.7 Histology slide showing section of ductal carcinoma *in situ* (immunohistochemical staining with IL-10) x 10

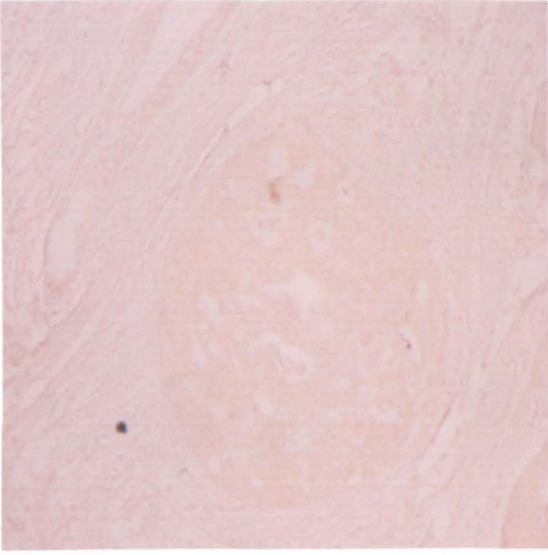


Fig 3.4.8 Histology slide showing section of ductal carcinoma *in situ* (immunohistochemical staining with IL-12) x 10

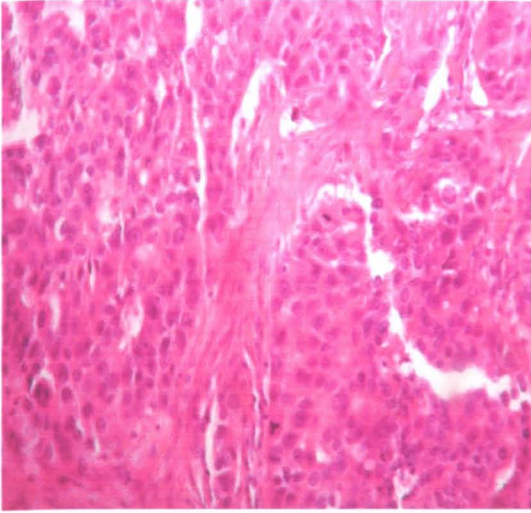


Fig 3.4.9 Histology slide showing section of invasive breast carcinoma (H&E) x 10

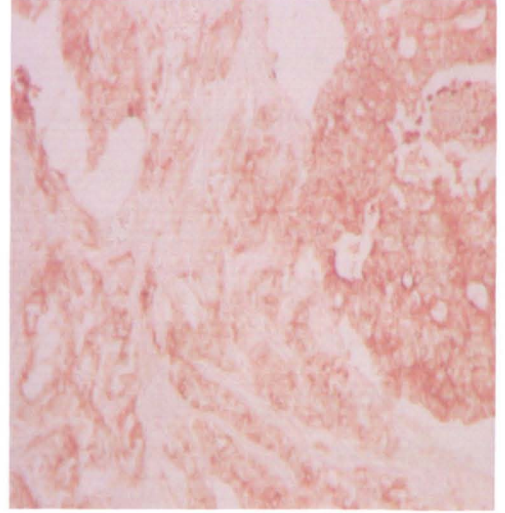


Fig 3.4.10 Histology slide showing section of invasive breast carcinoma (immunohistochemical staining with cytokeratin) x 10

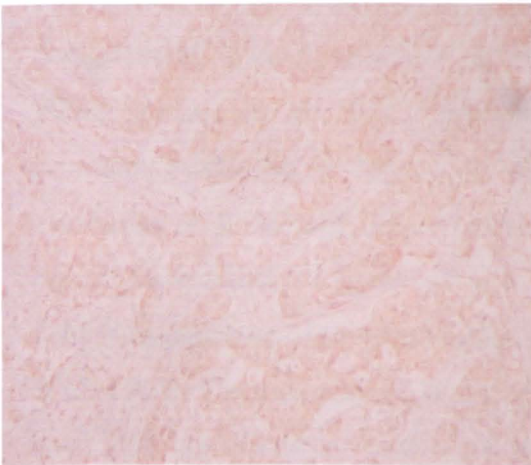


Fig 3.4.11 Histology slide showing section of invasive breast carcinoma (immunohistochemical staining with IL-10) x 10



Fig 3.4.12 Histology slide showing section of invasive breast carcinoma (immunohistochemical staining with IL-12) x 10

4. Discussion

4.1 Background

It is widely acknowledged that a degree of immune suppression is associated with cancer, but the mechanisms underlying the immune defects observed in cancer patients have not been fully elucidated (Haden, 2003; Dunn et al, 2002). Immunocompromised patients have been shown to be predisposed to carcinogenesis as seen in patients with HIV infection and transplant patients on immunosuppressive therapy. There is strong circumstantial evidence that the tumour growth can be actively controlled by the host immune system, however the limited effectiveness of anti-tumour immune response observed so far indicates that the ability of immune system to recognize and eliminate tumour cells might be impaired *in vivo* (Khong and Restifo, 2002).

A multitude of mechanisms are likely to be responsible for the immune defects of cancer patients and involve both direct and indirect immune inhibition by the tumour itself. Various modalities include production of immune inhibitory factors such as PGE₂ and TGF- β (Luo et al, 2000; Shah and Lee, 2000), reduction in CD4⁺ cell numbers, responsiveness to cytokines and proliferation in response to stimuli as has been demonstrated in cancer patients and animal models (Heimdal et al, 1999; Reichert et al, 1998; Young et al, 1997; De Paola et al, 2003). Tumour escape from host immunosurveillance is frequently attributed to the alteration of antigen presenting molecules on neoplastic cells or to the release of immunosuppressive cytokines. Many reports have indicated lower NK and LAK activities of patients with solid tumours and further impairment of these activities occurring in advanced stages of malignancy (Gastl et al, 1984). Tumour-derived humoral factors with potent immunosuppressive activities have been proposed as causes of failure of cell mediated cytotoxicity. Progressive tumour growth could lead to development of cellular immune deficiency involving both T cell lymphocytopenia and dysfunction (Heidecke et al, 2002). In one study, T cells from about one-third of head and neck squamous cell carcinoma (HNSCC) patients were reported to be unresponsive to CD3 stimulation *in vitro* (Shibuya et al, 2000).

The impairment of anti-tumour immunity is frequently associated with shift towards Th2 cytokine profile, resulting in unresponsiveness by the immune system, especially of T cells to autologous tumour cells. Studies have indicated that immune responses in

tumour bearers are biased in favour of a Th2 cytokine profile and inhibitory to anti tumour reactivity induced by Th1 responses. Th1 responses are characterised by production of IFN- γ , IL-2 and IL-12. Th2 responses are characterised by increased levels of IL-4, IL-6 and IL-10. Primary cultures of HNSCC have been reported to produce IL-4, IL-6 and GM-CSF (Mann et al, 1992). IL-6 can in turn stimulate production of IL-10 by tumour cells, such as human colon carcinoma cells (Suzuki et al, 2001). The cytokine profiles of tumour infiltrating T cells also seem to share this Th2 bias, for example, T cells that infiltrate cervical cancers are skewed toward the Th2 phenotype with increased production of IL-4 and IL-6 (Sheu et al, 2001).

IL-10 is a pleiotropic cytokine which exerts a variety of biological effects creating immunosuppressive microenvironment in favour of the tumour (Mocellin et al, 2001). IL-10 is one of the Th2 cytokines whose over expression is frequently reported in different cancer types. IL-10 compromises DC differentiation from stem cell precursors, maturation and functional status of DC by inhibiting antigen presentation, expression of co stimulatory molecules, IL-12 production and the induction of Th1 responses (Holland and Zlotnik, 1993). IL-10 also enhances spontaneous DC apoptosis, as well as their susceptibility to autologous NK cell lysis (Nabioulin et al, 1994). Through the downregulation of HLA class I and II molecules, intercellular adhesion molecule (ICAM)-1, CD80 and CD86 co stimulatory molecules, IL-10 may protect the tumour cells from CTL-mediated lysis (Yue et al, 1997). The loss of HLA class I expression may also be due to the IL-10 mediated down regulation of transporter associated with antigen processing (TAP)-1 and TAP-2 proteins in tumour cells (Seliger et al, 2002). Thus IL-10 is believed to play a key role in tumour development and metastasis. However, recent studies indicate that IL-10 indirectly suppresses the growth of certain tumours by inhibiting the infiltration of macrophages with tumour growth promoting activity via the decrease of macrophage derived angiogenic factors (Richter et al, 1993; Kundu et al, 1996). A high level of tumour associated macrophage infiltration aids in angiogenesis for tumour cells and has been shown to be significantly associated with poor prognosis in breast cancer (Leek et al, 1996). *In vivo* and *in vitro* studies have shown that IL-10 controls metastatic disease by down regulation of MHC Class I expression, leading to enhanced NK lysis of tumour cells (Kundu and Fulton, 1997). Thus, although generally considered an immunosuppressive molecule, IL-10 is demonstrated to possess immunostimulatory properties in several *in vitro* and *in vivo*

models. Hence IL-10 is seen to have two reciprocal roles: as a useful cytokine in the host and to provide an environment in which cancer cells will not be rejected. These conflicting findings reported on IL-10 effects on adaptive immunity continue to be an unresolved enigma and is the focus of many ongoing studies.

IL-12 is a cytokine that plays an essential role in the interaction between the innate and adaptive arms of the immune system (Trinchieri, 1995). IL-12 is a heterodimeric molecule composed of an α -chain (p35 subunit) and a β -chain (p40 subunit) linked by a disulfide bridge to form the biologically active 75 kDa heterodimer. Secretion of the isolated α -chain has never been detected; in contrast, the cells that produce the biologically active IL-12 heterodimer also secrete β -chain in free form in a 10-100 fold excess over the IL-12 heterodimer. Depending on the stimulus, consistent amounts of free β -chain is also seen to be produced in the absence of the heterodimer (Kobayashi et al, 1989; Wolf et al, 1991; D'Andrea et al, 1992).

IL-12 is an important inducer of Th1 responses produced by accessory cells during early antigenic stimulation and its production is regulated by a positive feedback mechanism mediated by Th1 cells through IFN- γ and a negative one by Th2 cells through IL-10 and IL-4 (Trinchieri et al, 1992). IL-12 is believed to play a fundamental role in activating anti-tumour immunity. It is a stimulatory factor for NK cells, and is produced primarily by monocytes/macrophages and to a lesser degree by B lymphocytes. Both *in vitro* and *in vivo*, IL-12 is a powerful inducer of T helper 1 type responses and an inhibitor of Th2 responses (Banks et al, 1995). Impairment of the immune system in the form of Th1/Th2 imbalance characterised by increased production of IL-10 and reduced production of IL-12 by peripheral blood mononuclear cells have been reported in breast cancer patients (Merendino et al, 1999). IL-12 level is also reported to be of prognostic value as patients with elevated blood concentrations of IL-12 have a higher survival rate than patients with low concentrations (Lissoni et al, 1998). In experimental tumour models, recombinant IL-12 treatment has been demonstrated to have a dramatic anti-tumour effect on transplantable tumours, on chemically induced tumours and in tumours arising spontaneously in genetically modified mice. It is seen to inhibit the growth and development of metastasis in various animal models such as the B16 melanoma model, the C26 colon carcinoma model, the TSA mammary carcinoma model, the SCK mammary carcinoma and K1735 melanoma models (Colombo and Trinchieri, 2002). Therapeutic effect of IL-12 has been investigated in various phase I & II trials involving

patients with renal ca, melanoma, colon ca, recurrent or refractory ovarian ca and cutaneous T cell lymphoma. Though initial results were encouraging, the modest clinical efficacy was associated with considerable toxicity (Atkins et al, 1997; Hurteau et al, 2001; Rook et al, 1999)

4.2 Hypothesis

As stated in the aims section, the objective of this study was to determine whether breast cancer patients had Th1/Th2 imbalance as one of the manifestations of immune impairment. The hypothesis was that, due to impairment of the immune system in breast cancer patients, serum IL-10 levels would be high and IL-12 levels would be low when compared to healthy controls as a result of skewing of the immune system in favour of Th2 cytokine profile. Like-wise, IL-12 production by PBMC was expected to be low in patients with breast cancer when compared to controls. Extrapolating this to the tumour micro environment, it was hypothesised that tumour infiltrating lymphocytes may be programmed to produce large amounts of IL-10 and this along with IL-10 production by the tumour would give rise to intense staining for IL-10 and absent or poor staining for IL-12 within the tumour vicinity on immunohistochemistry.

After surgery, due to the removal of the inhibitory influence of the tumour on the immune system, it was hypothesised that there will be a reversal in levels with a reduction in IL-10 and a rise in IL-12 levels. Similarly, IL-12 production by PBMC was expected to improve with the surgical removal of the tumour due to the absence of the suppressive effects of IL-10. As adjuvant therapy is generally thought to be beneficial to patients in terms of preventing recurrence and prolonging disease free interval, it was hypothesised that IL-10 levels would remain low and IL-12 levels would be sustained at high levels with an improvement in IL-12 production capacity of PBMC after completion of adjuvant therapy as a reflection of an improved state of immune surveillance.

In this study the levels of IL-10 and IL-12 in serum by ELISA, the production of IL-12 by PBMC and the expression of IL-10 and IL-12 in the tumour microenvironment by immunohistochemistry was determined. The relation, if any, of the various pathological factors (that have a bearing on the prognosis) such as tumour size, grade, lymph node involvement and oestrogen receptor status on the levels of IL-10 and IL-12 was also

analysed. The effects of therapy including that of surgery and adjuvant treatment including radiotherapy, chemotherapy and hormone therapy on the levels of these cytokines that represent the Th1 and Th2 cytokine family was also investigated.

4.3 Serum IL-10 and IL-12 levels in breast cancer patients

Serum IL-10 and IL-12 levels were measured in 91 patients with invasive disease, 11 patients with *in situ* disease and 31 healthy controls. Patients with *in situ* disease were also included in the study to determine whether the cytokine levels in this sub group of patients considered to have pre-invasive disease were different from patients with invasive carcinoma. IL-10 levels were not recordable (≤ 0) in a sizable number of patients and controls (35 patients with invasive disease, 8 patients with *in situ* disease and 17 healthy controls). In the case of IL-12, only one patient had undetectable levels of IL-12. The recording of negative values for IL-10 in ELISA has not been reported before in any other study. This may be due to faulty technique or faulty kit/reagents, but estimation of IL-10 in serum samples of patients with colorectal cancer and HNSCC using ELISA kits used in this study also reported negative values suggesting that factors other than technical may be responsible for this.

The levels of IL-10 expressed in terms of median (IQR) were: 1.32pg/ml (1.02-1.83) in patients with invasive disease, 1.83 pg/ml in patients with *in situ* disease and 1.45 pg/ml (0.93-3.08) in healthy controls. Statistical analysis showed that patients with invasive disease were more likely to record detectable levels of IL-10 ($P=0.045$). However, patients with *in situ* disease were found to be less likely to record detectable levels than controls. But attention must be drawn to the fact that the number of patients with non-invasive disease was small and hence this observation needs to be corroborated in studies with a larger number of samples. There was no statistically significant difference in IL-10 levels between patients and controls. This is contrary to the hypothesis that IL-10 levels would be high in breast cancer patients due to a Th2 bias. Also there was no significant association between IL-10 levels and prognostic factors such as tumour size, grade, lymph node involvement and oestrogen receptor status.

Many human tumour cell lines including malignant melanoma and human lung carcinomas express IL-10 spontaneously and this is thought to support directly the proliferation of tumour cells and thereby a possible mechanism of immune suppression

(Yue et al, 1997; Gastl et al, 1993). Production of IL-10 has also been reported in ovarian cancer, melanoma, skin cancer, head and neck cancer and hepatocellular carcinoma, suggesting that IL-10 reduces the function of tumour infiltrating lymphocytes and contributes to the tumour growth (Pisa et al, 1992; Smith et al, 1994; Kruger-krasagakes et al, 1994; Yamamura et al, 1993; Kim et al, 1995; Young et al, 1996). Plasma levels of IL-10 were found to be high in patients with ovarian cancer, non-hodgkins lymphoma, multiple myeloma and hepatocellular carcinoma (Pisa et al, 1992; Blay et al, 1993; Fortis et al, 1996; Merville et al, 1992; Chau et al, 2000). A study looking at IL-10 levels in patients with unresectable hepatocellular carcinoma reported a stage related increase in IL-10 levels with low LAK and NK activities. Patients with high IL-10 levels had significantly shorter survival and hence it was concluded that serum IL-10 concentration was a possible factor contributing to poor prognosis and low anti-tumour immunity in patients with unresectable hepatocellular carcinoma (Hattori et al, 2003). Malignant melanoma patients with metastasis have been reported to have high serum levels of IL-10 (Sato et al, 1996). Furthermore a negative correlation between circulating levels of IL-10 and prognosis has been observed in patients with solid or haematological malignancies (Moore et al, 2001).

Only two studies looking at serum IL-10 levels in breast cancer patients have been reported in literature. One study involved only 20 patients with half of the patients having metastatic disease. They reported high serum IL-10 levels in patients when compared to healthy controls and a statistically significant difference in IL-10 levels between patients and healthy controls and also between patients with and without metastasis (Merendino et al, 1996). Most of the patients without metastasis had nodal disease (7/10). Serum levels of IL-10 along with IL-6 and IL-8 were reported to be high in breast cancer patients in comparison with healthy women and this was found to have significant correlation with the clinical stage of the disease (Kozlowskik et al, 2003).

Patients with colorectal cancer (n=60) were reported to have significantly high levels of serum IL-10 when compared to healthy controls (n=30). In this study, patients with advanced disease (Duke's B & C) and large tumours (T3 & T4) had higher levels of IL-10 compared to patients with early disease stage. However, there was no such difference between node positive and node negative disease. The median tumour volume was 20.6 cm³ in node positive disease and 6 cm³ in node negative disease (O'Hara et al, 1998). In the present study, none of the patients had metastasis and most of the patients

had early stage disease with only a small number having nodal disease (17/83). The median tumour size was 2.3 cm. This might explain why there was no significant difference in IL-10 levels between patients and controls as most of the patients had low tumour burden. More-over, most of the studies looking into IL-10 levels in cancer patients had small sample sizes ranging between 10 and 40. The current study had 91 patients with invasive disease and 11 patients with *in situ* disease making it the largest study of its kind. The preliminary analysis (n=35) of this study revealed a trend towards high level of IL-10 which disappeared once the sample size increased to 91. This clearly demonstrates that studies looking into cytokine levels should be based on large patient samples to permit firm conclusions to be drawn.

The levels of IL-12 expressed in terms of median (IQR) were: 44.48 pg/ml (25.98-73.40) in patients with invasive disease, 38.73 pg/ml (28.51-55.09) in patients with *in situ* disease and 52.9 pg/ml (29.12-91.06) in healthy controls. Thus, though the levels of IL-12 were marginally lower than that in healthy controls, they were not statistically significant. This again did not agree with the hypothesis that IL-12 levels would be low in patients with breast cancer due to skewing of the immune system against the Th1 profile. There was also no evidence to suggest that known prognostic factors such as tumour size, lymph node involvement and oestrogen receptor status had any bearing on serum IL-12 levels. However, grade of the tumour seemed to have an inverse relationship with serum IL-12 levels as there was a statistically significant fall in serum IL-12 levels with increasing grade of the tumour.

Nakayama *et al* reported low serum levels of IL-12 in patients with advanced gastric (n=14) and colorectal cancer (n=15). This was again not statistically significant and all these patients had node positive disease with tumours breaching the serosa (Nakayama *et al*, 2000). Another study looking into serum IL-12 levels in 76 cancer patients (breast, gastrointestinal, uterine, ovarian, renal, bladder) reported elevated levels with a significant correlation with progression of the disease (Stage I & II vs Stage III & IV) . However, this positive correlation was not seen in breast cancer patients with values remaining in the same range in all groups (Kovacs, 2001). The present study seems to corroborate these findings although most of the patients had node negative early stage disease with low tumour burden. The emergence of grade as an important factor influencing serum IL-12 levels is significant as patients with grade III tumours are

known to be aggressive tumours with poor prognosis. The low IL-12 levels with increasing grade may be a manifestation of immune impairment by the tumour.

There was no difference in IL-10 and IL-12 levels between screening and symptomatic patients. Patients diagnosed at screening are expected to have early stage disease as the tumours are picked up before they become clinically palpable or symptomatic. Hence, the lack of significant difference in IL-10 and IL-12 levels is surprising and indicate that factors other than tumour size determine biological aggressiveness of the tumour and hence their effect on the immune system.

In a study involving 40 patients with solid cancer of whom 24 had distant metastasis, none had low serum IL-12 levels. On the other hand, patients with metastasis had high IL-12 levels compared to patients without metastasis and this was statistically significant (Lissoni et al, 1997). Within the metastatic group, one year survival was significantly higher in patients with high levels of IL-12 than in those with normal levels (Lissoni et al, 1998). These observations suggest that various tumours have different effects on the immune system and it is difficult to generalise the impact of the tumour on the Th1/Th2 cytokine profile. The high IL-12 levels in some patients with distant metastasis seem to indicate a turnaround in the immune system involving a switch towards Th1 cytokine profile and this is supported by the fact that patients with high IL-12 levels had better survival rates. This again underlines the fact that these seemingly contradictory findings must be validated by larger studies involving bigger sample sizes and long term follow up.

4.4 IL-12 production by PBMC in breast cancer patients

IL-12 production by PBMC after fixed SAC stimulation was measured in 73 patients with invasive disease and 8 healthy controls.

In this study, the IL-12 production capacity of PBMC as measured by the Biosource kit (p70 and p40) was found to be higher in patients with breast cancer (n=43) when compared to controls (n=5) though not statistically significant. On the other hand, the IL-12 production levels were low in breast cancer patients (n=30) when compared to controls (n=5) according to the R&D kit which measures only the biologically active heterodimer (p70) and again this was not statistically significant. It is a known fact that

the biological activity of IL-12 is associated with the p70 heterodimer; however, the cells produce IL-12p40 *in vitro* or *in vivo* up to 100 fold in excess over the IL-12p70 (Banks et al, 1995). Since the level of IL-12 production measured using the Biosource kit was high in cancer patients compared to healthy controls, it may be assumed that the p40 subunit is produced in large quantities in breast cancer patients. This finding is likely to be of little significance as the p40 subunit has little biological activity on its own. These results again run contrary to the hypothesis as it was hypothesised that the IL-12 production capacity of PBMC to be reduced in breast cancer patients due to the immunosuppressive effect of the tumour.

In a study involving 15 women with early stage breast cancer, IL-12 production by PBMC was reported to be significantly low when compared to healthy controls. In this study, PBMC was isolated from the non-monocyte cell population and stimulated using a different technique involving a combination of monoclonal antibody anti-IL-10, IFN- γ and lipopolysaccharide (LPS) from *E. coli* serotype was employed as the stimulus for IL-12 production. They concluded that PBMC of breast cancer patients had a defective IL-12 production capability probably as a result of immune impairment exerted by the tumour (Merendino et al, 1999). The different technique of PBMC isolation and stimulation may explain the discrepancy between this study and the findings obtained in our study. Similar reduction in IL-12 production capacity of PBMC were reported in patients with colorectal cancer (n=60) when compared to controls (n=30). This impairment was found to be more pronounced in patients with node positive disease, advanced stage (Duke's C compared to Duke's B) and large tumour size (T3, T4). This group employed a similar technique of PBMC stimulation using SAC as in the present study (O'Hara et al, 1998). However, these findings could not be replicated in this study as a reduction in IL-12 production capability of PBMC was not demonstrable. The use of stored PBMC for SAC stimulation may be a factor, but it might be worthwhile to repeat the experiment on fresh samples or using a different technique and try to replicate the results of the above cited studies in a larger sample size.

No analysis of a possible association between known prognostic factors such as size, grade, nodal involvement and oestrogen receptor status was done due to the small sample size.

4.5 IL-10 and IL-12 levels in the tumour microenvironment as assessed by immunohistochemistry

An attempt was made to assess IL-10 and IL-12 levels in the tumour microenvironment using immunohistochemistry and to analyse association, if any, with standard prognostic factors such as tumour size, grade, lymph node involvement and estrogen receptor status. In this study, most of the tumour specimens (n=21) showed mild to moderate staining for IL-10 although no correlation with known pathological factors was demonstrable albeit the small sample size. However, the intensity of staining for IL-12 could not be reported with confidence as it appeared that the staining was weak or negative.

In a study looking at expression of IL-10 in oral and oropharyngeal squamous cell carcinoma specimens by immunohistochemistry, 13/58 (22%) cases showed intense staining and 35/58 (60%) cases showed moderate positive staining for IL-10. No association between the intensity of staining and clinicopathological factors such as tumour size, lymph node involvement, clinical stage, occurrence of metastasis, incidence of recurrence and death was detected. However, patients with intense staining had a significantly low overall survival rate than those with moderate or negative staining (Fujieda et al, 1999). Tumour free sentinel lymph nodes (SLN) harvested from patients with breast cancer were found more likely to contain cells expressing IL-10 and to a lesser extent IL-12 than tumour containing SLN. Like-wise, a higher number of mature CD83-positive dendritic cells (DC) were found in tumour free SLN than in tumour containing SLN (Poindexter et al, 2004). IL-10 is known to affect immature DC by inhibiting upregulation of MHC class II and costimulatory molecules and expression of CD83 positive cells. Mature CD83 expressing DC are resistant to the effects of IL-10 (Jonuleit et al, 2001). On the other hand, IL-12 is a proinflammatory cytokine that drives DC towards activation of Th1 response (Heufler et al, 1996). Both these observations suggest that the tumour free SLN is immunologically competent and is potentially a site of tumour specific T cell activation. Future functional studies would determine whether the SLN is a site of immunosuppression, as suggested by the expression of IL-10, or of immune activation, as suggested by expression of IL-12.

Data from immunohistochemistry seem to partially support the hypothesis that IL-10 tends to be expressed by tumours in the microenvironment. Even though the staining for IL-12 was reported tentatively as being weak or negative with reservations on the quality of staining, this observation tends to point to the fact that IL-12 expression in the tumour vicinity is poor. This suggests that the immune response in the tumour microenvironment may be skewed towards a Th2 profile, although once again, this needs to be validated by larger studies.

4.6 Effect of various treatment modalities on levels of IL-10 and IL-12 in breast cancer patients

Part of the hypothesis was to study the impact of different treatment modalities on the immune system and to investigate its effect on the Th1/Th2 balance. It was hypothesised that after surgery, due to the removal of the inhibitory effects of the tumour on the immune system, IL-10 levels would decrease and IL-12 levels would show an increasing trend. After completion of adjuvant therapy in the form of radiotherapy, chemotherapy or hormone therapy, it was felt that due to the effects of these treatments on possible micrometastatic systemic disease, a manifestation of improved immunosurveillance would be continuing fall in IL-10 levels with a reciprocal increase in levels of IL-12.

4.6.1 Effect of surgical removal of tumour on levels of IL-10 and IL-12

IL-10 and IL-12 levels were measured in post-operative blood samples collected at a median interval of 21 days (IQR: 13-30) after surgery and before the commencement of adjuvant therapy. On statistical analysis, there was no significant difference between pre- and post-operative levels of IL-10 and IL-12 in patients with *in situ* disease (n=6), however the sample size was small. There was no change in IL-10 levels in patients with invasive disease (n=51) after surgery, but IL-12 levels showed a significant increase over pre-op levels after surgical removal of the tumour.

IL-12 production by PBMC was assessed in post-op blood samples collected at a median interval of 26 days after surgery (IQR: 21-35) and before the commencement of

adjuvant therapy. Analysis revealed no statistically significant difference between pre- and post-operative IL-12 production by PBMC in breast cancer patients (n=33).

It may be speculated that the rise in serum IL-12 levels after surgery is due to the effect of surgical trauma on the cytokine levels. But the post-operative blood samples were collected at least 14 days after surgery, as the inflammatory response of surgery is known to settle down within 7-10 days after a surgical procedure. No previous study has looked into the effect of surgery on IL-12 levels even though IL-10 levels have been reported to remain unchanged after surgical procedures such as open or laparoscopic hernia repairs (Jess et al, 2000). In our study, there was no significant difference in post-op IL-10 or IL-12 levels between patients who underwent mastectomy and those who underwent conservative surgery (wide local excision). Moreover, the IL-12 levels remained high even 20-70 days after surgery as illustrated in Fig 3.2.2. These findings suggest that the change in IL-12 levels appears to be a real effect rather than the effect of the inflammatory response of surgery.

In a study involving 10 patients with gastrointestinal tract tumours, serum IL-12 levels were found to fall after surgery (Lissoni et al, 1998). In the present study, there was no change in IL-10 levels, but IL-12 levels were found to significantly increase after surgery in patients with invasive disease. This observation agrees only partially with the hypothesis, according to which, there should be a fall in IL-10 levels and a rise in IL-12 levels after surgical extirpation of the tumour. Possible explanations might be that there is only a partial skewing of the immune response in favour of Th1 cytokine profile after surgery characterised by rise in IL-12 and static IL-10 levels. The fact that IL-10 levels do not fall after surgical removal of the tumour indicates that apart from the tumour, there may be other sources of IL-10, perhaps the cells of the immune system. An obvious additional source of IL-10 may be viable tumour cells in the systemic circulation or the dormant tumour cells at potential sites of metastasis and the bone marrow. Studies have shown that the bone marrow is a privileged site where tumour cells are controlled in a dormant state by the immune system. This reinforces the view that after surgical treatment of the primary, adjuvant systemic treatment should be instituted to neutralise the potentially lethal tumour cells in the system to prolong the disease free interval (Feuerer et al, 2001; Schirmacher et al, 2002). On the other hand, taking into account the potential immunostimulatory effects of IL-10, probably the static levels of IL-10 maintained after surgery may be beneficial to the host immune

system. It has been suggested that evidence of high IL-12 levels may have a favourable prognostic significance in terms of increased survival rates and stable disease (Lissoni et al, 1998). In the present study, 16 of the 51 patients with invasive disease showed a fall in IL-12 levels after surgery. It may be worthwhile to see whether this has any bearing on disease free interval and recurrence by conducting follow up studies. Studies to determine levels of IL-12 after surgery in other cancers are required before any definite conclusion can be deduced.

No appreciable change in IL-12 production capacity of PBMC after surgical removal of the tumour was demonstrated in this study. As discussed before, this observation must be placed in perspective against the background of the possible detrimental effect of storage and cryopreservation on the functional status of PBMC. This experiment needs to be repeated with fresh samples before definite conclusions can be made.

4.6.2 Effect of post - op adjuvant radiotherapy, chemotherapy and endocrine treatment on levels of IL-10 and IL-12

IL-10 and IL-12 levels were determined in blood samples of patients collected at a median interval of 77 days (IQR: 51-126) after completion of post-operative adjuvant radiotherapy and/or chemotherapy. Some of the patients who had oestrogen receptor positive tumours were on adjuvant endocrine treatment while the blood samples were collected. There was no significant change in serum levels of IL-10 and IL-12 after completion of adjuvant therapy in patients with *in situ* disease (n=5). Although there was no discernible change in IL-10 levels in patients with invasive disease (n=17), there was a trend towards a fall in IL-12 levels after adjuvant treatment in this group ($P=0.06$; Wilcoxon signed ranks test).

Chemotherapeutic agents such as 5-Fluorouracil are reported to increase serum levels of IL-12 in head and neck cancer patients (Okamoto et al, 2000). A study has reported that after concomitant chemo-radio-immunotherapy, cytotoxic activity and generation of cytokines such as TNF- α , IFN- γ , TGF- β and IL-12 *in vitro* by tumour infiltrating lymphocytes (TIL) is greatly increased in patients with oral squamous cell carcinoma. This shows that chemo-radio-immunotherapy modulates *in situ* cytokine generation which is advantageous for inhibition of tumour cell growth and activation of TIL

(Tetsuya et al, 2003). However, a study looking into IL-12 levels in 76 cancer patients (breast, gastrointestinal, uterine/ovarian, renal/bladder: 4 groups stage I & II, without and after chemo/radiotherapy; stage III & IV without and after chemo/radiotherapy) reported no alteration after chemo/radiotherapy in either stage (Kovacs, 2001). Patients with aggressive non-hodgkins lymphoma with high levels of serum IL-10 did not respond to induction chemotherapy and had poor outcomes (Stasi et al, 1994). IL-10 production by LPS stimulated alveolar macrophages in lung cancer patients has been reported to significantly improve after systemic chemotherapy. This may be seen as being detrimental or beneficial to the host immune system taking into consideration, the seemingly contradictory effects of IL-10. Since IL-10 is reported to be a potent inhibitor of tumour angiogenesis, systemic chemotherapy is suggested to augment this inhibitory activity (Yanagawa et al, 1999). In the present study, patients with invasive disease showed a fall in IL-10 after completion of adjuvant chemotherapy though this was not statistically significant. The IL-12 levels were also found to fall and this was statistically significant when compared to pre-op levels. Possible explanations may be that the immune system is in a dormant state as the adjuvant therapy has neutralised the systemic disease. It would be interesting to do serial measurements of IL-10 and IL-12 at follow up and to see whether any change in levels occur in the event of disease recurrence or development of metastasis. More detailed studies looking at effect of various adjuvant therapies such as radiotherapy, chemotherapy, endocrine therapy and Herceptin[®] individually and in combination on the levels of IL-10 and IL-12 and its relationship to disease free interval during follow up might help to define the role of these cytokines as prognostic markers.

5. Conclusion

There is a growing body of evidence to support the widely held view that patients with various malignancies have a significant degree of immune impairment. The immunosuppression associated with cancer is shown to escalate with stage of the disease and is believed to contribute to the progression of cancer (Woo et al, 2001). A multitude of mechanisms are likely to be responsible for the immune defects prevalent in cancer patients. One of these is believed to be through the skewing of the cytokine responses to a Th2 phenotype resulting in generation of cytokines such as IL-4 and IL-10 that are immunosuppressive of a cell-mediated response.

Studies have reported that there is a skewing of the immune system towards a Th2 cytokine profile in cancer patients. This may be complete (with elevated levels of Th2 cytokines such as IL-10 and low levels of Th1 cytokines such as IL-12) as has been reported in patients with colorectal cancer and a small study involving breast cancer patients (Merendino et al, 1999; O'Hara et al, 1998) or partial (elevated levels of Th2 cytokines without a concurrent decrease in Th1 cytokines) as reported in patients with head and neck squamous cell carcinoma and gastric cancer (Lathers et al, 2003; Wu et al, 1996). The aim of this study was to determine whether Th1/Th2 imbalance in the form of skewing of the immune response towards a Th2 bias is one of the manifestations of immune impairment in breast cancer patients.

This study is one of the largest of its kind involving almost 100 patients with breast cancer and 31 healthy controls. To summarise the findings:

- No change in serum IL-10 or IL-12 levels was detected in breast cancer patients with invasive disease or *in situ* disease when compared with healthy controls.
- Comparison of known prognostic factors such as tumour size, lymph node involvement, oestrogen receptor status and tumour grade with IL-10 and IL-12 levels did not show any significant association except for tumour grade which showed an inverse relationship to IL-12 levels, with increasing grade being associated with falling serum IL-12 levels.
- No difference in IL-12 production capacity of PBMC was detected between breast cancer patients and healthy controls.

- Immunohistochemistry for IL-10 in breast cancer specimens reported mild to intense staining for IL-10. As regards IL-12, the results were inconclusive as concerns were raised regarding the quality of the staining.
- After surgical removal of tumour, there was a significant increase in serum IL-12 levels with no change in IL-10 levels.
- No change in IL-12 production by PBMC was detected after surgery.
- After completion of adjuvant systemic therapy and commencement of hormone therapy, there was no discernible change in serum IL-10 levels, but serum IL-12 levels showed a significant fall when compared to pre-op levels.

Hence in this study, there was no skewing (partial or complete) towards a Th2 cytokine profile in breast cancer patients. Tumour grade was found to be the only known prognostic factor to have a correlation with serum IL-12 levels. The rise in serum IL-12 level following surgical removal of the tumour may indicate a partial skewing of the Th1/Th2 balance in favour of the Th1 cytokine profile which may be beneficial to the host immune system. The fall in serum IL-12 after completion of post-op adjuvant systemic therapy may be a manifestation of the long term impact on the immune system of these usually immunosuppressive treatment regimens. Previously studies have reported that patients who received chemotherapy had significant decrease in lymphocytes even 6 months after completion of chemotherapy (Caras et al, 2004). Specific anti-neoplastic therapy such as multi drug chemotherapy or radiotherapy has also been implicated in the impairment of several immune parameters (Heidecke et al, 2002; Steele, 2002). Considerable effort has been made to find modalities to overcome the immune deficiency of cancer patients to improve both anti-tumour immunity and general immune functions.

Thus this thesis describes the largest ever study on a cohort of breast cancer patients looking into levels of two important cytokines: IL-10 and IL-12 considered to be representative members of the Th2 and Th1 cytokine family. This is the first study of its kind which has looked into correlation between the levels of these cytokines and the recognised clinico-pathological factors (such as tumour size, grade, lymph node involvement and oestrogen receptor status) with prognostic significance. Also, this is the first study which has investigated the effect of treatment including surgery and adjuvant therapy such as radiotherapy and chemotherapy on the levels of these

cytokines. The information gained after analysing the data can be useful when devising immune therapy protocols involving these cytokines in the near future.

The results of this study contradict the findings reported in smaller studies involving breast cancer patients in terms of serum IL-10 and IL-12 levels and IL-12 production by PBMC. The sample size appears to be a critical factor and this should be taken into consideration before definite conclusions can be made. The role of IL-10 in the tumour immune response continues to be an unresolved enigma and this view seems to be reinforced in this study. The effect of the tumour on the production of IL-12 by PBMC needs to be further investigated. Issues regarding collection and storage of cells should be standardised as this can apparently have a major impact on the outcome of the experiments. The potential role of these cytokines as prognostic markers and markers of therapeutic efficacy should be further assessed using larger studies in other cancers and with longer follow-ups.

6. Future work

Over the last few decades, a wealth of evidence has been gathered to understand better the role that the immune system plays in the fight against cancer; and cytokines have been found to possess a key role. Breast cancer development and progression is influenced by intrinsic properties of the tumour cells, as well as by microenvironmental factors. An intensive interplay exists between the tumour cells on one hand, and inflammatory cells/cytokines/chemokines on the other (Ben-Baruch, 2003). Cytokine kinetics therefore represents key immunological phenomena occurring in the tumour microenvironment, where immune and malignant cells interact.

With hindsight and increased availability of time and resources, this study could have been more comprehensive regarding the IL-10 and IL-12 kinetics in breast cancer. The various angles include:

1. Majority of the patients in this study had early stage breast cancer. It might be interesting to investigate cytokine levels in patients with advanced disease and to determine whether the cytokine levels in this cohort of patients are different from those with early disease.
2. IL-10 and IL-12 are considered to be representative members of the Th2 and Th1 cytokine family. It would be interesting to look into a Th2 panel of cytokines which would include IL-4 and IL-6 apart from IL-10 and a Th1 panel of cytokines which would include IFN- γ and IL-2 in addition to IL-12. This would reveal a general trend, if any, of the Th1/Th2 balance in breast cancer patients at different stages.
3. As mentioned before, the PBMC production of IL-12 after SAC stimulation could not be confidently reported as the unstimulated wells also had high levels of IL-12. This may be due to issues related to storage and would need further validation by repeating the experiments on fresh samples. Different techniques of PBMC isolation and stimulation could also be investigated to determine whether this would yield different results. Also, it might be worthwhile to look into IL-10 production by PBMC, and to see whether this mirrors the trend seen in serum levels.

4. It would have been beneficial to have post-operative samples from controls undergoing surgery for benign breast conditions to determine any change in levels of IL-10 & IL-12. This would allow us to prove conclusively that change in serum IL-12 levels after surgery is a real effect due to removal of the tumour and not as a result of the inflammatory response to surgery.
5. It would be interesting to assess the functional status of PBMC such as generation of superoxide anion and candidacidal activity in breast cancer patients and at different stages of treatment as this would reveal the impact of these treatment modalities on the cytotoxic effector functions of the T cells.
6. Immunohistochemistry for IL-12 in tumour specimens could not be reported with confidence in our study. No previous study has reported data on IL-12 immunohistochemistry in breast tumour specimens. Future development of specific antibodies for immunohistochemical staining may increase the validity and success rate of obtaining reliable data regarding the IL-12 levels in the tumour microenvironment. Lymph nodes draining the tumour may be analysed by immunohistochemistry and comparison of metastatic and non metastatic lymph nodes would reveal variation if any, in the internal milieu of these lymph nodes.
7. The fall in serum IL-12 after completion of adjuvant therapy reported in this study may be due to the immuno-suppressive effects of these toxic systemic treatments. Therefore, it would be extremely useful to evaluate the immune status in cancer patients at different time points to identify the origin of immunosuppression and optimize the treatment for a maximum benefit with the fewest undesirable side effects. Evaluation of overall immunocompetence of cancer patients could help to guide and monitor the treatment and to identify candidates for specific immune intervention such as immune therapy.
8. Analysis at long term follow up would reveal whether there is any change in levels of IL-10 or IL-12 at time of recurrence or development of metastasis. It would also be interesting to see whether initial levels have a bearing on the disease free interval and the propensity to develop recurrence or metastasis.

This study is one of the largest of its kind with over 100 patients. Large sample size is essential to determine the real picture regarding cytokine kinetics in the tumour microenvironment. Future studies should specifically look into the problems encountered in our study including the negative values obtained in IL-10 ELISA, PBMC stimulation for IL-12 production and IL-12 immunohistochemistry in tumour specimens. Large studies with long term follow up will help us understand better the mimicry between the immune system and the tumour cells and extrapolate the findings to the clinical setting, thereby aiding us to devise ways of monitoring and neutralising the adverse effects of the tumour on the immune system. This also would help in devising new strategies in immune therapy involving these cytokines. Studies looking into the role of cytokines as adjuvants in cancer vaccines and in gene therapy have yielded promising results. With the advent of nanotechnology and development of micro drug delivery systems, the role of these cytokines in future immune therapy protocols look promising and will be an exciting area for future research.

Bibliography

Allan CP, Turtle CJ, Mainwaring PN, Pyke C, Hart DN (2004). "The immune response to breast cancer and the case for DC immunotherapy". *Cytotherapy* ; 6: 154-163.

Allred DC, Berardo MD, Prosser J, O'Connell P (1997). "Biologic and genetic features of in situ breast cancer". In: Silverstein MJ, ed. *Ductal carcinoma in situ of the breast*. Philadelphia: Williams and Wilkins: 37-49.

Althuis, MD, Dozier JM, Anderson WF, Devesa SS, Brinton LA (2005). "Global trends in breast cancer incidence and mortality". *International Journal of Epidemiology*; 34(2): 405-412.

Arduino S, Tessarolo M, Bellino R, Colombatto S, Leo L *et al* (1996). "Reduced IL-2 level concentration in patients with breast cancer as a possible risk factor for relapse". *European Journal of Gynaecological oncology*; 17(6): 535-537.

Atkins MB, Robertson MJ, Gordon M, Lotze MT, De Coste M *et al* (1997). "Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies". *Clinical Cancer Research*; 3(3): 409-417.

Bachelot T, Ray-Coquard I, Menetrier-Caux C, Rastkha M, Duc A, Blay JY (2003). "Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients". *British Journal of Cancer*; 88(11):1721-1726.

Banks E (2001). "Hormone replacement therapy and the sensitivity and specificity of breast cancer screening: a review". *Journal of Medical Screening*; 8(1): 29-34.

Banks RE, Patel PM, Selby PJ (1995). "Interleukin-12 ; a new clinical player in cytokine therapy". *British Journal of Cancer* ; 71(4): 655-659.

Ben-Baruch A (2003). "Host microenvironment in breast cancer development: inflammatory cells, cytokines and chemokines in breast cancer progression: reciprocal tumor-microenvironment interactions". *Breast Cancer Research*; 5(1):31-36.

Bendre MS, Montague DC, Peery T, Akel NS, Gaddy D, Suva LJ (2003). "Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease". *Bone*; 33(1):28-37.

Benoy I, Salgado R, Colpaert C, Weytjens R, Vermeulen PB, Dirix LY *et al* (2002). "Serum interleukin 6, plasma VEGF, serum VEGF, and VEGF platelet load in breast cancer patients". *Clinical Breast Cancer*; 2(4):311-315.

Benson JR (2004). "Role of transforming growth factor beta in breast carcinogenesis". *Lancet Oncology*; 5(4): 229-239.

- Beral V (2003). "Breast cancer and hormone-replacement therapy in the Million Women Study". *Lancet*; 362 (9382): 419-427.
- Beral V, Banks E, Reeves G (2002). "Evidence from randomised trials on the long-term effects of hormone replacement therapy". *Lancet*; 360 (9337): 942-944.
- Berg JW, Hutter RVP (1995). "Breast Cancer". *Cancer* ; 75: 257-269.
- Bergstrom A, Pisani P, Tenet V, Wolk A, Adami H (2001). "Overweight as an avoidable cause of cancer in Europe". *International Journal of Cancer*; 91: 421-430.
- Bhardwaj N, Seder RA, Reddy A, Feldman MV. "IL-12 in conjunction with dendritic cells enhances antiviral CD8+ CTL responses in vitro". *Journal of Clinical Investigation*; 98(3) :715-722.
- Bhatia S, Robison LL, Oberlin O, Greenberg M, Bunin G *et al.* (1996). "Breast cancer and other second neoplasms after childhood Hodgkin's disease". *New England Journal of Medicine*; 334(12): 745-751.
- Blanchard DK and Djeu JY (1988). "Protection of cultured human monocytes from lymphokine-activated killer mediated lysis by IFN- γ ". *Journal of Immunology*; 141: 4067-4073.
- Blidaru A, Bordea CI, Viisoreanu CG, Bordea M, Iliescu I *et al* (1998). "Interleukin-2 alterations in the immune status of patients with mammary neoplasm". *Romanian Journal of Physiology*; 35(1-2):127-134.
- Boyd NF, Stone J, Vogt KN, Connelly BS, Martin LJ, Minkin S (2003). "Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature". *British Journal of Cancer*; 89 (9): 1672-1685.
- Buelens C, Willems F, Delvaux A, Pierard G, Delville J *et al* (1995). "Interleukin-10 differentially regulates B7-1(CD80) and B7-2(CD86) expression on human peripheral blood dendritic cells". *European Journal of Immunology*; 25: 2668-2672.
- Butler WM, Cunningham J (2001). "Ethnic differences in breast cancer characteristics". *Breast Cancer Online*; 4(5).
- "Breast Cancer: Incidence rises while deaths continue to fall". www.statistics.gov.uk. [Accessed: 12/06/06].
- Byrne C, Connolly JL, Colditz GA, Schnitt SJ (2000). "Biopsy confirmed benign breast disease, postmenopausal use of exogenous female hormones, and breast carcinoma risk". *Cancer*; 89 (10): 2046-2052.
- Cai G, Kastelein RA, Hunter CA (1999). "IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18". *European Journal of Immunology*; 29: 2658-2665.

- Candido KA, Shimizu K, McLaughlin JC, Kunkel R, Fuller JA *et al* (2001). "Local administration of dendritic cells inhibits established breast tumor growth: implications for apoptosis-inducing agents". *Cancer Research*; 61(1): 228-236.
- Caras I, Grigorescu A, Stavaru C, Radu DL, Mogos I *et al* (2004). "Evidence for immune defects in breast and lung cancer patients". *Cancer Immunology, Immunotherapy*; 53(12): 1146-1152.
- Caudell EG, Mumm JB, Poindexter N, Ekmekcioglu S, Mhashilkar AM *et al* (2002). "The protein product of the tumor suppressor gene, melanoma differentiation-associated gene 7, exhibits immunostimulatory activity and is designated IL-24". *Journal of Immunology*; 168(12): 6041-6046.
- Caux C, Massacrier C, Vanbervliet B, Barthelemy C, Liu Y *et al* (1994). "Interleukin-10 inhibits T cell alloreaction induced by human dendritic cells". *International Immunology*; 6: 1177-1185.
- Cavallo F, Di Carlo E, Butera m, Verrua R, Colombo MP *et al* (1999). "Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin 12". *Cancer Research*; 59(2): 414-421.
- Chang C, Furue M, Tamaki K (1994). "Selective regulation of ICAM-1 and major histocompatibility complex class I and II molecule expression on epidermal Langerhans cells by some of the cytokines released by keratinocytes and T cells". *European Journal of Immunology*; 24: 2889-2895.
- Chau GY, Wu CW, Lui WY, Chang TJ, Kao HL *et al* (2000). "Serum IL-10 but not IL-6 is related to clinical outcome in patients with resectable HCC". *Annals of Surgery*; 231(4): 552-558.
- Chen Y, Thompson W, Semenciw R, Mao Y. (1999). "Epidemiology of contralateral breast cancer". *Cancer epidemiology, biomarkers & prevention*; 8 (10): 855-861.
- Chlebowski RT, Hendrix SL, Langer RD, Stefanick ML, Gass M *et al* (2003). "Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial". *Journal of the American Medical Association*; 289 (24): 3243-3253.
- Claus, EB, Risch N, Thompson, WD. (1994). "Autosomal dominant inheritance of early-onset breast cancer. Implications for risk prediction". *Cancer*; 73(3): 643-651.
- Collaborative Group on Hormonal Factors in Breast Cancer (2002). "Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease". *Lancet* ; 360 (9328): 187-195.
- Collaborative Group on Hormonal Factors in Breast Cancer (1997). "Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological

studies of 52,705 women with breast cancer and 108,411 women without breast cancer". *Lancet* ; 350 (9084): 1047-1059.

Collaborative Group on Hormonal Factors in Breast Cancer (1996). "Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53,297 women with breast cancer and 100,239 women without breast cancer from 54 epidemiological studies". *Lancet*; 347 (9017): 1713-1727.

Collaborative Group on Hormonal Factors in Breast Cancer (2001). "Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease" *Lancet*; 358 (9291): 1389-1399.

Colombo MP, Trinchieri G (2002). "Interleukin-12 in anti-tumour immunity and immunotherapy". *Cytokine and Growth Factor Reviews*; 13(2): 155-168.

Coughlin CM, Salhany KE, Wysocka M, Aruga E, Kurzawa H *et al* (1998). "Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis". *Journal of Clinical Investigation*; 101(6):1441-1452.

D'Andrea A, Rengaraju M, Valiante NM, Chehimi M, Kubin M *et al* (1992). "Production of natural killer cell stimulatory factor by peripheral blood mononuclear cells". *Journal of Experimental Medicine*; 176(5): 1387-1398.

Davis MM, Bjorkman PJ (1988). "T-cell antigen receptor genes and T-cell recognition". *Nature* ; 334 (6181): 395-402.

Del Prete G, De Carli M, Almerigogna F, Giudizi M, Biagiotti R *et al* (1993). "Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *Journal of Immunology*; 150: 353-356.

De Paola F, Ridolfi R, Riccobon A, Flamini E, Barazanti F *et al* (2003). "Restored T cell activation mechanisms in human tumour infiltrating lymphocytes from melanomas and colorectal carcinomas after exposure to interleukin-2". *British Journal of Cancer*; 88(2): 320-326.

De Waal Malefyt R, Yssel H, de Vries J (1993). "Direct effects of IL-10 on subsets of human CD4⁺T cell clones and resting T cells. Specific inhibition of IL-2 production". *Journal of Immunology*; 150: 4754-4765.

Derynck, R, Jarrett, JA, Chen, EY, Eaton DH, Bell JR *et al* (1985). "Human transforming growth factor- β c DNA sequence and expression in tumour cell lines". *Nature*; 316 (6030): 701-705.

Di Carlo E, Rovero S, Boggio K, Quaglino E, Amici A *et al* (2001). "Inhibition of mammary carcinogenesis by systemic interleukin-12 or p185neu DNA vaccination in Her-2/*neu* transgenic BALB/c mice". *Clinical Cancer Research*; 7(3S): 830s-837s.

- Dixon JM (2001). "Hormone replacement therapy and the breast". *British Medical Journal*; 323 (7326): 1381-1382.
- Dorsey R, Kundu N, Yang Q, Tannenbaum CS, Sun H *et al* (2002). "Immunotherapy with interleukin-10 depends on the CXC chemokines inducible protein-10 and monokine induced by IFN-gamma". *Cancer Research*; 62(9): 2606-2610.
- Dranoff G (2004). "Cytokines in cancer pathogenesis and cancer therapy". *Nature Reviews Cancer*; 4 :11-22.
- Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002). "Cancer immunoediting: from immunosurveillance to tumour escape". *Nature Immunology*; 3(11): 991-998.
- Early Breast Cancer Trialist's Collaborative Group (1998a). "Tamoxifen for early breast cancer: an overview of the randomised trials". *Lancet*; 351: 1461-1467.
- Early Breast Cancer Trialist's Collaborative Group (2005). "Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials". *Lancet*; 365: 1686-1717.
- Early Breast Cancer Trialist's Collaborative Group (2000). "Favourable and unfavourable effects on long-term survival of radiotherapy for early breast cancer: an overview of the randomised trials". *Lancet*; 355: 1757-1770.
- Emery J, Lucassen A, Murphy M. (2001). "Common hereditary cancers and implications for primary care". *Lancet*; 358(9275): 56-63.
- Endogenous Hormones and Breast Cancer Collaborative Group (2002). "Endogenous sex hormones and breast cancer in post menopausal women: reanalysis of nine prospective studies". *Journal of the National Cancer Institute*; 94 (8): 606-616.
- Endogenous Hormones Breast Cancer Collaborative Group (2003). "Body Mass Index, Serum Sex Hormones, and Breast Cancer Risk in Post menopausal Women". *JNCI Cancer Spectrum*; 95 (16): 1218-1226.
- Ewertz M, Duffy SW, Adami HO, Kvale G, Lund E *et al* (1990). "Age at first birth, parity and risk of breast cancer: a meta-analysis of 8 studies from the Nordic countries". *International Journal of Cancer*; 46 (4): 597-603.
- Fentiman IS (2002). "Breast cancer in older women". *Breast Cancer Online*; 5(8).
- Feurerer M, Rocha M, Bai L, Umansky V, Solomayer EF *et al* (2001). "Enrichment of memory T cells and other profound immunological changes in the bone marrow from untreated breast cancer patients". *International Journal of Cancer* ; 92(1): 96-105.
- Finbloom D, Winestock K (1995). "IL-10 induces the tyrosine phosphorylation of tyk2 and jak1 and the differential assembly of STAT1 and STAT3 complexes in human T cells and monocytes". *Journal of Immunology*; 155: 1079-1090.

- Fink MP (2001). "The role of cytokines as mediators of the inflammatory response". Townsend CM, Beauchamp RD, Evers BM, Mattox KL; Sabiston textbook of surgery 16th edition: WB Saunders: 28-30.
- Ford D, Easton DF, Stratton M, Narod S, Goldgar D *et al.* (1998). "Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families". American Journal of Human Genetics; 62(3): 676-689.
- Fortis C, Foppli M, Gianotti L, Galli L, Citterio G *et al* (1996). "Increased IL-10 serum levels in patients with solid tumours". Cancer Letters; 104(1): 1-5.
- Fujieda S, Sunaga H, Tsuzuki H, Fan GK, Saito H (1999). "IL-10 expression is associated with the expression of platelet derived endothelial cell growth factor and prognosis in oral and oropharyngeal carcinoma". Cancer letters ; 136(1): 1-9.
- Gaffen SL, Liu KD (2004). "Overview of interleukin-2 function, production and clinical applications". Cytokine; 28(3): 109-123.
- Gastl GA, Abrams JS, Nanus DM, Oosterkamp, Silber J *et al* (1993). "Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression". International Journal of Cancer ; 55(1): 96-101.
- Gastl G, Niederwiser D, Marth C, Huber H, Egg DE *et al* (1984). "Human large granular lymphocytes and their relationship to natural killer cell activity in various diseases states". Blood ; 64(1): 288-295.
- Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L *et al* (1998). "The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses". Annual review of immunology; 16: 495-521.
- Germann T, Rude E (1995). "Interleukin-12". International archives of allergy and immunology; 108(2): 103-112.
- Giannopoulos A, Constantinides C, Fokaeas E, Stravodimos C, Giannopoulou M *et al* (2003). "The immunomodulating effect of interferon-gamma intravesical instillations in preventing bladder cancer recurrence". Clinical Cancer Research; 9(15): 5550-5558.
- Gimmi CD, Morrison BW, Mainprice BA, Gribben JG, Boussiotis VA *et al* (1996). "Breast cancer-associated antigen, DF3/MUC1, induces apoptosis of activated human T cells". Nature Medicine; 2(12): 1367-1370.
- Goedegebuure PS, Eberlein TJ (2001). "Tumour biology and tumour markers". Townsend CM, Beauchamp RD, Evers BM, Mattox KL; Sabiston textbook of surgery 16th edition: WB Saunders : 475-476.
- Gollob JA, Mier JW, Veenstra K, McDermott DF, Clancy D *et al* (2000). "Phase I trial of twice weekly intravenous interleukin-12 in patients with metastatic renal cell cancer

or malignant melanoma: ability to maintain IFN-gamma induction is associated with clinical response". *Clinical Cancer Research*; 6:1678-1692.

Gunel N, Coskun U, Sancak B, Hasdemir O, Sare M *et al* (2003). "Prognostic value of serum IL-18 and nitric oxide activity in breast cancer patients at operable stage". *American Journal of Clinical Oncology*; 26(4): 416-421.

Gunel N, Coskun U, Sancak B, Gunel U, Hasdemir O, Bozkurt S (2002). "Clinical importance of serum interleukin-18 and nitric oxide activities in breast carcinoma patients". *Cancer*; 95(3): 663-667.

Haden JW (2003). "Immunodeficiency and cancer : prospects for correction". *International Immunopharmacology*; 3(8): 1061-1071.

Hamajima N, Hirose K, Tajima K, Rohan T, Calle EE *et al* (2002). "Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease". *British Journal of Cancer*; 87 (11): 1234-1245.

Hattori E, Okumoto K, Adachi T, Takeda T, Ito J *et al* (2003). "Possible contribution of circulating IL-10 to anti tumour immunity and prognosis in patients with unresectable HCC". *Hepatology research*; 27(4): 309-314.

Heidecke CD, Weighardt H, Feith M, Fink U, Zimmermann F *et al* (2002). "Neoadjuvant treatment of oesophageal cancer; immunosuppression following radiochemotherapy". *Surgery* ; 132(3): 495-501.

Heimdal JH, Aarstad HJ, Klemetsen B, Olofsson J (1999). "Peripheral blood monocuclear cell responsiveness in patients with head and neck cancer in relation to tumour stage and prognosis". *Acta Oto-Otolaryngologica*; 119(2): 281-284.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F (2003). "Principles of interleukin (IL)-6-type cytokine signalling and its regulation". *Biochemical Journal*; 374(1):1-20.

Heufler C, Koch F, Stanzl U, Topar G, Wysocka M *et al* (1996). "Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells". *European Journal of Immunology*; 26(3): 659-668.

Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y *et al* (1986). "Complimentary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin". *Nature*; 324(6092): 73-76.

Ho AS, Liu Y, Khan TA, Hsu DH, Bazan JF, Moore KW (1993). "A receptor for interleukin 10 is related to interferon receptors ". *Proceedings of the National Academy of Sciences of the United States of America*; 90:11267-11271.

Holland G and Zlotnik A (1993). "Interleukin-10 and Cancer". *Cancer Investigation*; 11(6): 751-758.

Howe GR, Hirohata T, Hislop TG, Iscovich JM, Yuan JM *et al.* (1990). "Dietary factors and risk of breast cancer: combined analysis of 12 case-control studies". *Journal of the National Cancer Institute*; 82 (7): 561-569.

<http://www.show.scot.nhs.uk/isd/cancer/cancer.html> 2004 ISD Online. Information and Statistics Division, NHS Scotland [Accessed: 20/06/06].

http://www.breastcancer.org/pathology_intro.html [Accessed : 20/06/06].

http://www.breastcancer.org/is_cancer_invasive.html [Accessed : 20/06/06].

http://www.breastcancer.org/testing_images.html [Accessed on 26/06/06].

<http://www.cancerresearchuk.org.html> Breast Cancer facts and statistics, Cancer Research UK [Accessed : 26/06/06].

<http://www-medlib.med.utah.edu/WebPath/BRESHTML/BREST020.html> [Accessed : 20/06/06].

<http://www-medlib.med.utah.edu/WebPath/BRESHTML/BREST008.html> [Accessed : 20/06/06].

<http://www-medlib.med.utah.edu/WebPath/BRESHTML/BREST011.html> [Accessed : 20/06/06].

Hunter DJ, Willett WC (1993). "Diet, body size, and breast cancer". *Epidemiologic Reviews*; 15 (1): 110-132.

Hunter DJ, Spiegelman D, Adami HO, Beeson L, can den Brandt PA *et al.* (1996). "Cohort studies of fat intake and the risk of breast cancer - a pooled analysis". *New England Journal of Medicine*; 334 (6): 356-361.

Hurteau JA, Blessing JA, De Cesare SL, Creasman WT (2001). "Evaluation of recombinant human interleukin-12 in patients with recurrent or refractory ovarian cancer: a gynaecologic oncology group study". *Gynecologic Oncology*; 82(1): 7-10.

Iglehart JD, Kaelin CM (2001). "Diseases of the breast". Townsend CM, Beauchamp RD, Evers BM, Mattox KL; Sabiston textbook of surgery 16th edition: WB Saunders : 563.

Iglehart JD, Kaelin CM (2001). "Diseases of the breast". Townsend CM, Beauchamp RD, Evers BM, Mattox KL; Sabiston textbook of surgery 16th edition: WB Saunders : 566.

Iglehart JD, Kaelin CM (2001). "Diseases of the breast". Townsend CM, Beauchamp RD, Evers BM, Mattox KL; Sabiston textbook of surgery 16th edition: WB Saunders : 570.

Iglehart JD, Kaelin CM (2001). "Diseases of the breast". Townsend CM, Beauchamp RD, Evers BM, Mattox KL; Sabiston textbook of surgery 16th edition: WB Saunders : 572-573.

Ivanovic V, Todorovic-Rakovic N, Demajo M, Neskovic-Konstantinovic Z, Subota V *et al* (2003). "Elevated plasma levels of transforming growth factor-beta 1 (TGF-beta 1) in patients with advanced breast cancer: association with disease progression". *European Journal of Cancer*; 39(4): 454-461.

Iwasaki T, Yamashita K, Tsujimura T, Kashiwamura S, Tsutsui H *et al* (2002). "Interleukin-18 inhibits osteolytic bone metastasis by human lung cancer cells possibly through suppression of osteoclastic bone-resorption in nude mice". *Journal of Immunotherapy*; 25 Suppl 1: S52-60.

Jablonska E (1998). "Serum levels of tumor necrosis factor alpha and production of this cytokine by polymorphonuclear cells in breast cancer patients". *Archivum Immunologiae et Therapiae Experimentalis*; 46(2): 93-96.

Jess P, Schultz K, Bendtzen K, Nielsen OH (2000). "Systemic inflammatory responses during laparoscopic and open inguinal hernia repair: a randomised prospective study". *European Journal of Surgery* ; 166(7): 540-544.

Jinquan T, Quan S, Jacobi H, Madsen H, Glue C *et al* (2000). "CXC chemokine receptor 4 expression and stromal cell-derived factor 1 alpha-induced chemotaxis in CD4+T lymphocytes are regulated by interleukin-4 and interleukin-10". *Immunology*; 99: 402-410.

Jinquan TCL, Gesser B, Matsushima K, Thestrup-Pedersen K (1993). "Human interleukin-10 is a chemoattractant for CD8+ T lymphocytes and an inhibitor of IL-8 induced CD4+ T lymphocyte migration". *Journal of Immunology*; 151: 4545-4551.

Jonuleit H, Schmitt E, Steinbrink K, Enk AH (2001). "Dendritic cells as a tool to induce anergic and regulatory T cells". *Trends in Immunology*; 22(7): 394-400.

Ju DW, Tao Q, Lou G, Bai M, He L *et al* (2001). "Interleukin 18 transfection enhances antitumor immunity induced by dendritic cell-tumor cell conjugates". *Cancer Research*; 61(9): 3735-3740.

Kamamura Y, Takahashi K, Komaki K, Monden Y (1998). "Effects of interferon-alpha and gamma on development of LAK activity from mononuclear cells in breast cancer patients". *Journal of Medical Investigation*; 45(1-4): 71-75.

Kang WK, Park C, Yoon HL, Kim WS, Yoon SS *et al* (2001). "Interleukin-12 gene therapy of cancer by peritumoural injection of transduced autologous fibroblasts: outcome of phase I study". *Human Gene Therapy*; 12(6): 671-684.

Khong HT and Restifo NP (2002). "Natural selection of tumour variants in the generation of tumour escape phenotypes". *Nature Immunology*; 3(11): 999-1005.

Kim J, Modlin RL, Moy RL, Dubinett SM, McHugh T *et al* (1995). "IL-10 production in cutaneous basal squamous cell carcinomas". *Journal of Immunology*; 155(4): 2240-2247.

- Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC *et al* (1989). "Identification and purification of natural killer cell stimulatory factor, a cytokine with multiple biologic effects on human lymphocytes". *Journal of Experimental Medicine*; 170(3): 827-845.
- Kobie JJ, Wu RS, Kurt RA, Lou S, Adelman MK *et al* (2003). "Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines". *Cancer Research*; 63(8):1860-1864.
- Koch F, Stanzl U, Jennewein P, Janke K, Heufler C *et al* (1996). "High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10". *Journal of Experimental Medicine*; 184:741-746.
- Kovacs E (2001). "The serum levels of IL-12 and IL-16 in cancer patients: relation to the tumour stage and previous therapy". *Biomedicine & Pharmacotherapy*; 55(2); 111-116.
- Kozlowski L, Zakrzewska I, Tokajuk P, Wojtukiewicz MZ (2003). "Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients". *Roczniki Akademii Medycznej W Bialymstoku*; 48: 82-84.
- Kriegler M, Perez C, DeFay K, Albert I, Lu SD (1988). "A novel form of TNF/Cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF". *Cell*; 53(1): 45-53.
- Kruger-krasagakes S, Krasagakis K, Garbe C, Schmitt E, Huls C *et al* (1994). "Expression of IL-10 in human melanoma". *British Journal of Cancer*; 70(6): 1182-1185.
- Kundu N, Beaty TL, Jackson M, Fulton AM (1996). "Antimetastatic and anti tumour activities of IL-10 in a murine model of breast cancer". *Journal of the National Cancer Institute*; 88(8): 536-541
- Kundu N and Fulton AM (1997). "Interleukin-10 inhibits tumor metastasis, downregulates MHC class I and enhances NK lysis". *Cellular immunology* ; 180(1): 55-61.
- Lathers DMR, Achille NJ, Young RI (2003). "Incomplete Th2 skewing of cytokines in plasma of patients with squamous cell carcinoma of the head and neck". *Human Immunology* ; 64(12): 1160-1166.
- Lauw FN, Pajkrt D, Hack CE, Kurimoto M, van Deventer SJ, van der Poll T (2000). "Pro inflammatory effects of IL-10 during human endotoxaemia". *Journal of Immunology*; 165(5): 2783-2789.

- Lawlor DA, Okasha M, Gunnell D, Smith GD, Ebrahim S (2003). "Associations of adult measures of childhood growth with breast cancer: findings from the British Women's Heart and Health Study". *British Journal of Cancer*; 89 (1): 81-87.
- Lebel-Binay S, Berger A, Zinzindohoue F, Cugnenc P, Thiounn N *et al* (2000). "Interleukin-18: biological properties and clinical implications". *European Cytokine Network*; 11(1):15-26.
- Lee P, Wang F, Kuniyoshi J, Rubiyo V, Stuges T *et al* (2001). "Effects of interleukin-12 on the immun response to a multi-peptide vaccine for resected metastatic melanoma". *Journal of Clinical Oncology*; 19(18): 3836-3847.
- Lee J, Fenton BM, Koch CJ, Frelinger JG, Lord EM (1998). "Interleukin 2 expression by tumor cells alters both the immune response and the tumor microenvironment". *Cancer Research*; 58(7):1478-1485.
- Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL (1996). "Association of macrophage infiltration with angiogenesis and prognosis in invasive carcinoma". *Cancer Research*; 56(20): 4625-4629.
- Leland P, Taguchi J, Husain SR, Kreitman RJ, Pastan I, Puri RK (2000). "Human breast carcinoma cells express type II IL-4 receptors and are sensitive to antitumor activity of a chimeric IL-4-Pseudomonas exotoxin fusion protein *in vitro* and *in vivo*". *Molecular Medicine*; 6(3): 165-178.
- Lin Y, Huang R, Chen L, Li S, Shi Q *et al* (2004). "Identification of interleukin-8 as estrogen receptor-regulated factor involved in breast cancer invasion and angiogenesis by protein arrays". *International Journal of Cancer*; 109(4): 507-515.
- Lissoni P, Mengo S, Mandala M, Mauri E, Brivio F *et al* (1998). "Physiopathology of IL-12 in human solid neoplasms; blood levels of IL-12 in early or advanced cancer patients and their variations with surgery and immunotherapy". *Journal of Biological Regulators & Homeostatic Agents* ; 12(1-2): 38-41.
- Lissoni P, Rovelli F, Pittalis S, Casati M, Perego MS *et al* (1997). "IL-12 in early or advanced cancer patients". *European Journal of Cancer* ; 33(10): 1703-1705.
- Luft T, Jefford M, Luetjens P, Hochrein H, Masterman KA *et al* (2002). "IL-1beta enhances CD40 ligand-mediated cytokine secretion by human dendritic cells (DC): a mechanism for T cell-independent DC activation". *Journal of Immunology*; 168(2): 713-722.
- Luo JS, Kammerer R, von Kleist S (2000). "Comparison of the effects of immunosuppressive factors from newly established colon carcinoma cell cultures on human lymphocyte proliferation and cytokine secretion". *Tumour Biology*; 21(1):11-20.
- Mann EA, Spiro JD, Chen LL, Kreutzer DL (1992). "Cytokine expression by head and neck squamous cell carcinomas". *American Journal of Surgery*; 164(6): 567-573.

- Manna PP, Mohanakumar T (2002). "Human dendritic cell mediated cytotoxicity against breast carcinoma cells *in vitro*". *Journal of Leukocyte Biology*; 72(2): 312-320.
- Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S (2000). "Escape of human solid tumours from T-cell recognition: molecular mechanisms and functional significance". *Advances in Immunology*; 74: 181-273.
- McIntosh, A., Shaw, C., Evans, G *et al* (2004). "Clinical guidelines and evidence review for the classification and care of women at risk of familial breast cancer". National Collaborating Centre for Primary Care/University of Sheffield. www.nice.org.uk [Accessed: 20/07/06].
- McKenzie T, Liu Y, Fanale M, Swisher SG, Chada S, Hunt KK (2004). "Combination therapy of Ad-mda7 and trastuzumab increases cell death in Her-2/neu-overexpressing breast cancer cells". *Surgery*; 136(2): 437-442.
- Merendino RA, Arena A, Capozza AB, Chillemi S, Mesiti M (1996). "Serum levels of interleukin-10 in patients affected by breast cancer". *Immunology letters* ; 53: 59-60.
- Merendino RA, Gangemi S, Misefari A, Arena A, Capozza AB *et al*(1999). "Interleukin-12 and interleukin-10 production by mononuclear phagocytic cells from breast cancer patients". *Immunology Letters*; 68(2-3) : 355-358.
- Merville P, Rousser F, Banchereau, Klein B, Betaillie R (1992). "Serum IL-10 in early stage multiple myeloma". *Lancet* ; 340(8834-8835): 1544-1545.
- Mocellin S, Marincola F, Rossi CR, Nitti D, Lise M (2004). "The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle". *Cytokine & Growth Factor Reviews*; 15(1): 61-76.
- Mocellin S, Wang E, Marincola FM (2001). "Cytokines and immune response in the tumour microenvironment". *Journal of Immunotherapy*; 24(5): 392-407.
- Moiseeva EV, Merkulova IB, Bijleveld C, Koten JW, Miroshnikov AI, Den Otter W (2003). "Therapeutic effect of a single peritumoural dose of IL-2 on transplanted murine breast cancer". *Cancer Immunology and Immunotherapy*; 52(8) :487-496.
- Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001). "Interleukin-10 and the interleukin-10 receptor". *Annual Review of Immunology*; 19: 683-765.
- Morrow M, Schnitt SJ (2000). "Lobular carcinoma in situ". In: Harris JR, Lippman ME, Morrow M, Osborne CK, eds. *Diseases of the Breast*, 2nd edition. Philadelphia: Lippincott, Williams and Wilkins: 377-382.
- Morrow M, Schnitt SJ, Harris JR (2000). "Ductal carcinoma in situ and microinvasive carcinoma". In: Harris JR, Lippman ME, Morrow M, Osborne CK, eds. *Diseases of the Breast*, 2nd edition. Philadelphia: Lippincott, Williams and Wilkins: 383-401.

Nabioullin R, Sone S, Mizuno K, Yano S, Nishioka Y *et al* (1994). "IL-10 is a potent inhibitor of tumour cytotoxicity by human monocytes and alveolar macrophages". *Journal of Leukocyte Biology*; 55(4): 437-442.

Nagai S, Toi M (2000). "Interleukin-4 and breast cancer". *Breast Cancer*; 7(3): 181-186.

Nakata A, Tsujimura T, Sugihara A, Okamura H, Iwasaki T *et al* (1999). "Inhibition by interleukin 18 of osteolytic bone metastasis by human breast cancer cells". *Anticancer Research*; 19(5B): 4131-4138.

Nakayama Y, Shibao K, Sako T, Ohta R, Onitsuka K, Hirata K *et al* (2000). "Serum levels of IL-12 in patients with gastrointestinal cancer". *Anticancer Research*; 20(1C): 635-640.

O'Connell J, Bennett MW, O'Sullivan GC, Collins JK, Shanahan F (1999). "The Fas counter attack: Cancer as a site of immune privilege". *Immunology Today* ; 20(1): 46-52.

O'Hara RJ, Greenman J, MacDonald AW, Gaskell KM, Topping KP *et al* (1998). "Advanced colorectal cancer is associated with impaired interleukin-12 and enhanced interleukin-10 production". *Clinical Cancer Research*; 4(8) : 1943-1948.

Ochsenbein A (2002). "Principles of tumour immunosurveillance and implications for immunotherapy". *Cancer Gene Therapy*; 9: 1043-1055.

Okamoto M, Ohe G, Oshikava T, Nishikava H, Furuichi S *et al* (2000). "Induction of cytokines and killer cell activities by cisplatin and 5-fluorouracil in head and neck cancer patients". *Anticancer Drugs* ; 11: 165-173.

Pedersen AE, Thorn M, Gad M, Walter MR, Johnsen HE *et al* (2005). "Phenotypic and functional characterization of clinical grade dendritic cells generated from patients with advanced breast cancer for therapeutic vaccination". *Scandinavian Journal of Immunology*; 61(2):147-56.

Petersson M, Charo J, Salazar-Onfray F, Noffz G, Mohaupt M *et al* (1998). "Constitutive IL-10 production accounts for the high NK sensitivity. Low MHC class I expression and poor transporter associated with antigen processing (TAP)-1/2 function in the prototype NK target YAC-1". *Journal of Immunology*; 161: 2099-2105.

Pisa P, Halapi E, Pisa EK, Gerdin E, Hising C *et al* (1992). "Selective expression of IL-10, IFN- γ and GM-CSF in ovarian cancer biopsies". *Proceedings of the National Academy of Sciences of the United States of America*; 89(16): 7708-7712

Poindexter NJ, Sahin A, Hunt KK, Grimm EA (2004). "Analysis of dendritic cells in tumour free and tumour containing sentinel lymph nodes from patients with breast cancer". *Breast Cancer Research* ; 6(4): 408-415.

- Pulaski BA, Smyth MJ, Ostrand-Rosenberg S (2002). "Interferon-gamma-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma". *Cancer Research*; 62(15): 4406-4412.
- Purohit A, Newman SP, Reed MP (2002). "The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer". *Breast Cancer Research*; 4(2): 65-69.
- Qin Z, Richter G, Schuler T, Ibe S, Cao X, Blankenstein T (1998). "B cells inhibit induction of T cell-dependent tumor immunity". *Nature Medicine* ; 4(5) :627-630.
- Reichert TE, Day R, Wagenr EM, Whiteside TL (1998). "Absent or low expression of the zeta chain in T cells at the tumour site correlates with poor survival in patients with oral carcinoma". *Cancer Research*; 58(23): 5344-5347.
- Repka T, Chiorean EG, Gay J, Herwig KE, Kohl VK *et al* (2003). "Trastuzumab and interleukin-2 in HER2-positive metastatic breast cancer: a pilot study". *Clinical Cancer Research*; 9(7): 2440-2446.
- Richter G, Kruger-Krasagakes S, Hein G, Huls C, Schmit E *et al* (1993). "IL-10 transfected into Chinese hamster ovary cells prevents tumour growth and macrophage infiltration". *Cancer Research*; 53(18): 4134-4137.
- Roberts AB, Sporn MB (1985). "Transforming growth factors". *Cancer Survey*; 4(4): 683-705.
- Roda-Navarro P, Arce I, Renedo M, Montgomery K, Kucherlapati R, Fenandez-Ruiz E (2000). "Human KLRP1, a novel member of the killer cell lectin-like receptor gene family: Molecular characterization, genomic structure, physical mapping to the NK gene complex and expression analysis". *European Journal of Immunology*; 30(2): 568-576.
- Rohrer JW, Coggin Jr JH (1995). "CD8 T cell clones inhibit anti tumour T cell function by secreting IL-10". *Journal of Immunology*; 155: 5719-5727.
- Rook AH, Wood GS, Yoo EK, Elenitsas R, Kao DM *et al* (1999). "Interleukin-12 therapy of cutaneous T-cell lymphoma induces lesion regression and cytotoxic T-cell responses". *Blood*; 94(3): 902-908.
- Rose DP, Boyar AP, Wynder EL (1986). "International comparisons of mortality rates for cancer of the breast, ovary, prostate, and colon, and per capita food consumption". *Cancer*; 58 (11): 2363-2371.
- Rosenberg SA, Yang JC, Restifo NP (2004). "Cancer immunotherapy: moving beyond current vaccines". *Nature Medicine*; 10(9): 909-915.
- Rousset F, Garcia E, Defrance T, Peronne C, Vezzio N, Hsu DH *et al* (1992). "Interleukin-10 is a potent growth and differentiation factor for activated human B lymphocytes". *Proceedings of the National Academy of Sciences of the United States of America*; 89(5): 1890-1893.

- Sabel MS, Skitzki J, Stoolman L, Eglilmez NK, Mathiowitz E *et al* (2004). "Intratumoral IL-12 and TNF-alpha-loaded microspheres lead to regression of breast cancer and systemic antitumor immunity". *Annals of Surgical Oncology*; 11(2):147-156.
- Sainsbury RC (2004). "The Breast". Russell RCG, Williams NS, Bulstrode CJK ; Bailey and Love's short practice of surgery 24th edition : Arnold : 824-826.
- Sainsbury RC (2004). "The Breast". Russell RCG, Williams NS, Bulstrode CJK ; Bailey and Love's short practice of surgery 24th edition : Arnold : 837.
- Sainsbury RC (2004). "The Breast". Russell RCG, Williams NS, Bulstrode CJK ; Bailey and Love's short practice of surgery 24th edition : Arnold : 840-842.
- Sainsbury RC (2004). "The Breast". Russell RCG, Williams NS, Bulstrode CJK ; Bailey and Love's short practice of surgery 24th edition : Arnold : 844.
- Sato T, McCue P, Masuoka K, Salwen S, Lattime EC *et al* (1996). "IL-10 production by human melanoma". *Clinical Cancer Research*; 2(8): 1383-1390
- Schirmacher V, Feuerer M, Beckhove P, Ahlert T, Umansky V (2002). "T cell memory, anergy and immunotherapy in breast cancer". *Journal of Mammary Gland Biology & Neoplasia* ; 7(2): 201-208.
- Schmid J, Weissmann C (1987). "Induction of mRNA for a serine protease and a β -thromboglobulin-like protein in mitogen-stimulated human leukocytes". *Journal of Immunology*; 139(1): 250-256.
- Schmitt E, Hoehn P, Hules C, Goedert S, Palm N *et al* (1994). "T helper type 1 development of naive CD4⁺ T cells requires the co-ordinate action of IL-12 and IFN- γ and is inhibited by TGF- β ". *European journal of immunology*; 24(4): 793-798.
- Schnitt SJ, Guidi AJ (2000). "Pathology of invasive breast cancer". In: Harris JR, Lippman ME, Morrow M, Osborne CK, eds. *Diseases of the Breast*, 2nd edition. Philadelphia: Lippincott, Williams and Wilkins: 425-470.
- Schreiber GH, Schreiber RD (2003). "Interferon- γ ". In Thomson AW, Lotze MT (eds). *The Cytokine Handbook*. London: Academic Press, 567-601.
- Seliger B, Cabrera T, Garrido F, Ferrone S (2002). "HLA class I antigen abnormalities and immune escape by malignant cells". *Seminars in Cancer Biology*; 12(1): 3-13.
- Shah AH and Lee C (2000). "TGF- β based immunotherapy for cancer: breaching the tumour firewall". *Prostate* ; 45(2): 167-172.
- Shak S (1999). "Overview of the trastuzumab (Herceptin) anti-Her2 monoclonal antibody clinical program in Her-2 overexpressing metastatic breast cancer". *Seminars in Oncology*; 26:71.

- Sheu BC, Lin RH, Lien HC, Ho HN, Hsu SM, Huang SC (2001). "Predominant Th2/Tc2 polarity of tumor infiltrating lymphocytes in human cervical cancer". *Journal of Immunology*; 167(5): 2972-2978.
- Shibata Y, Foster LA, Kurimoto M, Okamura H, Nakamura RM *et al* (1998). "Immunoregulatory roles of IL-10 in innate immunity: IL-10 inhibits macrophage production of IFN-gamma inducing factors but enhances NK cell production of IFN-gamma". *Journal of Immunology*; 161: 4283-4288.
- Shibuya TY, Wei WZ, Zormeier M, Ensley J, Sakr W *et al* (2000). "Anti CD3/anti CD28 bead stimulation overcomes CD3 unresponsiveness in patients with head and neck squamous cell carcinoma". *Archives of Otolaryngology - Head Neck Surgery*; 126(4): 473-479.
- Slamon DJ and Clarke GM (1988). "Amplification of c-erbB-2 and aggressive human breast tumours". *Science*; 240: 1795-1798.
- Smith DR, Kunkel SL, Burdick MD, Wilke CA, Orringer MB *et al* (1994). "Production of IL-10 by human bronchogenic carcinoma". *American Journal of Pathology*; 145(1): 18-25
- Smith-Warner SA, Spiegelman D, Adami HO, Beeson WL, van den Brandt PA *et al.*(2001). "Types of dietary fat and breast cancer: a pooled analysis of cohort studies". *International Journal of Cancer*; 92 (5): 767-774.
- Stasi R, Zinzani PL, Galienucci P, Lauta VM, Dmasio E *et al* (1994). "Detection of soluble IL-2 receptor and IL-10 in the serum of patients with aggressive non-hodgkins lymphoma". *Cancer*; 74: 1792-1800.
- Steele TA (2002). "Chemotherapy induced immunosuppression and reconstitution of immune function". *Leukemia Research*; 26(4): 411-414.
- Steinbrink K, Jonuleit H, Muller G, Schuler G, Knop J, Enk AH (1999). "Interleukin-10 treated human dendritic cells induce a melanoma-antigen specific anergy in CD8+ T cells resulting in a failure to lyse tumour cells". *Blood*; 93: 1634-1642.
- Stewart TH (1996). "Evidence for immune facilitation of breast cancer growth and for the immune promotion of oncogenesis in breast cancer". *Medicina* ; 56(S): 13-24.
- Stewart TH, Heppner GH (1997). "Immunological enhancement of breast cancer". *Parasitology* ; 115(S) : 141-153.
- Su ZZ, Madireddi MT, Lin JJ, HYoung CS, Kitada S *et al* (1998). "The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice". *Proceedings of the National Academy of Sciences of the United States of America*; 95(24):14400-14405.
- Sun H, Gutierrez P, Jackson MJ, Kundu N, Fulton AM (2000). "Essential role of nitric oxide and interferon-gamma for tumour immunotherapy with interleukin-10". *Journal of Immunotherapy*; 23(2): 208-214.

- Suzuki S, Mita S, Kamohara H, Skamoto K, Ishiko T, Ogawa M (2001). "IL-6 and IFN- γ regulation of IL-10 production by human colon carcinoma cells". *International Journal of Oncology*; 18(3): 581-586.
- Taga K, Mostowski H, Tosato G (1993). "Human interleukin-10 can directly inhibit T cell growth". *Blood*; 81: 2964-2971.
- Tanner JM (1973). "Trend towards earlier menarche in London, Oslo, Copenhagen, the Netherlands and Hungary". *Nature* ; 243 (5402): 95-96.
- Tao M, Li B, Nayini J, Sivaraman S, Song S, Larson A *et al* (2001). "In vivo effects of IL-4, IL-10 and amifostine on cytokine production in patients with acute myelogenous leukaemia". *Leukemia and Lymphoma*; 41(1-2): 161-168.
- Terres G, Coffman RL (1998). "The role of IL-4 and IL-10 cytokines in controlling an anti tumour response in vivo". *International Immunology*; 10(6): 823-832.
- Tetsuya Y, Tsuyoshi K, Eisaku U, Tatemoto Yukhiro T, Tokio O (2003). "Characteristic cytokine generation patterns in cancer cells and infiltrating lymphocytes in oral squamous cell carcinomas and the influence of chemoradiation combined with immunotherapy on these patterns". *Oncology*; 64(4): 407-415
- Thompson AM and Dewar JA (2002). "Disorders of the breast". Cuschieri A, Steele RJC, Moosa AR; *Essential Surgical Practice 4th edition*: Arnold : 71.
- Thompson AM and Dewar JA (2002). "Disorders of the breast". Cuschieri A, Steele RJC, Moosa AR; *Essential Surgical Practice 4th edition*: Arnold : 76.
- Thompson AM and Dewar JA (2002). "Disorders of the breast". Cuschieri A, Steele RJC, Moosa AR; *Essential Surgical Practice 4th edition*: Arnold : 84.
- Trinchieri G (1994). "IL-12: a cytokine produced by antigen presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes". *Blood*; 84: 4008-4027.
- Trinchieri G (1995). "Interleukin-12: a pro inflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen specific adaptive immunity". *Annual Review of Immunology*; 13: 251-276
- Trinchieri G, Wysocka M, D'Andrea A, Rengaraju M, Aste-Amezaga M *et al* (1992). "Natural killer cell stimulatory factor or IL-12 is a key regulator of immune response and inflammation". *Progress in Growth Factor Research*; 4(4): 355-368.
- Tsuge T, Yamakawa M, Tsukamoto M (2000). "Infiltrating dendritic/Langerhans cells in primary breast cancer". *Breast Cancer Research and Treatment*; 59(2):141-152.

van den Brandt PA, Spiegelman D, Yaun SS, Adami HO, Beeson L *et al* (2000). "Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk". *American Journal of Epidemiology*; 152 (6): 514-527.

Venetsanakos E, Beckman I, Bradley J, Skinner JM (1997). "High incidence of interleukin 10 mRNA but not interleukin 2 mRNA detected in human breast tumours". *British Journal of Cancer*; 75(12):1826-1830.

Vora M, Romero LI, Karasek MA (1996). "Interleukin-10 induces E-selectin on small and large blood vessel endothelial cells". *Journal of Experimental Medicine*; 184:821-829.

Wang H, Czura CJ, Tracey KJ (2003). "Tumor Necrosis Factor". In Thomson AW, Lotze MT (eds). *The Cytokine Handbook*. London: Academic Press, 837-860.

Wang P, Wu D, Siegel MI, Egan RW, Motasim Billah M (1994). "IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells". *Journal of Immunology*; 153: 811-816.

Weinberg A, Betensky RA, Zhang L, Ray G (1998). "Effect of shipment, storage, anticoagulant and cell separation on lymphocyte proliferation assays for Human Immunodeficiency Virus infected patients". *Clinical & Diagnostic Laboratory Immunology*; 5(6): 804-807.

Welsch CW (1992). "Relationship between dietary fat and experimental mammary tumorigenesis: a review and critique". *Cancer Research*; 52 (7S): 2040s-2048s.

Wolf SF, Temple PA, Kobayashi M, Young D, Dicig M *et al* (1991). "Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells". *Journal of Immunology*; 146(9): 3074-3081.

Wolfram RM, Budinsky AC, Brodowicz T, Kubista M, Kostler WJ *et al* (2000). "Defective antigen presentation resulting from impaired expression of costimulatory molecules in breast cancer". *International Journal of Cancer*; 88(2):239-244.

Woo EY, Chu CS, Goletz, Schlienger K, Yeh H *et al* (2001). "Regulatory CD4+CD25+T cells in tumours from patients with early stage non small cell lung cancer and late stage ovarian cancer". *Cancer Research*; 61(12): 4766-4772.

Wooster R, Bignee G, Lancaster J, Swift S, Seal S *et al.*(1995). "Identification of the breast cancer susceptibility gene BRCA2". *Nature*; 378 (6559): 789-792.

Wu CW, Wang SR, Chao MF, Wu TC, Lui WY *et al* (1996). "Serum interleukin-6 levels reflect disease status of gastric cancer". *American Journal of Gastroenterology*; 91(7): 1417-1422.

Xie KP (2001). "Interleukin-8 and human cancer biology". *Cytokine Growth Factor Reviews*; 12(4): 375-391.

Yamamura M, Modlin RL, Ohmen JD, Moy RL (1993). "Local expression of anti-inflammatory cytokines in cancer". *Journal of Clinical Investigation*; 91(3): 1005-1010.

Yanagawa H, Takeuchi E, Suzuki Y, Hanibuchi M, Haku T *et al* (1999). "Production of interleukin-10 by alveolar macrophages from lung cancer patients". *Respiratory medicine* ; 93(9): 666-671.

Yokoe T, Iino Y, Morishita Y (2000). "Trends of IL-6 and IL-8 levels in patients with recurrent breast cancer: preliminary report". *Breast Cancer*; 7(3):187-190.

Young MR, Wright MA, Pandit R (1997). "Myeloid differentiation treatment to diminish the presence of immune suppressive CD34+cells within human head and neck squamous cell carcinomas". *Journal of Immunology*; 159(2): 990-996.

Young MR, Wright MA, Lozano Y, Matthews JP, Benefield J, Prechel MM (1996). "Mechanisms of immune suppression in patients with head and neck cancer: influence on the immune infiltrate of the cancer". *International Journal of Cancer* ; 67(3): 333-338.

Yue FY, Dummer R., Geersten R, Hofbauer G, Laine E *et al* (1997). "Interleukin-10 is a growth factor for human melanoma cells and down regulates HLA class I, HLA class II and ICAM-1 molecules". *International Journal of Cancer*; 71(4):630-637.

Zakrzewska I, Kozlowski L, Wojtukiewicz M (2002). "Value of interleukin-8 determination in diagnosis of benign and malignant breast tumour". *Polski Merkuriusz Lekarski*; 13(76): 302-304.

Zielinski CC, Budinsky AC, Wagner TM, Woldfram RM, Kostler WJ *et al* (2003). "Defect of tumour necrosis factor-alpha (TNF-alpha) production and TNF-alpha-induced ICAM-1-expression in BRCA1 mutations carriers". *Breast Cancer Research and Treatment*; 81(2): 99-105.

Appendix

Primary tumour (P)

- T0** No evidence of primary tumour
- Tis** Carcinoma in situ: ductal carcinoma in situ, lobular carcinoma in situ, or Paget's disease of the nipple with no tumour
- T1** Tumour 2 cm or less in greatest dimension
- PTmic** Microinvasion 0.1 cm or less in greatest dimension
- T1a** Tumour more than 0.1 cm but not more than 0.5 cm in greatest dimension
- T1b** Tumour more than 0.5 cm but not more than 1 cm in greatest dimension
- T1c** Tumour more than 1 cm but not more than 2 cm in greatest dimension
- T2** Tumour more than 2 cm but no more than 5 cm in greatest dimension
- T3** Tumour more than 5 cm in greatest dimension
- T4** Tumour of any size with direct extension to (a) chest wall or (b) skin, only as described below
- T4a** Extension to chest wall
- T4b** Oedema (including peau d'orange) or ulceration of the skin of breast or satellite skin nodules confined to same breast
- T4c** Both T4a and T4b
- T4d** Inflammatory carcinoma

Regional lymph nodes (N)

- N0** No regional lymph node metastasis
- N1** Spread to movable ipsilateral axillary lymph nodes
- N2** Spread to ipsilateral axillary lymph node fixed to one another or to other structures
- N3** Spread to ipsilateral internal mammary lymph node(s)

Pathological classification (pN)

pN0	No regional lymph node metastasis
pN1	Metastasis to movable ipsilateral axillary lymph nodes
pN1a	Only micrometastasis (none larger than 0.2 cm)
pN1b	Metastasis to lymph nodes, any larger than 0.2 cm
pN2	Metastasis to ipsilateral axillary lymph nodes that are fixed to one another or to other structures
pN3	Metastasis to ipsilateral internal mammary lymph nodes

Distant metastasis (M)

M0	No distant metastasis
M1	Distant metastasis (including to ipsilateral supraclavicular lymphnodes)

Appendix 1. The 1999 TNM classification system (Iglehart and Kaelin, 2001).