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The Immune System and Breast Carcinoma: Implications of Dietary and Other Associated Factors

by

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A Dissertation Submitted in Partial Fulfillment of the Requirements of the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming to the required standard

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ABSTRACT

Introduction: A review of animal and human studies demonstrates that the immune system is a major factor in both the enhancement and inhibition of malignant tumour growth. Macrophages, one of the most durable and versatile immune cells, may be key to this immune duality. Macrophages have been observed in particularly high concentrations in and around breast tumours. It has been suggested that these cells generally aid tumour growth, unless activated by an acute infections, immunomodulators or other means.
Study I: Using immunohistochemistry and computer-aided image analysis, macrophage concentrations in and around breast tumours were examined. Other pathological tissues were also examined for comparative purposes. Macrophage density was found to correlate positively with the Modified Bloom Richardson (MBR) grade ($r = 0.41$) and MBR subscore ($r = 0.44$), suggesting that macrophage concentrations increase as tumours become more aggressive. Similar infiltrations of macrophages were observed in lung, prostate and hyperplastic thyroid tissues; although in these latter tissues, macrophages were generally confined to the tumour periphery.
Study II: Iodine has been shown to play many roles in normal human physiology. In addition to its incorporation into thyroid hormones, iodine also has antibiotic and anti-tumour properties. Epidemiological studies of iodine in breast cancer have not been conducted. In this pilot case-control study, whole blood levels of 10 trace elements (Br, Cr, Fe, I, Mb, Mg, Mn, Se, V, and Zn) and their association with breast cancer was investigated. Other general, medical and dietary characteristics were examined as well. In comparison with iodine levels in Japan, iodine levels in the population under study were considerably lower, with a mean of 28.4 μg/l and a range of 19-35 μg/l. In the univariate logistic regression analysis, a number of significant associations with breast cancer were observed. A high education status (OR = 0.31) and high iron status (OR = 0.15) were associated with reduced risks, whereas previous hysterectomy or ovariectomy was associated with an increased risk of breast cancer (OR = 3.64). In the adjusted multivariate analysis, a high iron status remained associated with a reduced risk (OR = 0.01) and a history of breast pain with an increased risk (OR = 11.25).

Conclusion: Understanding the duality of immune function, not only provides insight into cancer progression, but offers two primary avenues for treatment. First, one may down-regulate immune reparative activities, which aid tumour growth. This may be accomplished by using immunosuppressants. A second approach is to take advantage of the large population of tumour-associated immune cells, particularly macrophages, and stimulate these cells into their defensive activities. A wide variety of infectious agents may be used to stimulate this response. Finally, iodine is one immunomodulator that may be used to enhance immune activity for treatment, or alternatively, prevent tumour growth through long-term intake; unfortunately, blood iodine levels noted in this study would be too low to afford protection.

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ABBREVIATIONS USED

aFGF, acidic fibroblast growth factor
BBD, benign breast disease
BCG, bacillus Calmette Guérin
bFGF, basic fibroblast growth factor
BMI, body mass index
CI, confidence interval
CIAS, computed-aided image analysis system
CS, cellulose sulphate
CT, computed tomography
D, deiodinase
DAB, diaminobenzadine
DBPMAF, vitamin D3 binding protein-derived macrophage activating factor
DCIS, ductal carcinoma in situ
DMBA, dimethylbenz[*a*]anthracene
ECM, extracellular matrix
EGF, epidermal growth factor
EORTC, European Organization for Research and Treatment of Cancer
G-CSF, granulocyte colony-stimulating factor
GM-CSF, granulocyte-macrophage colony-stimulating factor
HAF, human angiogenic factor
HB-EGF, heparin-binding epidermal growth factor
HGF, hepatocyte growth factor
I-, iodide
I_2, diatomic iodine
IBD, inflammatory bowel disease
ICP-MS, inductively coupled mass spectrometry
IFN, interferon
IGF, insulin-like growth factor
IgG, immunoglobulin
IL, interleukin
LLC, Lewis lung carcinoma
MALT, mucosal-associated lymphoid tissue
MBR, modified Bloom-Richardson
MCP, macrophage chemotactic protein
M-CSF, macrophage colony-stimulating factor
MDSF, monocyte-derived scattering factor
m/e, mass to charge ratio
MEIA, microparticle enzyme immunoassay
MMP, matrix metalloproteinase
mRNA, messenger ribonucleic acid
NBT, nitro-blue tetrazolium
NCIC, National Cancer Institute of Canada
NHANES, National Health and Nutrition Examination Survey
NIS, sodium-iodide symporter

NK, natural killer
OI-, hypoiodite
OR, odds ratio
PBS, phosphate buffered saline
PCOS, polycystic ovary syndrome
PD-ECGF, platelet-derived endothelial cell growth factor
PDGF, platelet-derived growth factor
RDA, recommended daily allowance
RR, relative risk
RT-PCR, reverse transcriptase-polymerase chain reaction
SCN, thiocyanate
SD, Sprague-Dawley rats
SE, standard error
SEER, Surveillance Epidemiology End Result Registry
SMPBC, Screening Mammography Program of British Columbia
SSKI, superstaturated potassium iodide
T3, triiodothyronine
T4, thyroxine
TAM, tumour-associated macrophage
tPA, tissue type plasminogen activator
TGF, transforming growth factor
TNF, tumour necrosis factor
TP, thymidine phophorylase
TRH, thyrotropin-releasing hormone
TSH, thyroid-stimulating hormone
VEGF, vascular endothelial growth factor
VPF, vascular permeability factor
UIC, urinary iodine concentration
uPA, urokinase type plasminogen activator
WHO, World Health Organization

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GENERAL INTRODUCTION

In Canada, cancer is the leading cause of premature death, responsible for almost one-third of all potential years of life lost. The potential years of life lost due to cancer in Canada is approximately 895 000, whereas that due to heart disease is 610 000. Solid tumour carcinomas (*i.e.* cancers of epithelial cell origin) are by far the most common causes of cancer death (National Cancer Institute of Canada 2000). Lung cancer is now the most common cause of mortality from cancer in both men (69.5 per 100 000 annually) and women (36.2 per 100 000), followed by prostate cancer (29.7 per 100 000) for men and breast cancer (27.0 per 100 000) for women. The annual death rate for lung cancer has declined in the last ten years for men, while it has shown a progressively strong increase in women for more than 30 years. These variations are largely due to changes in smoking habits and could be prevented. The mortality rate for prostate cancer has increased gradually during the past 30 years. In contrast, the breast cancer mortality rate has changed little over the last 30 years, although a modest decline has been observed over the last ten years. Mortality rates for men and women in solid tumour carcinomas in Canada are illustrated in Figures 1 and 2.

The major causative factors for the development of breast carcinoma remain to be determined. Both incidence and mortality rates are known to vary considerably between different countries. However, immigration studies have suggested that there is a strong causative or protective role for dietary, lifestyle and/or environmental factors (Ziegler et al 1993). The significant temporal changes in breast cancer mortality over the last 50 years support such conclusions (Figure 3).

Figure 1. Age-adjusted mortality rates for major solid tumour carcinomas in males, Canada 2000. Adapted from NCIC, Canadian Cancer Statistics 2000.

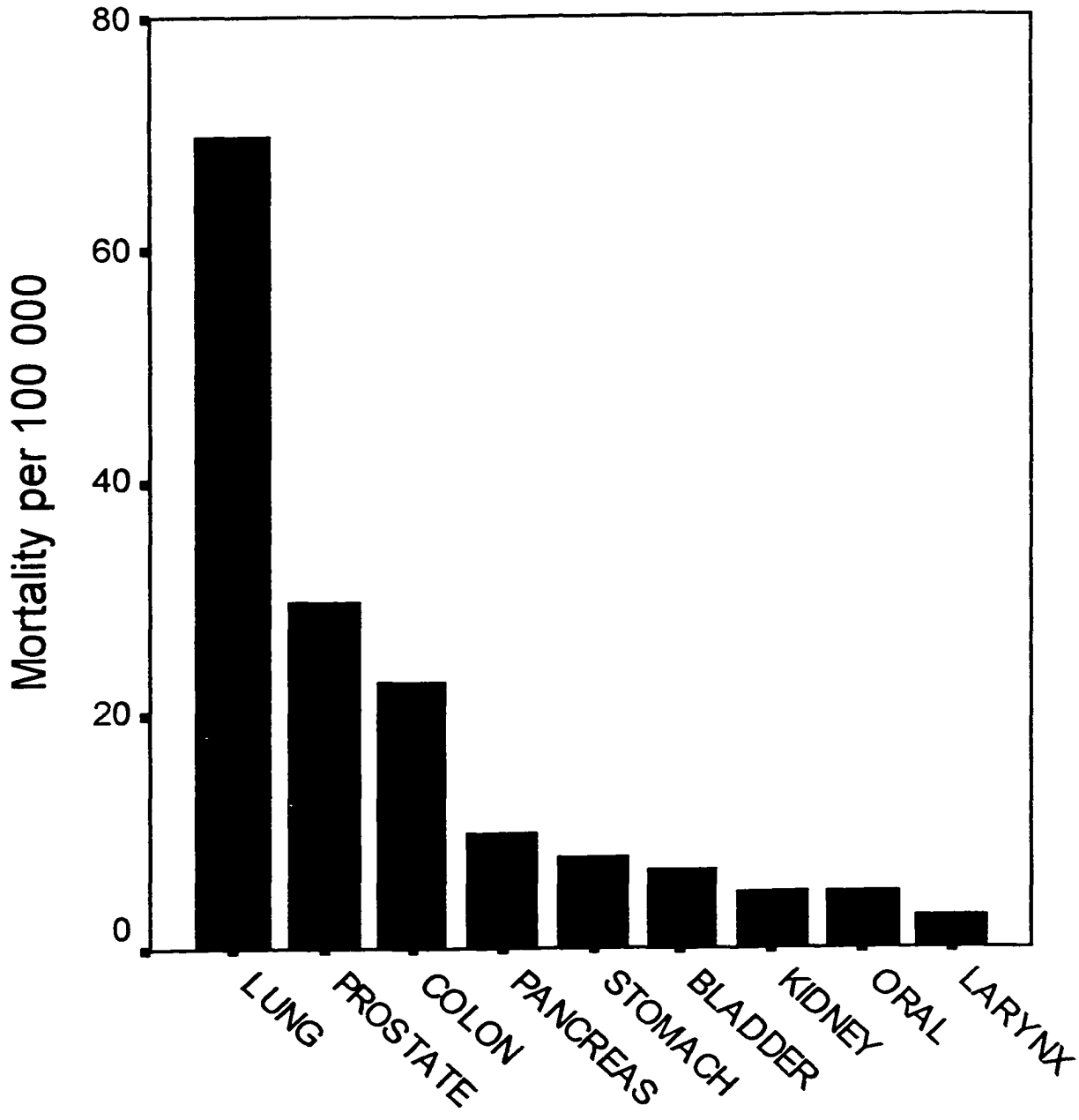


Figure 2. Age-adjusted mortality rates for major solid tumour carcinomas in females, Canada 2000. Adapted from NCIC, Canadian Cancer Statistics 2000.

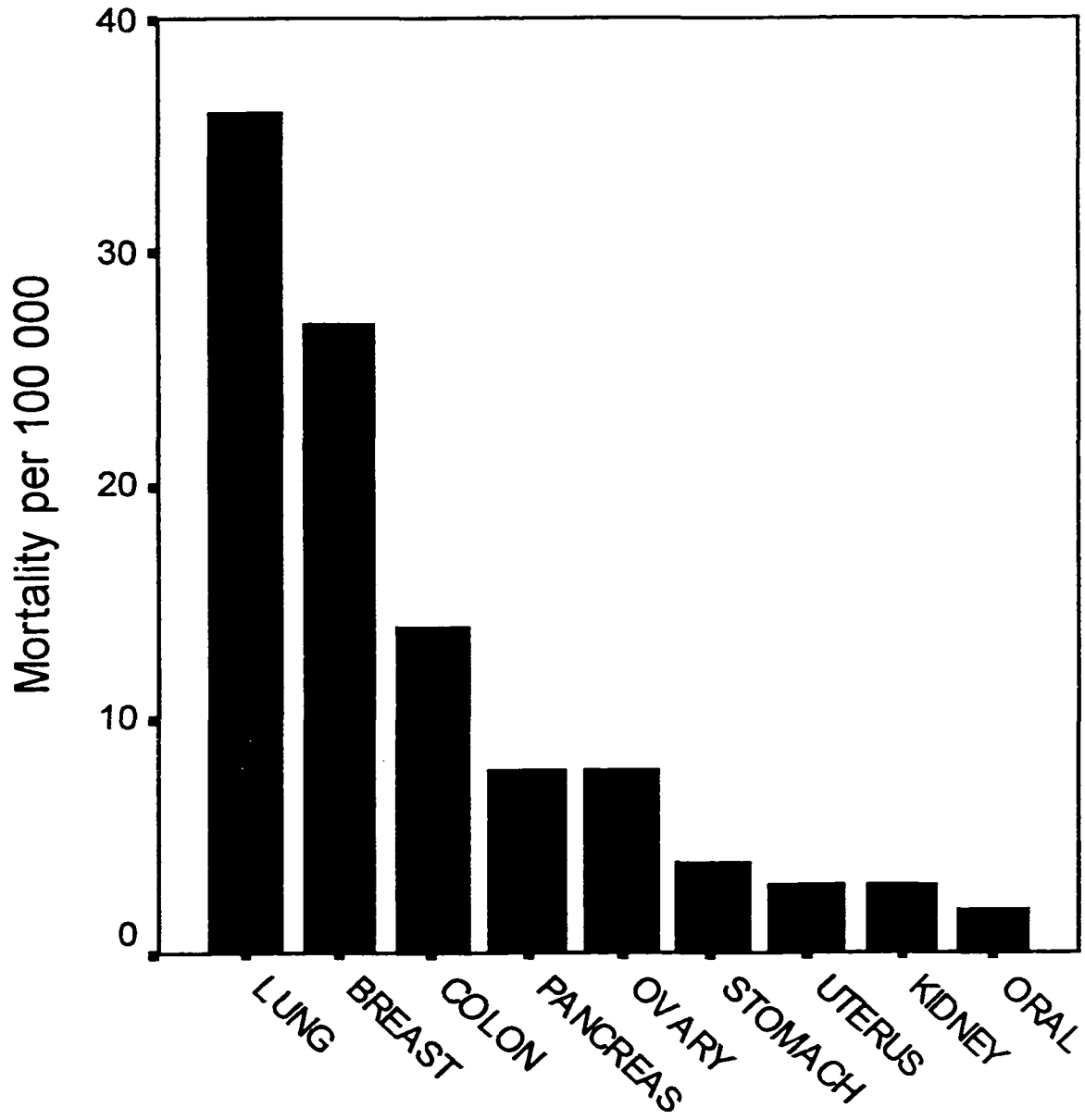
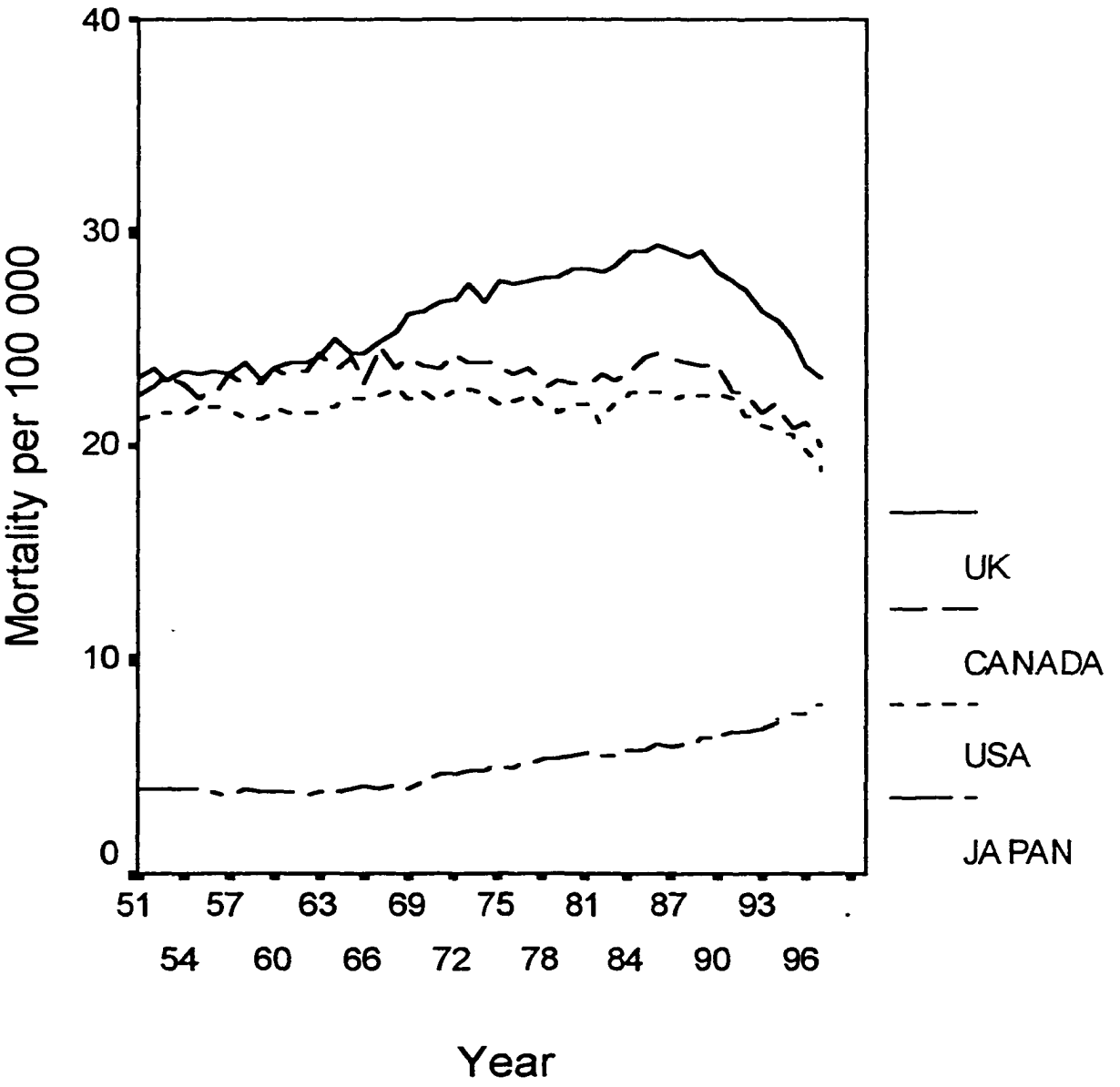


Figure 3. Age-adjusted breast cancer mortality rates in select western nations and Japan. It should be noted that the recent decline in breast cancer rates in the UK, USA and Canada all preceded the widespread introduction of mammography. Adapted from the WHO Cancer Mortality Databank 2000.



Aspects of breast cancer biology

Breast cancer is one of the most thoroughly studied cancers and provides a useful model for the development of solid tumours in general. The majority of breast cancers arise from epithelium of the terminal ducts or ductules. These terminal structures are the most biologically active areas in the breast and where milk is produced and stored during pregnancy and lactation. Breast cancers are believed to arise from several precursor conditions: hyperplastic tissue (high rate of proliferation, normal cellular morphology) that develops into atypical hyperplasia (rapid cell division, abnormal cellular morphology), and finally, into ductal carcinoma in situ (DCIS). DCIS refers to a stage where the terminal duct structures become filled with cancerous cells, but still have an unbroken basement membrane surrounding the tumour. DCIS is considered a carcinoma when this basement membrane has been breached, and is then called infiltrating DCIS.

Two key classification systems, tumour stage and grade, are used to describe the aggressiveness of these infiltrating breast cancers. In brief, tumour stage (Table 1) classifies cancer by size, nodal status (dispersion into regional lymph nodes), and metastasis (spreading beyond the breast or lymph nodes). Tumour grade (Table 1) classifies cancer by size, morphology and proliferation rate. These classification systems are used for prognostic information (*i.e.* predicting how advanced and aggressive a disease may be) and to guide the physician and patient in choosing the most appropriate treatment protocol. Other solid tumours have similar precursor stages and each have individualized grading and staging systems.

The stage at which breast cancer becomes a systemic disease has long been a matter of controversy (van Netten et al 1995). New techniques for detecting lymph node and

Table 1. Staging and grading of breast carcinomas.[a]

Stage	
I	Tumour less than 2 cm, no lymph node metastasis
II	Tumour more than 2 cm or lymph node involvement
III	Matting of lymph nodes, or skin, nipple or chest wall involvement
IV	Any distant metastasis
Grade	
I	Well differentiated (non-aggressive)
II	Moderately differentiated
III	Undifferentiated (highly aggressive)

a. A more detailed outline of staging and grading systems is presented in Appendix I.

distant metastasis have altered the current understanding of this disease. For example, lymph node metastasis has traditionally been analyzed by standard histochemical or immunohistochemical techniques. However, more recent advances in molecular biology, such as reverse transcriptase-polymerase chain reaction (RT-PCR), have been adapted for lymph node analysis and these techniques have significantly increased the number of patients who are diagnosed with positive lymph nodes. Molecular methods are based on the detection of either mutations in oncogenes and tumor suppressor genes (*e.g.* Ki-ras and p53 genes) or the mRNA expression of tissue-specific and tumor-associated genes. mRNA species targeted in these assays encode cytokeratins, prostate-specific antigen, prostate-specific membrane antigen, carcinoembryonic antigen, and polymorphic-epithelial mucin. This work has demonstrated that the spreading of this disease, in its early stages, is more common than previously recognized. These changes also increase the number of patients that will receive more aggressive treatment — although it is uncertain whether such patients will benefit from these changes. The use of bone marrow biopsies is another advance that has increased the sensitivity of detecting metastases. In a study by Diel and colleagues (1996), women undergoing breast cancer surgery also received a bone marrow biopsy at two sites on each anterior iliac crest. Surprisingly, as many as 30% of patients with stage I and 42% of patients with stage II cancers had positive bone marrow biopsies. Stage I tumours are generally considered non-aggressive, and the fact that only two needle biopsies per patient could detect metastases in such a high number of patients, suggests that a more thorough examination would likely reveal positive metastases in the majority of such patients. Analogous studies conducted in patients with lung and prostate cancer have also found evidence of an early dissemination of disease (Pantel et al 1995, Pantel et al 1996). If the majority of early

stage cancer patients already have distant metastases, one must ask what this means with respect to treatment? Unfortunately, whereas such advances in medical research and technology have increased our diagnostic capabilities, only minimal gains have been observed in overall patient survival.

The dual role of the immune system

Injury stimulates the chain of events in the wound healing process (Clark et al 1976, Dvorak 1986, Wilson 1997). Platelets initiate the injury-induced growth-factor response, and in turn, these cytokines attract inflammatory cells, fibroblasts, endothelial and other cell types involved in wound healing at the site of trauma. These infiltrating cells can then be stimulated to proliferate and produce other growth factors and cytokines that mediate the healing process. Thus, the types and concentrations of cytokines, as well as, the types of cells in the wound, are in a dynamic state during the wound healing process. In general, normal wound healing can be divided into three consecutive phases: (1) hemostasis and inflammation (days 0 to 3 after injury); (2) re-epithelialization and granulation (days 3 to 14); and (3) scar tissue remodeling (days 7 to 30 after injury). However, cancer cells express similar receptors and respond to the same factors as do the body's natural epithelial cells during tissue injury. In fact, it would be surprising if neoplasms were not affected by the immune-stimulating pathways of wound healing during their stages of growth and invasion.

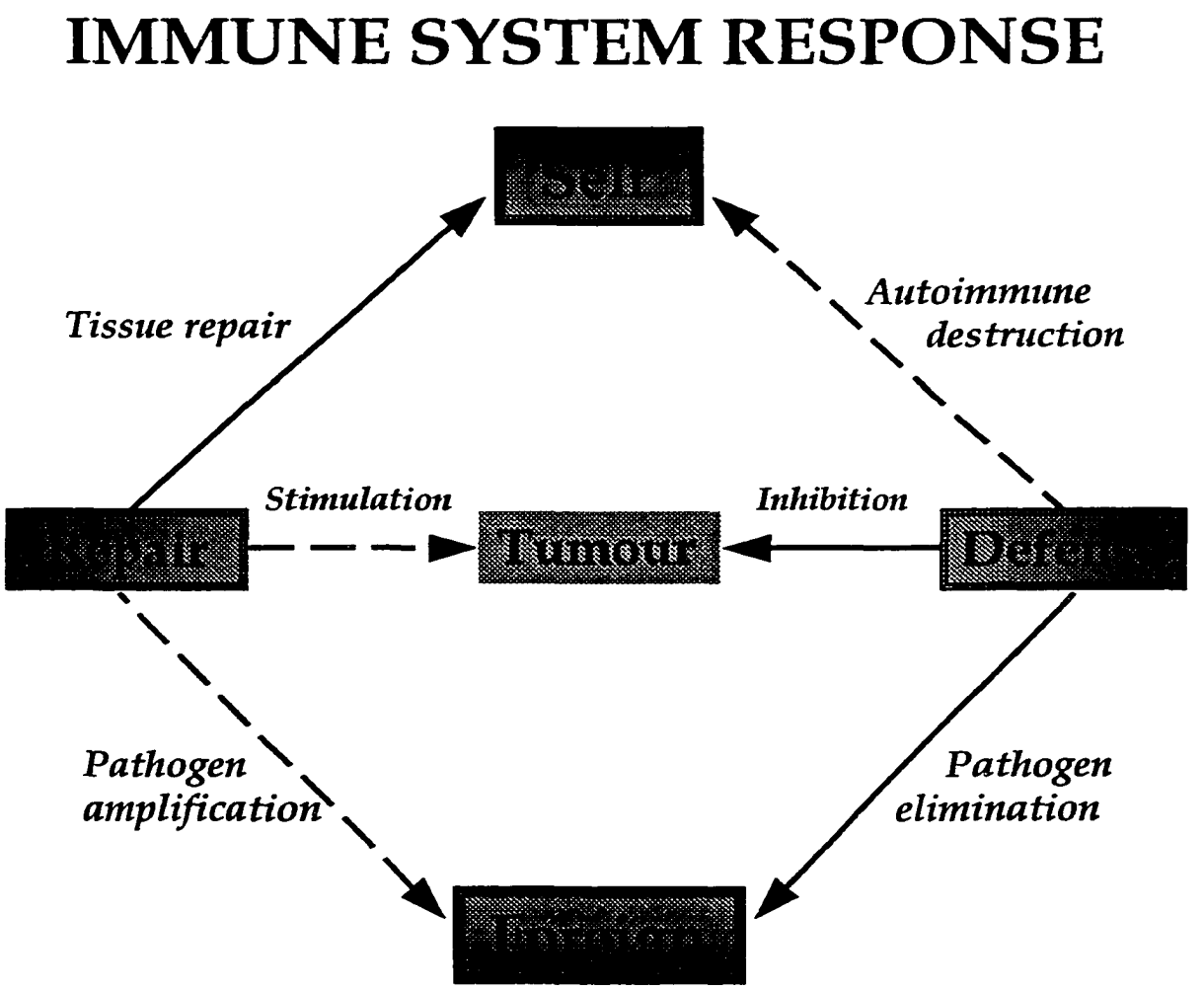
Our work suggests that the immune system plays two key roles in the body: *defense* and *repair* (Oleszczuk et al 1994, van Netten et al 1999). The well-studied *defensive* role becomes active during infection, where cytotoxic cells seek out and destroy invading pathogens. Although the *reparative* process is also well-known, less emphasis has been

placed on the role the immune system plays in mediating this process. We believe that these roles are intimately linked and often occur simultaneously. When a tumour arises (in many respects part foreign and part self), the relative balance of these two arms will determine its outcome. If the defensive arm predominates the tumour will regress, yet if the reparative arm prevails it will progress. An illustration of this duality in function is presented in Figure 4.

In many respects, tumours are analogous to unhealing wounds (Haddow 1972). This is due to the similar behaviour seen in the activities of neoplastic cells and of those cells involved in normal wound healing (Dvorak 1986). Normal epithelium and inflammatory cells (leukocytes) may transiently develop the attributes of tumour tissue during the wound healing process: enhanced cellular proliferation, chemotactic and invasive activities; increased production of matrix-degrading proteases and motility factors; and the production of vasogenic (blood/lymphatic vessel) inducing factors (Whalen 1990, Cann et al 1995b).

As an example, the duality of immune system functions can be seen in the role played by macrophages during fertilized egg implantation in the uterus. Macrophages are among the most common bone marrow-derived cells in the human decidua (*i.e.* the uterine mucous membrane), being found near the implantation site (Hunt and Pollard 1992) and near allogeneic trophoblasts at early stages of pregnancy (Beer and Billingham 1974). Grafts to the pregnant uterus survive much longer than grafts in other locations, favouring a local immune-suppressive rather than immune-enhancing role of utero-placental macrophages (Beer and Billingham 1974, Hunt and Pollard 1992). Growth-stimulation and immune tolerance appear to be the dominant effects, with an emphasis on tissue repair rather than destruction. In fact, functional studies of uterine macrophages

Figure 4. Diagram showing the immune system response to self and foreign antigens. A malignancy expressing both self and foreign moieties could elicit an abnormal response from the host. Factors such as sterile trauma at the tumour site (blunt trauma, surgery, *etc.*) could exacerbate this aberrant response. Normal responses (solid lines); abnormal responses (dashed lines).



suggest that these cells contribute in a variety of ways to the survival of the semiallogeneic fetus (Hunt and Pollard 1992). The trophoblast, in turn, secretes macrophage colony-stimulating factor (M-CSF), a potent macrophage chemoattractant and growth factor that does not activate macrophage cytotoxic functions (Pollard et al 1991). In contrast, rejection of the trophoblast is considered to be primarily due to decidual macrophage cytotoxic activities (Haddad et al 1997).

The analogy between the uterine trophoblast and cancerous growth (both to a certain degree part foreign and part self) was first put forward by the embryologist, Beard (1902). This functional duality may explain how malignant cells are continually able to divide and invade surrounding tissues in spite of the high concentrations of tumour-associated leukocytes (van Netten et al 1992, van Netten et al 1993a).

The immune system and cancer

Solid tumours and their metastases are infiltrated by large numbers of tumour-associated leukocytes (Bast 1989). These are heterogeneous populations of cells, consisting of variable proportions of helper, suppressor and cytotoxic T cells, B cells, natural killer (NK) cells, and macrophages. Although the presence of host inflammatory cells within or at the periphery of solid tumours has long been recognized, their biological and clinical significance remain the subject of conflicting reports. In view of their normally defensive role *in vivo*, leukocyte infiltration into tumours was originally believed to herald an immune response to the growing malignancy. Unfortunately, spontaneous regression of tumours is a *rare* event, suggesting that once in the tumour milieu, the immunocompetence of these cells has been compromised. Such evidence of compromised leukocyte defensive functions is further supported by histological studies

(van Netten et al 1992, Leek et al 2000, Balkwill and Mantovani 2001). Morphological alterations of tumour cells suggestive of leukocyte-induced damage are absent or extremely rare — in spite of the close contact generally observed between leukocytes and tumour cells.

Some of the earliest evidence suggesting that the immune system could potentially enhance cancer growth came from studies associating sterile injury and tumour progression. Due to the fact that leukocytes concentrate at sites of wounding, one might expect that these inflammatory cells could potentially *inhibit* (due to defensive activities) or *enhance* (due to reparative activities) tumour growth at the wound site. A brief review of the research in this area is instructive in this respect.

Infection and tumour remission

In a recent review of current immunotherapy regimens, it was stated that "immunotherapy applied to patients with established tumors rarely leads to an objective response" (Forni et al 2000). Yet historically, tumour regression associated with an immune response was not an unusual phenomenon. Why is it that focused modern immunotherapy treatments seem to fail, while in the past nature appeared to succeed?

In the last several centuries, hundreds of case reports have been published on the 'spontaneous' remission of cancer following acute microbial infections (reviewed by Christensen 1959, Hoption Cann et al 2001a). Some of these infections have included diphtheria, gonorrhea, hepatitis, influenza, malaria, measles, small pox, syphilis, tuberculosis, as well as, various other pyogenic and non-pyogenic infections (reviewed in Nauts 1980). An early example was a case reported by the French surgeon Le Dran (1742). A 15 year old patient had extensive inoperable cancer of the left breast. The

tumor ulcerated and gangrene developed. Within two days the entire tumor sloughed off with profuse hemorrhaging and later suppuration. The wound healed after five weeks, but the disease recurred causing death eight months later. Similarly, Trnka (1783) described a patient with breast cancer who developed tertian malaria (associated with chills, fever and sweating). The illness was associated with a complete remission after a few weeks. Benefits arising from such intratumoural infections was not an uncommon observation as the physician Quesnay (1749) stated of one patient "this mortification could have been advantageous to the patient, for it could, as we have seen sometimes, destroy the whole tumor, procuring a salutary amputation without pain."

Such coincidental remissions were probably the impetus for the active use of infections as a cancer treatment. Such treatments have been noted throughout history from many cultures. For example, in 1752, Amoureaux treated a patient with an ulcerated malignant breast by applying a septic dressing. The patient developed a fever and severe inflammation with suppuration, complete regression was noted in four weeks (Amoureaux 1760). In 1794, a similar approach was tried by Robert in a patient with extensive metastatic breast cancer. He made a small incision in the center of a large breast lesion (77 cm in circumference) and applied dressings soaked with gangrenous discharges. This led to a rapid destruction of the entire tumour mass and metastatic lesions, with complete healing by four months. The patient was reported to be entirely well 18 years later (Robert 1812). Similarly, other physicians were known to deliberately establish multiple 'issues' (suppurating sores) following cancer surgery. These issues were generally situated in the tumor, its periphery or in the arms (Dupré de Lisle 1774, Vautier 1813). An analogous method was used by Verneuil (1886). After cancer surgery, he would leave the incision open or loosely approximated with drainage, where suppuration would then

ensue. A student of Verneuil stated that "I was often struck by the slowness with which recurrence developed in such cases . . . I asked myself if suppuration, in eliminating the traces of cancer which had escaped the knife, did not play a role in delaying recurrence, and if therein lay the secret of success" (Thiery 1909). In addition to these treatments, some investigators induced tumor regression by injecting patients with other infectious agents such as malaria (Rovighi 1903) and syphilis (Alquié 1851, Didot 1851-52).

Another interesting example of active treatment was reported by White (1768). He mentioned the existence of a lady in Hungerford, England who used toads to treat women with breast cancer. The method was described as follows (Goldsmith 1774). A toad is placed in a linen bag with only its head exposed, which is then applied directly to the tumour. It is stated that the toad then sucks on the lesion for a number of hours, increasing in size and then eventually dropping off dead. Often a dead toad would be allowed to remain on the breast lesion by the application of a bandage for several weeks. Toads would be applied constantly or at least once daily for several weeks, depending on the patient. The treatment could continue for up to several months.

Pennant (1777) provides the details of one case, a woman who received a crush on her breast by the fall of a pail. She subsequently developed five wounds (from the ulcerating tumour) on her breast, causing her great pain. Over time she was described as being reduced to a skeleton, her left side and stomach swollen, and a tumour appeared on her neck that made swallowing difficult. The treatment began and she received seven toads in five days. By the fifth day, it was reported that she could now swallow with ease. She continued this treatment for several months and during this time her wounds healed and the swelling abated. The last account, four months after beginning treatment, was that the patient was feeling much better.

These basic 'immunotherapies' gained some general acceptance in the 1800's. In his treatise on breast cancer, Tanchou (1844) commented that "it is remarkable that after hemlock [a common cancer treatment at that time] it is gangrene that caused the largest number of cures. Gangrene may be considered as a therapeutic agent, whether it occurs spontaneously or is induced medically." Similarly, Walshe (1844) in his text on cancer treatment viewed such therapy as a practical application of a phenomenon that had been observed for centuries, and concluded that "the inoculation of the matter of common and hospital gangrene has been practiced, with the design of imitating the natural processes of cure." Cruveilhier (1864) also promoted these measures stating that a beneficial inflammation may be produced spontaneously or "induced by incisions or irritating applications. There results a melting away or gangrene of the affected tissues, followed by complete sloughing and a radical cure." Other prominent physicians of that era who induced infections often abstained from using surgical excision as part of the treatment. For example, Blake of New York published the pamphlet entitled, "Cancers cured without the use of the knife". He stated that the treatment brought on a discharge from the malignancy after a few days, which continued abundantly until the cancer disappeared (Blake 1858).

However, as a result of the growing popularity of Joseph Lister's methods for aseptic surgery in the late 1800's and early 1900's (Lister 1906), septic 'immunotherapy' soon fell into disfavour. Unseasoned cancer surgeons failed to appreciate its therapeutic value. Thus, as the prevention of postoperative infections gained further acceptance, the idea that cancer surgery should be distinct from other types of surgery was lost.

William Coley, a surgeon at New York Memorial (Sloan-Kettering) Hospital, was the first researcher to make a systematic study of the entire immunotherapy approach and

treated the largest series of patients in this manner (Nauts et al 1953, Hoption Cann et al 2001a). Unaware of the previous work in this area, Coley noted coincidental tumour regression in some of his patients who developed bacterial infections. Based on these initial observations, Coley attempted to reproduce these results by infecting his cancer patients with live bacteria (Coley 1893). An interesting example of his first treated case, a patient with a sarcoma (tumour arising from muscle, bone or connective tissue), was reported as follows:

> The patient was an Italian, 35 years of age, operated upon previously [five operations]. At the latter operation the growth was found too extensive to remove. At the time of my first inoculation [of bacterial culture] the tumor of the neck was growing, and the right tonsil was the seat of a tumor the size of a hen's egg and almost completely blocking up the pharynx. Solid food could not be taken, and liquids frequently regurgitated through the nose.
>
> The patient's condition was very bad. He was emaciated and cachectic. The dangers attendant upon attack of erysipelas [*Streptococcus pyogenes*] were explained to him, and in view of the hopelessness of his condition and the impossibility of obtaining further surgical relief, he consented to erysipelas inoculation.
>
> The inoculations were continued at short intervals during May and a part of June. Slight local and constitutional reaction followed the inoculations, the tumor of the neck diminished in size, and the general condition improved. The tonsil tumor was also smaller and the voice much better.
>
> Five decigrammes of a fresh culture were injected into the tumor substance. Up to this time he had had no attack of true erysipelas, the slight local reaction passing away in from 24 to 48 hours, and the temperature thereupon becoming normal.
>
> Within an hour he had severe pain, nausea, vomiting, and a chill lasting 40 minutes. His temperature rose to 105° [F], and within 12 hours a patch of perfectly typical erysipelas the size of the palm of the hand appeared on the neck. This gradually extended over the face and head, and met upon the opposite side.

> The disease ran the usual course, and I made little effort to check it, save to apply some ichthyol [mineral oil antiseptic] upon the forehead to prevent its extending to the scalp (which, I may add, it failed to do). At the end of 10 days the pulse and temperature had become normal. The tumor of the neck began to break down on the second day, and discharged until the end of the attack. At the end of two weeks the tumor of the neck had disappeared, and there remained only the induration from the previous operations. The appetite began soon to improve and he gained rapidly in flesh and strength.
>
> The patient's general condition at present (nearly two years) is very good, although he is suffering from a confirmed morphine habit which he had contracted previous to the inoculations.

In spite of the unfortunate 'morphine habit', the patient was followed for a further seven years and remained free from recurrence. Coley went on to report further successes with bacterial inoculations, however, in the pre-antibiotic era, the problems associated with this approach soon became obvious. Erysipelas was not easy to control once it began and, perhaps surprisingly, it was not all that easy to induce in the first place, some patients requiring repeated injections and others never developing an infection. Subsequently, Coley developed a vaccine consisting of extracts of killed gram negative *Streptococcus pyogenes* and gram positive *Serratia marcescens*, which became known as 'Coley's toxins' (Coley 1906). These toxins produced many of the symptoms of bacterial infections, such as fever and chills, without the need to worry about producing an actual infection.

Although Coley is often credited as the father of cancer immunotherapy, few investigators have ever closely examined his results. A recent retrospective analysis compared patients treated with Coley's toxins (1890-1960) to that of patients from the Surveillance Epidemiology End Result (SEER) registry (1980's) for cancers of the breast, ovaries, kidneys, and soft-tissue sarcomas (Richardson et al 1999). In comparing those treated with Coley's toxin and those from the SEER registry, the authors concluded that

the risk of death within 10 years was not significantly different for any of the cancers studied. These results are rather surprising considering the fact that Coley's vaccine was developed at only a nominal cost, that most cases were considered inoperable, and that this experimental work began over 100 years ago.

Throughout his career Coley stressed that the technique of administration was crucial to its curative effect, while the precise formulation was of secondary importance — he used more than 15 different formulations during his career. Martha Tracy (Beebe and Tracy 1907), a researcher who made many of the vaccine formulations for Coley and who experimented with a wide range of killed bacterial vaccines on animal tumors, observed that the most effective formulations were those that produced both local and systemic reactions.

A key aspect that Coley found to be necessary for tumor regression, was the induction of a mild to moderate fever. He would thus gauge dosage levels according to individual patient responses and increase the dose as necessary to avoid vaccine tolerance. Other factors that he found critical to a patient's long term survival included: direct vaccine injection into the tumor or metastases, frequent vaccine injections (daily or every other day) during the first month or two, and a prolonged follow-up to prevent recurrence (Coley 1906). Ensuring a prolonged follow-up was the most difficult aspect. Due to space limitations, patients would often be referred to their personal physician after a week to one month of treatment. In general, these physicians, and in many cases the patient, would not fully comprehend the importance of follow-up treatments or how these treatments should be carried out. Two other points of interest that he observed were that the toxins led to a marked relief of pain, where patients could often discontinue using narcotics; and, as these injections often followed surgery, there was an

extraordinary enhancement of wound healing and even bone regeneration (Nauts et al 1953). Similar observations on infectious amelioration of cancer pain and enhancement of wound healing has been reported by others (Verneuil 1883, Verneuil 1886, Mohr 1888). In contrast to Coley's findings, a number of recent studies using Coley's toxins, but not his technique, have correspondingly shown less impressive and often disappointing results (Chandler et al 1969, Tang et al 1991). Although these latter studies used Coley's formulation, they did not administer daily intratumoural injections or use dose levels that could consistently induce symptoms (*e.g.* fever, chills) as Coley had advised.

Cancer and infectious diseases have often been considered mutually exclusive states of health. Laurence (1858) stated that "as a rule, it will be found that cancerous patients have otherwise been remarkably free of disease." Similarly, Lambotte (1896) observed that antecedent erysipelas and other suppurative diseases rarely occurred in the cancer patient and that "these maladities, by their vaccinal action protect against cancer." He based his conclusions on an examination of the records of the Belgian Surgical Society, in which he found that antecedent pyogenic infection occurred in only 5% of 600 cases of cancer as compared to 80% of non-cancerous patients of the same age, sex and occupation.

In this respect, it is interesting to note that after the Second World War, antibiotics came into routine use for presurgical sterilization. Cohn and colleagues (1960, 1965) have shown in rabbits that the antibiotic control of colonic bacterial flora significantly increased the incidence of tumour growth in the wound site following surgery. Zwaveling (1962) made similar observations in mice and concluded that "current surgical methods (preoperative surgical disinfection) . . . increase the possibility of growth of

tumor cells that have spilled in the wound." In humans, Black (reported by Herter and Slanetz 1967) from the Mayo Clinic noted that subsequent to the discontinued use of preoperative antibiotics (a six and a half year period), local malignant recurrences following colon cancer surgery were "reduced greatly, far below the usually reported rates."

There are still a few reports in the literature on cancer regression in patients who develop a concomitant infection. Some more recent work has shown a significantly better overall survival in patients who develop coincidental infections subsequent to cancer surgery and/or radiotherapy (Miller et al 1971, Ruckdeschel et al 1972). In a case-control study by Kolmel and colleagues (1999), they compared the history of severe infections in melanoma patients with population controls. Overall melanoma risk was significantly reduced in subjects with a history of infections associated with a body temperature above 38.5° C. The protective effect depended on the type of infection: pulmonary tuberculosis, 86%; sepsis, 77%; pneumonia, 55%; *Staphylococcus aureus*, 46%; and influenza and related infections, a 35% risk reduction. Furthermore, a dose-response relationship was observed with increasing numbers of recorded infections and fever height. Similarly, Mastrangelo and colleagues (1998) reported on a significant negative correlation between the decline in mortality from infectious disease and the rise in cancer mortality in Italy during the first half of the 20th century. Variations in infectious diseases were noted to precede variations in cancer mortality. For example, a 2% reduction in infectious illnesses was followed by a 2% increase in cancer mortality, with a latency period of approximately 10 years. Of course, this association may be confounded by many factors such as changes in cancer treatment, diagnostic accuracy, or changes in exposure to cancer causative or

preventive factors; still, such alterations would have been less notable in the first half of the 20th century.

Further support of such reports come from studies showing that the removal of key immunological organs correlates with an increase in cancer risk. For example, a history of appendectomy has been associated with an increased risk for colon cancer and to a lesser degree cancer of the stomach, pancreas, lung, breast and cervix (McVay 1964, Vobecky et al 1983). Other similar increased risks have been noted between: leukemia with appendectomies and tonsillectomies (Bierman 1968, Schuz et al 1999); tonsillectomy with laryngeal cancer (Sokic et al 1995); and appendectomy with rectal carcinoma (Jarebinski et al 1989).

Overall, however, reports of 'spontaneous' regressions following infections have become rare. Decline in the use of Coley's toxin came about after his death in 1936. At this time, radiation and chemotherapy became mainstays of treatment as they required less individualization and the results were more predictable. Unfortunately, it soon became apparent that these new forms of treatment led to cures of a very short duration (Boyland 1963, Davis and Larionov 1964). Chemotherapy, and to varying degrees radiation, are highly immunosuppressive, and therefore infections in the cancer patient cause little immunostimulation, and in any case, are rapidly treated with antibiotics. Thus, it is not surprising that reports of 'spontaneous' regression following infections have become rare.

Coley's work did, however, lead to an eventual blossoming of immunotherapy research — experimental studies attempting to isolate antitumour agents from pathogenic organisms or human tissues. Such research has focused on isolating specific compounds responsible for activating the immune system and then using these agents for cancer

treatment. An extremely diverse range of immunological treatments are now under study. Cancer vaccines are being tested in humans and animals systems where irradiated whole tumour cells or tumour derived-proteins, peptides, nucleic acids or carbohydrates are given intravenously or directly injected into the tumour itself (Miles 1997). There are a number of phase I (toxicity), II (efficacy) and III (effectiveness) studies presently underway and include those examining the activities of a variety of cytokines or combination of cytokines, including: IFN-α, IFN-β, IFN-γ, IL-1, IL-2, IL-4, IL-6, IL-10, IL-12 in various human carcinomas (Skalla 1996, Wheeler 1996, Dalgleish 2000). Some of these treatments are being used to prime the patient's immune cells, others are used in combination with standard chemotherapy, and some as stand-alone treatments.

In general, modern immunotherapy investigators have focused on the development of more precise tumor-specific, immune-stimulating antigens; however, reductionism and antigen specificity does not always translate into improved therapeutic results. For example, in a recent study of stage IV melanoma patients, a peptide vaccine to the melanoma–specific tumor antigen, gp100, was combined with Freund's adjuvant with or without IL-2 (Lee et al 1999). The vaccine without IL-2 produced *specific* T cell clones against this antigen in 91% of patients, ***but no tumor responses*** (0/11); however, only 16% of patients produced T cell clones in the group with IL-2, yet the response rate was 42% (13/31). Such results suggest that a more refined approach, where the tumor antigenic repertoire is simple and well-defined may be less useful to patients.

Stimulation of cancer during sterile injury

The medical community has not infrequently ignored the beliefs of laymen simply because no satisfactory scientific explanation could be found for these beliefs. No better example

of this can be found than in the discovery of the origin of tuberculosis. For hundreds of years, it was generally believed that tuberculosis was a contagious or infectious disease. However, leading medical authorities in the 1800's denied such an association. They based their opinion on innumerable statistics, the chief of which included those on staff at the Brompton Home for Tuberculosis. It was shown that in 35 years (1846-81) not a single doctor or nurse had contracted tuberculosis (Coley and Higinbotham 1933). In the following year, Koch discovered the tubercle bacillus.

In a similar fashion, it has long been believed by the lay public that injury may cause cancer or enhance its growth. Yet, physical injury as a causative factor or co-carcinogen for tumour progression has long been a contentious issue (Weiss 1990). Still, if inflammation occurs at the exact site of injury and within a few days, weeks or months thereafter develops characteristics typical of cancer, it seems illogical to nullify or minimize the influence of injury on the development of this disease. Although it is true that injuries often occur without the subsequent development of cancer, the high proportion of cancer patients with previous site-specific injuries appears to support a causal influence. Coley used the analogy that if 50 people were plunged into an icy pond and only two developed pneumonia, one might say that because the 48 remained well, the shock and exposure were not causative factors in the development of the pneumonia in the two who had contracted it (Coley and Coley 1925). Quite to the contrary, the exposure to cold simply lowered the resistance of the body and prepared a favorable soil for the development of the infection. One of the difficulties in resolving this question of causation is distinguishing between those patients who become aware of a tumour because of the injury and those whose disease has been augmented by the injury.

In contrast to infection, during sterile injury only immune reparative functions are required. There is a large body of literature from the 19th century associating physical injury with the subsequent aggressive growth of malignant tumours. One of the earliest and most comprehensive series of case studies comes from Lowenthal's (1894-95) paper on "The traumatic origin of tumors." His work was based on a careful analysis of 800 collected cases that included a wide variety of tumour types. Of these 800 cases, 135 refer to traumatic carcinoma of the female breast. The time elapsing between single trauma and the subsequent presentation of tumours was as follows: 71.1 % within one month or less; 17.4 % greater than one month up to one year; and 11.6 % greater than one year.

Coley (1911) reported on a series of 120 breast carcinomas, where 42.3 % of subjects reported a history of single antecedent trauma. The following is a typical example from his series of case reports:

> Mrs GHC, age 54 years (March 1908); family history good. Two and a half years ago, patient slipped on a rug and fell heavily to the floor, striking the right thumb against the right breast, so severely that it caused dislocation of the thumb. A few weeks (less than a month) afterward, a tumor developed at the upper and inner side of the breast at the exact site of the injury. Finally, six months after, breast and axillary glands were removed by a very extensive operation. A few weeks after the operation there appeared a reddish colored thickening along the whole cicatrix [surgical scar] accompanied by oedema of the arm. Examination, March, 1908, showed very extensive local recurrence with metastasis in the lung and pleura.

Others studies followed and many of those focusing on breast cancer were reviewed by Coley and Higinbotham (1933). McWilliams, in a study of one hundred consecutive cases of carcinoma of the breast observed at Presbyterian Hospital, found a history of

antecedent local trauma in 44% of subjects. In a large case study by Janet Lane-Claypon, one the foremost English authorities on breast cancer at that time, the histories of 508 cases of breast cancer (1924-26) were examined and she concluded that there was a definite history of previous local injury in 136 cases, 26.8%. Unfortunately, much of this knowledge has been lost due to the greater emphasis that has focused on cancer genetics and other causative factors.

Similarly to physical injury, wounding caused by the surgical removal of tumours is known to be a potent enhancer of the growth of residual disease. Ryall (1907) first described this dilemma in his cancer patients:

> In those cases where, after the attempted removal of cancerous tissues, the disease not only appears locally but sometimes seems to recur with increased fury and virulence, in fact, in cases such as the latter, it seems as if one has stirred up a hornet's nest, the post-operative history showing the disease recurring in and around the wound with extraordinary virulence and in an alarming extent, the virulence of such recurrences sometimes being so intense as to appear almost like some inflammatory malignant disease.

Over the last century, numerous other reports have followed illustrating cases where surgical intervention subsequently enhanced the growth dynamics of tumours (Fisher and Fisher 1959, Simpson-Herren et al 1976, Gunduz et al 1979, Mabuchi 1985). A number of these reports (O'Rourke et al 1993, Siriwardena and Samrji 1993, Walsh et al 1993, Berends et al 1994, Nduka et al 1994) have focused the negative impacts resulting from the recently developed laparoscopic technique when used in cancer surgery. In this procedure, a small incision is made (*i.e.* into the abdominal cavity), the tumour is excised, and then extracted through the narrow surgical opening. The difficulty arises when shed malignant cells from the primary tumour become lodged in the incision site during extraction. Gleason and colleagues (1993) reported three cases of abdominal wall

metastases from ovarian cancer after laparoscopy. In one case, a tumour mass eight centimeters in diameter developed in the abdominal incision site within two weeks of surgery. This illustrates how aggressive a cancer can be under the right circumstances, as the tumour would have had to develop from a single cell or microcluster of malignant cells and proliferate at an incredible rate to reach this mass in such a short time period. Another case reported by Watson (1995) illustrates how few cells are required for seeding of a surgical wound. He presents the case of a patient with gastric obstruction confirmed to be pancreatic carcinoma by computed tomography. To achieve gastric drainage, the patient underwent laparoscopic gastroenterostomy [surgical formation of a passage between the stomach and small intestine] — with the pancreatic tumour neither manipulated nor visualized. Subsequent to surgery, however, tumour nodules developed in the incision site. The author suggested that insufflation of the peritoneal cavity (used to allow greater surgical access within the abdomen) may have disseminated already free intraperitoneal tumour cells into the incision site. Finally, Wang and colleagues (1999) comprehensively reviewed reports where metastases followed surgery and concluded that the incision site was a primary area for recurrence even when distant from the tumor excised. Although metastases may occur in any form of cancer surgery, the previous examples illustrate how potentially hazardous a healing wound can be.

Any modifications to these procedures to minimize growth enhancement can improve patient survival. One positive modification in breast surgery has been the trend towards lumpectomy (removal of the tumour and a small margin of surrounding tissue) over mastectomy (complete breast removal). At the 10 year follow-up of the European Organization for Research and Treatment of Cancer (EORTC) Trial, there was no difference in overall survival (66% for mastectomy patients and 65% for patients with

breast-conserving therapy) (van Dongen et al 2000). One would expect that the more comprehensive surgery (*i.e.* mastectomy) would have resulted in a better overall survival since more malignant cells would have been removed. In contrast, the lack of a survival advantage may suggest that smaller wounds (*i.e.* lumpectomy), which also require shorter periods of time to heal, may stimulate residual tumour regrowth to a lesser extent than larger wounds (*i.e.* mastectomy). Thus, the extent of wounding may be a more important factor in disease recurrence than the actual number of malignant cells left behind after surgery.

The fact that surgical wounding can enhance tumour growth has also been supported by work in animal systems. Baker and colleagues (1989) determined that the number of cancer cells necessary for tumour take after subcutaneous injection was significantly reduced by a factor of ten when cancer cells were injected into a surgically-induced flank wound. Similarly, Murphy and colleagues (1988) demonstrated an enhanced efficiency of tumour take with hematogenously injected tumour cells after a surgically-produced injury. The probability of intra-arterially injected sarcoma cells colonizing the muscle of a laparotomy wound was 1000 times more likely than if the muscle was not traumatized. Using a murine mammary adenocarcinoma model, Murthy and Scanlon (1993) demonstrated that tumour cells injected intravenously *never* implant in the spleen. However, after injury to the lower pole of the spleen, these cancer cells implanted precisely at the site of surgical wounding in the spleen in 76% of cases. A predilection for growth at sites of wounding has also been demonstrated with surgical trauma to kidney, colon, liver and bone (Ammirati et al 1989, Murthy and Scanlon 1993, Lee et al 1994a). Tumour take was dependent on the cancer cells being administered at the time of, or shortly after, the surgical injury. In contrast, as the wound aged, the surgical site

became increasingly refractory to tumour implantation. When healing was complete, injected tumour cells had no affinity for the old surgical site (Murthy et al 1989).

Sporadic reports and reviews in the literature continue to appear associating trauma, from seat belt injuries (Dawes et al 1986, McInerney 1987), diagnostic trauma (Weiss 1990, van Netten et al 1999) and other forms of injury (Qi et al 1994, Widhe and Widhe 2000), with the subsequent appearance of a tumour or the aggressive progression of previously dormant or benign tumours; however, the association between these two factors remains a matter of controversy (van Netten and Cann 1996, van Netten et al 1997).

Immune activation of cancer during injury

The question arises as to whether this enhanced progression is simply due to a localized release of growth factors in response to injury or if some specific immune-stimulating mechanism is at play. Immune cells infiltrate into sites of wounding irrespective of whether or not infection has occurred (Clark et al 1976, Thakral et al 1979). Klebs, in the late 19th century, was one of the first authors to speculate that immune cells could actually stimulate cancer growth, suggesting that these cells had a "fructifying" influence that caused cancer cells to multiply (Beatson 1896). Animals studies along these lines were first carried out by Jones and Rous (1914). In their early experimental studies, a variety of chemical, biological and inert materials were used to induce inflammation in the peritoneum of mice. Peritoneal tissue was subsequently inoculated with tumour cells and a much greater tendency for implantation was observed in mice with inflamed tissue over untreated animals. They concluded from these investigations that "the secondary localization of tumours at points of injury is referable to the presence at such points of a very cellular connective tissue which may come more readily than the normal to the

support and nourishment of tumour cells." This "cellular connective tissue" following injury is typical of immune cell infiltration. More recently, in a study by Van den Brenk and colleagues (1974), cellulose sulphate (CS) was used to induce inflammation in lung tissue in rats. It was found that the CS treatments significantly increased the survival and growth of tumour cells lines (Walker-256 and Yoshida-P388) in the inflamed lung whether the cells were injected intravenously or directly into the treated tissue. The growth of these cell lines was neither enhanced nor inhibited by CS treatment *in vitro*. In contrast, administration of the anti-inflammatory steroid, dexamethasone (which impairs immune cell function) was shown to counter the effect of CS by inhibiting both tumour growth and survival.

A variety of leukocytes have been implicated as important mediators of tumour growth enhancement during sterile injury. In a study by Picard and colleagues (1986), the investigators showed that tumour growth could be significantly enhanced by the concurrent implantation of fibroblasts with cancer cells. Tang and colleagues (1990) examined breast cancer specimens for oncogene expression (c-*erbB2*, *int-2*, and c-*myc*). A high expression of c-*erbB2* was associated with estrogen receptor negative and progesterone receptor negative tumours (*i.e.* aggressive hormone-independent tumours). Surprisingly, a strong association was also observed between oncogene amplification and dense B and T lymphocyte infiltration. The correlation was even stronger when only high levels of amplification were considered. In a similar experiment by Pupa and colleagues (1996), they found a high correlation between macrophage infiltration and c-*erbB2* expression.

Research by Peoples and colleagues (1995) examined the ability of T lymphocytes to stimulate tumour growth. Tumour-infiltrating lymphocytes were shown to produce heparin-binding epidermal growth factor (HB-EGF) and basic fibroblast growth factor (bFGF) *in vitro* under non-specific conditions and *in vivo* in tumours visualized by immunohistochemistry. At the same time, growth factors derived from tumour-infiltrating

lymphocytes both stimulated tumour cell division (*i.e.* HB-EGF) and were angiogenic *in vitro* (*i.e.* bFGF). Other immune cells have been implicated in this process as well. Rowse and colleagues (1995) examined the association between NK cells and the Shionogi mouse mammary tumour. The investigators found that mice with the largest tumours had the most dense intratumoural NK cell infiltration. In contrast, administration of antibodies that impair NK cell function was shown to inhibit tumour growth in these mice. Seung and colleagues (1996) examined the effect of whole body irradiation on subsequent tumour challenge in nude mice. Even when the site of tumour implantation was shielded, whole body irradiation before the tumour challenge inhibited subsequent tumour growth significantly. The interval of inhibition correlated with the depletion of circulating leukocytes, which did not return to normal until 12 to 21 days after irradiation. These results were consistent with an earlier experiment by Seung and colleagues (1995) in mice, where tumour challenge was inhibited by predepleting immune cells with anti-leukocyte antibodies (Gr-1+).

This dissertation is partitioned into two chapters designed to gain insight into the role the immune system plays in aiding or impeding tumour growth, with breast cancer as the model tumour type. In addition, discussions of the practical applications of this research will be presented throughout. A considerable body of evidence exists to show that macrophages may be the key immune cell type involved in mediating tumour growth, and this has been examined in the first chapter. In contrast, the second chapter focuses on other associated factors, such as diet, lifestyle, *etc.*, that may modify (beneficially or detrimentally) interactions between the immune system and cancer.

CHAPTER 1: *Immunohistochemical and computed-aided image analysis of macrophage content in breast carcinoma*

INTRODUCTION

Macrophages and cancer progression

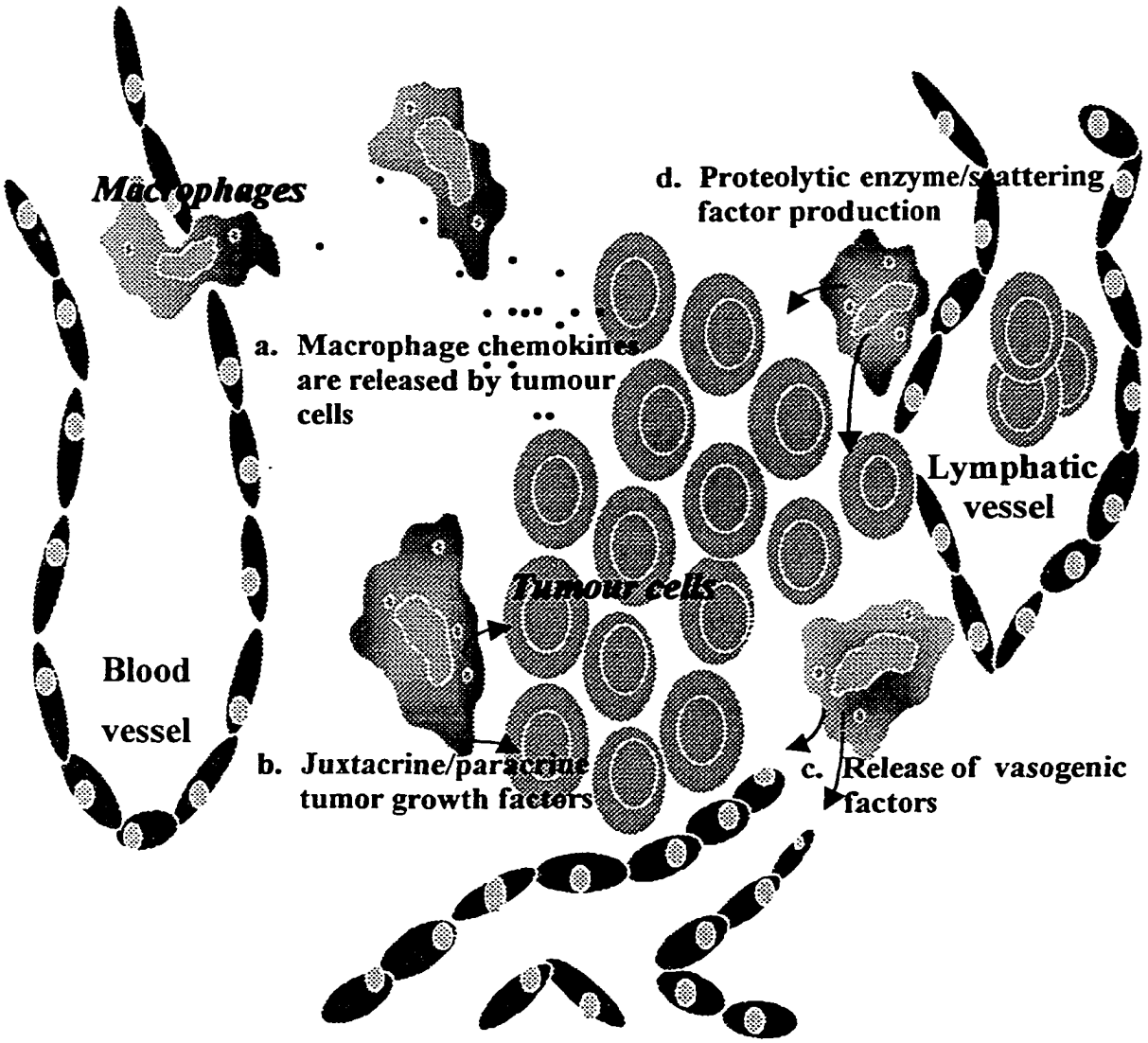
Macrophages belong to the mononuclear phagocyte system. These cells form a heterogeneous cell population with differing developmental and functional stages. They have a common progenitor cell with granulocytes (*i.e.* basophils, neutrophils and eosinophils) in the bone marrow. While in the bloodstream these cells are referred to as monocytes. When they are specifically recruited into tissues (*i.e.* during injury or infection), they are referred to as macrophages. Macrophages that perform tissue-specific metabolic functions often bear special names, for example: alveolar macrophages (lung), dendritic cells (lymph nodes), Kupffer cells (liver), Langerhans cells (skin), microglial cells (brain), osteoclasts (bone), foam cells (debris-laden macrophages in various tissues), *etc.* Inflammatory infiltrates have been shown to contain several differentiation stages of macrophages (Rutherford et al 1993). Thus, macrophages are present ubiquitously in all tissues, and in the case of inflammation, certain subtypes are recruited from the blood-borne monocytes.

Macrophages play an important role in tissue homeostasis. These immune cells produce a variety of secretory molecules, such as small lipid derivatives, growth-promoting factors, complement proteins and cytolytic agents necessary for the maintenance of normal tissue physiology (Metzer and Nacy 1989). A number of studies have shown that stimulated macrophages can be tumouricidal (Urban and Schreiber 1983, Nestel et al 1984, Fogler and Fidler 1985), however, chronic stimulation is

required as the state of activation for tumour killing is transient (Ruco and Metzer 1978, Poste and Kirsh 1979). *In vitro* studies have shown that a wide range of tumour cell lines (carcinomas, leukemias, melanomas and sarcomas) secrete macrophage chemokines and that the production of these chemotactic factors by tumour cells correspond to the degree of infiltration by macrophages (Bottazzi et al 1983). This capacity for tumours to attract macrophages is central to the contention that macrophages promote tumour emergence and that some host inflammatory responses encourage tumour growth (Van den Brenk et al 1974). Macrophages stimulate new vessel formation (Polverini et al 1977b, Polverini and Leibovich 1984), produce matrix-degrading proteases (Henry et al 1983), and secrete cellular growth and scattering factors (Jiang et al 1995) (Figure 5). Thus, the two views on the role of macrophages within tumours (enhancing or inhibiting growth) are not mutually exclusive, but may depend on the biology of the tumour itself (Inoue and Nelson 1984), the subsets of monocytes attracted (Rutherford et al 1993), as well as the macrophage state of activation (Gabizon et al 1980), their rates of turnover and absolute numbers within the tumour.

Finally, it is worth noting several reasons for studying macrophage accumulation during cancer growth. First, effective immunotherapy may depend upon the content of macrophages within the tumour. For example, the efficacy of bacillus Calmette Guérin (BCG) treatment has been linked to tumour macrophage content (Hopper and Pimm 1976). Second, alterations in macrophage traffic induced by tumours may alter host resistance, increasing susceptibility to infection — a frequent cause of death in the cancer patient. Third, the use of therapeutic agents that can inhibit certain macrophage reparative or chemotactic activities may, in turn, lead to tumour regression (Richter et al 1993, Vukanovic et al 1995a).

Figure 5. Tumour cell subversion of macrophage repair activities. (a) Chemokines released from tumour cells attract macrophages. (b) Macrophages release growth factors that can stimulate malignant cell proliferation. (c) Secretion of angiogenic and lymphagenic factors by macrophages leads to neovascularization via endothelial cell migration and/or proliferation. (d) Proteolytic factors expressed by macrophages aid in tumour expansion and invasion into the stroma; scattering factors promote malignant cell dissemination through associated lymphatics.



Macrophage content in tumours

One approach to investigating the role of macrophages in human oncology is through their quantitation in tumour material obtained at surgery. This approach has been hampered by the difficulty of identifying macrophages in standard histological preparations of tumours. This impediment has led to the development of various markers to assist in their identification. However, problems have arisen due to cross reactions with other cell types or a failure to detect all tissue macrophages (Lwin et al 1985, Steele et al 1985). In these early studies, macrophage content in tumours was generally reported to be in the range of 10 to 20% (Talmadge et al 1981). The development of antibodies to the CD68 antigen was an important step forward as it had a high specificity for all cells of the mononuclear phagocyte system. This included those macrophages in the peripheral blood, bone, brain, dermis, gut, kidney, liver, lymph nodes, spleen, thyroid, and other glandular tissues.

However, the macrophage content in animal and human tumours may be quite variable and this must be considered when using animal systems as a model for macrophage infiltration into human neoplasms. For example, Bucana and colleagues (1992) compared the macrophage content in murine and human cancers growing subcutaneously in nude mice. The pattern of tumour-associated macrophage (TAM) distribution differed between human and mouse tumours. Regardless of histological classification, TAMs were uniformly distributed throughout all murine tumours. In implanted human tumours, TAMs were found on the periphery of the lesions and in association with fibrous septae. In contrast, histological examinations by Kelly and colleagues (1988) of human breast cancer biopsies found macrophages to be distributed throughout the tumour with only a transient tendency to collect at the tumour periphery. In addition, a quantitative analysis was performed on tumour macrophage density. Areas of tumour necrosis, which contain phagositizing macrophages, were avoided. In benign breast disease (fibrocystic disease or DCIS), macrophages represented 25% of cells within the tumour, whereas malignant

tumours contained a significantly higher mean of 53% macrophage positive cells. The range for both groups was wide, however, suggesting considerable individual variability. Other studies examining the relative contribution of macrophages to the entire inflammatory cell infiltrate, have found macrophages to be the dominant immune cell type (Gottlinger et al 1985, van Ravenswaay Claasen et al 1992, Leek et al 2000).

Similar large infiltrations of macrophages have been observed in other solid carcinomas including: bladder (Hanada et al 2000), colon (Takahashi et al 1996), lung (Arenberg et al 2000), thyroid (Scarpino et al 2000), and uterine cancer (Salvesen and Akslen 1999).

Tumour production of chemokines

A wide variety of leukocyte chemokines have been isolated from tumour cells, a large number of which act on macrophages (Table 2). Bottazzi and colleagues (1983) first demonstrated that many tumour cell lines actively secrete macrophage chemokines at varying concentrations. Subsequent studies focused on the isolation and characterization of these compounds (Wang et al 1986, Mantovani et al 1992). However, the work by Martinet and colleagues (1991) was the first to study such factors in human cancers *in vivo*. They evaluated the release by cancer cells of macrophage chemotactic activity in patients with lung cancer through the collection of malignant pleural effusions (*i.e.* those containing cancer cells). Interestingly, significantly greater amounts of macrophage chemotaxins were quantified in malignant pleural effusions in comparison to plasma from the same patients and to nonmalignant pleural effusions. Furthermore, regarding lung cancer, the levels of macrophage chemotaxins were greater in pleural effusions from patients with adenocarcinoma (a cancer characterized by a high inflammatory cell infiltration), than in pleural effusions from patients with small cell carcinoma (a cancer characterized by a limited inflammatory cell infiltration).

Table 2. Tumour-derived chemokines involved in the regulation of tumour-infiltrating macrophages.[a]

Cytokine	Role
Macrophage chemotactic protein (MCP-1, -2, -3)	Recruitment via chemotaxis
Monocyte colony-stimulating factor (M-CSF)	Promotion of survival, proliferation, maturation chemotaxis, downregulation of oxidative burst
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Recruitment via interaction with endothelium and chemotaxis, inhibition of cytotoxicity
Vascular permeability factor (VPF)	Downregulation of recruitment and activation, systemic anti-inflammation

a. Adapted from Bottazzi et al 1983, Wang et al 1986, Mantovani et al 1992, Martinet et al 1991, Elgert et al 1998.

Acero and colleagues (1984) studied the effects of hydrocortisone treatment on the growth of murine sarcomas (mFS6 and MN/MCA1) and carcinomas (3LL and M109). Hydrocortisone inhibits the recruitment of mononuclear phagocytes to sites of inflammation, but does not affect the viability and proliferative capacity of macrophages. In this study, hydrocortisone treatment inhibited the growth of these tumours and was found to reduce macrophage content of the four murine tumours to less than half the control value. The effects of hydrocortisone on macrophage content and tumour growth and metastasis were similar in mice with defective lymphocyte activity (nude or thymectomized) or defective NK cell activity (beige or antiasialo GM1-treated mice) and in controls, suggesting that macrophages played a key role in the immune-mediated tumour stimulation. This work also suggested that the maintenance of macrophage levels in growing tumours is dependent upon the entry into neoplasms of circulating monocytes. In vitro, hydrocortisone treatment did not alter sarcoma cell growth, although it did mildly inhibit carcinoma growth.

The cytokines, M-CSF and GM-CSF, induce cellular proliferation and chemotaxis in macrophages and the latter for macrophages and granulocytes. These chemokines have been shown to be crucial for complete wound healing by attracting these immune cells to the site of injury and, in turn, upregulating the production of paracrine growth factors (Raderer et al 1997, Wu et al 1997). In an analysis of human breast carcinomas, Tang and colleagues (1992) found a high degree of staining for M-CSF protein in areas of obvious stromal invasion by macrophages, while absent or trace staining was observed in intraductal carcinoma. In situ hybridization experiments confirmed the production of M-CSF mRNA by tumour cells. In contrast, the M-CSF receptor protein (fms) was observed in areas of macrophage infiltration. More recently, Allione and colleagues (1994) gene-transduced mammary adenocarcinoma cells to produce cytokines and then examined the ability of these engineered cells to form tumours in mice. Those cells transduced with the GM-CSF gene, the granulocyte/macrophage chemokine, were the

most aggressive and showed the highest percentage of tumour takes when tumour cells were injected subcutaneously. However, those cells engineered to produce IL-10, a cytokine that impairs macrophage function, were one of the least aggressive cell lines. Of course, one must keep in mind the limitations of such animal experiments, as cells injected into animal systems are not necessarily comparable to the development of spontaneous human tumours (van Netten et al 1993b).

Stimulation by growth factors

In addition to stimulating cellular division, most growth factors have a number of pleotropic activities. Peptide growth factors act locally through juxtacrine or paracrine cell stimulation. The ultimate response of a target cell to a particular growth factor is determined not just by the combination of other factors present, but also its concentration and the cellular context into which the stimulus is received. Evidence from *in vitro* co-cultures of macrophages and tumour cells has suggested that their associations, rather than being inhibitory, may facilitate tumour growth and migration (Munzarova and Kovarik 1987, van Netten et al 1993a). Such evidence suggests that macrophages may release cytokines that have direct stimulatory effects on tumour cell proliferation.

The ability of macrophages to stimulate tumour growth was demonstrated by Davies and colleagues (1994). The investigators showed that an intraperitoneal inoculum of more than 1000 murine mammary carcinoma cells was required to establish a metastatic nidus. However, by inducing a macrophage-rich ascites plentiful in growth factors, as few as ten cells were adequate for tumour take. Similarly, Alexander and colleagues (1988) injected lung carcinoma cells into rats and observed that traumatizing tissues by procedures such as surgical incision of the abdomen, gut anastamosis, placing a stitch in the kidney, or compression of the liver greatly enhanced the likelihood of metastatic development. Metastases were most pronounced when the wound was made between eight to two days prior to injection. However, little effect was observed if the trauma

was induced immediately before or after the injection of cancer cells. This temporal pattern of metastatic establishment corresponds to the peak presence of macrophages in wounded tissue (Leibovich and Ross 1975). During the early phase of healing, there is an influx of neutrophils that reaches a maximum at two days and then rapidly declines. At this time, macrophages begin to appear in the wound, reaching a plateau at days three to four, and persisting for some eight days.

O'Sullivan and colleagues (1993) examined the origin of epidermal growth factor (EGF) production in breast carcinoma. Although secretion of EGF by normal or malignant epithelial cells was not observed, use of a cytokine release assay demonstrated that EGF was secreted by cells with a characteristic morphological and immunophenotypic profile of macrophages. Other important growth factors secreted by TAMs include L-arginine derived polyamines, such as ornithine (Mills et al 1992, Nathan 1987). This TAM orchestrated secretion of growth factors is analogous to the normal activities carried out by macrophages in healing wounds (Wilson 1997). In the latter setting, macrophages are pivotal to the healing process, acting as director cells, and producing growth factors to stimulate tissue repair and re-epithelialization during wound healing process.

Matrix-degrading proteases

Numerous conditions are necessary for the successful outcome of tumour invasion and metastasis. One factor appears to be the ability of a tumour to degrade the surrounding connective tissue barriers of the host through the action of proteolytic enzymes (Jones and DeClerck 1980). Investigations have been devoted to the capacity of tumour cells to produce and secrete proteases that might play a role in tumour invasion and metastasis. However, the governing factors that regulate this process, although probably critical in determining the degree of invasiveness of any given tumour, are to a large degree unknown. Due to the fact that malignant tumours are infiltrated in varying degrees by

normal cell types from the host (*e.g.* lymphocytes, fibroblasts, macrophages, *etc.*), the possibility that these host cells may have a regulatory role must be considered. Local degradation of the extracellular matrix (ECM) entails more than opening up a way for migrating cells. The ECM has been suggested to have a controlling role in a variety of physiological and biochemical functions. Macrophages can secrete a variety of enzymes and cytokines that cause changes in the molecular and mechanical structure of the ECM. Furthermore, some evidence of macrophage transformation into fibroblasts and fibroblasts into endothelial cells has been demonstrated, which may further add to the macrophage role in tumour development (van Netten et al 1993a).

In co-cultures with normal fibroblast cells, macrophages were shown to be the primary regulators and stimulators of fibroblast enzymatic breakdown of connective tissue (Laub et al 1982, Laub and Vaes 1982). Henry and colleagues (1983) examined the interactions between metastatic Lewis lung carcinoma cells (LLC) and normal mouse peritoneal or bone marrow macrophages. From the parental cell population, a series of homogeneous, clonal cell subpopulations that differed markedly in their ability to produce lung metastases after intramuscular implantation into mice were examined for further study. *In vitro*, neither the mouse macrophages nor clonal LLC subpopulations were able to spontaneously degrade type I collagen. In contrast, the co-culture of LLC cells with macrophages on a collagen substrate stimulated the production of collagenase and other proteases. Similar degrees of stimulation were observed for a given cancer cell population when the co-cultures involved macrophages that were either syngeneic or allogeneic to the tumour cells, or when the macrophages had been collected from intact mice or from mice bearing an intramuscular tumour of the same cancer cell type, or when bone marrow-derived macrophages were used instead of resident peritoneal macrophages. Tumour cell-conditioned media did not stimulate collagen degradation when it was added to cultures of macrophages, but macrophage-conditioned media could be substituted for living macrophages to stimulate, to a certain extent, collagen

degradation or collagenase secretion by tumour cells. The reduced stimulation of collagen degradation caused by the macrophage-conditioned media as compared to that seen in co-cultures may suggest that cell-to-cell contact between macrophages and tumour cells may be an important component in this process.

Macrophages are a rich source of metalloproteases (Nathan 1987, Adams and Hamilton 1992) and serine proteases, such as tissue type and urokinase type plasminogen activator (tPA and uPA) (Adams and Hamilton 1992, Hildenbrand et al 1995). These enzymes can degrade ECM molecules, modulate mechanical structures and liberate ECM-bound growth factors. As these proteolytic enzymes are capable of degrading nearly all components of the ECM, control mechanisms are necessary. Thus, macrophages also synthesize tissue inhibitors of metalloproteases and serine proteases (Wohlwend et al 1987). Macrophage secretion of degradative enzymes also leads to the release of several soluble growth factors — granulocyte colony-stimulating factor (G-CSF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF) — stored in the ECM that are bound, for example, to heparin-like glycosaminoglycans.

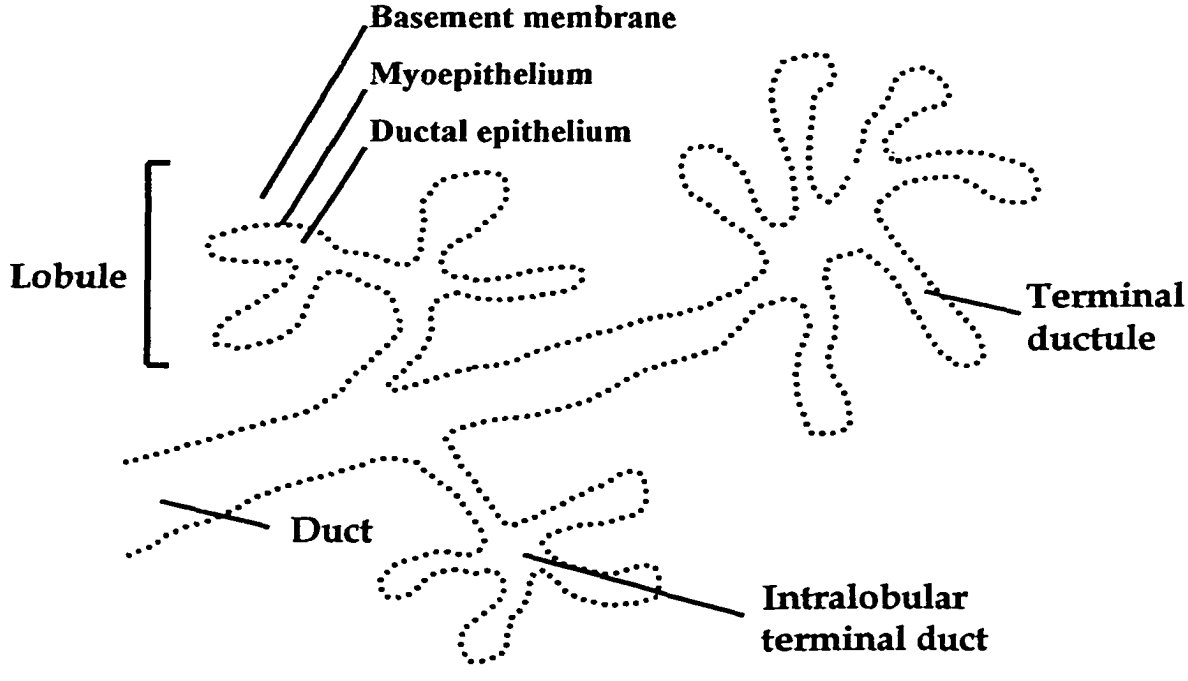
In a study of human breast cancers using in situ hybridization, Heppner and colleagues (1996) found that macrophages were the primary producers of the metalloproteinase, metalloelastase. Nielsen and colleagues (1997) also studying breast cancer conclusively demonstrated matrix metalloproteinase-9 (MMP-9) expression in macrophages using double immunoflorescence staining. In support of this work, other studies have confirmed that macrophages, but not tumours cells, were the key source of MMP-9 in colorectal cancer (Swallow et al 1996, Ring et al 1997). Ring and colleagues (1997) found that MMP-9 positive macrophages were positively correlated with poor tumour differentiation (*i.e.* tumour aggressiveness). Swallow and colleagues demonstrated that a co-culture of macrophages and a highly metastatic colorectal cell line (SW620-S5) significantly increased macrophage production of both MMP-2 and MMP-9. In contrast, the co-culture of monocytic cells and a prostatic cell line (LNCaP) led to tumour cell

production of the metalloproteinase, matrilysin (Klein et al 1997). Tumour matrilysin secretion was shown to be induced by monocyte IL-2β production. In a study of lung tumour biopsies, Kawano and colleagues (1997) found gelatinase A to be expressed by macrophages as well as some endothelial cells and fibroblasts. Finally, in a study of gastric adenocarcinoma, dual immunohistochemistry revealed that macrophages produced membrane type metalloproteinase-1 (MT1-MMP), an activator of pro-gelatinase A (Ohtani et al 1996).

Besides macrophages, endothelial cells also synthesize various proteases by which they locally degrade the capillary basement membrane (van Hinsbergh et al 1991). Macrophage secretion of cytokines (G-CSF, bFGF, TGF-β, TNF-α) can in turn modulate secretion and activation of endothelial proteases. For example, G-CSF and bFGF increase activity of plasminogen activator in endothelial cells and TGF-β decreases it (Flaumenhaft et al 1992).

One final point should be considered, however. The idea that malignant ductal epithelial cells in the breast are contained by the myoepithelium and basement membrane that surrounds these malignant cells may be incorrect (Figure 6). The definition of ductal carcinoma in situ, considered a benign lesion if there is no microscopic evidence of invasion, is based on this concept. Yet, one must take into account the normal activities of these ductal cells. During pregnancy, as the system of ducts rapidly expands to accommodate eventual milk production, these same ductal epithelial cells rapidly divide while simultaneously inducing considerable expansion of the surrounding myoepithelium and basement membrane. Why would a malignant cell at an early stage of differentiation be any different? Although basement membrane material is normally produced by myoepithelial cells, it can also be produced by cancer cells (Raymond and Leong 1991). In patients with DCIS, who have metastases to the axillary lymph nodes, these metastatic lesions may also show a well-formed basement membrane that stains positively for basement membrane constituents such as laminin and type 4 collagen (Querci della

Figure 6. Ductal structure in the breast. The ductal glandular epithelium lines the duct lumen and produces and secretes components for milk production. A secondary layer is formed by the myoepithelium, which contracts the ductules to facilitate excretion of milk from the lobules to the main ducts. These cells are then lined by a supportive basement membrane through which nutrients flow from the vascular network and surrounding fatty tissue.



Rovere and Levene 1995). These observations rule out the possibility that these neoplastic cells are simply growing in pre-existing glandular ducts. Therefore, in light of the preceding evidence, DCIS may reflect a less differentiated, but not contained, carcinoma. In this respect, macrophages may facilitate the early expansion of this DCIS network, consisting of intraductal malignant cells and its associated basement membrane.

Tumour neovascularization

In healthy tissue, neovascularization is a rare phenomenon. In contrast, tumours and inflamed stromal tissues are constantly being remodeled to accommodate neovessel formation. The development of new capillaries proceeds through a series of steps (Ausprunk and Folkman 1977). The sequence of events for endothelial cells begins with destruction of the basement membrane and local degradation of the ECM. This allows endothelial cells to migrate by extending cytoplasmic buds in the direction of chemotactic factors. Similarly, neoplastic and inflamed tissues contain highly permeable capillaries that allow exudation of fibrin, which serves as a migratory matrix for endothelial and other cells (Liu et al 1990). For elongation of new capillaries, migrating endothelial cells must be replaced by newly dividing endothelial cells. Eventually, migration and mitosis have to be stopped while capillary sprouts differentiate into mature capillaries with a new basement membrane.

Tumour neovascularization was initially believed to be induced by tumour cells themselves (reviewed by Folkman 1985). However, over the past 20 years research from a variety of fields has suggested that macrophages may be the key mediator of this process. Such activation of macrophages has biological relevance. The proliferation rate of endothelial cells in normal tissues is very low (Hobson and Denekamp 1984). In growth and repair processes, neovascularization is up-regulated for brief periods and then downregulated or completely inhibited. Persistent neovascularization, on the other hand, is a characteristic feature of malignant tumours and chronic inflammatory diseases.

Macrophages play a prominent role in eliciting vasogenesis (new blood and lymphatic vessel formation) through the release of growth-regulatory molecules that control neovascular growth, tissue remodeling, and repair. A variety of pathological neovascular processes are in fact believed to be mediated by macrophages including duodenal and stomach ulcers, endometriosis and rheumatoid arthritis (Battegay 1995, Taylor et al 1997). Thus, in healthy organisms neovascularization is under tight control. Cells able to induce neovascularization should be expected to remain inactive unless specifically activated. Many of the macrophage-derived angiogenic factors are indeed synthesized and released only by macrophages.

Polverini and colleagues (1977a, 1977b) were one of the first groups to demonstrate that macrophages may be required for neovessel formation *in vivo*. During delayed hypersensitivity reactions in the skin of guinea pigs, it was found that the highest degree of endothelial proliferation corresponded to the time of maximal mononuclear cell infiltration (Polverini et al 1977a). In a separate experiment, neovascularization was assayed in the normally avascular cornea of the guinea pig eye (Polverini et al 1977b). Control materials (paraffin oil, thioglycolate, macrophage collection media, culture media) induced angiogenesis in 6% of the corneas tested. Purified non-activated peritoneal macrophages showed activity in 8% of corneas. In contrast, macrophages stimulated with paraffin oil or thioglycolate induced a neovascular response in 75%. Furthermore, no corneal neovascularization occurred when activated lymphocytes or neutrophils were implanted. Other subsequent studies confirmed that macrophages need to be activated to exert angiogenic activity (Koch et al 1986, Meyer et al 1989).

Support for the work by Polverini and colleagues was simultaneously published by Evans (1977) who found that mice depleted of monocytes showed a strong reduction of tumour vascularization in implanted syngeneic fibrosarcomas. Similarly, Mostafa and colleagues (1980) showed that lymphoid neoplastic tissues exhibited angiogenic activity *in vitro* and *in vivo* only when macrophages were present. Knighton and colleagues

(1983) reported that macrophages cultured in a low oxygen environment exhibit enhanced angiogenic activity. The authors suggested that the low oxygen environment of tumours might serve to enhance macrophage-mediated angiogenic activity. Further work by Polverini and Leibovich (1984) using unstimulated tumour-associated macrophages showed that these cells were potent inducers of neovascularization. In contrast, purified tumour cell suspensions showed only a weak stimulation of microvascular proliferation both *in vitro* and *in vivo*. Thus, an association with tumour cells *in vivo* may be a natural stimulus of macrophage angiogenic activity.

Macrophages produce several factors, other than proteases, that induce the migration of endothelial cells (Table 3). Most of these factors also support other stages of the neovascularization process such as endothelial cell mitosis or differentiation. However, two factors have been isolated that appear to have predominantly chemotactic effects: human angiogenic factor (HAF) and angiotropin. Their migratory effects are sufficient for initial neovascularization as migrating cells can form sprouts without proliferating (Sholley et al 1984). Macrophages have also been implicated in laying down the fibrin matrix that supports neovessel formation. In a study of malignant lymphoid tissues, Costantini and colleagues (1992) found that the key proteins of the coagulation pathway (*i.e.* tissue factor, factor VII, factor X, and factor VIIa) were restricted to tissue macrophages. Furthermore, double-labeling techniques revealed fibrin in direct apposition to the infiltrating macrophages. Thus, both the systemic and local hypercoagulability associated with malignant disease is likely attributable to macrophage induction of procoagulant activity.

Macrophages have now been implicated in the neovascularization of many solid tumours. For example, in a study of patients with colon cancer, Takahashi and colleagues (1996) found a significant association between tumour-associated macrophage staining and both platelet derived endothelial cell growth factor (PD-ECGF, also known as thymidine phosphorylase) expression and vessel counts. Herrmann and colleagues

Table 3. Factors secreted by macrophages that stimulate neovascularization.[a]

Cytokine	Effect on endothelial cells
Acidic fibroblast growth factor (aFGF)	Chemotaxis, mitosis and tube formation
Angiotrophin	Stimulates chemotaxis, promotes capillary tube formation
Basic fibroblast growth factor (bFGF)	Chemotaxis, mitosis and tube formation
Granulocyte colony-stimulating factor (G-CSF)	Chemotaxis, mitosis and tube formation
Granulocyte/macrophage-CSF (GM-CSF)	Chemotaxis, mitosis and tube formation
Hepatocyte growth factor (HGF)	Chemotaxis
Human angiogenic factor (HAF)	Chemotaxis
Interferon (IFN)-α	Tube formation
Insulin-like growth factor (IGF)-1	Mitosis and tube formation
Interleukin (IL)-8	Chemotaxis and mitosis
Platelet-derived growth factor (PDGF)	Chemotaxis and mitosis
Substance P	Chemotaxis and mitosis
Thymidine phosphorylase (TP)	Chemotaxis
Transforming growth factor (TGF)-α	Mitosis
Transforming growth factor (TGF)-β	Chemotaxis and mitosis
Tumor necrosis factor (TNF)-α / nitric oxide	Chemotaxis, permeability and vasodilation
Vascular endothelial growth factor (VEGF)	Chemotaxis and mitosis

a. Adapted from Sunderkotter et al 1994, Battegay 1995, Liles and Van Voorhis 1995, Elgert et al 1998.

(1994) studying human follicular cell thyroid carcinoma (an aggressive variant of thyroid cancer) noted that increased macrophage infiltration was positively associated with dedifferentiation and vascularization. A study of bladder cancer by Hanada and colleagues (2000) showed that macrophage density was positively correlated with microvessel counts, while negatively associated with overall survival.

Although it has been shown that tumours or associated host cells can stimulate angiogenesis, it has been suggested that lymphagenesis (a term we coined for lymphatic vessel formation) does not occur in tumours (Folkman 1995). During wounding or other inflammatory conditions, the lymphatic system regenerates concomitantly with the blood vascular system in all tissues except the brain, retina and bone marrow. These latter tissues, however, do contain non-endothelialized prelymphatic channels that empty into the true lymphatics outside of these tissues (Casley-Smith 1980). Thus, we suggested that it is contrary to normal human physiology that such a process would not exist in association with tumours (Cann et al 1995b); in fact, more recent evidence suggests tumour-induced lymphagenesis does indeed occur (van Netten et al 1996, van Netten et al 1998). Furthermore, upon gross examination, tumours tend to be distinctly white, yellow or gray in contrast to more pink or reddish adjacent tissues (JP van Netten, personal communication). This suggests that although tumours are highly vascular, vessels containing red blood cells are relatively uncommon. As macrophages are key mediators of the angiogenic process, it is probable that they would also mediate solid tumour lymphagenesis.

Invasion and motility factors

Macrophages are known to express a range of motility factors or 'motogens' that are secreted during wound healing and promote the invasion of normal cells into injured tissue (Stoker and Gherardi 1991). Such factors may also promote the invasion and

metastasis of tumour cells when in the presence of macrophages. Two of the most potent motility factors produced by macrophages are the hepatocyte growth factor/scatter factor (HGF/SF) and the monocyte-derived scattering factor (MDSF) (Jiang et al 1993). Other factors secreted by macrophages including interleukin (IL)-1, IL-6 and transforming growth factor (TGF)-β have been shown to increase breast cancer cell motility (Verhasselt et al 1992, Jiang et al 1993). The prominent macrophage infiltration into tumour tissue and the close interactions observed between these two cell types (van Netten et al 1992, van Netten et al 1993a) may facilitate the stimulation of tumour cell functions such as invasion and metastasis through the release of these factors.

In a study by Jiang and colleagues (1995), a co-culture system was developed to study macrophage effects on the motility of tumour cells. In their co-culture, both cell types were physically separated by a porous Matrigel membrane. Human colon cancer (HT115, HRT18 and HT29) and breast cancer (MCF-7, ZR751 and MDA-MB-231) cell lines were co-cultured with human monocytes derived from the peripheral blood of healthy subjects or the monocyte cell line, U937. *In vitro* colonies of colon or breast cancer cells were shown to subsequently scatter and invade the Matrigel substrate after the addition of macrophages. In addition, the rate of tumour cell growth increased when these cells were co-cultured with macrophages. The pretreatment of macrophages with IL-4 or IL-10 (factors that inhibit macrophage chemotaxis and cytokine production) significantly reduced macrophage-stimulation of tumour cell growth, motility and invasion. IL-10 showed the strongest inhibitory effect on macrophages. In contrast, little or no effect was observed when tumour cells were treated with IL-10 or IL-4 alone. Therefore, macrophage interactions with tumour cells could be a crucial factor to augment the invasive and metastatic potential of a tumour.

Purpose of immunohistochemical analysis

In the first part of this study, an analysis of macrophage content in breast carcinomas was undertaken to examine the association between these immune cells and tumour aggressiveness. This involved the quantitative immunohistochemical identification of macrophage populations in breast tumours. This data was then correlated with the modified Bloom-Richardson grade, the most commonly used breast cancer grading system used to predict disease aggressiveness and tumour prognosis. The data was also compared to tumour cell mitosis to examine the influence of macrophage density on malignant cell proliferation. Finally, a qualitative analysis of macrophages in other pathological tissues was undertaken for comparative purposes. A computer aided image analysis system was introduced into this investigation in order to develop a more expedient, yet accurate, technique for quantitatively determining macrophage density.

METHODS AND MATERIALS

Ethical approval

The study to immunohistochemically analyze macrophage content in breast tumours was approved by the Capital Health Region Research Review and Ethical Approval Committee (Appendix II).

Tissue specimens

Specimens were obtained from the Department of Pathology at Royal Jubilee Hospital from September 1997 to August 1998. To preserve cellular morphology, breast tumour specimens were fixed in formalin immediately following surgery and subsequently embedded in paraffin wax. Samples were stored at room temperature until sectioning. In this study, thirty breast tumour specimens were examined in total. Representative specimens of lung cancer, prostate cancer, and thyroid goiter were collected and handled in a similar manner.

Immunohistochemical procedures

The macrophage antigen, CD68, and mitotic index antigen, Ki-67, were visualized using the mouse monoclonal antibody KP1 (DAKO, Glostrup, UK) and the rabbit polyclonal Ki-67 (DAKO), respectively. Using poly-l-lysine (Sigma Diagnostics, St Louis, MO) coated slides, representative 4 μm sections incorporating the tumour core to periphery were cut from paraffin-embedded blocks of breast carcinoma tissue. Serial sections were cut in order that regions of macrophage staining could be correlated with subsequent sections stained for mitotic cells. Cut sections were allowed to adhere in a 56° C oven overnight. The slides were then deparaffinized and rehydrated through immersion in

xylene (3 x 5 minutes), 100% ethanol (2 x 5 minutes), 95% ethanol (5 minutes) and distilled water (5 minutes). This was followed by microwave treatment (in pH 6.0 citric acid buffer), high power for nine minutes in a high-pressure container to enhance antigen retrieval before staining. To block endogenous peroxidase activity, sections were incubated for 30 minutes in 0.3% hydrogen peroxide diluted in methanol. Slides were then covered with normal horse serum (Vector Laboratories, Burlingame, CA) diluted 1:25 for 30 minutes in order to inhibit any non-specific binding of antibodies. The primary antibodies were added to separate sections for 45 minutes at the following dilutions: CD68 (1:150), Ki-67 (1:200). After application of the primary antibody, sections were treated with a bi-specific biotinylated horse anti-rabbit/mouse immunoglobulin (IgG) (Vector Laboratories) for 30 minutes. This was followed by incubation with a horseradish peroxidase-avidin biotin complex (Vector Laboratories) for 30 minutes. Staining was carried out with diaminobenzadine (DAB), which produces a brown precipitate. The solution was made 2 to 3 hours in advance and stored in the dark — one DAB tablet (Sigma Diagnostics) in 12 ml buffer. Immediately before use, the DAB solution was vortexed, filtered and 220 µl of 3% hydrogen peroxide was added. The tissue sections were incubated with DAB solution under a light source for 2 to 10 minutes for colour development. Phosphate buffered saline (PBS, pH 7.5) was used for all dilutions and for rinsing slides (2 x 5 minutes) between each step, and all incubations were performed at room temperature. A non-ionic detergent (Nonidet P-40, Sigma Diagnostics) was added to the PBS buffer at a concentration of 0.01% to enhance antibody penetration and reduce background staining. Finally, sections were counterstained with nuclear fast red (Vector Laboratories) or blue Mayer's hematoxylin (BDH Inc, Toronto) and coverslipped in Univert aqueous mountant (Hopkin and

Williams, Essex, UK). Control sections were treated identically, excepting the primary antibody.

Intratumoural macrophage / mitotic cell density determination

Density of staining was determined by light microscopy in invasive tumour areas that contained the most macrophages or mitoses per area (*i.e.* hot spots of more intense staining). Intratumoural hot spots were found by scanning the tumour at low power 25X (10X eyepiece/2.5X objective). A computer-aided image analysis system (CIAS — Optimas 5.1) at high power (100X) was then used to measure the total area of staining in a given field (five computer screens) for a total of 0.67 mm^2 per tumour per antigen. This field size has been suggested as an optimal size for tumour analysis (Weidner and Folkman 1996).

A second analysis was performed on a subgroup of five breast tumours to examine if a positive correlation existed between serial areas of macrophage and mitotic-stained areas, respectively. For each tumour, six regions were chosen randomly from control sections for study. Matching regions, on serial sections, stained with antibodies to CD68 and Ki-67, were then examined by the Optimas imaging system. A total of 2.0 mm^2 was comparatively analyzed per tumour per antigen.

Analogous immunohistochemical staining was performed on other pathological tissues (lung and prostate carcinoma, and hyperplastic goiter) to determine if similar processes were occurring in these abnormal tissues. With respect to the lung carcinoma, tissues were stained with the same primary antibodies (CD68 and Ki-67), however, glucose oxidase-labeled avidin (Jackson Immunoresearch, West Grove, PA) with a nitro-blue tetrazolium (NBT, Vector Laboratories) substrate was used to highlight the antigens of interest. NBT forms a purple/blue precipitate upon conversion by glucose oxidase. These three tissue types were analyzed qualitatively.

Statistical analysis

The following relationships were examined in the breast carcinoma samples: (1) *hot spots* — correlations were examined between (a) tumour diameter (*i.e.* longest diameter) and CD68/Ki-67, (b) modified Bloom-Richardson grade and CD68/Ki-67, (c) modified Bloom-Richardson subscore and CD68/Ki-67 and; (2) *matched sets* — correlations in staining density between matched regions of CD68 and Ki-67. The levels of significance for each correlation were determined using a 95% confidence interval. The Pearson correlation coefficent was used as the distribution of outcomes was found to be approximately linear and normally distributed. Correlations with a P value ≤ 0.05 (two-tailed) were considered significant. All calculations were performed using the statistical software SPSS version 9.0.

RESULTS

Statistical analyses were summarized in Table 4. Briefly, no relationship was observed between breast tumour diameter and the density of CD68 macrophage staining (Figure 7). In contrast, Ki-67 mitotic staining density was significantly ($r = 0.64$, $P < 0.001$) associated with tumour diameter (Figure 8). A significant relationship was found between macrophage staining and the modified Bloom-Richardson grade ($r = 0.41$, $P = 0.035$) (Figure 9); and similarly, the association between mitotic staining and the modified Bloom-Richardson grade ($r = 0.68$, $P < 0.001$) (Figure 10). Finally, the modified Bloom-Richardson subscore, from which the tumour grade is calculated, was significantly correlated with macrophage density ($r = 0.44$, $P = 0.023$) (Figure 11), and a highly significant association was observed with mitotic staining and the modified Bloom-Richardson subscore ($r = 0.78$, $P < 0.001$) (Figure 12).

As seen in Figure 13, a significant association ($r = 0.75$, $P < 0.001$) was found between matched regions from serial sections stained for CD68 and Ki-67. This data was accumulated from a random subgroup analysis of five breast tumours with modified Bloom-Richardson grades I to III.

Macrophage staining tended to reach its greatest density at the tumour periphery and beyond, where isolated invasive tumour cells could be seen (Figure 14). Within the tumour, macrophage staining was less dense and generally confined to the narrow stromal areas around abundant tumour cell microclusters (Figure 15a). The exception was in central necrotic areas within tumours, where dense clusters of macrophages are often seen phagocytizing debris. Staining for dividing cells, in contrast, was more homogeneous. There was generally denser staining towards the tumour perimeter with fewer areas of distinct clustering (Figure 15b).

A tumour of low histologic grade in Figure 16 illustrates the association between cellular proliferation (Figure 16a) and macrophage density (Figure 16c); yet, in general,

Table 4. Linear regression analysis of macrophage and mitotic cell associations with breast tumour prognostic factors.

Correlation Variables	Correlation Coefficient (r)	*P* Value[a]
Hot spot analyses		
Macrophage density *vs*:		
Tumour diameter	0.08	0.678
MBR grade	**0.41**	0.035
MBR subscore	**0.44**	0.023
Mitotic cell density *vs*:		
Tumour diameter	**0.64**	< 0.001
MBR grade	**0.68**	< 0.001
MBR subscore	**0.78**	< 0.001
Matched set analysis		
Macrophage *vs* mitotic cell density	**0.75**	< 0.001

a. Correlation coefficients in bold are significant

Figure 7. Non-significant association between macrophage staining (CD68 antigen) and breast tumour diameter. (n = 28, r = 0.08, $P < 0.678$)

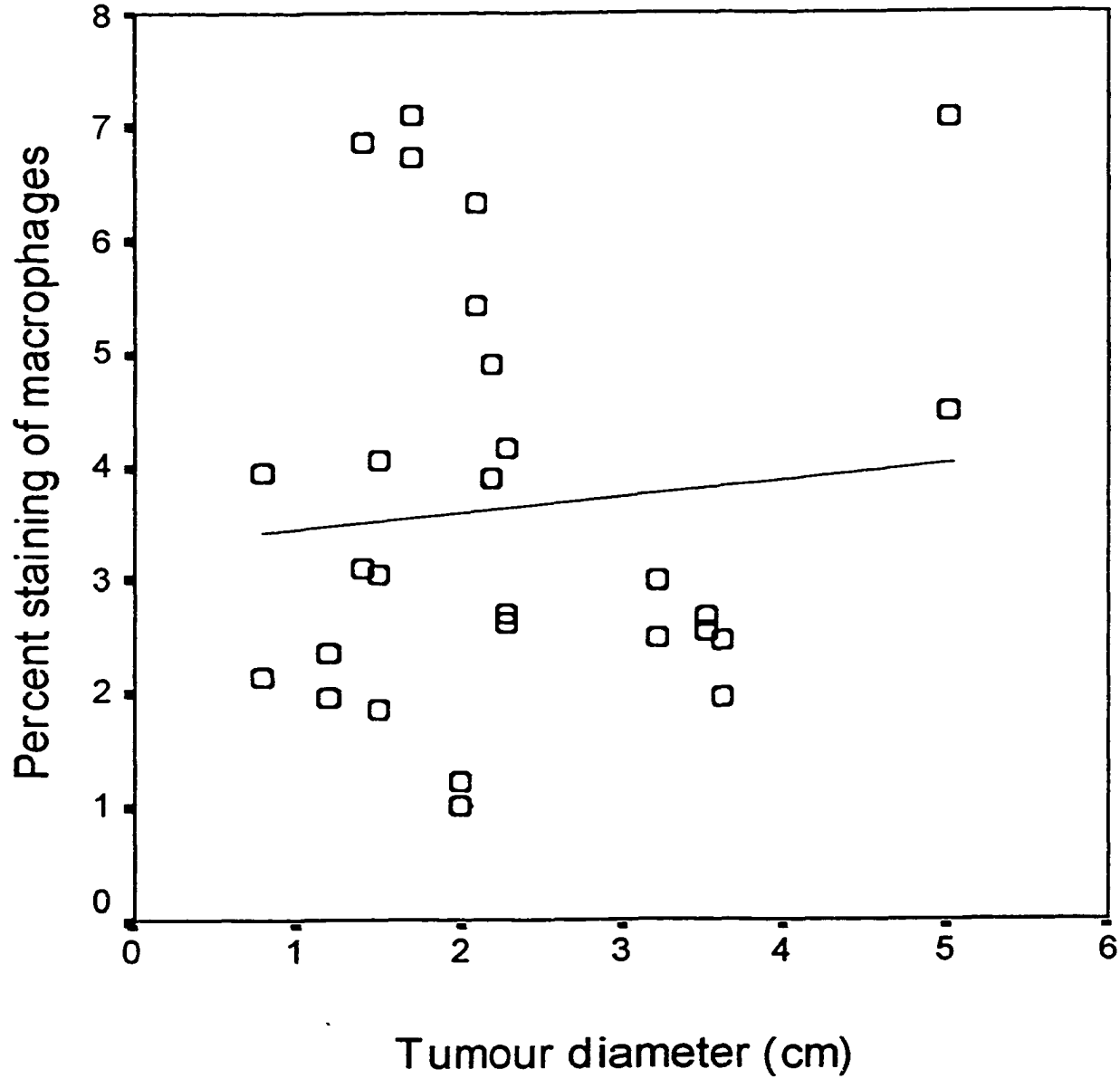


Figure 8. Association between staining of mitotic cells (Ki-67 antigen) and breast tumour diameter. ($n = 28$, $r = 0.63$, $P < 0.001$)

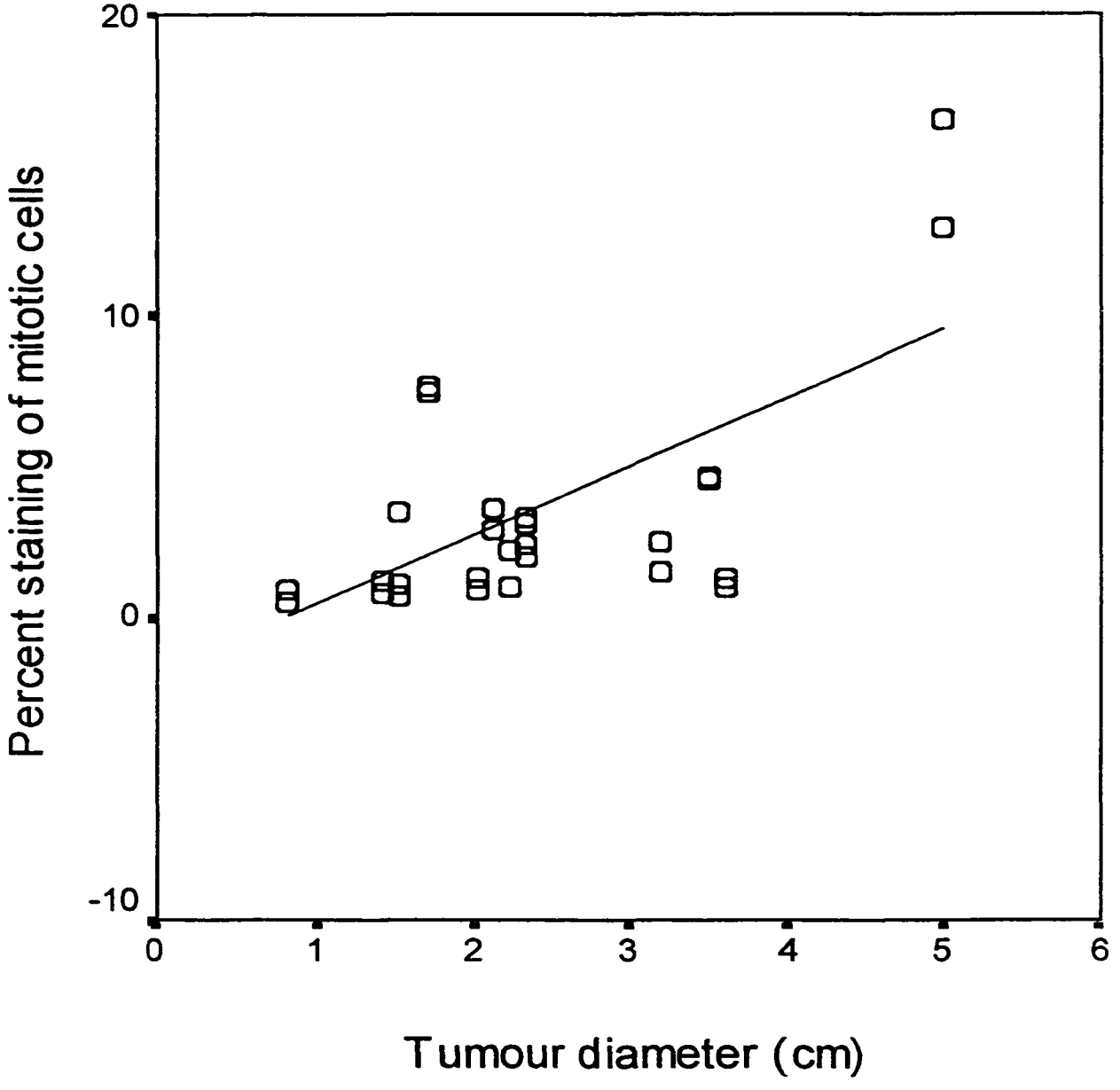


Figure 9. Association between staining of macrophages (CD68 antigen) and the modified Bloom-Richardson grade. (n = 27, r = 0.41, $P < 0.035$)

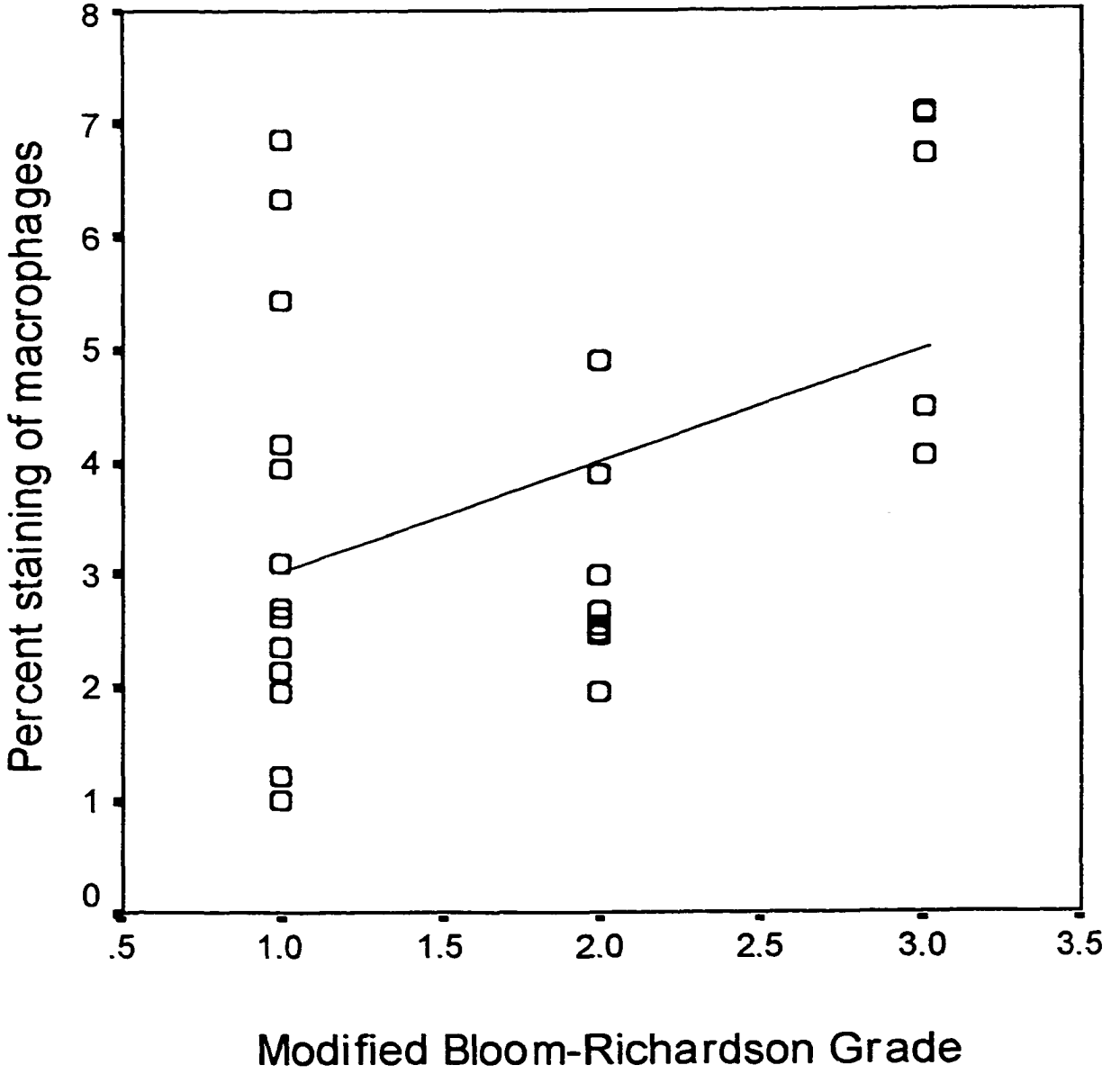


Figure 10. Association between staining of mitotic cells (Ki-67 antigen) and the modified Bloom-Richardson grade. (n = 27, r = 0.68, $P < 0.001$)

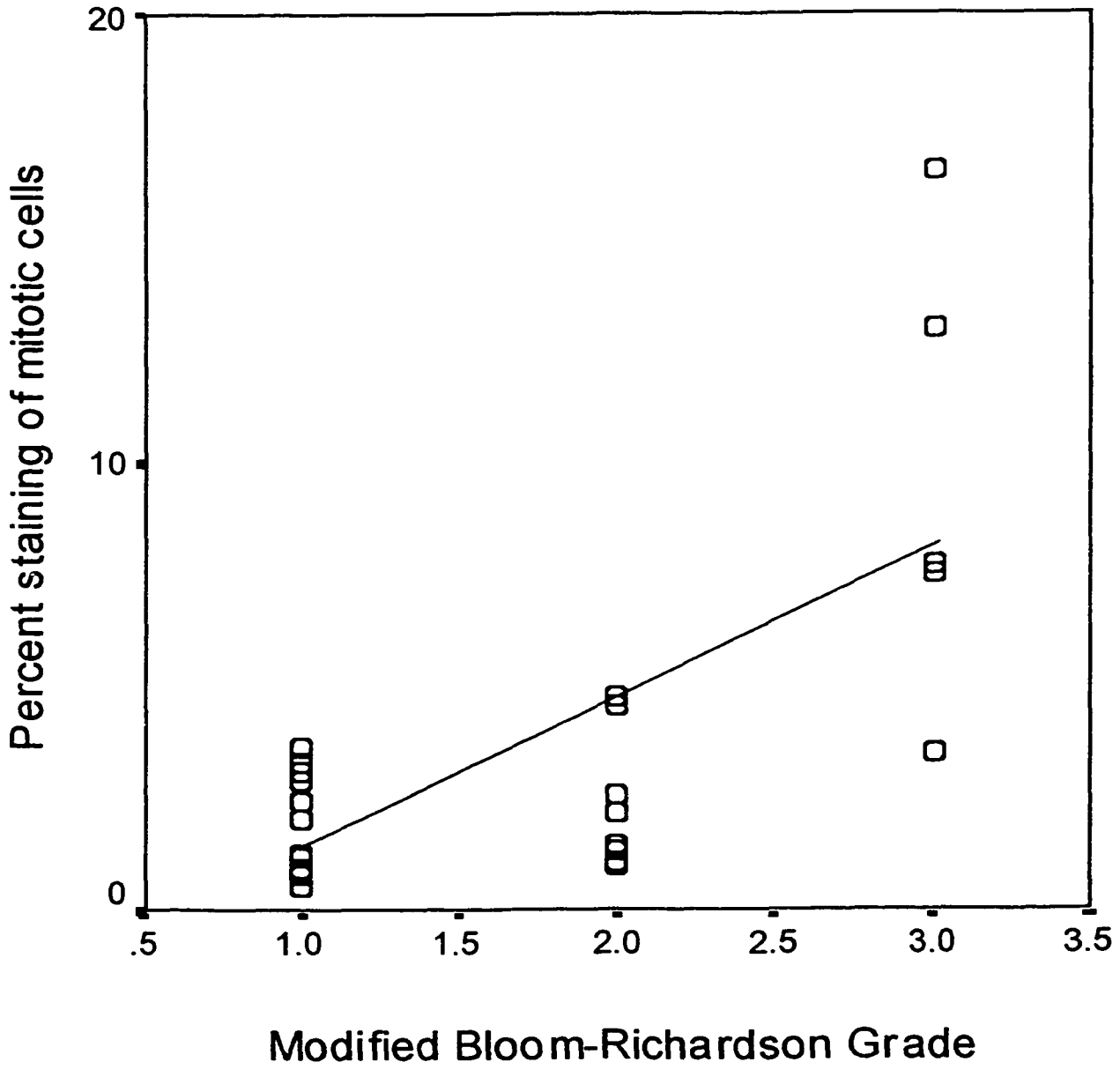


Figure 11. Association between staining of macrophages (CD68 antigen) and the modified Bloom-Richardson subscore. (n = 27, r = 0.44, $P < 0.023$)

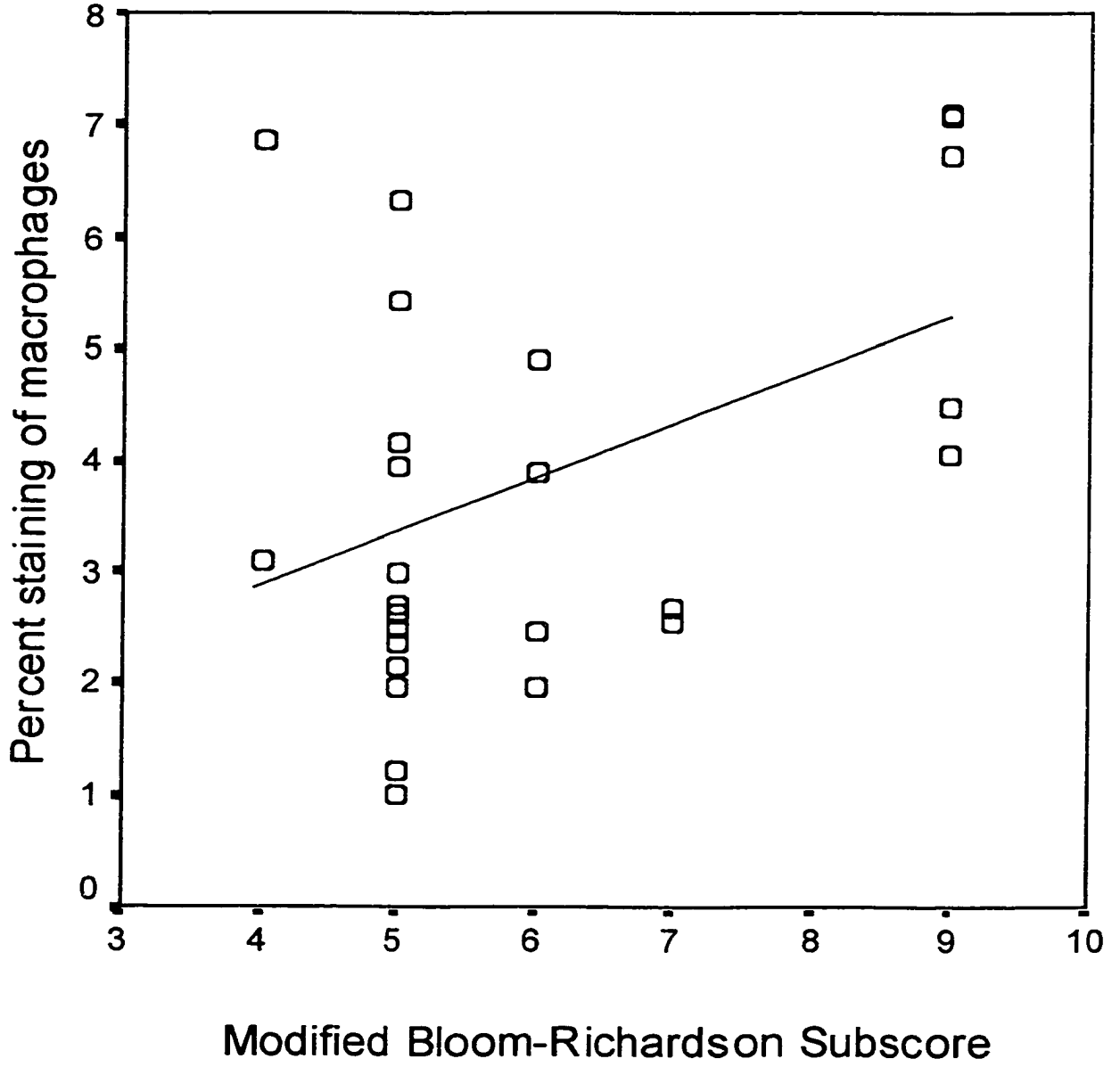


Figure 12. Association between staining of mitotic cells (Ki-67 antigen) and the modified Bloom-Richardson subscore. (n = 27, r = 0.78, $P < 0.001$)

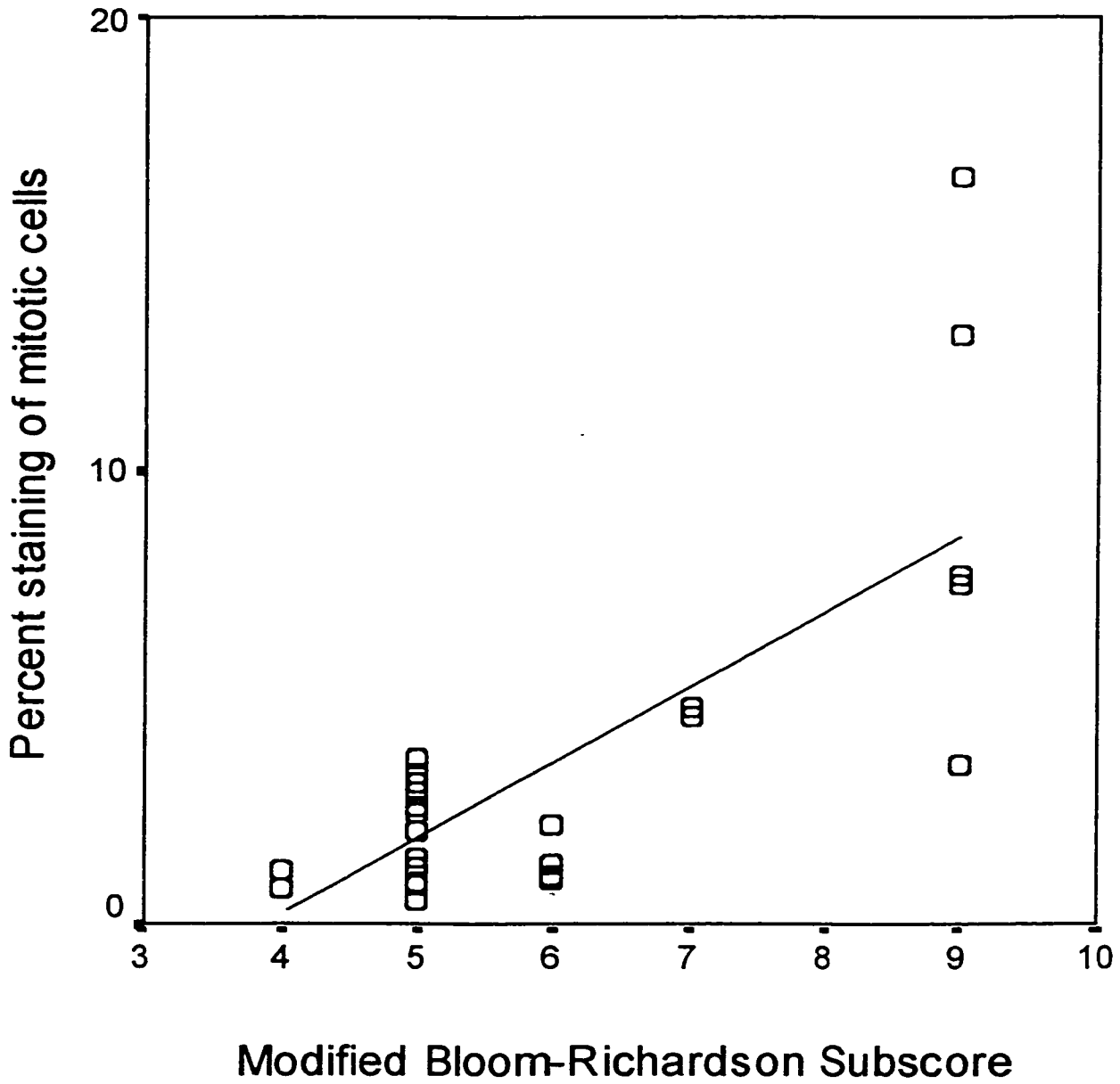


Figure 13. Association between staining of macrophages (CD68 antigen) and mitotic cells (Ki-67 antigen). (n = 5 tumours, 30 observations, r = 0.75, $P < 0.001$)

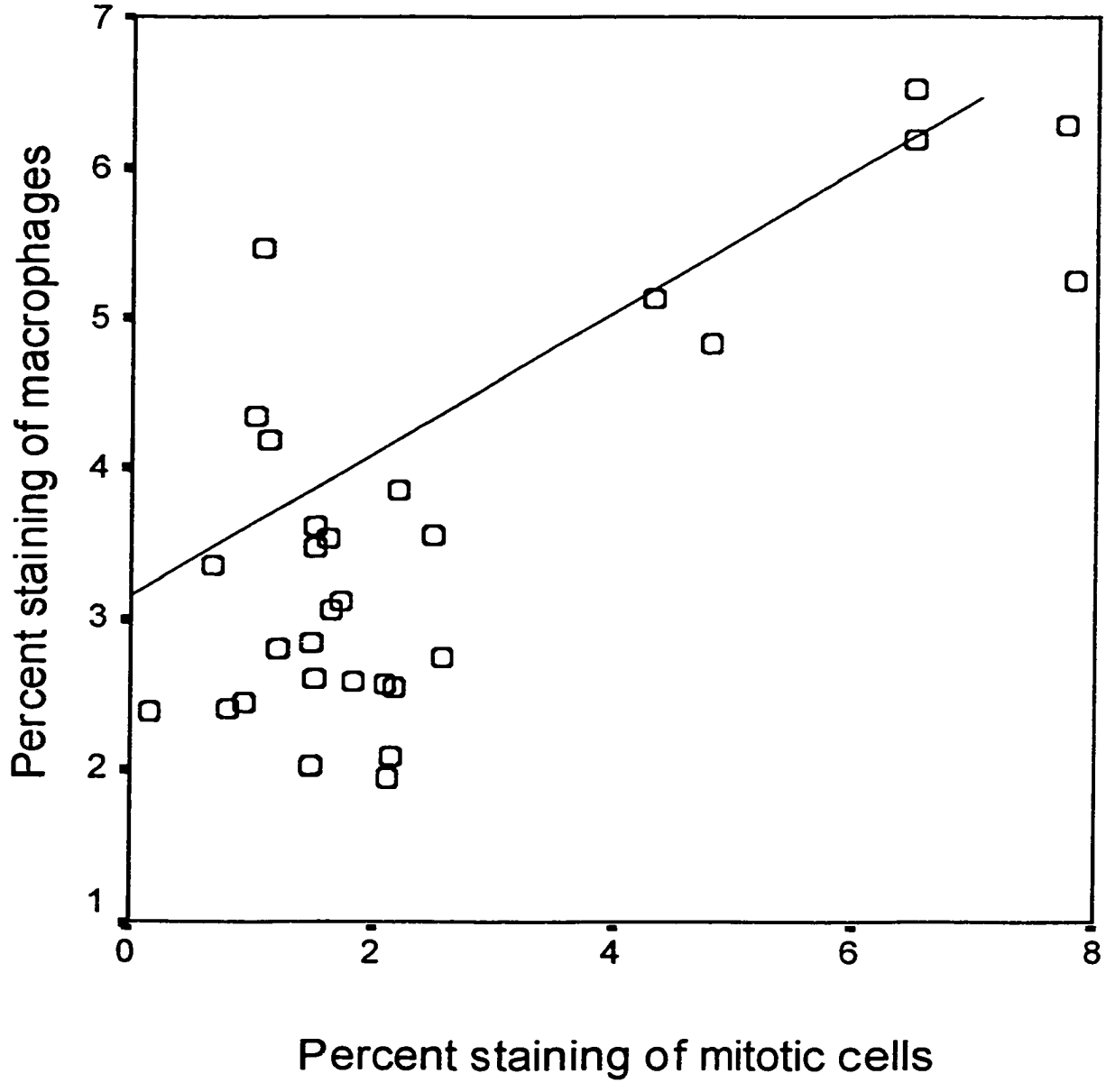


Figure 14. MBR grade III breast carcinoma shows a serial section with (a) and without (b) staining for CD68 antigen. As seen in the photomicrograph, there is a tendency for macrophages to concentrate at the tumour periphery (arrows).

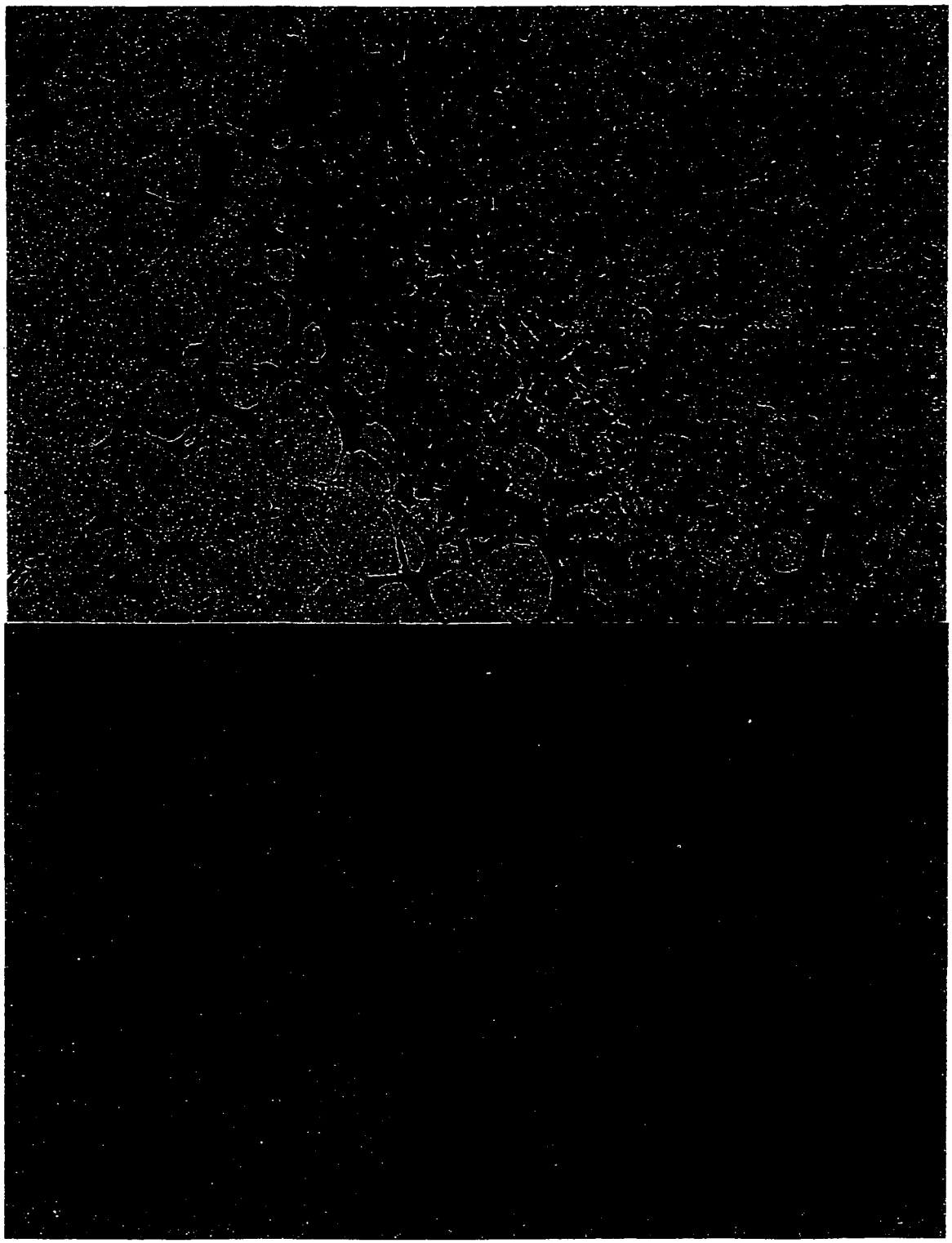


Figure 15. MBR grade III breast carcinomas: (a) this photomicrograph illustrates macrophage abilities for infiltrating within the tumour core and filling gaps between tumour microclusters (arrows); (b) staining for Ki-67 antigen shows the high degree of proliferative activity in this aggressive tumour.

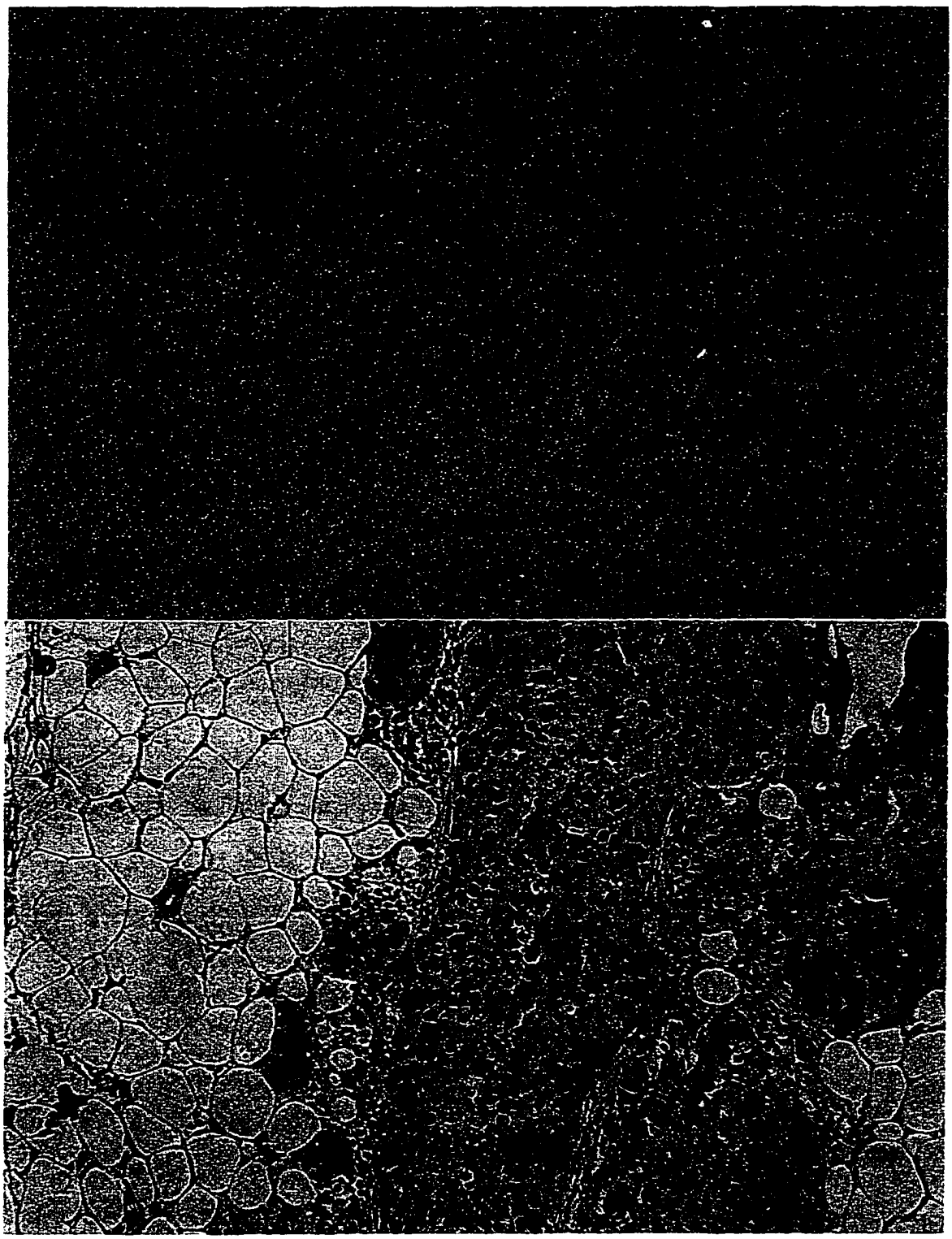
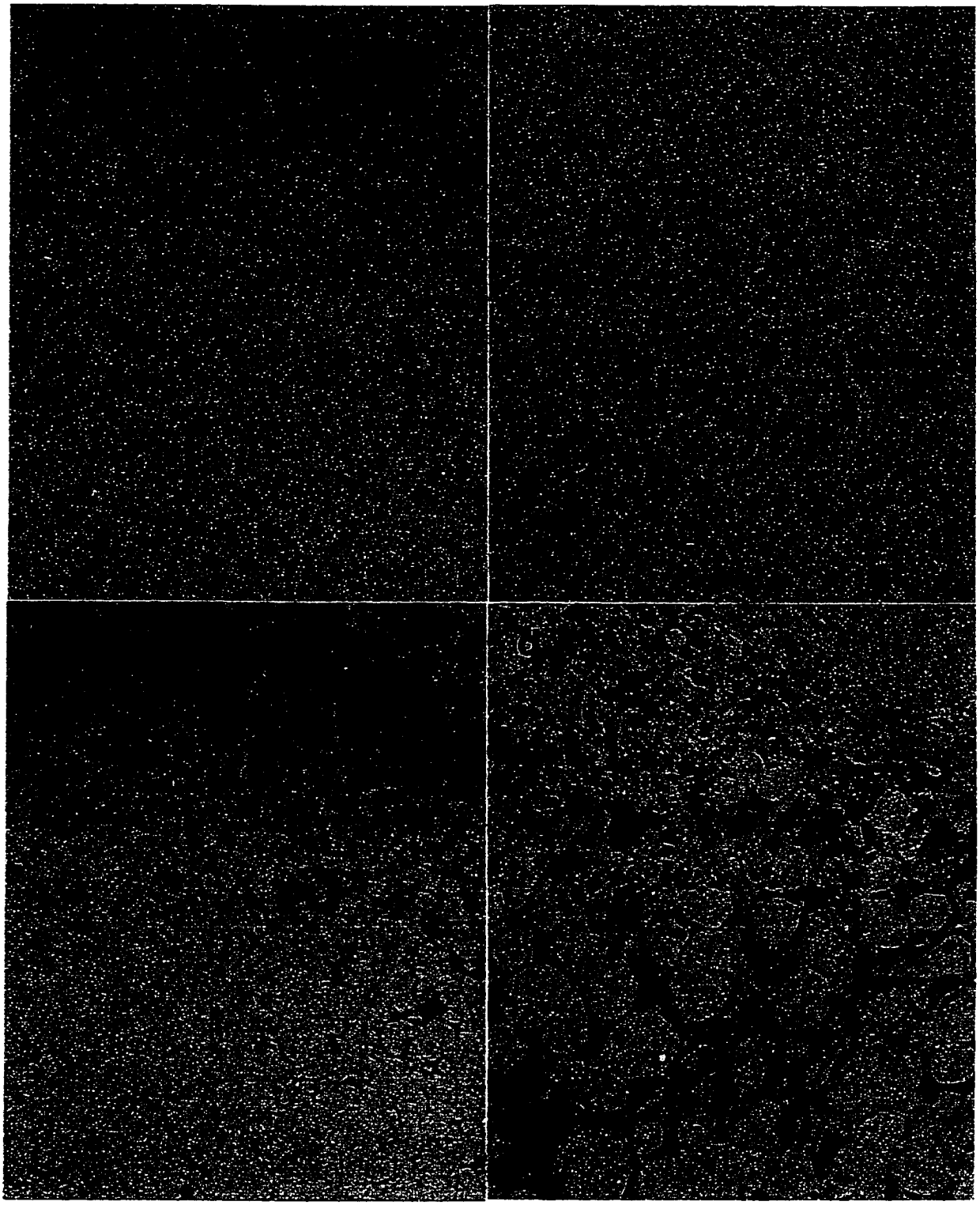


Figure 16. MBR grade I breast tumour infiltrating surrounding tissue stroma and fat. Staining for dividing cells shows mild proliferative activity (a and b). Serial sections stained for CD68 antigen show considerable macrophage infiltration (c and d).



under higher magnification mitotic activity (Figure 16b) was minimal in areas of dense macrophage infiltration (Figure 16d), although cell division was often elevated in tumour cells adjacent to such regions.

Examinations of lung tumours suggested that macrophages play an important role in this tumour type as well. Unlike breast carcinomas, where macrophage penetration into the tumour structure itself is common, infiltration was sparse in the lung adenocarcinomas examined (Figure 17a). However, macrophage infiltration of the lung tumour periphery was common and often quite dense (Figure 17b). Large macrophage clusters were often observed at the tumour edge with smaller infiltrative components seen as well. In the prostatic carcinoma samples examined, macrophages appeared to be confined primarily to the acellular stroma that surrounds the invasive cancer cells. The clustering of macrophages in prostate tumours did not appear to be distinctly associated with the tumour perimeter and these cells were generally not within the tumour structure itself. Finally, in hyperplastic goiter, macrophages tended to associate in cellular areas often clustering around, but not distinctly within, areas with high mitotic indices (Figure 18).

Figure 17. Poorly differentiated non-small cell lung carcinoma. In central areas of the tumour, macrophages (stained blue) are less prevalent (a); in contrast, macrophage clustering at the tumour periphery can be quite prominent (b).

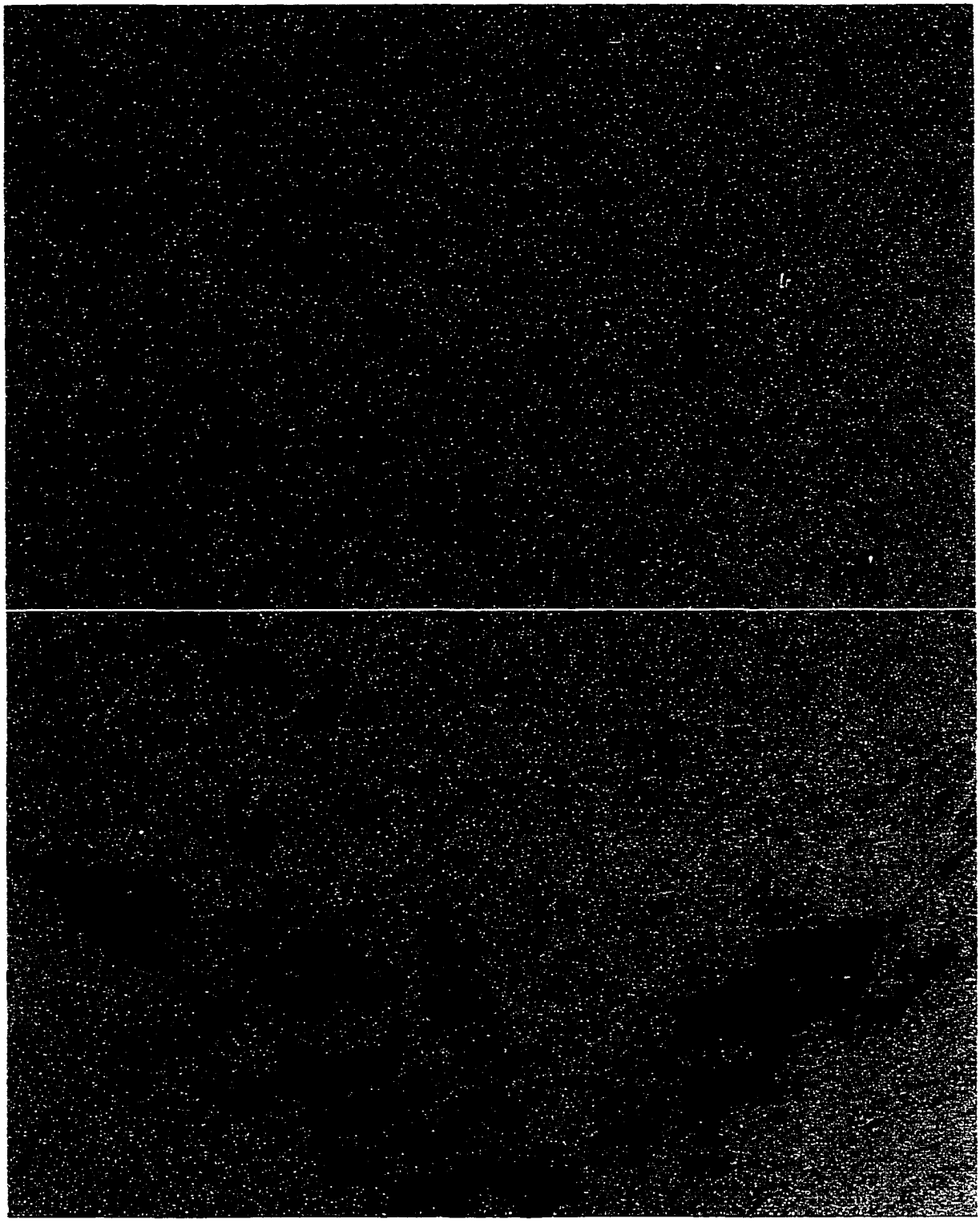
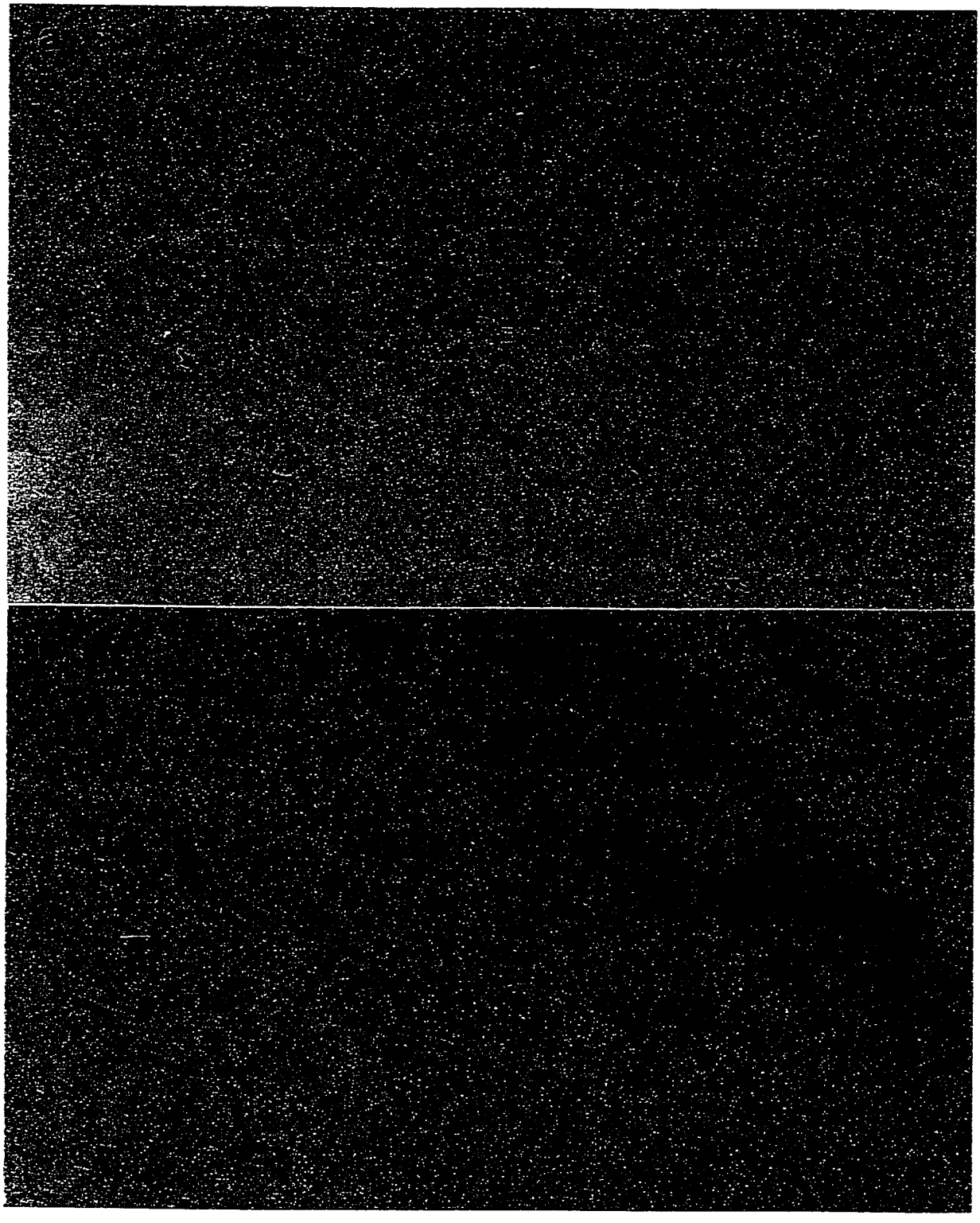


Figure 18. Microsection of a multinodular goitre. In these photographs (a and b) areas of hyperplasia can be seen (large arrows) along with colloid follicles (small arrows). The accumulation of proteinaceous colloid (normally where thyroid hormones are stored) may result from prolonged iodine deficiency, or infrequently, from genetic disease. Mitotic cell staining (a) is seen primarily within hyperplastic regions. Macrophages (b) are generally seen at the periphery and sporadically throughout colloid areas.



DISCUSSION

The macrophage antigen, CD68, targeted in this study has a wide specificity for macrophage subtypes including blood monocytes, liver Kupffer cells, lung alveolar macrophages, and skin Langerhans cells among others. CD68 antigen is a 110kD intracellular glycoprotein primarily associated with macrophage cytoplasmic granules and membranes. The function of CD68 is unknown, but lysosomal glycoproteins are major components of lysosomal membranes and may protect the membranes from attack by hydrolases.

In this study, macrophage staining density in breast cancer tissue was found to correlate significantly with the modified Bloom-Richardson grade and subscore, but not with tumour diameter. Although the sample size was small, the positive correlation between macrophage density and poor prognosis has now been confirmed in other solid cancers — bladder (Hanada et al 2000), endometrium (Salvesen and Akslen 1999), lung (Takanami et al 1999) — as well as a recently published study on breast cancer (Leek et al 2000).

A significant association was found between Ki-67 staining and tumour diameter, as well as, highly significant correlations with both the modified Bloom-Richardson grade and subscore. The Ki-67 antigen is a cell cycle and tumour growth marker that is only present in cycling cell nuclei and is variably expressed in the different phases (G1, S, G2, and M) of the cell cycle (Gerdes et al 1991). The association of mitotic index with tumour diameter demonstrated that tumour diameter was an inferior prognostic indicator in comparison to tumour grade. The modified Bloom-Richardson grading scheme employed in this study is the most common in use.

Most studies of Ki-67 antigen expression in breast carcinomas have utilized frozen section immunohistochemistry or flow cytometry on fresh tissue. Fresh or frozen tissue may not be available on all tumors, and this is especially true of small tumours that are

usually entirely fixed and embedded to preserve the morphological detail needed to make an accurate diagnosis of malignancy. The high-pressure microwave antigen retrieval system used in this study was able to enhance staining intensity and the significant association between staining density and tumour grade suggests that paraffin-embedded tissue may be equally suitable with this method.

The analysis of matched serial sections found a significant association between CD68 and Ki-67 staining density. This potential association may suggest several possibilities: (1) that macrophages stimulate tumour cell division through juxtacrine or paracrine growth factor (*i.e.* TGF-β, EGF) production; (2) tumour cell division attracts macrophages, but tumour proliferation is not stimulated by the macrophage presence; or (3) it is the macrophages themselves that are dividing. This first and second hypotheses may be equally possible and cannot be ruled out from this analysis. However, as outlined in the introduction, macrophages can stimulate tumour cell division *in vitro* (Munzarova and Kovarik 1987, van Netten et al 1993a), and observationally, macrophage phagocytic activity was only noted in the necrotic tumour core and not along the rapidly dividing tumour growth front. The third hypothesis (that macrophages themselves are dividing) seems unlikely due to the fact that under high power magnification, it is highly unusual to observe anything more than minimal Ki-67 staining in macrophage clusters — often none is apparent. This suggests that the majority of macrophages are infiltrating into, rather than dividing at, the tumour site. The observations of other pathological tissue types suggest that macrophages may play a similar and at the same time distinct role in the progression of these other diseases.

Macrophages and cancer treatment

In light of the preceding information, several potential avenues exist for the immunomodulation of macrophages in cancer therapy. First, activation of macrophage cytotoxic functions would allow one to utilize the considerable population of macrophages

already present in tumours and their metastases to induce cancer regression. In contrast, a second strategy would involve the inhibition of macrophage tumour-stimulating (repair) activities. Finally, a third strategy relates to the role that dietary deficiencies may play in enhancing both the abnormal proliferation of breast epithelium and macrophage infiltration. This final strategy will be discussed in greater detail in Chapter 2.

Enhancement of macrophage defense

Many recent experiments in animal systems have taken advantage of the high prevalence of macrophages in solid tumours. For example, Korbelik and colleagues (1997) examined the synergistic effects of administering photophrin (a light-activated toxin) and vitamin D3 binding protein-derived macrophage activating factor (DBPMAF, a macrophage-specific stimulator) on mice with subcutaneously transplanted squamous cell carcinoma. The photodynamic therapy alone was curative to one quarter of the tumours treated, however, when used in combination with DBPMAF, the regimen boosted cures to 100%.

In humans, BCG is one of the few immunotherapies in standard use, where it is employed primarily for the treatment of bladder cancer. Most controlled clinical trials suggest that BCG is more effective than either anthracycline, doxorubicin, or the alkylating agent, thiotepa (standard chemotherapeutic agents for bladder cancer) (Cummings 1991). Research examining the mechanisms for BCG's antitumour activity have emphasized the importance of macrophages. Experimental studies have shown that BCG-induced suppression of tumours is a host-mediated response since these organisms are not directly cytotoxic to tumour cells *in vitro*. However, BCG therapy is successful in immunosuppressed animals. Rat tumours xenotransplanted into athymic nude mice, which have deficient lymphocyte activity, may be inhibited in their growth through the use of BCG therapy (Pimm and Baldwin 1975). In contrast, treatment of animals with particulate silica, a type known to be selectively toxic for macrophages, abrogates BCG-

mediated tumour suppression both in athymic mice and rats (Chassoux and Salomon 1975, Hopper and Pimm 1976), suggesting that macrophages play a primary role in this type of immunotherapy. Furthermore, it has been shown that enrichment of tumour deposits with syngeneic macrophages facilitates tumour suppression with regionally applied BCG (Hopper and Pimm 1976). For example, the transplanted 4-dimethylaminoazobenzene-induced rat hepatoma normally contains 10% macrophages, and only a small number of tumour cells are prevented from growth when injected subcutaneously in an admixture with BCG (Hopper and Pimm 1976). However, when normal rat peritoneal macrophages are added to the tumour cell inocula to give a 50% final macrophage content, the maximum number of hepatoma cells prevented from growth by admixture with BCG is raised at least 20 times. Addition of macrophages alone had no inhibitory effect on tumour growth. More recent work supports the major role for macrophages in BCG-induced antitumour activity (Akaza et al 1993, Maes and Cocito 1996). Such macrophage activation has also been implicated as the key mechanism for tumour inhibition of another non-specific immunotherapeutic agent, Coley's toxin. Tang and colleagues (1991) reported that the presurgical use of Coley's toxin in patients with hepatocellular carcinoma significantly increased macrophage activity, as well as, macrophage tumour infiltration in comparison to untreated subjects. Hepatic tumours that were initially inoperable, became operable subsequent to treatment at the 'second look' surgery.

Inhibiting macrophage repair

In contrast to the preceding research, experiments are also being carried out that inhibit macrophage function. For example, the immunomodulator, linomide, has been used effectively in animal systems against breast, lung, melanoma, prostate and seminal vesicle cancer (Borgstrom et al 1995, Joseph et al 1996, Ziche et al 1998). Although the precise method of action is unclear, linomide has been shown to suppress macrophage

production of acidic and basic FGF (aFGF and bFGF) and vascular endothelial growth factor (VEGF), as well as, inhibiting macrophage migration (Vukanovic and Isaacs 1995b, Joseph et al 1996). Thalidomide, another agent that inhibits macrophage production angiogenic factors, also suppresses tumour growth but to a lesser degree than linomide (Joseph and Isaacs 1998). IL-10 is another factor that impairs macrophage function, including cytokine production (Fiorentino et al 1991), and it has been shown to inhibit tumour growth in various animals systems (de Waal Malefyt et al 1991, Kundu et al 1996). Therefore, these studies demonstrate that macrophage inhibition may directly or indirectly impair tumour progression and/or associated vascular development.

CHAPTER 2: *A case-control study of iodine, selenium and other associated factors in patients with newly diagnosed breast carcinoma*

INTRODUCTION

Iodine has a number of key roles in normal human physiology, yet aside from its activity in thyroid hormones, little attention has been paid to the extrathyroidal functions of this essential trace element. In this second chapter, an examination is conducted on the physiological role of iodine with respect to the endocrine and immune systems, as well as how these activities may relate to that sub-component of the immune system, the macrophage. In the following sections, 'iodine' is used as a general term to refer this element or compounds that contain this element; however, when specific iodine compounds are known or relevant to the discussion more precise terminology will be used.

Iodine and thyroid function

The thyroid gland is the primary site for iodine uptake and storage. Few glands exert such a significant and diverse influence on the whole body as the thyroid. It controls the metabolism and function of nearly every gland, organ and cell in the body. Iodide (I^-) is concentrated in the thyroid under the influence of thyroid stimulating hormone (TSH or thyrotropin), a hormone secreted by the anterior pituitary. Once in the thyroid, iodide is oxidized by thyroperoxidase and the electron acceptor, hydrogen peroxide. The oxidized iodine is then organified to tyrosyl residues of thyroglobulin. This is followed by a coupling of iodinated tyrosyl residues (*i.e.* iodotyrosines) within thyroglobulin, forming

tetra-iodothyronine (thyroxine or T4) and to a lesser degree tri-iodothyronine (T3). These thyroglobulin-bound hormones are stored in the colloid region within thyroid follicles and released from the thyroid as needed (depending on blood T3, T4 and TSH levels). During transport through the bloodstream, thyroid hormones carried primarily by the thyroid hormone transport proteins, thyroxine-binding globulin, transthyretin and albumin; although a small fraction of thyroid hormones are unbound, where they are taken up by the cells of the body. The levels of T3 and T4 in the blood provide negative feedback to the hypothalamus. Thus, low thyroid hormone levels induce the hypothalamus to secrete the tripeptide thyrotropin-releasing hormone (TRH), which in turn, stimulates pituitary secretion of TSH. It is this hypothalamic-pituitary-thyroid axis that maintains primary regulatory control over blood thyroid hormone levels.

Thyroid hormones play an absolutely crucial role in controlling the metabolic activity of bodily organs. The physician Coindet (1820) was the first to note that iodine was effective in alleviating the symptoms of goitrous subjects. Further evidence of the importance of thyroid hormones became clear in the late 1800's when thyroidectomy (surgical excision of the thyroid gland) was introduced as a treatment for hyperthyroidism and goiter. Although the operation alleviated symptoms, patients subsequently became puffy-faced, slow of mind and socially non-functional (*i.e.* they became hypothyroid) (Reverdin 1882, Kocher 1883). Insufficient thyroid hormone production can lead to a wide spectrum of symptoms including: apathy, coarse skin and hair, cold sensitivity, depression, fatigue, forgetfulness, swollen face, hands and feet, muscle and joint pain, reduced heart rate, susceptibility to infections, excessive menstrual bleeding in women, and in the elderly, a common factor in dementia (Ganguli et al 1996).

In general, however, the thyroid is capable of maintaining normal hormone levels even under extremes of iodine intake. Euthyroidism has been shown to be common even when iodine intake is an order of magnitude higher than the recommended daily allowance of 150 μg/day (Konno et al 1993a), as well as when intake is insufficient (< 100 μg/day)

(Hollowell et al 1998). Under conditions of excess, kidney iodine excretion is enhanced. Under conditions of insufficiency, the thyroid becomes highly specialized at iodine absorption, through increased TSH production. However, in the latter case, this enhanced incorporation of iodine into the thyroid and thyroid hormones occurs at the expense of extrathyroidal uses of this element.

It should be noted, however, that an increase in iodine uptake in a highly deficient individual (*i.e.* $\leq$ 50 μg/day) may adversely effect thyroid function. This has been observed in Europe and developing countries when iodization programs are first introduced. The change in iodine intake can lead to autoimmune thyroid disease, referred to as 'Jod-Basedow syndrome'. The supranormally enhanced iodine uptake of deficient thyroids leads to iodine-induced oxidative damage, generally in subjects with other dietary deficiencies.

Abnormalities of thyroid hormone metabolism, primarily reduced T3 levels, are commonly seen in a wide variety of nonthyroidal illnesses, including: severe infections, inflammatory conditions, trauma, major surgery, and starvation. This phenomenon is known as 'euthyroid sick syndrome' or 'low T3 syndrome' (McIver and Gorman 1997). Little is known about the function of this decreased thyroidal activity on the host-parasite relationship during infectious disease. One purpose would be a reduction of tissue energy requirements while metabolic activity is redistributed to immune functions (*i.e.* generating a fever, leukocyte proliferation, antibody production, *etc.*). A study by Little (1985) appears to support this hypothesis. He demonstrated that rats infected with *Streptococcus pneumoniae* develop euthyroid sick syndrome (significantly reduced T3 and T4 levels), yet daily administration of T4 (to normalize thyroid hormone levels) significantly increased total animal deaths as compared to controls. Thus, as we will see in greater detail later, the iodine of thyroid hormones and extrahormonal iodine play very distinctive roles.

Iodine and immune function

The effects of iodine on specific immune functions have been studied for over 100 years. Heinz (1899) stated that iodides made leukocytes more active. Rothschild (1913) published the idea that iodine stimulated phagocytosis *in vitro*. Similarly, Kolmer and colleagues (1916) showed that the sera of persons and rabbits receiving iodides had increased power for phagocytosis. Iodine is known to concentrate at sites of inflammation and it is likely that factors released from sites of injury or infection increase iodine uptake (Sternberg et al 1955, Bakheet et al 1999). For example, it has been shown that histamine can enhance iodide uptake and trapping in extrathyroidal tissues (Scott and Peng 1955, Brown-Grant et al 1965). Conversely, thyroidal iodine uptake during acute infections in mice has been shown to be reduced by half in comparison to control animals. This may relate to the euthyroid sick syndrome discussed earlier, where iodine assumes an extrahormonal role. Thus, there may be a variety of factors secreted from sites of inflammation that enhance iodine uptake, where iodine plays a nonhormonal physiological role in resolving the inflammation or infection. In order to examine the immune-enhancing effects of iodine, Stone and Willis (1967) injected heat killed *Corynebacterium acnes* subcutaneously into human volunteers. An iodide patch or a control saline patch was placed over the injection sites. The control sites with saline and *C. acnes* resulted in mild inflammation, while in the test sites with iodide and *C. acnes*, significant inflammation and tenderness was noted. Furthermore, the latter subjects also reported some systemic joint discomfort, another sign of immune activation.

Seaweed, rich in iodine, was employed in Chinese medicine for the treatment of tuberculosis (MacGowan 1872). Some Inuit populations in Greenland were known to consume large quantities of seaweed, and Hoygaard (1938) observed in those regions where seaweed was commonly consumed, tuberculosis was rare. Furthermore, in such regions the disease was surprisingly benign in the small number of Inuit who were infected (Hoygaard 1938). Le Gac used iodine-rich seaweeds in combination with antibiotics for

the treatment of multiple sclerosis (Le Gac et al 1963), a disease now believed to have a bacterial etiology (Hoption Cann et al 2000).

The specific use of iodides for the treatment of various infectious diseases began soon after its discovery by Bernard Courtois in the residue of burnt seaweed in 1811. It was stated to be beneficial when used both topically and systemically for the treatment of syphilis and tuberculosis (Lugol 1829). In the 20th century, it was shown that iodine inactivated tubercle bacilli (Jobling and Petersen 1915) and the syphilis spirochete (Burke 1935) through iodination of unsaturated fatty acids on the bacterial cell surface, which in turn inhibited their metabolic activities. Woody and Avery (1948) confirmed the effectiveness of iodine against tuberculosis in guinea pigs, where combined streptomycin/iodine regimens were significantly more effective than streptomycin alone. It was postulated that the iodine may also reduce the fibrosis around granulomas (mass of chronically inflamed tissue associated with an infective process), and thus facilitate the penetration of streptomycin. Kolmer (1953) found a similar synergism between penicillin/iodine regimens in the treatment of syphilis. Oral therapy with supersaturated potassium iodide (SSKI) solution was later used to treat a broad spectrum of fungal infections including: blastomycosis, chromoblastosis, cryptococcus, murcormycosis, pythiosis, sporotrichosis, and zygomycosis (Stone 1971). Oral iodine therapy has also proven effective in the treatment of a variety of inflammatory conditions that have an unknown, but generally considered infectious etiology. Such conditions include: erythema multiforme, erythema nodosum, nodular vasculitis, Sweet's syndrome, and Villanova-Panol panniculitis (Horio et al 1980, 1983, Marani and Venturi 1986) and more recently asthma (Kawano and Noma 1995, 1996). Similarly, regular iodine baths (balneotherapy) have been shown to be effective in resolving symptoms of probable infectious diseases, including gout and osteoarthritis (Kamenskaia and Fedorova 1990). Finally, iodine has also become one of the most commonly used topical antimicrobial agents in hospitals, in the form of povidone-iodine (Gottardi 1999).

Increased secretion of inorganic iodine has been noted in a number of mucosal tissues including the salivary glands, gastric mucosa and uterine cervix (von Kaulla et al 1957, Brown-Grant 1961). We have suggested that iodine functions in mucosal tissues as a natural antimicrobial protectant (Cann et al 1999). In fact, Banerjee and Datta (1982) have shown that haloperoxidase activity in the stomach is 3.5-fold higher and in the salivary gland 2-fold as high as that in the thyroid. Macrophages, as well as neutrophils, can use iodine in antimicrobial defense via the peroxidase-H_2O_2-halide system (Akaki et al 2000, Neill and Klebanoff 1988). Haloperoxidases, in conjunction with hydrogen peroxide, oxidize iodide to the more toxic hypoiodite (OI^-) (Majerus and Courtois 1992). Other effector molecules that may be used by this system include: bromide, chloride and thiocyanate (SCN^-); although iodide appears to have the most potent antimicrobial activity (Neill and Klebanoff 1988, Majerus and Courtois 1992). The discovery and characterization of a specific transporter for iodine, the sodium-iodide symporter (NIS), has further confirmed the importance of iodine in mucosal tissues (Cann et al 1999). In addition to being highly expressed in the thyroid, especially goitrous tissue (Joba et al 1999), other tissues such as the salivary glands and stomach express NIS (Spitzweg et al 1998, Vayre et al 1999).

Iodine and cancer

The sodium-iodide symporter has also been noted in some hormone-dependent tissues, such as the breast, ovaries, prostate and testes — frequent sites for cancer development (Smanik et al 1997, Spitzweg et al 1998). A study by Tazebay and colleagues (2000) found the constitutive form of NIS to be expressed in over 80% of 23 invasive breast cancers compared to none of eight control samples. In contrast, the highly promoted breast cancer marker, Her2/*neu*, is only expressed in about 33% of breast cancers (Tazebay et al 2000). This evidence suggests that the iodine symporter plays a more significant role in the development of this disease. More recently, other potential iodine

transporters have been isolated, including the general anion exchangers of the SLC4 and SLC26 families (Lohi et al 2000). Pendrin, an interesting iodine transport protein from the latter family has recently been characterized, but it has only been studied in the thyroid and cochlea (Scott et al 1999). Unlike NIS, pendrin appears to be not only linked with iodine transport, but also oxidation and organification (Scott et al 1999). A mutation in the pendrin gene is associated with a rare condition called Pendred's syndrome. This condition characterized by deafness and thyroid anomalies, although other pathologies have not been well characterized. Ozluk and colleagues (1998) recently reported on a patient with simultaneous thyroid and breast cancer who had Pendred's syndrome. Could these multiple primary cancers have arisen due to genetically-induced iodine deficiency in these tissues? In the following paragraphs, we will examine how iodine may be involved in cancer progression.

Traditional eastern Asian medicine has long used seaweeds, rich in iodine, as a cancer treatment (Kun 1989, Zhang 1989). Beatson (1896) was one of the first investigators to report on the use of desiccated thyroid for cancer treatment. He gave his breast cancer patients large daily doses of desiccated thyroid (1000 mg/day), an extract which contains iodine as well as iodinated thyroid hormones (T3 and T4). Desiccated thyroid is administered in tablet form. His studies were supported by Loeser (1954) who used thyroid extract to treat uterine and ovarian cancer, in addition to breast cancer. Both investigators reported on the ability of the desiccated thyroid to shrink tumours or result in their necrosis. Crile (1955) observed similar results using desiccated thyroid to treat metastatic thyroid cancer. He demonstrated that regular treatment with thyroid extract (130-260 mg/day) could lead to a regression of tumours of the neck, as well as, a disappearance of lung metastases (as seen on chest x-rays). With these early thyroid extract preparations, however, it is difficult to estimate the actual iodine dose received by these patients due to wide variations in potency (Taylor 1961). By the late 1950s, purified thyroid hormones (T3 and T4) tablets became commercially available, and thus, it was

considered unnecessary to use desiccated thyroid in the treatment of hypothyroidism. Under the assumption that T3 or T4 were the active ingredients of thyroid extract, clinical studies were carried out using these hormones (in doses ranging from 0.02 to 0.10 mg/day) for the treatment of breast cancer. The *objective* results were generally disappointing and it was concluded that thyroid hormones were ineffective against cancer (Gardner et al 1962, Emery and Trotter 1963, Lyons and Edelstyn 1965). In contrast, some investigators noted that *subjective* improvements were "frequent and often striking" (Gardner et al 1962) and local disease recurrence was more common in the treatment groups (Lyons and Edelstyn 1965). Unfortunately, these investigators overlooked the fact that the small doses of purified T3 or T4 were not analogous to the large doses of desiccated thyroid used in earlier studies, which also contain a considerable amount of iodine.

The first case of cancer treated with iodine was reported by Pozzi (1878, 1904). His patient was a woman with ovarian cancer, who presented with severe abdominal distention and pain. She was initially given a 100 g injection of tincture of iodine into the peritoneal cavity, which caused fever and further abdominal distention lasting for 36 hours. Her abdomen was tapped for fluid several times and she remained well for a year, whereafter her symptoms returned. Another intra-abdominal injection was given, this time 150 g of tincture of iodine, followed by a violent reaction lasting 15 days. She remained well for a further year, although her abdomen was tapped for fluid several more times. At this time, an exploratory operation was performed, confirming malignancy. Following partial tumour excision (the disease being too extensive to remove in its entirety), she developed an infection that caused fever and abdominal pain for several weeks. After this time, she improved rapidly. She died of a recurrence of her disease 25 years after surgery. In this case, iodine appeared to slow disease progression in addition to activating a potent immune response. However, the infection following surgery seemed largely responsible for her prolonged survival.

After disappointing results using surgery and radiation treatment for cancer, a London physician, Forbes Ross (1912), experimented with dietary interventions to treat cancer. He believed that potassium deficiency was a major factor in cancer development and prescribed potassium citrate and phosphate to correct this deficiency. Interestingly, he also prescribed a weekly dose of five grains of potassium iodide (approximately 35 mg/day of iodine) and claimed remarkable success in inoperable patients. Furthermore, he prescribed this regimen to all his other patients (*i.e.* those without cancer) and claimed that over a 15 year period no patient under his care had contracted cancer (Ross 1912). Unfortunately, he died at a young age, 46, before his treatments could be validated by others. Coley (1925) experimented with the direct application of a potassium iodide solution to cancers; however, the added effectiveness of such treatment to his many successful cases was unclear as iodine was used in combination with other therapies.

More recently, however, Silecchia and colleagues (1994) reported on a patient with a rare lymphangioma (a tumour composed of lymphatic vessels) of the peritoneum, thigh and gluteal region that serendipitously regressed following use of the iodine-dense contrast agent, Lipiodol. In order to visualize the malignancy, the investigators administered the Lipiodol dye before a computed tomography (CT) scan. However, at the time of the CT scan, diffuse microembolization of the lesions was observed, and the patient was discharged three days later without any sign of disease. A follow-up at 18 months showed no evidence of malignancy.

The preceding examples illustrate that are a number of interesting, but largely anecdotal reports, which exist to suggest that iodine may be effective against cancer. Unfortunately, there are no modern conventional cancer regimens that include the use of iodine. Nevertheless, there are many current alternative therapies where iodine, desiccated thyroid or seaweed are employed.

Purpose of the study of dietary and other associated factors

Due to the lack of research examining a role for iodine in human breast cancer, epidemiological studies are needed. The measurement of thyroid hormones may not be the ideal method for determining iodine status as deficient iodine intake may lead to hyperthyroidism (Hintze et al 1991) or hypothyroidism (Weaver et al 1976) and conversely, excessive iodine intake may also be associated with either hyperthyroidism (Ishizuki et al 1989) or hypothyroidism (Konno et al 1994). The direct measure of iodine content in food from dietary studies may be compromised by iodine-containing compounds that are poorly absorbed, such as the commonly used food colorants erythrosine and rose bengal (Katamine et al 1987); furthermore, studies that estimate iodine intake through dietary questionnaires may not reflect the regional variations of important sources for this element (Lee et al 1994b, Valeix et al 1999). Urinary iodine levels can be highly variable, reflecting only short term iodine intake, and particularly in smaller studies, are not a reliable measure of iodine status (Konno et al 1993b). Whole blood, plasma or serum specimens may better measures of iodine status; however, whole blood samples (which account for cellular iodine as well) are more stable and may best reflect long term iodine status, as has been shown for selenium (Longnecker et al 1996). In case-control studies involving surgery, where blood or urine samples are used as indictors of iodine status, one must be careful of specimen contamination by iodine-containing surgical disinfectants (*i.e.* povidone-iodine); samples are ideally collected in case subjects before such exposure. Prospective cohort studies can avoid this artifact, however, they are considerably more expensive and require many years of follow-up. Correlational studies which found a negative association between selenium levels and many human cancers (Shamberger et al 1976, Schrauzer et al 1977) have been the stimulus for many subsequent studies of selenium and cancer; however, studies simultaneously examining iodine and selenium status in patients with breast cancer have not been conducted and would be of considerable interest.

The intake of iodine and other associated dietary factors is easily modifiable, and thus understanding the role these factors play in cancer progression may provide some immediate practical applications. Iodine, and its interactions with selenium, have not been investigated in human studies for breast cancer. Thus, this investigation provides a unique opportunity to examine the associations between these important co-factors and this disease. If such protective associations are found, then one could test/hypothesize relatively inexpensive ways of preventing this disease, through dietary changes or nutritional supplements, in the hope of changing the natural history of the disease. Notably, this study will provide insight into the range of *whole blood* iodine levels in the population under study and how comparable it is to Japan where iodine intake is considered high and where breast cancer rates are low. The normal *serum* range for iodine in the USA is considered to be between 0 to 30 μg/l (Hunt et al 1980), while in Japan it is reported to be between 20 to 90 μg/l (Aiba et al 1999).

In addition, a multiple logistic regression model will be developed with breast cancer as the *outcome* variable. Trace element levels (*exposure* variables), measured in whole blood (iodine and selenium, as well as, bromine, chromium, iron, magnesium, manganese, molybdenum, vanadium, and zinc), will be divided into tertiles (*i.e.* high, moderate and low). Using the case-control study design, this regression model facilitates the development of approximate measures of risk (*i.e.* odds ratios); for example, to determine whether a particular factor is associated with an increased or decreased risk of disease and to what degree. Other general, medical, and dietary factors will be analyzed as well, with adjustment for potentially confounding factors.

METHODS

This pilot study was designed to investigate the association between breast cancer, trace elements, and other associated factors in women 40 to 70 years of age. A multivariate logistic regression model was used to compare data between 21 patients with newly diagnosed breast cancer and 30 control subjects. Participants were recruited between January and December 2000.

Study Population and Eligibility Criteria

Entry Criteria	General	Cases	Controls
Eligibility	• Age range 40-70 years • Signed informed consent form (Appendix III and IV)	Newly diagnosed breast carcinoma	Normal mammographic screen
Ineligibility	• An increase (≥200 µg/day) in iodine containing medications, foods or supplements within the last year	• *Previous* history of breast cancer or ductal carcinoma in situ • Patients who have received other associated treatments (chemo- or hormonal therapy or radiation) for breast cancer prior to the collection of blood samples.	History of breast cancer or ductal carcinoma in situ

Subject Evaluation

Investigations	
General Information and Medical History	Age, height, weight, occupation, education, ethnic background, physical activity, age at menarche/menopause, reproductive history, menstrual irregularities, family and personal history of breast or other cancers, other medical conditions, medication use (hormone replacement therapy, oral contraceptives, *etc.*), and for case subjects, breast cancer pathology
Dietary Questionnaire	Consumption of major food groups, dietary changes, use of vitamin/mineral supplements, alcohol/smoking habits (Appendix V and VI)
Clinical Chemistry	*Whole blood analysis:* iodine, selenium, bromine, chromium, iron, magnesium, manganese, molybdenum, vanadium, and zinc

Ethical approval

The Capital Health Region (Victoria, BC, Canada) Research Review and Ethical Approval Committee, responsible for research conducted at Royal Jubilee Hospital and Victoria General Hospital, and the University of Victoria's Human Research Ethics Committee were consulted and ethical approval was obtained (Appendix VII and VIII).

Recruitment

Case subjects were recruited from the surgical scheduling departments of Royal Jubilee Hospital and Victoria General Hospital. Patients were obtained from a list of women who have had positive (for malignancy) breast cancer biopsies and were scheduled for breast cancer surgery. Control subjects were recruited through the Victoria Screening

Mammography Program of British Columbia (SMPBC) and from physician offices in Victoria.

Participant assessment

A nurse investigator was responsible for interviewing patient and control subjects, including obtaining a signed informed consent form, recording participant general, medical and dietary histories, and organizing the collection of whole blood specimens.

Whole blood analysis

Whole blood analysis is considered to be a more stable determinant of long term trace element intake and usage, as has been shown for selenium (Longnecker *et al* 1996), and was therefore chosen for this study. Whole blood samples were collected from study participants (before surgery for case subjects) at the time of interview and stored at –70° C until the time of analysis. Samples were then digested with nitric acid in metal-free polypropylene tubes. Elemental analysis of whole blood elemental levels was carried out in conjunction with Elemental Research Inc (Vancouver, BC) using inductively coupled plasma mass spectrometry (ICP-MS) as described previously (Nuttall *et al* 1995). ICP-MS is a sensitive and comprehensive technique for multielemental analysis of trace elements in solution. It combines an ion source (ICP), a well-proven analytical source that operates at a temperature in excess of 8000 K and the linear quadrupole mass spectrometer (MS) used as a detector to separate the elements and their isotopes for subsequent detection and measurement.

The analytical process is as follows (Montaser 1998). Nitric acid digested samples are introduced through a peristaltic pump to a nebulizer, which produces a very fine aerosol within a spray chamber. The aerosol is first carried by a stream of argon gas into the

central injector of a quartz plasma torch and then to the high temperature plasma where the elements transported are desolvated and vaporized. Dissociation is virtually complete during transit through the plasma core and elements with a first ionization energy less than 10 eV are fully ionized. Ions are extracted from the central channel of the plasma at the sampling interface consisting of a 1 mm aperture in a water-cooled cone. The ions are transmitted through the reduced pressure stage behind the sampling interface, through a second cone, referred to as a skimmer and into an ion lens region, which operates at a further reduced pressure. The ion lens causes the focused and energy corrected ion beam to pass into the quadrupole mass filter where the ions are separated according to their mass to charge ratio (m/e). All the separated ion species are detected sequentially by a continuous dynode channeltron detector placed at the exit to the mass filter; this is accomplished by scanning the mass range from lithium at m/e 6 through uranium at m/e 238. The scan process is controlled by varying the radio frequency voltage applied to rods within the quadrupole mass filter. The pulses of ions are amplified and accumulated in a high capacity multi-channel analyzer/scaler then subsequently processed by a microcomputer.

Statistical analysis

In the analysis of group (*i.e.* cases and controls) comparability, parameter means were assessed using the independent samples t-test for equality of means, with a two-tailed level of significance. Means differing with a P value ≤ 0.05 were considered significantly different. The correlations between parameter pairs were examined using the Pearson correlation coefficient. The Pearson coefficent was used as the distribution of outcomes

was found to be approximately linear and normally distributed. Correlations with a P value ≤ 0.05 (two-tailed) were considered significant.

The relationships of whole blood elemental levels and breast cancer were analyzed by logistic regression, while controlling for possible confounding variables such as sociodemographic characteristics, comorbidity, and disease specific variables. Appropriate categorical and continuous measures of association were computed depending on the data types.

In logistic regression, there is one binary outcome (dependent) variable — breast cancer (yes or no) — and several independent or explanatory variables (age, education, iodine levels, *etc.*). The effect of the explanatory variables is used to assess the distribution of the outcome variable and to separate the effect of each from the others, when possible. Separating the effect of each variable is accomplished by adjustment, where possible confounding variables (age, body mass index (BMI), education, *etc.*) are fixed or kept constant. Power considerations are used to determine whether to add potential confounding variables to the model or not. For example, the power of the analysis can be increased by dropping variables from the model when their coefficients are not significantly different from zero. However, if a variable has a significant effect on the outcome, then including it in the model may improve power.

The odds ratios (OR) associated with logistic coefficients are calculated relative to the default value, 1. By convention, the lowest, smallest or null category is generally set as the reference category to which the higher categories are compared. Thus, odds ratios greater than 1 represent factors that increase the risk of disease, and conversely, those less than 1 represent factors associated with a reduced risk of disease. For example, in examining the risk of lung cancer in smokers and non-smokers, non-smokers would be the

default or reference category (*i.e.* OR = 1). In one recent study, it was found that female smokers had a relative odds ratio of almost nine (*i.e.* OR = 8.94), or one can conclude that smoking was associated with a nine-fold increase in lung cancer risk in comparison to non-smokers (Agudo et al 2000). In contrast, a case-control study in Germany (Chang-Claude et al 2000) found that prolonged breast feeding ($\geq$ 25 months) was associated with a reduced risk for developing breast cancer (*e.g.* OR = 0.5), or we can say a 50% reduction in risk compared to women who breastfed for less than 25 months. Thus, the logistic coefficient can be interpreted as the change in the log odds (*i.e.* log [probability of event / probability of no event]) associated with a one unit change (*e.g.* non-smoking to smoking, low to medium, medium to high, *etc.*) in the explanatory variable.

ORs approximate relative risks (RR) under certain assumptions. The RR estimates the magnitude of an association between exposure and a disease, as well as, the likelihood of developing the disease in the exposed group relative to the non-exposed group. In a case-control study, it is usually not possible to calculate the rate of disease development given the presence or absence of an exposure. Thus, in case-control studies one approximates the RR by calculating the ratio of the odds of exposure among the cases to that among the controls. ORs provide a valid estimate of the RR when cases of disease are newly diagnosed, when prevalent cases are not included in the control group, and when selection of cases and controls is not based on exposure status.

In logistic regression, the parameters of the model are estimated using the maximum-likelihood method. That is, the coefficients (explanatory variables) that make the observed results most 'likely' are selected. Univariate analysis is performed by including only one independent (explanatory) variable of interest in the model. Multivariate analysis is used

to study the association between disease and explanatory variables, while controlling for potential confounders included in the model (*e.g.* age, BMI, *etc.*).

The logistic regression model for disease status was derived using commonly accepted statistical modeling principles. Model checking diagnostics for goodness-of-fit and model valuation were performed. Given that this was a case-control study, the model does not provide strong conclusions about causality, however, it may expose some interesting relationships that will warrant further clinical study. All analyses were performed on SPSS 9.0.

RESULTS

General participant characteristics can potentially confound the analysis when the two groups are unevenly matched in such characteristics. Thus, a general review of such factors and an analysis of their association with the outcome (*i.e.* breast cancer or no breast cancer) is initially presented.

Comparison of case and control general and dietary characteristics

A general comparison of the two study groups (21 cases, 30 controls) is presented in Table 5. Although the control group tended to be younger than the case group (50.8 years *vs* 53.4 years), this difference was not significant ($P = 0.24$). Similarly, there was no significant diffenrence in the distribution of premenopausal and postmenopausal subjects between groups ($P = 0.84$). No significant differences were noted in the mean weight, height or body mass index (BMI) of case and control subjects. Those reporting a first degree relative with breast cancer was slightly higher in the case group (38.1% *vs* 36.7%), but not significantly so ($P = 0.92$). In contrast, control subjects had a higher degree of education ($P = 0.02$) than case subjects. Oral contraceptive use (past or present) was higher in control subjects (60.0% *vs* 38.0%), although this was not significant ($P = 0.13$). A history of benign breast disease and breast pain tended to be more common in case subjects, but this was not significant ($P = 0.24$ and $P = 0.07$). Similarly, thyroid disease was more common in women with breast cancer (9.5% *vs* 6.7%) as was inflammatory bowel disease (19.0% *vs* 10.0%) and previous hysterectomy / ovariectomy (47.6% *vs* 20.0%), however, only the latter factor was significantly higher in case subjects

Table 5. General characteristics of the case and control base sample.

Characteristic	Cases ($n = 21$)	Controls ($n = 30$)	P[a]
Age at entry	53.4	50.8	.238
Menstrual status			.842
Premenopausal	42.9%	40.0%	
Postmenopausal	57.1%	60.0%	
Weight (kg)	72.4	69.2	.359
Height (m)	1.64	1.66	.252
BMI[b] (kg/m^2)	26.9	25.3	.187
Breast cancer (1^{st} degree relative)	38.1%	36.7%	.919
Education level attained			**.019**
< High school	9.5%	0%	
High school	52.4%	33.3%	
> High school	13.0%	66.7%	
Oral contraceptive use	38.0%	60.0%	.129
Parity			.094
0	19.0%	33.3%	
1-2	47.7%	16.7%	
≥ 3	33.3%	50.0%	
History of BBD[b]	42.9%	26.7%	.236
History of breast pain	47.6%	23.3%	.073
History of thyroid disease	9.5%	6.7%	.715

History of IBD[b]	19.0%	10.0%	.365
History of anemia	14.3%	36.7%	.081
Previous hysterectomy/ovariectomy	47.6%	20.0%	**.037**
Exercise frequency			**.039**
Low	20.0%	3.3%	
Moderate	30.0%	23.3%	
High	50.0%	73.3%	
Alcohol consumption	40.0%	56.7%	.257
Smoking			.603
Never / past[c]	85.0%	90.0%	
Current	15.0%	10.0%	

a. Independent samples t-test for equality of means (2-tailed); bold indicates significant difference

b. BMI, Body mass index; BBD, benign breast disease; IBD, inflammatory bowel disease

c. Quit > 3 years previous

($P = 0.04$). A history of anemia, by comparison, was more common in women without breast cancer, but this did not reach statistical significance ($P = 0.08$). Regular alcohol consumption was more common in women without breast cancer (56.7% *vs* 40.0%), while smoking was more common in women with breast cancer (15% *vs* 10%); however, neither factor was significantly different between groups. Finally, exercise frequency was significantly higher in controls than cases ($P = 0.04$). Although controls in general tended to be younger, this was not likely an age effect as no significant correlation between age and exercise frequency was observed ($r = 0.06$, $P = 0.67$).

In the univariate logistic regression analysis (Table 6), a higher degree of education was significantly associated with a reduced risk for breast cancer (OR = 0.31), while those women who had a previous hysterectomy / ovariectomy were at a significantly increased risk (OR = 3.64). As the present study group was not matched for age or BMI, these potential confounding factors were incorporated into the multivariate model. Those variables significant in the univariate analysis (i.e. education level, history of hysterectomy / ovariectomy) were also included as was parity, which showed a consistent trend in the univariate analysis and improved the precision of the multivariate model. In this latter model (Table 6), education level and previous hysterectomy / ovariectomy were no longer significant. However, a history of breast pain was associated with a significantly increased risk (OR = 11.25).

With respect to nutritional factors, frequency of fruit, vegetable and whole grain consumption was higher in controls than in cases, yet only the difference in fruit intake was significant ($P = 0.04$). Controls also tended to eat more meat, fish and use more table salt than cases, but not significantly so. By comparison, consumption of dairy foods, and processed meats tended to be higher in cases, but no factor was significant. Regular

Table 6. Odds ratios (OR) and 95% confidence (CI) intervals for general breast cancer risk factors.[a]

Risk factors	Crude OR[b] (95% CI)	Adjusted OR[c] (95% CI)
Menopausal status		
Pre	1.00	1.00
Post	0.89 (0.29-2.76)	0.13 (0.02-1.05)
Education level attained		
≤ High school	1.00	1.00
> High school	**0.31** (0.10-0.98)	0.36 (0.10-1.35)
1st degree relative with breast cancer		
No	1.00	1.00
Yes	1.06 (0.34-3.36)	0.60 (0.14-2.60)
Oral contraceptive use		
Never	1.00	1.00
Ever	0.41 (0.13-1.29)	0.36 (0.10-1.38)
Age at first birth		
≤ 30	1.00	1.00
> 30	0.25 (0.03-2.51)	0.49 (0.40-6.00)
Parity		
0	1.00	1.00
1-2	1.67 (0.41-6.82)	1.64 (0.34-8.02)
> 2	3.50 (0.69-17.89)	4.52 (0.69-29.76)

Breastfeeding		
No	1.00	1.00
Yes	0.95 (0.26-3.55)	1.38 (0.27-7.10)
History of BBD[d]		
No	1.00	1.00
Yes	2.06 (0.63-6.74)	4.40 (0.82-23.64)
History of breast pain		
No	1.00	1.00
Yes	2.99 (0.90-9.96)	**11.25** (1.84-68.99)
History of thyroid disease		
No	1.00	1.00
Yes	1.47 (0.19-11.39)	0.81 (0.06-11.74)
History of IBD[d]		
No	1.00	1.00
Yes	2.12 (0.42-10.65)	1.91 (0.30-12.07)
History of anemia		
No	1.00	1.00
Yes	0.29 (0.07-1.20)	0.20 (0.03-1.40)
Previous hysterectomy/ovariectomy		
No	1.00	1.00
Yes	**3.64** (1.05-12.56)	3.38 (0.69-16.57)

Exercise frequency		
low	1.00	1.00
moderate	0.21 (0.02-2.48)	0.18 (0.01-4.20)
high	0.11 (0.01-1.15)	0.10 (0.01-1.91)
Alcohol use		
No	1.00	1.00
Yes	0.51 (0.16-1.61)	0.45 (0.12-1.77)
Smoking		
Never / past[e]	1.00	1.00
Yes	1.59 (0.29-8.79)	0.41 (0.03-4.86)

a. Groups are compared to lowest category (*i.e.* where OR = 1), ORs in bold are significant

b. Univariate analysis

c. Adjusted for age, BMI, education, parity, and previous hysterectomy/ovariectomy

d. BBD, benign breast disease; IBD, inflammatory bowel disease

e. Quit > 3 years previous

vitamin intake was more common in controls (80% *vs* 55%), but the difference was not quite significant ($P = 0.06$). Regular consumption of caffeinated products (*i.e.* coffee and tea) was identical in both groups. Neither frequency of fruit consumption ($r = -0.19$, $P = 0.18$) nor vitamin intake ($r = -0.23$, $P = 0.11$) was significantly correlated with age.

Other correlations noted that reached significance included a positive association between vegetable intake and exercise frequency ($r = 0.29$, $P = 0.04$), with negative correlations between vegetable and meat intake ($r = -0.28$, $P = 0.05$), weight and exercise frequency ($r = -0.28$, $P = 0.05$) and meat and whole grain consumption ($r = -0.30$, $P = 0.03$). Surprisingly, height was associated with both salt intake ($r = 0.37$, $P = 0.01$) and intake of processed meats ($r = -0.32$, $P = 0.02$). Correspondingly, salt and processed meat consumption had a negative, but not quite significant association ($r = -0.26$, $P = 0.07$). Finally, although not quite significant ($P = 0.07$), caffeine intake was positively correlated with stress ($r = 0.26$). However, due to the large number of correlations and small sample size, some associations may be due to chance.

In the logistic regression analysis of dietary factors (Table 7), no variable was significant in either the univariate or multivariate (adjusted for age, BMI, education, parity and previous / hysterectomy as previously) model. Fruits and vegetables showed a trend towards reduced risks in the univariate analysis, but this was not sustained in the multivariate analysis. Whole grain and meat consumption tended to be associated with reduced risks in both models. Use of table salt as well as regular vitamin usage tended to be associated with reduced risks.

Table 7. Odds ratios (OR) and 95% confidence (CI) intervals for breast cancer dietary factors.[a]

Dietary factor	Crude OR[b] (95% CI)	Adjusted OR[c] (95% CI)
Fruit		
low	1.00	1.00
moderate	0.54 (0.04-6.77)	1.16 (0.05-26.94)
high	0.18 (0.01-2.32)	0.35 (0.01 - 9.39)
Vegetables		
low	1.00	1.00
moderate	0.47 (0.14-1.59)	0.58 (0.13-2.70)
high	0.59 (0.08-4.50)	1.23 (0.13-11.94)
Grains and cereals		
Mostly whole grains	1.00	1.00
Mostly refined grains	0.50 (0.14-1.79)	0.78 (0.16-3.92)
Meat intake		
low	1.00	1.00
moderate	0.30 (0.03-2.77)	0.11 (0.01-2.30)
high	0.48 (0.09-2.46)	0.25 (0.04-1.79)
Seafood		
low	1.00	1.00
moderate	1.77 (0.44-7.06)	0.92 (0.18-4.64)
high	0.80 (0.11-6.10)	0.59 (0.06-5.73)

Milk and dairy products		
low	1.00	1.00
moderate	1.56 (0.39-6.24)	0.95 (0.19 - 4.68)
high	1.50 (0.22-10.22)	1.39 (0.18-10.83)
Table salt		
low	1.00	1.00
moderate	0.49 (0.11-2.26)	0.57 (0.09-3.64)
high	0.49 (0.11-2.26)	0.48 (0.08-2.81)
Vitamin usage		
No	1.00	1.00
Yes	0.31 (0.09-1.07)	0.58 (0.12-2.79)

a. Groups are compared to lowest category (*i.e.* where OR = 1)

b. Univariate analysis

c. Adjusted for age, BMI, education, parity, and previous hysterectomy/ovariectomy

Case and control elemental status

In this population, molybdenum blood levels were below the limit of detection (1 μg/l) in 86 % of subjects examined, and therefore, these levels were not statistically analyzed. Furthermore, two participants reported a significant (> 200 μg/day) increase in the intake of iodine-containing products within the last year and were thus excluded from the analysis as this was not representative of their usual iodine intake. Both subjects were cases and had the two highest iodine levels (41 and 45 μg/l) of the population under study.

In Table 8, a summary of mean trace elemental levels is presented. Cases had significantly higher selenium levels (220.0 μg/l *vs* 193.7 μg/l) than controls. In contrast, mean iron levels were higher in controls than cases (504 166.6 μg/l *vs* 479 761.9 μg/l). No other values were significantly different.

In the univariate logistic regression analysis (Table 9), moderate iron levels were associated with a nonsignificant reduced risk and correspondingly, high levels were associated with a further significant reduction in risk (OR = 0.15). No other values were significant, although bromine showed a trend towards an increased risk, while vanadium tended to be associated with a reduced risk of breast cancer. In the multivariate analysis (Table 9), adjusted for age, BMI, education, parity and previous / hysterectomy, only iron remained significantly associated with reduced risks both for moderate (OR = 0.07) and high (OR = 0.01) iron levels. Vanadium continued to show a trend towards a reduced risk, but again this was not significant.

Correlations noted with trace elements included a positive association between age and magnesium levels (r = 0.41, $P = 0.003$), and correspondingly, magnesium was positively associated with postmenopausal status (r = 0.422, $P = 0.002$). Magnesium was also

Table 8. Mean elemental levels of the case and control base sample.

Elements	Cases (SE)[a]		Controls (SE)		P[b]
Iodine	30.05	(1.02)	27.33	(0.82)	0.051
Selenium	220.00	(10.00)	193.67	(4.30)	**0.010**
Bromine	8 298.10	(657.61)	7 083.67	(399.80)	0.102
Chromium	62.67	(7.27)	58.67	(2.36)	0.605
Iron	479 761.90	(8282.77)	504 166.67	(8414.42)	**0.050**
Magnesium	34 495.24	(551.51)	34 006.67	(593.22)	0.567
Manganese	12.94	(1.04)	11.28	(0.46)	0.112
Vanadium	6.00	(0.09)	6.08	(0.08)	0.481
Zinc	7 178.57	(179.37)	7 560.67	(362.15)	0.409

a. SE, standard error

b. Independent samples t-test for equality of means (P values in bold are significant)

Table 9. Odds ratios (OR) and 95% confidence intervals (CI) for whole blood elemental status with respect to breast cancer risk.[a]

Elemental status (category range)[b]	Crude OR[c] (95% CI)	Adjusted OR[d] (95% CI)
Iodine		
low (≤ 26)	1.00	1.00
moderate (27-31)	0.72 (0.16-3.20)	0.53 (0.09 – 3.07)
high (≥ 32)	3.90 (0.91-16.79)	2.46 (0.40-15.24)
Selenium		
low (≤ 190)	1.00	1.00
moderate (200-210)	0.83 (0.19-3.58)	0.57 (0.10-3.09)
high (≥ 220)	2.81 (0.73-10.77)	4.37 (0.80-23.93)
Bromine		
low (≤ 6130)	1.00	1.00
moderate (6140-7660)	1.68 (0.41-6.96)	1.94 (0.34-11.27)
high (≥ 7670)	2.70 (0.66-11.09)	1.53 (0.29 - 7.97)
Chromium		
low (≤ 50)	1.00	1.00
high (≥ 60)	1.17 (0.35-3.86)	1.41 (0.32-6.29)
Iron		
low (≤ 469 000)	1.00	1.00
moderate (470 000-517 000)	0.45 (0.12-1.72)	**0.07** (0.01-0.71)
high (≥ 518 000)	**0.15** (0.03-0.71)	**0.01** (0.00-0.14)

Magnesium		
low (≤ 32 600)	1.00	1.00
moderate (32 700-35 800)	4.06 (0.95-17.43)	2.25 (0.40-12.49)
high (≥ 35 900)	2.53 (0.57-11.26)	1.66 (0.30 - 9.08)
Manganese		
low (≤ 9.7)	1.00	1.00
moderate (9.8-13.2)	0.31 (0.06-1.48)	0.08 (0.01-1.02)
high (≥ 13.3)	2.62 (0.66-10.48)	2.05 (0.39-10.77)
Vanadium		
low (≤ 5.8)	1.00	1.00
moderate (5.9-6.2)	0.63 (0.17-2.36)	0.82 (0.17-4.13)
high (≥ 6.3)	0.33 (0.08-1.40)	0.35 (0.06-1.97)
Zinc		
low (≤ 6770)	1.00	1.00
moderate (6780-7640)	2.06 (0.52-8.17)	2.68 (0.49-14.78)
high (≥ 7650)	1.00 (0.25-4.08)	0.55 (0.09 - 3.42)

a. Groups are compared to lowest category (*i.e.* where OR = 1), ORs in bold are significant

b. Ranges for trace elements are in μg/l

c. Univariate analysis

d. Adjusted for age, BMI, education, parity, and previous hysterectomy/ovariectomy

positively associated with salt intake (r = 0.40, $P = 0.013$), while surprisingly, iodine was not (r = -0.04, $P = 0.78$). Chromium was positively associated with vegetable intake (r = 0.45, $P = 0.012$). Vanadium was positively associated with fruit intake (r = 0.33, $P = 0.02$), with magnesium (r = -0.34, $P = 0.014$) and iron (r = -0.29, $P = 0.04$) negatively associated. However, iron was not associated with a history of anemia (r = -0.08, $P = 0.60$), but was associated with past or present oral contraceptive use (r = 0.29, $P = 0.04$).

The correlation amongst trace elements is presented in Table 10. Iron was positively correlated with magnesium (r = 0.55, $P < 0.001$) and zinc (r = 0.34, $P = 0.02$), and negatively with vanadium (r = -0.32, $P = 0.02$). Chromium was positively correlated with manganese (r = 0.67, $P < 0.001$) and vanadium (r = 0.65, $P < 0.001$). Finally bromine was positively correlated with zinc (r = 0.38, $P = 0.01$).

Table 10. Correlations amongst whole blood trace element levels.[a]

		Br	Cr	Fe	I	Mg	Mn	Se	V	Zn
Br	Pearson corr.	1.000								
	Sig. (2-tailed)									
	n	51								
Cr	Pearson corr.	-.050	1.000							
	Sig. (2-tailed)	.794								
	n	30	30							
Fe	Pearson corr.	-.039	-.217	1.000						
	Sig. (2-tailed)	.785	.249							
	n	51	30	51						
I	Pearson corr.	-.039	.325	-.190	1.000					
	Sig. (2-tailed)	.799	.080	.181						
	n	51	30	51	51					
Mg	Pearson corr.	-.071	-.215	**.551**	-.106	1.000				
	Sig. (2-tailed)	.623	.255	**.000**	.460					
	n	51	30	**51**	51	51				
Mn	Pearson corr.	.106	**.674**	.002	.232	-.055	1.000			
	Sig. (2-tailed)	.458	**.000**	.989	.101	.701				
	n	51	**30**	51	51	51	51			
Se	Pearson corr.	.042	-.005	.037	.115	.115	.272	1.000		
	Sig. (2-tailed)	.769	.979	.795	.421	.423	.053			
	n	51	30	51	51	51	51	51		
V	Pearson corr.	-.122	**.648**	**-.323**	.069	-.167	.104	-.105	1.000	
	Sig. (2-tailed)	.395	**.000**	**.021**	.633	.243	.467	.463		
	n	51	**30**	**51**	51	51	51	51	51	
Zn	Pearson corr.	**.376**	-.137	**.340**	.016	.077	.008	.015	-.179	1.000
	Sig. (2-tailed)	**.007**	.469	**.015**	.912	.591	.958	.919	.208	
	n	**51**	30	**51**	51	51	51	51	51	51

a. Correlations in bold are significant

DISCUSSION

Case-control studies

Case-control studies have become the most common analytical epidemiologic study design encountered in medical literature due to their advantage in evaluating diseases that occur many years following relevant exposures in a timely and cost-effective manner (Hennekens and Buring 1987). Still, these investigations, sometimes referred to as retrospective studies, have some inherent limitations that should be considered. First, the participant information questionnaire requires accurate recall for the validity of the data; yet, a certain degree of recall or reporting bias may arise when a participant is required to provide details of past events. This type of bias was not likely a critical factor in this study, however, because the key variables examined were determined objectively (*e.g.* height and weight were measured during the interview, elemental levels were measured analytically, disease status histologically) or would likely have equal subjectivity for both case and control groups (age, education, reproductive history, *etc.*). A second disadvantage is that the data is collected at a time when both the exposures and the disease have already occurred, thus, one assumes that the factors measured represent antecedent exposures. This is not always the case. Although whole blood levels of trace elements are more stable measures of long-term elemental status, than urinary levels for example, the whole blood levels do not necessarily represent the elemental intake of a subject 10 or 20 years previously. A woman may change her dietary habits after being diagnosed with cancer. Yet in the present study, blood samples were collected from incident cases (*i.e.* newly diagnosed). Thus, samples were collected after a positive biopsy, but before

surgery or other cancer treatments. In contrast, most studies of this nature recruit more easily accessible prevalent cases (diagnosed from previous years) where treatment associated changes or dietary changes may be significant.

Sample size considerations

It must be considered that this case-control study is only a pilot study and the participant numbers are small (21 cases and 30 controls). Conclusions that may be drawn from this initial sample are consequently limited. The initial difficulties involved in recruiting subjects and the associated costs of these delays are largely responsible for the small sample size. Therefore, those values that are found to be significant may lose significance upon further analysis, when a larger sample is obtained. We are presently involved in an expansion of this study and at a future date will publish our analysis of the data when a larger participant sample has been obtained (*i.e.* 100 cases and 100 controls)

General and dietary characteristics

Although the two study groups were not matched for age or BMI, no significant differences in the two groups were found. Controls tended to have more education than cases and education was associated with a reduced risk in the univariate analysis. Although a higher education was once considered a risk factor for breast cancer, more recent studies have not supported this association, and in fact, suggest that the trends in risk associated with education have become inverted (Martikainen and Valkonen 2000). Exercise frequency was higher in controls and in the logistic regression analysis there was a trend towards a reduced risk, although not significant. This coincides with recent evidence suggesting regular exercise may reduce the risk of breast cancer (Verloop et al

2000). A history of breast pain was significantly associated with an increased risk of breast cancer in the multivariate analysis and a previous study in France found a similar association (Plu-Bureau et al 1992). Thyroid disease and inflammatory bowel disease were associated with increased risks, however, not significantly so and the incidence of these conditions was low in both groups. A previous hysterectomy / ovariectomy was significantly more common in cases and was associated with a significant increased risk in the univariate analysis. Although an ovariectomy is generally considered a preventative operation for breast cancer, in this study most women reported undergoing this procedure in their 40's and 50's for menstrual or hormonal irregularities, and thus, such prolonged irregularities may have been a contributing factor in the subsequent disease development. An abnormal hormonal profile has been associated with an increased risk for breast cancer (Coulam et al 1983).

No significant associations were seen between fruits, vegetables, whole grains or vitamin intake and breast cancer, although the trend was towards protection. These results correspond to most published studies which show no or only a weak protective association between these factors and breast cancer (La Vecchia and Chatenoud 1998, Welp et al 1998).

Elemental status: iodine and selenium

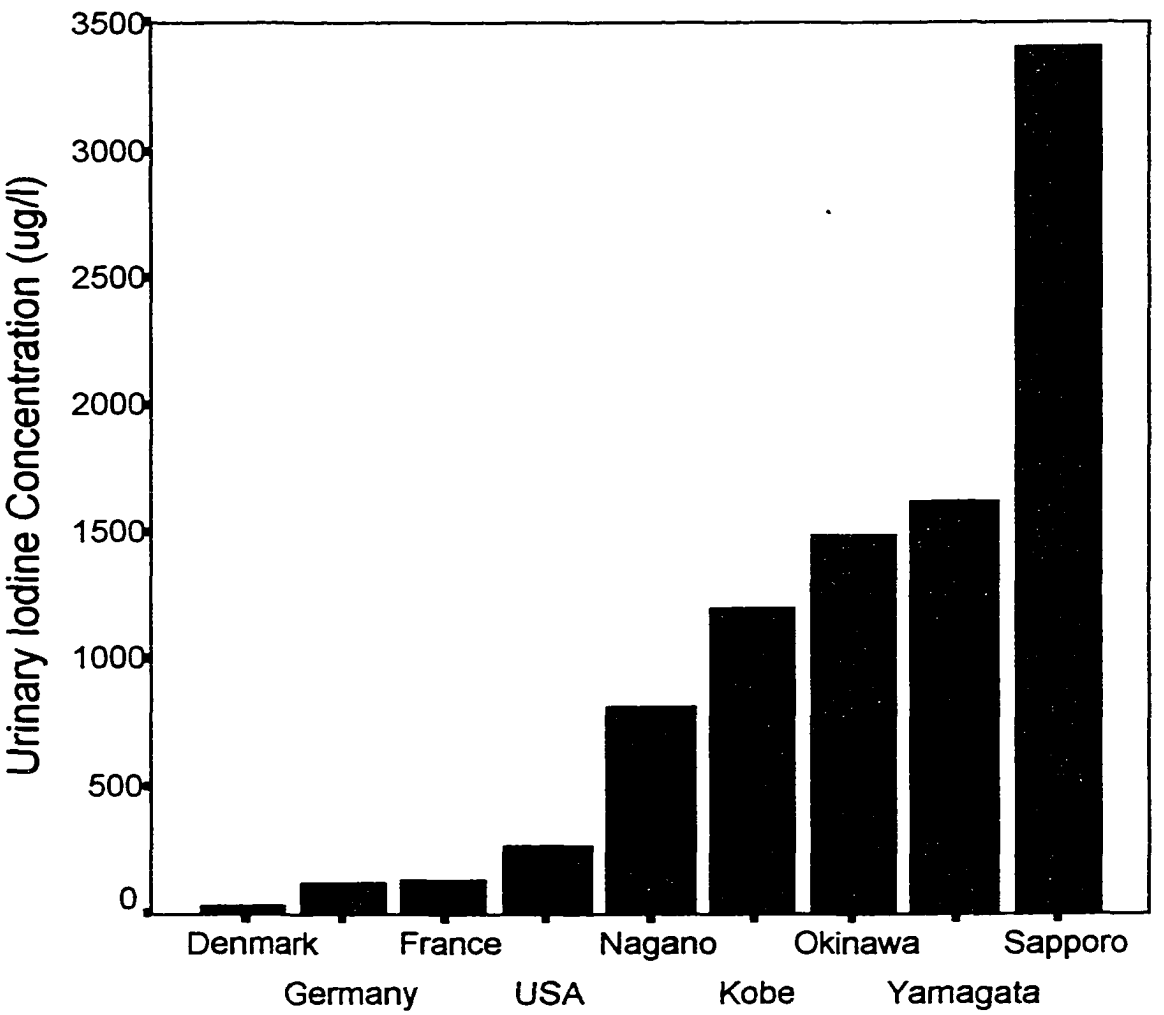
In this study, the range of iodine levels (19-36 μg/l) was surprisingly narrow and the mean difference in iodine levels between groups was small (Table 8). Measuring elemental levels in whole blood as a surrogate measure for trace element intake is a relatively new method for estimating long term elemental status (Longnecker et al 1996). Although more accurate than dietary questionnaires, or urinary, serum or plasma measures, whole

blood measurements are uncommon, and thus, comparisons to trace element levels found in others studies is limited. This is particularly true for iodine. There are, however, a number of large studies of iodine levels where urinary iodine is used as is surrogate marker for iodine intake. These studies demonstrate that iodine intake in Japan is significantly higher than that seen in the west (Figure 19). In Japan, breast cancer is comparatively rare (Figure 3), as is the precursor condition, benign breast disease. In contrast, breast cancer and benign breast disease are common in North America where iodine intake is comparatively low. Thus, this study highlights that fact that without a sufficiently large range of iodine levels, it may be impossible to determine its significance in the development of this disease. In contrast, an analogous study conducted in Japan would be the ideal setting to test this hypothesis, as the range in iodine levels is considerable. One study of Japanese women in Hawaii found a significantly higher level of seaweed consumption in controls than in patients with breast cancer (Nomura et al 1978). Unfortunately, neither dietary iodine intake nor blood or urinary levels were determined.

An analogy to this problem may be seen in the association between salt and gastric cancer. In Japan and other countries where gastric cancer and its precursor lesions are common, there has been little epidemiological evidence showing that salt intake is associated with this disease. In contrast, studies conducted in western countries where gastric cancer and associated precursor lesions are uncommon (and salt intake is comparatively low, but ranging widely) have provided consistently strong support for an association (Wong and Lam 1998).

Surprisingly, whole blood iodine levels were not significantly correlated with table salt use. The question on dietary table salt usage was designed as surrogate marker of iodine intake (iodized salt is not used in processed foods). It may be that this question did not

Figure 19. Comparison of mean urinary iodine levels between western nations (Denmark, France, Germany, USA) and various regions in Japan (Kobe, Nagano, Okinawa, Sapporo and Yamagata). Adapted from the International Council for the Control of Iodine Deficiency Disorders Database 2000, Hollowell et al 1998, Konno et al 1993b, and Nagata et al 1998.



provide an accurate estimation of salt usage. Alternatively, table salt may not be a primary source of iodine in the diet of the subjects in this study. In support of this evidence, most subjects reported minimal or no usage of table salt on meals or in cooking. Furthermore, other poorly bioavailable sources of iodine are common to the North American diet (as discussed further in a later section). No other dietary measure was associated with blood iodine levels.

The other major component of this study was to examine selenium levels, as selenium can affect iodine metabolism, in addition to playing a role in a number of antioxidant enzymes. It can be seen that the mean selenium levels were higher in cases than controls, although no consistent trend was seen in the logistic regression analysis. The protective role for selenium against cancer appears to be particularly prominent in those countries where selenium intake low (*i.e.* < 150 μg/l in whole blood), for example, many regions in China and some European countries (Qi et al 1994, Knekt et al 1990). In this study, selenium levels were comparatively high and probably adequate in both groups. Thus, finding no evidence for protection in this population is not unexpected.

Elemental status: other trace elements

Iron levels were found to be higher in controls than cases, in spite of the more common history of anemia in the former group. This may have resulted from anemia-associated dietary changes in the controls. For example, controls tended to eat more meat and fish. In addition, more controls were postmenopausal than cases, and thus may have had relatively less menstruation-associated iron loss. Similarly, controls were more likely to have used or be using oral contraceptives which are known to increase iron levels (Jensen and Speroff 2000) and which were positively associated with iron levels in this study. Due

to the fact that high iron levels are generally associated with oxidative stress and DNA damage, it seems paradoxical that iron could protect against breast cancer; however, a previous case-control study in England reported similar results (Cade et al 1998). Further studies are required to determine the relative role of long-term iron status on the development of this disease.

One point of interest was the fact that blood iron levels were found to be almost 70 times higher than blood zinc levels. This is an interesting point because the recommended daily allowance (RDA) for zinc and iron are similar (approximately 10-15 mg/day) and highlights the importance of red blood cell heme iron for oxygen transport. It should be noted, however, that intake and uptake are not necessarily the same, and may vary as a result of elemental bioavailability and the state of an individual's gastrointestinal tract. Mean bromine levels (7584 μg/l) were also higher than zinc levels (7403 μg/l). There is no RDA for bromine as it is not considered an essential element, although animal studies have suggested that it may be important for growth (Nielsen 1993). Bromine tended to be associated with an increased risk, although not significantly so. Sources of bromine may include seafood, as an additive in some baked goods and fruit juices, and it is often found in various pharmaceutical drugs.

Vanadium showed a non-significant trend toward protection in both the univariate and multivariate analysis. The biological function of vanadium has only recently received attention. In its pervanadate form, it may catalyze the oxidation of halide ions or stimulate the phosphorylation of receptor proteins (Nielsen 1993). Vanadium also possesses insulin-mimetic or insulin-enhancing properties and is being used in experimental studies for the treatment of diabetes (Goldwaser et al 2000).

Finally, a number of significant inter-elemental correlations were found as outlined in Table 10. Such correlations may arise when a food source is rich in both correlated elements. For example, meat is an excellent source of both iron and magnesium, which were correlated. Alternatively, vitamin and mineral supplements may be a good source of two associated elements such as chromium and manganese.

Iodine and selenium intake in the UK, USA and Japan

Due to the narrow range of iodine levels in the population under study, its role in breast cancer could not be fully elucidated. However, considerable data exists to support a protective role for iodine against breast cancer. Selenium, in turn, is a critical factor in iodine metabolism. Therefore, the following in-depth review of this supportive evidence may aid in the design of future studies of these factors with respect to the development of breast and other cancers.

In countries with a moderate iodine intake such as the UK and the USA, the average intake level in adults (estimated from total diet studies) is approximately 166 (Lee et al 1994b) and 209 (Pennington et al 1996b) μg/day, respectively. The major sources of iodine in the UK (Lee et al 1994b) and USA (Pennington et al 1996a), respectively, include: 46% and 19% from dairy products (largely from iodophor sanitizing agents, veterinary medicines, and organic feed supplements), 7% and 31% from grains and cereals (iodate dough stabilizers and the food colourants erythrosine and rose bengal), 35% and 13% from meat and meat products (feed supplements, veterinary medicines and food colourants) and iodized salt (common in the USA) where dietary levels can vary considerably. The bioavailability and bioactivity of these differing sources of iodine is important because it can complicate estimates of the actual physiological usage of this

element. For example, erythrosine (tetraiodofluorescein) has been shown to interfere with normal iodine metabolism by inhibiting T4 to T3 conversion, and in turn causing a compensatory increase in TSH (Jennings et al 1990, Capen 1994).

Measurement of the urinary iodine concentration (UIC) in morning spot urine samples is an alternative method for estimating daily iodine intake. Japan has a comparatively high iodine intake as shown in a recent study by Nagata et al (1998) where the mean iodine intake in four different regions ranged from 810 to 1620 μg/l. In the Northern city of Sapporo, the average UIC was 3400 μg/l, as determined in a study of 4138 men and women (Konno et al 1993b). In contrast, the mean iodine level determined from the Third National Health and Nutrition Examination Survey (NHANES III) in the USA was 265 μg/l (Hollowell et al 1998).

Seaweed consumption is a key source of iodine in Japan and is used with almost every meal: as a garnish or vegetable, in sushi, soups, salads, in sweet cakes and jellies, as a tea, in sauces and flour, and in powdered form as a condiment. *Porphyra* (nori), *Undaria* (wakame), and *Laminaria* (kombu) are some of the most popular varieties, with an iodine content that we have found may range from 80 to 2500 μg/g (Cann et al 1998, van Netten et al 2000). Other seafoods are, to a lesser degree, another important source of iodine in Japan. In contrast to seafood, most foods contain less than 1 μg/g of iodine (Pennington et al 1996a). Selenium intake is similar in the USA and Japan (approximately 100-150 μg/day) (Pennington et al 1996b, Hirai et al 1996), but much higher than in the UK (less than 100 μg/day) (Hirai et al 1996, Rayman 1997). Important selenium sources include seafood, meat and meat products, whole grains and cereals (Pennington et al 1996a, Hirai et al 1996).

Epidemiology of benign breast disease

High grade fibrocystic disease (*i.e.* ductal or lobular hyperplasia, but especially atypical hyperplasia) is generally believed to be a precursor to ductal carcinoma in situ (DCIS) and subsequent invasive/metastatic carcinoma. Other symptoms of benign breast disease, including cyclical mastalgia (Plu-Bureau et al 1992) and apocrine cysts (Bruzzi et al 1997) have also been associated with an increased breast cancer risk. In the USA, it has been estimated that between 50 to 90% of women experience fibrocystic disease during their lifetime (Love et al 1982, Devitt 1986). A rate so high that some have suggested that this condition no longer be classified as a disease (Love et al 1982, Devitt 1986). However, downgrading the disease status simply due to prevalence estimations is questionable when this condition, in populations at low risk for breast cancer, is so much less common (Wang and Fentiman 1985). Gravelle and colleagues (1991) found that healthy British women had significantly less low-risk (low density) and a greater proportion of high-risk (high density) breast parenchymal patterns than Japanese women. Furthermore, immigration studies suggest that these breast parenchymal patterns may be influenced by nongenetic factors. For example, Sasano and colleagues (1978) found that the prevalence of breast epithelial hyperplasia was similar between Japanese women (18.4%) and Japanese issei Hawaiians (immigrant generation) (14.5%), but significantly lower than nisei Hawaiians (second generation) (51.4%).

In estradiol treated rats, iodine deficiency has been shown to lead to pathological changes similar to that seen in benign breast disease: cystic changes, periductal fibrosis and lobular hyperplasia (Strum 1979, Eskin et al 1986, 1995). Although iodine deficient, the thyroid parameters (*i.e.* free and total T4, and TSH) remained normal (Eskin et al 1986). Conversely, dietary iodine reintroduction has been shown to reverse pathological changes

in mammary tissue (Eskin et al 1995). The iodine-deficient mammary tissues were found to have a heightened affinity for iodine uptake. Similarly in human studies, where ^{131}I was administered to women with and without breast disease, a significantly greater uptake was observed in dysplastic and malignant breast tissue (Eskin et al 1974). Thus, iodine deficiency appears to enhance mammary-tissue sensitivity to estrogen.

In humans, several studies have shown that iodine-containing desiccated thyroid (Daro et al 1964) or T4 (Estes 1981, Peters et al 1985) were effective in reducing mastalgia, as well as other symptoms of benign breast disease (Daro et al 1964, Estes 1981). Iodine supplementation has also been examined in women with this disease. Surprisingly, all of the early studies were carried out in Russia (Bobrov 1964, Vishnyakova et al 1964, Bobrov 1969, Buianov et al 1971, Sidorenko and Shal'neva 1973). In one of the larger studies by Vishnyakova and Muravieva (1966), a beneficial effect was reported in 71.7% of patients. In support of this research, other investigators (Kotliarov and Talipov 1973, Sidorenko and Egor'kova 1978) have noted diminished blood iodine levels in patients with benign breast disease as compared to control subjects. More recently in Canada and the USA, a large clinical trial was conducted and found that iodine supplementation significantly reduced the prevalence of breast cysts, fibrous tissue plaques and breast pain (Ghent et al 1993) — thus, demonstrating that this precursor disease may be treatable through dietary modifications. In light of this evidence, the comparatively low iodine levels observed in the present study may explain why benign breast disease is so common in the west.

Epidemiology of breast cancer

In the developed world, Japan has one of the lowest age-adjusted breast cancer mortality rates, 6.6 per 100,000 (WHO 2000). In the UK and USA by comparison, these rates are 27.7 and 22.0, respectively (WHO 2000). Unfortunately, the breast cancer mortality rate in Japan has been increasing (Figure 3) and it has been suggested that "westernization" of the diet may be responsible for this trend (Tominaga and Kuroishi 1997). Evidence for a dietary link is further supported by the rise in breast cancer incidence seen in Japanese immigrants to the USA, and their successive generations, whose rates gradually increase to that of white women in the USA (Ziegler et al 1993). A high iodine status may be one of the key dietary factors protecting against the development of breast cancer in Japanese women.

Epidemiological studies investigating this potential association are lacking. One correlational study in Spain found a significant positive association between regional iodine intake and regional breast cancer mortality rates (Serra Majem et al 1988); however, because correlational studies do not link exposure with disease in individual subjects and because one cannot control for confounding factors, firm conclusions cannot be drawn from this study.

As with prevention, a role for iodine in the treatment of breast cancer needs to be investigated. As mentioned previously, traditional eastern Asian medicine has long used iodine-rich seaweeds as a cancer treatment to 'soften' tumours and 'reduce' nodulation (Kun 1985, Zhang 1989). Recent work with animal systems seems to support an antitumour effect for iodine. For example, Teas and colleagues (1984) found that dimethylbenz[*a*]anthracene (DMBA)-treated Sprague-Dawley (SD) rats receiving a 5% diet of the brown kelp *Laminaria* (a rich source of iodine), developed significantly fewer

mammary adenocarcinomas and had a significantly longer delay in time to tumour onset. In an analogous study by Funahashi and colleagues (1999), SD rats were given 0%, 1% or 5% diet of the seaweed *Undaria* for eight weeks following exposure to DMBA. At the end of eight weeks, the mammary tumours increased four fold in the control group (0%), with little or no increase in the latter two groups (*i.e.* 1% and 5%). Although not significantly different from the 1% group, the 5% group showed the greatest degree of tumour suppression.

Similarly, iodine itself may suppress tumour cell division. It is has been shown to significantly inhibit proliferation of the MCF-7 breast cancer cell line in comparison to control cultures (Ando et al 1995), and this effect that was enhanced when iodine treatment was combined with progesterone (medroxy-progesterone acetate). Analogous experiments in SD rats showed a similar suppressive effect on the growth of DMBA-induced mammary tumours (Kato et al 1994, Funahashi et al 1996). Again, this suppressive activity was enhanced when iodine treatment was combined with progesterone (Funahashi et al 1996). The suppressed tumors were found to have a significantly higher mean iodine content (95 times higher) than non-suppressed tumors (Funahashi et al 1996). The enhancement of iodine uptake by progesterone has been observed in other hormone-dependent tissues including the uterus and oviduct (Brown-Grant 1972, Cann et al 1999).

Reproductive factors and breast cancer risk

During the follicular phase of the menstrual cycle, breast epithelium is largely composed of small lobules and a few terminal duct structures with intralobular stromal condensation; in the luteal phase, however, the ductal epithelium, lobules and terminal duct structures increase in size (Markopoulos et al 1988). It has been shown that resting human breast

tissue can absorb iodine and secrete iodinated proteins from the terminal ductules and intralobular ducts (Strum et al 1983). However, dysplastic and malignant breast tissue has been shown to have an enhanced ability to take up iodine (Eskin et al 1974). Similarly, in patients with iodine-deficient goiter, the thyroid shows enhanced iodine uptake, indicating that in both tissues insufficient iodine may be an underlying cause of pathological growth.

During pregnancy and lactation, hormonal stimulation of the mammary gland leads to glandular differentiation with dramatically enhanced iodine absorption and organification (Shah et al 1986). It is interesting to note that this iodine absorption occurs in the same ductal epithelium (Strum 1979, Eskin et al 1995) where the majority of breast cancers arise (Russo and Russo 1997). This preferential uptake of free iodine in breast tissue may also explain the reduction in nodularity and tissue density that is often observed following pregnancy and lactation (Haagensen 1971). Thus, a link may exist between enhanced breast iodine uptake during pregnancy/lactation and the subsequent reduction of breast cancer risk. A number of studies have also shown that early parity and lactation are associated with a reduction in breast cancer risk (Kelsey et al 1993). It has been suggested that these reproductive periods may be protective against breast cancer because of the lobular differentiation that occurs during these stages (Russo and Russo 1997). It may be that increased iodine uptake may also play a pivotal role in this differentiation process. For example, most studies in Asia have found lactation to be protective in preventing subsequent breast cancer development in both premenopausal and postmenopausal women (Yoo et al 1992, Ross and Yu 1994, Yang et al 1997, Zheng et al 2001). In contrast, studies conducted in North America and Europe have generally shown breastfeeding to be protective only in premenopausal women (Newcomb et al 1994, Katsouyanni et al 1996), or not at all (Thomas and Noonan 1993, Michels et al 1996).

The reproductive characteristics more common in these western countries (*i.e.* later age at first birth, shorter duration of breastfeeding and less frequent daily breastfeeding (London 1994, Yang et al 1997, Zheng et al 2001)), as well as, a lower level of iodine intake may be responsible for these differences (Cann et al 2000).

The thyroid and breast cancer

Although a number of tissues have iodine-concentrating capabilities (*e.g.* salivary glands, stomach, cervix, *etc.*), only in the thyroid and breast is iodine known to be organified for storage (Brown-Grant 1961). In the thyroid, this occurs via thyroperoxidase oxidation of iodide (I-), which subsequently binds to tyrosyl residues (Taurog 1996). In an analogous manner, lactoperoxidase organifies iodine in the breast — a process particularly active during pregnancy and lactation (Shah et al 1986, Taurog 1996). In subjects with iodine-deficient goiter, iodine administration has been shown to be effective in reducing thyroid size (Hintze and Kobberling 1992). Similarly, iodine treatment of patients with benign breast disease has been shown to cause a significant bilateral reduction in breast size, in addition to causing a remission of disease symptoms (Ghent et al 1996). This evidence suggests that iodine plays an important role in the maintenance of both normal thyroid and breast physiology. A similar mechanism for this observation may be operative in both tissues. For example, in recent years a second pathway for iodine organification has been described and involves iodine incorporation into lipid molecules. These iodolipids have been isolated from thyroid tissue and have been shown to be key regulators of thyroid cell proliferation and metabolism (Dugrillon 1996). Iodide peroxidases (*e.g.* thyroperoxidase / lactoperoxidase) catalyze the iodination of lipids (Boeynaems et al 1981). One such compound derived from arachidonic acid, 6-iodo-5-hydroxy-eicosatrienoic acid (δ-

iodolactone), was found to be a potent inhibitor of human thyroid follicular cell proliferation *in vitro* (Dugrillon et al 1994) and to induce goiter regression in rats *in vivo* (Pisarev et al 1994). These or similar compounds may also play a role in the proliferative control of breast tissue.

Another possible link has been made by examining the relationship between thyroid dysfunction and breast cancer (Takatani et al 1989, Giani et al 1996, Yokoe et al 1996, Strain et al 1997) — although controversy exists over this association (Goldman 1990). In a study in Ireland, Smyth (1993) found a significantly greater mean thyroid volume in patients with breast cancer compared to controls. A previous study by Smyth et al (1988) showed that as many as 30% of the population had iodine excretion values corresponding to less than half the minimum daily requirement. In addition, combined selenium and iodine deficiency in rats has been shown to cause an increase in thyroid size over that caused by iodine deficiency alone (Beckett et al 1993) and also may be a factor in these human subjects. Thus, iodine deficiency, in combination with other dietary factors, may be associated with the spectrum of thyroid dysfunction patterns often observed in patients with breast cancer.

Selenium and thyroid hormone homeostasis

Sediment concentrations of selenium throughout the world vary widely and are deficient in many soils (Diplock 1993). Selenium deficiency in humans has been shown to effect the metabolism of thyroid hormones (Olivieri et al 1995). This is due to the fact that all three thyroid hormone deiodinases (D1, D2, D3) are selenoenzymes (Salvatore et al 1996). D1 catalyzes the conversion of T4 to the more active tri-iodothyronine (T3), providing the majority of plasma T3. D2 is responsible for intracellular T4 to T3 conversion and may

supply a significant level of T3 to peripheral tissues (Salvatore et al 1996). The other selenoenzyme, D3, is involved in T3 and T4 inactivation. In each case, free iodine is released upon conversion of these hormones.

In addition to its role in deiodinases, selenium, in the form of selenocysteine, is a key component in the active site of numerous antioxidant proteins — glutathione peroxidase (four subtypes), and thioredoxin reductase, selenoprotein P, and selenoprotein W — as well as selenoprotein synthetase (involved in selenoprotein synthesis) and sperm mitochondrial capsule protein (involved in spermatogenesis) (Burk and Hill 1999; Holben and Smith 1999).

Selenium plays a crucial role in the maintenance of normal thyroid physiology. For example, *iodine* supplementation alone, in a selenium and iodine deficient animal model, was shown to cause irreversible thyroid gland fibrosis (Contempre et al 1995, Hotz et al 1997). The selenoenzyme glutathione peroxidase was implicated as a necessary component for thyroidal protection during iodine supplementation (Contempre et al 1995). In contrast, *selenium* supplementation alone, in human subjects with combined selenium and iodine deficiency, has been shown to cause an aggravation of iodine deficiency and hypothyroidism (Contempre et al 1991, Contempre et al 1992, Hofbauer et al 1997). These studies demonstrate the complex interplay between these two essential elements.

Animal studies have shown selenium to be protective at both the initiation and post-initiation stages of mammary tumor development (Vernie 1984, El-Bayoumy 1994). With respect to human breast cancer, selenium status has been estimated using a variety of biological samples. Early correlation studies, which estimated regional selenium levels from forage crops (Shamberger et al 1976), dietary intakes and whole blood (Schrauzer et

al 1977), suggested that a high selenium status may protect against breast cancer. This work has been followed by a number of case-control and prospective cohort studies; a summary of those studies reporting odds ratios (OR) or relative risks (RR) are presented in Table 11. These studies suggest that selenium may afford protection against breast cancer in regions where selenium intake is most deficient.

Iodine and ovarian function

A second, but not mutually exclusive mechanism to the iodolipid hypothesis, may depend on the influence of iodine on ovarian hormone production. An abnormal ovarian hormone profile has been observed in women with polycystic ovary syndrome (PCOS) (Cheung and Chang 1995). This abnormal secretion of hormones, in turn, has been associated with an increased breast cancer risk (Coulam et al 1983). Overt PCOS may be diagnosed and surgically treated at an early stage, and thus, preclude any increase in breast cancer risk. However, studies have shown that the incidence of PCOS may vary from 0.6% to as high as 92%, depending on the diagnostic criteria (Insler and Lunenfeld 1991). These data suggest that many women may harbour occult ovarian cystic disease, and thus, be at an increased risk for breast cancer.

No studies in humans have examined the role of iodine in the maintenance of normal ovarian physiology, however, animal studies have been conducted. Studies in bovines found a high prevalence of ovarian cystic disease in regions where dietary iodine was considered deficient (Afiefy et al 1970, Dzhambazov 1976). Interestingly, iodine supplementation of cows with cystic ovaries has been shown to cause a regression of disease (Dzhambazov and Nikolaev 1974, Dzhambazov et al 1975). Similar investigations by Sushkova and colleagues (1972) have shown that iodine administration can improve

Table 11. Selenium status and breast cancer risk

Study location	Cases	Selenium biomarker	Comparison groups	OR or RR (95% CI) highest *vs* lowest[a]	References
			Case-control		
Boston, MA	38	Erythrocytes	Tertiles	2.0 (0.8-5.0)	Meyer 1987
Netherlands	92	Erythrocytes	Quartiles	1.1 (0.5-2.5)	van't Veer et al 1987
	92	Plasma		0.5 (0.2-1.1)	
	124	Toenails		0.9 (0.5-1.7)	
Denmark	36	Serum	Tertiles	1.0 (0.3-2.9)	Overvad et al 1991
Sweden	441	Plasma	Tertiles	**0.4** (0.2-0.9)	Hardell et al 1993
Tianjin, China	244	Whole blood	Quartiles	**0.1** (0.0-0.4)	Qi et al 1994
Europe[b]	326	Toenails	Tertiles	1.0 (0.6-1.5)	van't Veer et al 1996
Finland	289	Toenails	Quintiles	0.7 (0.3-1.5)	Mannisto et al 2000
			Prospective cohort		
Netherlands	61	Toenails	Quartiles	1.1 (0.5-2.9)	van Noord et al 1987
USA	434	Toenails	Quintiles	1.1 (0.7-1.7)	Hunter et al 1990
Finland	90	Serum	Quintiles[c]	0.5 (P>0.05)	Knekt et al 1990
England	46	Plasma	Quartiles	1.3 (0.5-3.4)	Overvad et al 1991
Netherlands	270	Toenails	Quintiles	0.8 (0.6-1.3)	van den Brandt et al 1994
Missouri	105	Serum	Quartiles	0.9 (0.4-1.8)	Dorgan et al 1998

a. Odds ratios (OR) and relative risks (RR) are for age-matched subjects or with adjustment for age, and adjustment for other risk factors. Values in bold are significant.

b. Germany, Netherlands, Northern Ireland, Spain and Switzerland.

c. Four higher quintiles *vs* lowest.

ovarian cystic disease in rats. In support of these observations, Vishnyakova and Muravieva (1966) found that iodine therapy in women with fibrocystic disease led to a normalization of the ovarian cycle, as well as estrogen and progesterone levels. Thus, iodine deficiency may lead to ovarian cystic disease, abnormal ovarian hormone production, and over time, an increased risk for breast cancer.

Benign breast disease and infection

A third hypothesis relates to a possible infectious etiology for benign breast disease. In their study of iodine administration for this disease, Ghent and colleagues (1983) noted an unusual side effect of the iodine treatment. Women experienced an increase in breast pain during the first month of treatment, which the authors speculated was due to tissue remodeling as the fibrosis gradually regressed. However, such symptoms appear to be curiously similar to a phenomenon known as the 'Jarisch-Herxheimer reaction'.

Components of this phenomenon were first described by Jarisch (1895) and later Herxheimer (1902) who were using mercury to treat patients with syphilis. In their patients, it was noted that there was a temporary (several hours to several days) worsening of disease symptoms following mercurial treatment. Many years later when antibiotics came into widespread use, this phenomenon was again observed in patients with *Brucella melitensis* bacteremia (Sprink et al 1948) and in those treated for typhoid fever (Reilly et al 1950). Similar reactions have been noted when antibiotics were administered to patients with autoimmune diseases, such as multiple sclerosis (Le Gac et al 1964) and rheumatoid arthritis (Wyburn Mason 1976), and it is a well-known side effect of the treatment of *Borrelia* infections (*i.e.* louse-borne relapsing fever and Lyme disease) (Fekade et al 1996). The Jarisch-Herxheimer reaction is now believed to be due

to toxemia arising when injured or killed bacteria release their constituents into tissues and the bloodstream. These breakdown products, in turn, provoke a sudden and exaggerated inflammatory response that is characteristic of the symptoms of the disease being treated (Beutler and Munford 1996). Often this immune response may be severe enough to produce a fatal reaction. Yet, generally, the reaction subsides once the offending organism has been cleared from the bloodstream and affected tissues.

What further evidence exists to demonstrate that fibrocystic disease has an infectious etiology? As has been mentioned in the introduction, iodine has antimicrobial properties against bacteria, fungi, *etc.* Thus, if fibrocystic disease were an infection, iodine could theoretically destroy these pathogens. Surprisingly, some support for this infectious hypothesis comes from research on breast implants. It was observed that women who had undergone this reconstructive surgery developed increasing fibrosis around the implant over time, leading to capsular contracture. Initially, it was believed that this was simply due to a foreign body reaction and largely unavoidable; however, Burkhardt and colleagues (1981) noted significantly less capsular contraction occurring in those patients who received intraluminal antibiotics with their implants. Patients not receiving this medication were often given antibiotics postoperatively if breast inflammation and tenderness formed (symptoms frequently heralding the onset of capsular contracture). Such treatment reduced the inflammation, and to varying degrees, the tissue fibrosis. More recent research has substantiated the infectious hypothesis for capsular contraction, and coincidentally, iodine (in the form of povidone-iodine) has become the most common intraluminal antibiotic employed during this procedure (Adams et al 2000). It is generally used in combination with other antibiotics such as gentamicin or polymyxin B.

Although the fibrosis arising from implants is analogous to fibrocystic disease, it is not the same condition. Would antibiotics be similarly effective against fibrocystic disease? Wyburn Mason (1978) experimentally administered the antibiotic, tinidazole, to patients with fibrocystic disease. Tinidazole is a 5-nitro-imidazole antibiotic used in the treatment of bacterial and protozoal infections. He noted that patients initially developed inflammation, tenderness and swelling in nodular areas — symptoms characteristic of a Jarisch-Herxheimer reaction. These symptoms gradually disappeared with a marked improvement in the fibrocystic condition after one to two months of treatment. Conversely, in healthy women, the drug produced no effect.

As far back as 1899, the pathologist George Adami stated his belief that in many chronic fibroid conditions, subinfection will be found to play a definite part (Adami 1899). Yet despite evidence to support his conclusions, modern researchers continue to focus on the investigation of symptomatic treatments (*e.g.* anti-inflammatory agents) for fibroid conditions. In the case of fibrocystic disease, the beneficial effects of iodine may, in part, be a result of its microbicidal properties.

With respect to breast cancer, it would not be too surprising if such subclinical, chronic infections play a role in the etiology of this disease. For example, recent evidence has shown that the bacteria, *Helicobacter pylori*, is one of the primary pathogens involved in the development and progression of gastric adenocarcinomas and gastric mucosal-associated lymphoid tissue (MALT) lymphomas (Hoption Cann et al 2001b, Pakodi et al 2000). Moreover, administration of a wide variety of antibiotic regimens can induce the complete regression of gastric adenomas (precursors to adenocarcinoma) (Gotoda et al 1999) and MALT B and T cell lymphomas (Hoption Cann et al 2001b, Bariol et al 2001). Similarly, other infections have been associated

with carcinoma-like diseases — *Entamoeba histolytica* and cervical, colon and rectal tumours (Wyburn-Mason 1966, ten Seldam 1970, McClatchie and Sambhi 1971); *Bartonella* infections and sweat gland and squamous cell tumours (Arias-Stella et al 1987) — all of which regress following antibiotic treatment.

Iodide vs diatomic iodine

Dietary iodine is generally in the form of iodide (I^-) or iodine bound to organic molecules such as tyrosine or histidine. It has been suggested, however, that diatomic iodine (I_2) is more effective in treating benign breast disease than is iodide (Ghent et al 1993, Eskin et al 1995). It was long considered that I_2 is reduced to I^- in the small intestine before absorption, and thus oral ingestion of either form would be equivalent; yet several investigations suggest otherwise. A difference in the metabolism of these two forms of iodine has been noted with respect to thyroid hormone levels. The daily injection of I_2 into thyroidectomized animals increases plasma thyroxine levels, metabolic rates and prevents hypothyroidism; however, injection of inorganic I^- increases plasma thyroxine levels only slightly, without preventing hypothyroidism (Barker and Lipner 1948, Williams 1955). More recently, Sherer and colleagues (1991) examined the administration of equivalent doses of $^{125}I^-$ and $^{125}I_2$ in rats with intact thyroids. Similar to the previous studies, plasma thyroxine levels were shown to increase significantly in rats receiving $^{125}I_2$, while no change occurred in rats receiving $^{125}I^-$. In rats receiving $^{125}I_2$, most of the radioactive iodine (^{125}I) was found to associate with serum proteins and lipids ($^{125}I_2$ was not detectable), while rats receiving $^{125}I^-$ had significantly higher levels thyroidal ^{125}I

(Thrall et al 1992a). *In vitro*, it was shown that only $^{125}I_2$ incubation with di-iodotyrosine (T2), T3 or reverse T3 in phosphate buffered saline resulted in the formation of T4 (Thrall et al 1992b). Thus, due to its reactive nature, I_2 may react with organic molecules directly, while I^- would require an enzymatic reaction (*i.e.* via iodide peroxidases) for organification. In the gastrointestinal tract and blood, the more reactive I_2 could form T4, subverting control of the thyroid. In contrast, I^- would primarily be absorbed by the thyroid, which only produces thyroid hormones as needed while storing any excess iodine. Therefore, over the short term, diatomic iodine may produce more immediate results over iodide treatment of benign breast disease, if an oxidation reaction is required (*i.e.* to hypoiodite, an iodolipid, or other beneficial iodinated compound). However, the long-term effects of I_2 ingestion are unknown.

Iodine and macrophages in breast cancer

Macrophages are known to infiltrate into tissues where an abnormality has arisen. Fibrocystic breast tissue, a precursor to breast cancer, is no exception. Several studies of hyperplastic and cystic breast tissue have confirmed that macrophages accumulate in the stroma, ductal lumen, and intercalate between duct epithelial cells (King et al 1984, Shousha et al 1987, Dabbs 1993), although macrophage concentrations are less than that seen in breast cancers (Nestor and Cochran 1987). The analogy between the development of iodine-deficient goiter (benign hyperplasia of the thyroid gland) and breast cancer progression is of particular interest. Studies of hyperplastic thyroid tissue have found high concentrations of macrophages not found in normal tissues (Herrmann et al 1994). This former condition has been associated with the subsequent development of

several types of aggressive thyroid cancers (*i.e.* follicular and anaplastic carcinoma) (Bakiri et al 1998). At its early stages, goiter can be treated with iodine supplementation which causes glandular regression, along with a subsidence of the inflammatory infiltration (Hintze and Kobberling 1992, Many and Denef 1992). Fortuitously, iodine supplementation has also been shown to cause a remission of benign breast disease — an important risk factor for subsequent breast cancer development. Thus, the maintenance of their normal physiology seems dependent on an adequate supply of this element.

Macrophages are also an important factor in both progression and resolution of these conditions. As has been seen from the present study, macrophage density increases with breast tumour aggressiveness, and macrophages are particularly concentrated in areas of high tumour mitotic activity. In a population with an inadequate iodine supply, such as the one presently studied, macrophages cytotoxic activities may be inadequate and tissue pathology may develop. To fully elucidate the role of iodine as a factor in this process, however, further studies are required in populations with a higher and more widely ranging iodine intake. Japanese women, who eat a diet plentiful in seafood (*i.e.* iodine rich foods), have one quarter the breast cancer mortality rate as that seen in the west (Cann et al 1998), and thus, would be the ideal population for such studies.

OVERVIEW

In the introductory sections of this dissertation, the supporting conceptual and experimental citations were obtained largely from historical literature. This is due to the fact that many key concepts with respect to cancer development and progression (*i.e.* that pertaining to the immune system and immunomodulators such as iodine) have been forgotten. Yet, such ideas are more relevant today than ever. With modern research increasingly more focused on molecular and genetic techniques, one may often overlook the broader picture of disease, crucial to its full understanding. In the historical studies examined, however, the observation of the whole spectrum of disease symptoms was paramount and such observations correspondingly produced an appreciation of many factors that may affect cancer development. With the synthesis of modern and historical investigations, one can then piece together a puzzle and therefore construct a more focused picture of cancer, its etiology, and avenues for prevention and treatment.

Through the present research, a more lucid picture of cancer etiology has come to light through the fusion of newly discovered and re-discovered pieces of this puzzle. In this final section, I wish to summarize the key discoveries of my research with a discussion of the impact it may have on the prevention and treatment of breast and other cancers.

1. *Dual role of the immune system.* The immune system is an integral component in the development of cancer, being both a primary factor in its progression and regression (*see* Figure 4). As we have suggested, it is the dual nature of this immune activity (defense and repair) that leads to this two-pronged effect (Hoption Cann et al 2001a). A basic

awareness of this functional duality is the starting point for the understanding and control of this disease. The following points illustrate the importance of this concept.

2. *Injury and tumour progression.* Following sterile injury (*e.g.* physical trauma, surgery or other medical procedures) to a tumour, immune defensive functions are not required. However, the reparative functions of the immune system are immediately set in motion. This includes the initial recruitment of immune cells to the injured tissue, with subsequent immune-mediated growth stimulation and vascular regeneration. Sterile trauma from injurious procedures such as mammography (van Netten and Cann 1996, van Netten et al 1997, van Netten et al 1999) may turn dormant tumours into an invasive disease. With a compressive force up to 45 lbs (200 N), this may not only induce injury and bruising, but may aid in malignant cell dissemination. Thus, it is not surprising that the recently published Canadian randomized study (Miller et al 2000), a review of one of the largest screening programmes, the Swedish mammography programme (Stahle and Sjonell 1999), and a review of all previous randomized trials (Gotzsche and Olsen 2000) failed to show any protective benefit arising from mammographic screening. We have recommended that women with breast pain in particular should not undergo this procedure (van Netten et al 1997, van Netten et al 1999).

Other common sources of breast injury such as seat belt trauma following a car accident may accelerate the growth of previously dormant tumours (van Netten and Cann 1996). Due to our research and due to personal knowledge of a number of women who developed such aggressive trauma-induced cancers, we developed a symmetrical seat belt system that would not cause breast injury during a car accident. This design is now under review by a major automobile manufacturer.

3. *Infection and tumour progression.* It is the particular response of the immune system to infection that ultimately shapes its role with respect to cancer development. Chronic infections generally reflect a failed response by the immune system (reparative rather than defensive arm) and the subsequent prolonged inflammation facilitates malignant transformation. In fact, we have shown that the same infection may elicit both immune defensive and reparative responses during the prolonged course of disease (Hoption Cann et al 2001a). Co-infection may be one factor that can reawaken the defensive arm of this system. More importantly, we have reported that treatment with antibiotics (to eliminate the organism driving this response) may induce the regression of pre-malignant as well as some malignant tumours (Hoption Cann et al 2001b) and I am personally aware of one such case. Thus, more research is required on the effectiveness of these more benign pathogen-targeting treatments, with less emphasis on toxic tumour cell-targeting treatments such as radiation and chemotherapy.

4. *Infection and tumour regression.* In contrast to the previous example, tumour regression in association with an acute infection reflects an effective immune response (the defensive arm). I have reviewed those factors associated with an enhancement of this natural response to infection (*e.g.* direct tumour injection, increasing the dosage to avoid tolerance, gauging the dose by patient symptoms, a prolonged follow-up, *etc.*) and its ability to reduce pain and accelerate healing. Furthermore, I provided an outline of how one can use this information for the development of an effective immunotherapy treatment for cancer (Hoption Cann et al 2001a). Again, I am personally aware of two patients who experienced infection-associated tumour regression, as well as, the ability of such infections to eliminate cancer-associated pain.

5. *The macrophage and cancer.* There are many aspects of macrophage functional activity which can aid in cancer progression, including the facilitation of tumour cell proliferation, invasion, metastasis and tumour neovascularization (*see* Figure 5). In my initial study, macrophage density was found to correspond to breast tumour aggressiveness, and this association has been confirmed by other recent studies in breast and other cancers. Practical applications of this phenomenon for cancer treatment include re-educating these tumour-associated macrophages by inducing their cytotoxic activities (as outlined previously), or in contrast, suppression of those macrophage reparative activities that aid tumour progression. Linomide and thalidomide are two such compounds in the latter category that are now under study.

6. *The macrophage and vasogenesis.* Macrophage induction of vasogenesis (blood and lymphatic vessel development) is critical for both tumour nutrition and metastasis. Research in this area, however, has focused solely on the blood vascular system (*i.e.* angiogenesis). Our investigations suggested that tumours can induce lymphagenesis and we supported this finding with the following analogy (Cann et al 1995). Macrophage induction of lymphagenesis is a natural process that arises during infection. In an infected wound, the body immediately seals off the blood vascular system, while macrophages induce lymphagenesis for removal of debris and foreign agents to regional lymph nodes. Tumours can mimic wounds by secreting signaling molecules that instruct macrophages to stimulate lymphatic cell proliferation and vessel formation, and these lymphatic channels become a primary route for tumour invasion and metastasis. Although our hypothesis and research (Cann et al 1995, van Netten et al 1996) was initially met with considerable skepticism (Folkman 1996), a recent series of studies now confirm this work (Mäkinen et

al 2001, Mandriota et al 2001, Skobe et al 2001, Stacker et al 2001). Practical applications of this phenomenon include the development of specific agents that can suppress macrophage production of lymphagenic factors (van Netten et al 1998, Hoption Cann et al 2001c).

7. *Iodine and immune function.* We promoted the concept that iodine could enhance the cytotoxicity of immune cells and discussed its importance in normal mucosal defense against infections (Cann et al 1999). Through the macrophage halide-H_2O_2-peroxidase system, iodine can be oxidized to hypoiodite (or other toxic forms), which can then bind and impair the functional activity of bacterial cell membranes. However, this antimicrobial activity can only occur if iodine intake is sufficient, in deficient states, the thyroid sequesters the majority of dietary iodine. The population of the present breast cancer study appears to be in this latter category.

8. *Iodine and benign breast disease.* Several hypotheses were explored as to the reason for iodine's beneficial effect on the breast cancer precursor, benign breast disease (Cann et al 2000). First, that benign breast disease is an infectious disease and that iodine enhances the cytotoxicity of macrophages associated with these infectious regions of the breast. The low blood iodine levels observed in the present study suggest that iodine intake of this population is insufficient to prevent the development of this disease. Experimental use of antibiotics has been observed to improve symptoms of benign breast disease. Further support for this hypothesis is the fact that this precursor disease is so uncommon in Japan where iodine intake is three to five times higher.

Second, in animal and human studies, iodine appears to beneficially modify sex hormone levels. Thus, it may be that insufficient iodine intake will, over a prolonged period, lead to hormonal changes that favour the development of this disease.

Third, the development of breast pathology is in many ways analogous to the development of thyroid goiter (associated inflammation, recruitment of macrophages, and a high expression of the sodium/iodide symporter suggesting iodine deficiency). Correspondingly, thyroid size has been observed to be larger in patients with breast cancer than in those without. Iodine administration, in turn, causes a reduction in the size and pathology of both conditions. Iodolipids are known to be key factors in suppressing thyroid cell proliferation and may serve the same function in breast tissue as well.

One or all of these phenomena may be principally involved, yet in any case, the evidence presented (animal and human studies) supports the concept that benign breast disease develops when iodine is insufficient, and conversely, subjects who increase their iodine intake can experience a regression of this disease.

9. *Iodine and breast cancer.* The preceding points are also pertinent to the prevention and treatment of breast cancer. Iodine or iodine-containing products (*e.g.* seaweed, desiccated thyroid) have long been used in cancer treatment (Cann et al 2000). Some hormones, such as progesterone, are known to enhance iodine uptake in breast tissue and may be used synergistically with iodine for treatment of this disease (Cann et al 1998). Based on this research, we have developed a study to test this hypothesis in patients with breast cancer using iodine supplements in combination with the progesterone analogue, megestrol acetate (a third line therapy for breast cancer).

To further elucidate the role of iodine in the development of this disease, an analogous study to the present breast cancer study needs to be conducted in a population with a wider range of iodine intake. The Japanese population would be ideal, as salt is not iodized in Japan. Therefore, women who eat a diet rich in seafood, particularly seaweed, would have a high intake of iodine, while women who did not eat such foods would not. Such a study would also allow for one to approximate the iodine levels required for protection against this disease.

10. *Extrathyroidal activities of iodine.* Although emphasis has been placed on role of iodine with respect to breast cancer, I have also examined a more expansive role for this element in the development of a variety of diseases. Its beneficial effects have been observed in other cancers, cardiovascular diseases, a wide range of infectious diseases, autoimmune conditions, and some diseases with an unknown etiology (Cann et al 2000, Hoption Cann et al 2000). A wider physiological role for iodine needs to be explored; further research into its accumulation, oxidation, organification and functional activity in nonthyroidal tissues should aid in elucidating these less well recognized functions.

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APPENDIX I

TNM system of classification for breast tumour staging.

T1 tumour is 2 cm or less

T2 tumour is more than 2 cm

T3 tumour is more than 5 cm

T4 tumour any size with extension into chest wall or skin

N0 no lymph node involvement

N1 metastasis to moveable nodes

N2 metastasis to fixed nodes

N3 metastasis to internal mammary lymph nodes

MX metastasis cannot be assessed

M0 no distant metastasis

M1 distant metastasis

Stage (a combination of TNM sub-categories)

I Tumour less than 2 cm, no lymph node metastasis

II Tumour more than 2 cm or lymph node involvement

III Matting of lymph nodes, or skin, nipple or chest wall involvement

IV Any distant metastasis

Modified Bloom-Richardson grading scheme.[a]

Tumour tubule formation:

if > 75% of tumour cells arranged in tubules, then score 1;

if > 10%, and < 75%, then score 2;

if < 10%, then score 3.

Number of mitoses:

Via low power scanning (X100), locate most mitotically active area of tumour and proceed to high power (X400):

if < 10 mitoses in 10 high-power fields, then score 1;

if ≥ 10, and < 20 mitoses, then score 2;

if > 20 mitoses per 10 high-power fields, then score 3.

Nuclear pleomorphism:

If cell nuclei are uniform in size and shape, relatively small, have dispersed chromatin patterns, and are without prominent nucleoli, then score 1;

If cell nuclei are somewhat pleomorphic, have nucleoli, are of intermediate size, then score 2;

If cell nuclei are relatively large, with prominent nucleoli or multiple nucleoli, coarse chromatin patterns, and vary in size and shape, then score 3.

Final combined Bloom-Richardson (BR) grade:

Add the score from tubule formation plus the number of mitoses plus nuclear pleomorphism to obtain the BR subscore:

If BR subscore is 3, 4, or 5, then BR grade is low (I);

If BR subscore is 6 or 7, then BR grade is intermediate (II);

If BR subscore is 8 or 9, then BR grade is high (III);

a. Adapted from Elston 1987.

GREATER VICTORIA
HOSPITAL SOCIETY

2101 Richmond Avenue
Victoria, B.C. V8R 4R7

Telephone: Area Code 604
595-9699

November 23, 1993

Dr. Johann van Netten
Psysiologist
Department of Laboratory Medicine
Greater Victoria Hospital Society

Dear Dr. van Netten:

Research: A Proposal to Study the Involvement of Macrophages in the Metastatic Process of Infiltrating Ductal Carcinomas of the Breast

As Co-Chair of the Research Review and Ethical Approval Committee, I am pleased to inform you that approval to proceed with the above noted protocol has been granted by the Corporate Executive Committee.

Yours sincerely,

Pat Coward
Vice President - Patient Care

PC/lp

APPENDIX III

Patient Information and Informed Consent Patient No. □□□

A Case-Control Study of Trace Element Levels in Patients with Newly Diagnosed Breast Disease

I am told by my doctor that I may have some form of breast disease. I understand that I am consenting to take part in a study that compares my blood trace element levels with those who have not been diagnosed with breast disease, but are of a similar age. I may have my urine collected during a 24 hour period and have this sample analyzed for several hormones that will also be compared to control subjects. Also, I will be asked for some general background information and for my dietary and medical history.

Purpose, Design and Potential Benefits

The purpose of this study is to determine whether the blood trace element levels differ in women with and without breast disease. We hypothesize that an insufficient intake of some trace elements may be a risk factor for breast disease, however, research must be carried out to confirm this hypothesis. The results of this study will be submitted for publication to reputable medical journals. If differences are found between the two groups, adjustment of essential trace elements may be able to prevent or delay the development of breast disease. Furthermore, this research could lead to the development of diagnostic tests for identifying subjects who could reduce their breast disease risk through dietary changes.

Tests

If I agree to take part in this study, the following will be required:

- Blood tests
- 24 hour urine sample
- General background information
- Dietary questionnaire
- Medical history

A blood sample will be taken to determine my blood levels of trace elements and hormones. Two tubes of blood (about one tablespoon) will be required for these tests. The needles used to take blood might be uncomfortable. I might get a bruise, and *rarely*, an infection at the site of needle puncture. In addition, a urine sample may be required encompassing a 24 hour period.

I will be asked to complete general information and dietary questionnaire. Some of the questions are of a personal nature, and I can refuse to answer if I wish. Furthermore, I understand that information about my medical history (*i.e.* history of any significant health conditions that may be associated with diet) will also be needed for the study.

Confidentiality

The investigators involved in this study will keep strictly confidential all information from my medical records, dietary history, blood/urine tests and any other information that I may provide. Only the study investigators will have access to the data. My name will not be

given to anyone except the researchers doing this study. Any identifying information will be kept in a locked filing cabinet behind locked doors. The results of this research may be presented at meetings or in publications; however, my anonymity will be protected by using a code number to identify results obtained from each participant. All participant data will be stored after completion of the study for future analyses, while the biological samples will be destroyed following testing.

Voluntary Participation

I have talked with the project nurse coordinator about the study and she has answered my questions. I may ask more questions about the study at any time. No monetary compensation has been offered to me.

I understand that my participation in this study is voluntary and that I may withdraw from the study at any time, without explanation. My refusal to participate will not affect the services I receive. If I do decide to withdraw, the information and samples I have provided will be destroyed at my request.

I am aware that the principal investigators, Dr. Hans van Netten (Clinical) at (xxx) xxx-xxxx and Dr. James Houston (Medical) at (xxx) xxx-xxxx, will be available to answer any questions I might have concerning this study.

If I have any concerns about my treatment by study personnel or my rights as a research subject, I may telephone Dr. Ernie Higgs, Medical Director of the Capital Health Region at (xxx) xxx-xxxx, who is not involved in this study.

By signing below, I agree to participate in this study. I understand that I will receive a copy of the information and consent form.

Signature of Research Subject	Name (please print)	Date
Signature of Nurse Coordinator	Name (please print)	Date

APPENDIX IV

Participant Information and Informed Consent Participant No. □□□

A Case-Control Study of Trace Element Levels in Patients with Newly Diagnosed Breast Disease

I understand that I am being asked to participate in a study that compares my blood trace elements levels to the levels of subjects of a similar age who have been diagnosed with breast disease. I may be asked to collect my urine during a 24 hour period and have this sample analyzed for several hormones that may be compared to case subjects. Also, I will be asked for some general background information and for my dietary and medical history.

Purpose, Design and Potential Benefits

The purpose of this study is to determine whether the blood levels of trace elements differ in women with and without breast disease. We hypothesize that an insufficient intake of some trace elements may be a risk factor for breast disease, however, research must be carried out to confirm this hypothesis. The results of this study will be submitted for publication to reputable medical journals. If differences are found between the two groups, adjustment of essential trace elements may be able to prevent or delay the development of breast disease. Furthermore, this research could lead to the development of diagnostic tests for identifying subjects who could reduce their breast disease risk through dietary changes.

Tests

If I agree to take part in this study, the following will be required:

- Blood tests
- 24 hour urine sample
- General background information
- Dietary questionnaire
- Medical history

A blood sample will be taken to determine my blood levels of trace elements and hormones. Two tubes of blood (about one tablespoon) will be required for these tests. The needles used to take blood might be uncomfortable. I might get a bruise, and *rarely*, an infection at the site of needle puncture. In addition, a urine sample may be required encompassing a 24 hour period.

I will be asked to complete general information and dietary questionnaire. Some of the questions are of a personal nature, and I can refuse to answer if I wish. Furthermore, I understand that information about my medical history (*i.e.* history of any significant health conditions that may be associated with diet) will also be needed for the study.

Confidentiality

The investigators involved in this study will keep strictly confidential all information from my medical records, dietary history, blood/urine tests and any other information that I may provide. Only the study investigators will have access to the data. My name will not be

given to anyone except the researchers doing this study. Any identifying information will be kept in a locked filing cabinet behind locked doors. The results of this research may be presented at meetings or in publications; however, my anonymity will be protected by using a code number to identify results obtained from each participant. All participant data will be stored after completion of the study for future analyses, while the biological samples will be destroyed following testing.

Voluntary Participation

I have talked with the project nurse coordinator about the study and she has answered my questions. I may ask more questions about the study at any time. No monetary compensation has been offered to me.

I understand that my participation in this study is voluntary and that I may withdraw from the study at any time, without explanation. My refusal to participate will not affect the services I receive. If I do decide to withdraw, the information and samples I have provided will be destroyed at my request.

I am aware that the principal investigators, Dr. Hans van Netten (Clinical) at (xxx) xxx-xxxx and Dr. James Houston (Medical) at (xxx) xxx-xxxx, will be available to answer any questions I might have concerning this study.

If I have any concerns about my treatment by study personnel or my rights as a research subject, I may telephone Dr. Ernie Higgs, Medical Director of the Capital Health Region at (xxx) xxx-xxxx, who is not involved in this study.

By signing below, I agree to participate in this study. I understand that I will receive a copy of the information and consent form.

Signature of Research Subject	Name (please print)	Date
Signature of Nurse Coordinator	Name (please print)	Date

APPENDIX V

Patient information questionnaire Date:_____

Patient number: □□□

A. General Information		
Surname	Given Name	Middle Initial
Street Address		
City	Province	Postal Code
Home Telephone	Work Telephone	
Date of Birth (day/month/year)	Height	Weight
Date of sample collection:	Blood ____/____/____	Urine ____/____/____

B. BACKGROUND INFORMATION

1. Primary occupation (or previous occupation if retired):_____

2. Education level:
   ☐ Less than high school ☐ High school graduate ☐ Greater than high school

3. Ethnic background:_____

C. ENDOCRINE HISTORY

1. Menstrual status: . . . . . ☐ Premenopausal ☐ Postmenopausal

2. Age of menopause: . . . . . _____

3. Use of hormone replacement therapy within last six months?. . . . . ☐ Yes ☐ No
   If yes, which type:_____ How many years:_____

4. Menstrual cycle history:. . . . . ☐ Regular ☐ Irregular
Cycle length (days):_____ Length of flow (days):_____

5. Age of first period: . . . . . _____

6. Painful periods? (during premenopausal years) . . . . . ☐ Yes ☐ No
☐ Mild ☐ Moderate ☐ Severe

7. History of amenorrhea? (Scant menstrual bleeding) . . . . . ☐ Yes ☐ No
Age:_____ No. of months:_____(not including pregnancy)

8. History of menorrhagia? (Excessive menstrual bleeding) . . . . . ☐ Yes ☐ No
Age:_____ No. of months:_____

9. Birth information: . . . . . ☐ Single ☐ Twin Weight:_____lbs

10. Intrauterine exposure to hormones? . . . . . ☐ Yes ☐ No
☐ Diethylstibestrol (DES) ☐ Oral contraceptives ☐ Progesterone ☐ Other

11. Oral contraceptive use? . . . . . ☐ Yes ☐ No
Number of years:_____ Type:_____

12. Use of fertility drugs? . . . . . ☐ Yes ☐ No Type:_____ No. of times used:_____

13. Previous pregnancy? . . . . . ☐ Yes ☐ No
Age at *first* full term pregnancy:_____

14. Number of *full* term pregnancies: . . . . . _____

15. Breastfeeding first child? . . . . . ☐ Yes ☐ No
No. of months:_____

16. History of fibrocystic breast disease? . . . . . ☐ Yes ☐ No
During premenopausal years: ☐ Mild ☐ Moderate ☐ Severe
During postmenopausal years: ☐ Mild ☐ Moderate ☐ Severe

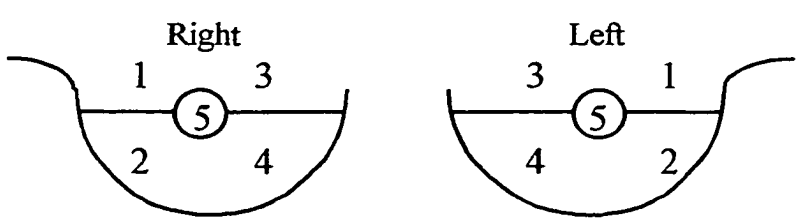
17. Breast pain? . . . . . ☐ Yes ☐ No
During premenopausal years: ☐ Mild ☐ Moderate ☐ Severe
☐ Chronic ☐ Premenstrual ☐ Other
During postmenopausal years: ☐ Mild ☐ Moderate ☐ Severe

D. FAMILY HISTORY

1. First degree blood relative with breast cancer? . . . . . ☐ Yes ☐ No
   Who?_____
2. Family history of blood relatives with other cancers? . . . . . ☐ Yes ☐ No
   Who?_____
3. Positive genetic testing in family member? . . . . . ☐ Yes ☐ No
   ☐ *BRCA1* ☐ *BRCA2* ☐ *ATM* ☐ Other

E. BREAST CANCER

1. Discovered by: ☐ Self ☐ Physician ☐ Screening mammography ☐ Ultrasound
2. Any significant physical *injury* to site of tumour prior to *diagnosis*? . ☐ Yes ☐ No
   ☐ Less than 1 month ☐ 1-3 months ☐ 3-6 months ☐ Greater than 6 months
   Explain: _____
   _____
   _____
   _____
3. Position of tumour: . . . . . Side:_____ Quadrant:_____



4. Date of surgery: . . . . . (day/month/year) ____/____/____
5. Size of tumour: . . . . . _____
6. Axillary nodes: . . . . . ☐ Positive ☐ Negative

7. Sentinel node: . . . . . ☐ Positive ☐ Negative

8. Clinical stage: . . . . . _____

9. Clinical grade: . . . . . _____

10. Histologic type: . . . . . _____

11. Estrogen receptor: . . . . . ☐ Positive ☐ Negative

F. MEDICAL HISTORY

1. History of previous breast cancer? . . . . . ☐ Yes ☐ No

2. Positive genetic testing? . . . . . ☐ Yes ☐ No
   ☐ *BRCA1* ☐ *BRCA2* ☐ *ATM* ☐ Other

3. Have you ever attended *screening* mammography? . . . . . ☐ Yes ☐ No

4. History of other cancers? . . . . . ☐ Yes ☐ No
   If yes, please list type and age at diagnosis:_____
   _____

5. Current medications: _____

6. Preventative therapies:_____

7. Stress in life (last 10 years), scale: 1 (low stress) to 10 (high stress) . . . . . _____

8. Severe episodes of physical/metabolic/emotional stress in last two years? ☐ Yes ☐ No

History of other major illness or disease:

9. Anemia . . . . . ☐ Yes ☐ No
   During which time period? ☐ Premenopausal years ☐ Postmenopausal years
   Type (*i.e.* Pernicious/B_{12}, iron deficiency, etc): _____

10. Autoimmune disease . . . . . ☐ Yes ☐ No
    If yes, please list type:_____

11. Cardiovascular disease . . . . . ☐ Yes ☐ No
    Explain:_____

12. Severe depression? . . . . . ☐ Yes ☐ No

13. Diabetes? . . . . . ☐ Yes ☐ No
Type:_____ Age of onset:_____

14. Hysterectomy? . . . . . ☐ Yes ☐ No
Age:_____

15. History of bowel disorders? . . . . . ☐ Yes ☐ No
(*i.e.* inflammatory/irritable bowel, Crohn's disease, ulcerative colitis)

16. History of thyroid disease? . . . . . ☐ Yes ☐ No
(*i.e.* thyroid enlargement, cysts, hypo/Hashimoto's thyroiditis, hyper/Graves' disease)
Are you presently taking thyroid medication? . . . . . ☐ Yes ☐ No
If yes, numbers of years:_____ Type:_____ Amount/day:_____
Original diagnosis and treatment:_____
_____

17. Ovariectomy . . . . . ☐ Yes ☐ No

18. Ovarian cysts or polycystic ovarian syndrome . . . . . ☐ Yes ☐ No

19. Stomach/peptic or duodenal ulcers . . . . . ☐ Yes ☐ No

20. Other:_____
_____

G. Diet and Lifestyle

Fruits and Vegetables

1. Servings of fruit per day: . . . . ☐ Less than one ☐ 1-2 ☐ 3-5 ☐ 6 or more

2. Servings of vegetables per day: . . ☐ Less than one ☐ 1-2 ☐ 3-5 ☐ 6 or more

Grains and Cereals

3. Do you eat mostly: . . . . . ☐ Whole grains (*i.e.* whole wheat bread, brown rice, etc)
☐ Enriched grains (*i.e.* white bread or rice, pastries, etc)

Meat and Meat Products

4. Do you eat meat? . . . . . ☐ Yes ☐ No
If yes, times per week: ☐ Less than once ☐ 1-2 ☐ 3-5 ☐ 6 or more

5. Do you eat processed meats (*i.e.* sausages, luncheon meats, etc)? . . . . ☐ Yes ☐ No
If yes, times per week: ☐ Less than once ☐ 1-2 ☐ 3-5 ☐ 6 or more

6. Do you eat seafood? . . . . . ☐ Yes ☐ No
If yes, times per week: ☐ Less than once ☐ 1-2 ☐ 3-5 ☐ 6 or more

Milk and Dairy Products

7. Do you consume milk or dairy products? . . . . . ☐ Yes ☐ No
If yes, times per day: ☐ Less than once ☐ 1-2 ☐ 3-5 ☐ 6 or more

Salt Intake

8. Do you use table salt (either in cooking or at the table): . . . . . ☐ Yes ☐ No
☐ Very few meals ☐ Only some meals ☐ With most meals ☐ With every meal

Vitamin, Mineral or Herbal Supplements

9. Do you take any of the following supplements on a *regular* basis? . . . . ☐ Yes ☐ No
☐ Multivitamin/mineral supplement
☐ Other vitamins or minerals (please list with amount/day):_____
_____
☐ Herbal supplements (please list):_____

Other Lifestyle Factors

10. Do you use iodine-containing products? (*i.e.* Lugol's sol., kelp, seaweed, algae, Betadine® products, Flor-Essence®) ☐ Rarely or never ☐ Sometimes ☐ Most of the time
Type:_____

11. Do you use zinc or selenium containing (*i.e.* anti-dandruff) shampoos? . ☐ Yes ☐ No
(*i.e.* Head & Shoulders®, Selsun Blue®, etc) Type:_____

12. Have you used cough syrup or an expectorant in the last month? . . . ☐ Yes ☐ No

13. Do you consume organic foods? ☐ Rarely or never ☐ Sometimes ☐ Most of the time

14. Drinking water, which do you use most? ☐ Filtered ☐ Bottled ☐ Unfiltered tap water

15. Do you consume cola products? ☐ Rarely or never ☐ Sometimes ☐ Often

16. Coffee/tea? (caffeinated only) . . . . . ☐ Yes ☐ No
Cups/day:____

17. Alcohol? . . . . . ☐ Yes ☐ No
Quantity:_____ Type:_____

18. Smoking? . . . . . ☐ Yes ☐ No
Quantity:_____ Quit, number of years?_____

19. Exercise (hours per week):. . . . . ☐ Less than one ☐ 2-3 ☐ 4 or more
Type: _____

Has your diet or lifestyle changed significantly

20. Over the last six years? . . . . . ☐ Yes ☐ No

21. Over the last three years? . . . . . ☐ Yes ☐ No

If yes, in what ways (*i.e.* increase/decrease in salt intake, vitamin usage, exercise, etc)

_____

_____

APPENDIX VI

Control subject questionnaire Date:_____

Participant number: □□□

A. General Information		
Surname	Given Name	Middle Initial
Street Address		
City	Province	Postal Code
Home Telephone	Work Telephone	
Date of Birth (day/month/year)	Height	Weight
Date of sample collection:	Blood ____/____/____	Urine ____/____/____

B. Background Information

1. Primary occupation (or previous occupation if retired):_____

2. Education level:
   ☐ Less than high school ☐ High school graduate ☐ Greater than high school

3. Ethnic background:_____

C. ENDOCRINE HISTORY

1. Menstrual status: . . . . . ☐ Premenopausal ☐ Postmenopausal

2. Age of menopause: . . . . . _____

3. Use of hormone replacement therapy within last six months? . . . . . ☐ Yes ☐ No
   If yes, which type:_____ How many years:_____

4. Menstrual cycle history:. . . . . ☐ Regular ☐ Irregular
Cycle length (days):_____ Length of flow (days):_____

5. Age of first period: . . . . . _____

6. Painful periods? (during premenopausal years) . . . . . ☐ Yes ☐ No
☐ Mild ☐ Moderate ☐ Severe

7. History of amenorrhea? (scant menstrual bleeding) . . . . . ☐ Yes ☐ No
Age:_____ No. of months:_____(not including pregnancy)

8. History of menorrhagia? (excessive menstrual bleeding) . . . . . ☐ Yes ☐ No
Age:_____ No. of months:_____

9. Birth information:. . . . . ☐ Single ☐ Twin Weight:_____lbs

10. Intrauterine exposure to hormones? . . . . . ☐ Yes ☐ No
☐ Diethylstibestrol (DES) ☐ Oral contraceptives ☐ Progesterone ☐ Other

11. Oral contraceptive use? . . . . . ☐ Yes ☐ No
Number of years:_____ Type:_____

12. Use of fertility drugs? . . . . . ☐ Yes ☐ No
Type:_____ No. of times used:_____

13. Previous pregnancy? . . . . . ☐ Yes ☐ No
Age at *first* full term pregnancy:_____

14. Number of *full* term pregnancies: . . . . . _____

15. Breastfeeding first child? . . . . . ☐ Yes ☐ No
No. of months:_____

16. History of fibrocystic breast disease? . . . . . ☐ Yes ☐ No
During premenopausal years: ☐ Mild ☐ Moderate ☐ Severe
During postmenopausal years: ☐ Mild ☐ Moderate ☐ Severe

17. Breast pain? . . . . . ☐ Yes ☐ No
During premenopausal years: ☐ Mild ☐ Moderate ☐ Severe
☐ Chronic ☐ Premenstrual ☐ Other
During postmenopausal years: ☐ Mild ☐ Moderate ☐ Severe

D. FAMILY HISTORY

1. First degree blood relative with breast cancer? . . . . . ☐ Yes ☐ No Who?

_____

2. Family history of blood relatives with other cancers? . . . . . ☐ Yes ☐ No Who?

_____

3. Positive genetic testing in family member? . . . . . ☐ Yes ☐ No
☐ *BRCA1* ☐ *BRCA2* ☐ *ATM* ☐ Other

E. Medical History

1. Any history of significant physical *injury* to the breast? . . . . . ☐ Yes ☐ No
☐ Less than 1 month ☐ 1-3 months ☐ 3-6 months ☐ Greater than 6 months
Explain: _____
_____
_____
_____

2. History of previous breast cancer? . . . . . ☐ Yes ☐ No

3. Positive genetic testing? . . . . . ☐ Yes ☐ No
☐ *BRCA1* ☐ *BRCA2* ☐ *ATM* ☐ Other

4. History of other cancers? . . . . . ☐ Yes ☐ No
If yes, please list type and age at diagnosis: _____
_____

5. Current medications: _____

6. Preventative therapies: _____

7. Stress in life (last 10 years), scale: 1 (low stress) to 10 (high stress) . . . . . _____

8. Severe episodes of physical/metabolic/emotional stress in last two years? ☐ Yes ☐ No

<u>History of other major illness or disease:</u>

9. Anemia . . . . . ☐ Yes ☐ No
During which time period? ☐ Premenopausal years ☐ Postmenopausal years
Type (*i.e.* Pernicious/B_{12}, iron deficiency, etc): _____

10. Autoimmune disease . . . . . ☐ Yes ☐ No
If yes, please list type:_____

11. Cardiovascular disease . . . . . ☐ Yes ☐ No
Explain:_____

12. Severe depression . . . . . ☐ Yes ☐ No

13. Diabetes . . . . . ☐ Yes ☐ No
Type:_____ Age of onset:_____

14. Hysterectomy . . . . . ☐ Yes ☐ No
Age:_____

15. History of bowel disorders? . . . . . ☐ Yes ☐ No
(*i.e.* inflammatory/irritable bowel, Crohn's disease, ulcerative colitis)

16. History of thyroid disease? . . . . . ☐ Yes ☐ No
(*i.e.* thyroid enlargement, cysts, hypo/Hashimoto's thyroiditis, hyper/Graves' disease)
Are you presently taking thyroid medication? . . . . . ☐ Yes ☐ No
If yes, numbers of years:_____ Type:_____ Amount/day:_____
Original diagnosis and treatment:_____
. . . . _____

17. Ovariectomy . . . . . ☐ Yes ☐ No

18. Ovarian cysts or polycystic ovarian syndrome . . . . . ☐ Yes ☐ No

19. Stomach/peptic or duodenal ulcers . . . . . ☐ Yes ☐ No

20. Other:_____
_____

F. DIET AND LIFESTYLE

Fruits and Vegetables

1. Servings of fruit per day: . . . . . ☐ Less than one ☐ 1-2 ☐ 3-5 ☐ 6 or more

2. Servings of vegetables per day: . . . ☐ Less than one ☐ 1-2 ☐ 3-5 ☐ 6 or more

Grains and Cereals

3. Do you eat mostly: . . . . . ☐ Whole grains (*i.e.* whole wheat bread, brown rice, etc)
☐ Enriched grains (*i.e.* white bread or rice, pastries, etc)

Meat and Meat Products

4. Do you eat meat? . . . . . ☐ Yes ☐ No
If yes, times per week: ☐ Less than once ☐ 1-2 ☐ 3-5 ☐ 6 or more

5. Do you eat processed meats (*i.e.* sausages, luncheon meats, etc)? . . ☐ Yes ☐ No
If yes, times per week: ☐ Less than once ☐ 1-2 ☐ 3-5 ☐ 6 or more

6. Do you eat seafood? . . . . . ☐ Yes ☐ No
If yes, times per week: ☐ Less than once ☐ 1-2 ☐ 3-5 ☐ 6 or more

Milk and Dairy Products

7. Do you consume milk or dairy products? . . . . . ☐ Yes ☐ No
If yes, times per day: ☐ Less than once ☐ 1-2 ☐ 3-5 ☐ 6 or more

Salt Intake

8. Do you use table salt (either in cooking or at the table): . . . . . ☐ Yes ☐ No
☐ Very few meals ☐ Only some meals ☐ With most meals ☐ With every meal

Vitamin, Mineral or Herbal Supplements

9. Do you take any of the following supplements on a *regular* basis? . . . ☐ Yes ☐ No
☐ Multivitamin/mineral supplement
☐ Other vitamins or minerals (please list with amount/day):_____
_____
☐ Herbal supplements (please list):_____

Other Lifestyle Factors

10. Do you use iodine-containing products? (*i.e.* Lugol's sol., kelp, seaweed, algae, Betadine® products, Flor-Essence®) ☐ Rarely or never ☐ Sometimes ☐ Most of the time
Type:_____

11. Do you use zinc or selenium containing (*i.e.* anti-dandruff) shampoos? . ☐ Yes ☐ No
(*i.e.* Head & Shoulders®, Selsun Blue®, etc) Type:_____

12. Have you used cough syrup or an expectorant in the last month? . . ☐ Yes ☐ No

13. Do you consume organic foods? ☐ Rarely or never ☐ Sometimes ☐ Most of the time

14. Drinking water, which do you use most? ☐ Filtered ☐ Bottled ☐ Unfiltered tap water

15. Do you consume cola products? ☐ Rarely or never ☐ Sometimes ☐ Often

16. Coffee/tea? (caffeinated only) . . . . . ☐ Yes ☐ No
Cups/day:____

17. Alcohol? . . . . . ☐ Yes ☐ No
Quantity:_____ Type:_____

18. Smoking? . . . . . ☐ Yes ☐ No
Quantity:_____ Quit, number of years?_____

19. Exercise (hours per week):. . . . . ☐ Less than one ☐ 2-3 ☐ 4 or more
Type: _____

<u>Has your diet or lifestyle changed significantly</u>

20. Over the last six years? . . . . . ☐ Yes ☐ No

21. Over the last three years? . . . . . ☐ Yes ☐ No

If yes, in what ways (*i.e.* increase/decrease in salt intake, vitamin usage, exercise, etc)

_____

_____

Capital Health Region
Building Partnerships for Better Health

Telephone: (250) 370-8801

RESEARCH REVIEW AND ETHICAL APPROVAL COMMITTEE

APPROVAL FORM

Reference Number #99-44

Proposal Title: A Case Control Study of Iodine, Selenium and Sex Hormone Levels in Patients with Newly Diagnosed Breast Cancer

Title of Researcher(s): Dr. J.P. van Netten

Date of Review: June 14, 1999

Research protocol dated May 1999, and informed consent, have been approved (until study completion).

Date: August 9, 1999

Joe Murphy, B.Sc.Pharm., M.B.A.
Co-Chair, Research Review and Ethical Approval Committee

Position: Regional Director, Clinical Support Services

NOTE:
Any significant changes in the proposal should be reported to the Chairperson for Research Review and Ethical Approval Committee's consideration, in advance of implementation of such changes.
The Research Review and Ethical Approval Committee is organized and operates according to the applicable laws and regulations, as required by section 5.11.1 of the Therapeutic Products Programme/ICH Good Clinical Practice: Consolidated Guideline, dated 19 September 1997.

University of Victoria
Human Research Ethics Committee

CERTIFICATE OF APPROVAL

Principal Investigators	Department/School	Supervisor
Dr. J.P. van Netten **Faculty**	**BIOL**	

Co-investigator(s):
Stephen Cann, Student Co-ordinator
Norma Christou, Project Nurse Co-ordinator, Royal Jubilee Hospital

Title: Iodine, Selenium and Hormone Levels in Patients with Breast Cancer

Project No.	Start Date	End Date	Approval Date
272-99	**01 Sep 99**	**31 Aug 00**	**30 Aug 99**

Certification

This is to certify that the University of Victoria Ethics Review Committee on Research and Other Activities Involving Human Subjects has examined the research proposal and concludes that, in all respects, the proposed research meets appropriate standards of ethics as outlined by the University of Victoria Research Regulations Involving Human Subjects.

J. Howard Brunt,
Associate Vice-President, Research

This Certificate of Approval is valid for the above term provided there is no change in the procedures. Extensions/minor amendments may be granted upon receipt of "Request for Continuing Review or Amendment of an Approved Project" form.

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