BIOCHEMICAL CHANGES IN BREAST CANCER

BY

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I. INTRODUCTION

The human body is an intricate structure built with billions of tiny bricks of living units, called cells, that constantly grow, age and get repaired (Meenakshi, 1980). Biologists have long known that the control of cell division is one of the most basic aspects of multicellular existence (Powar, 1983). Throughout embryological development, as well as through all of adult life, many differentiated cells have the choice to divide or not and only if a programmed series of correct decisions are made can the respective organisms continue to function normally. Interwined with any study of cell proliferation is the nature of cancer. This collection of horrific diseases by definition involves cells that divide when they should not, usually to produce the contiguous cellular masses called tumours (Watson et al., 1987).

A cancer cell results from a permanent genetic change in an otherwise normal somatic cell. Such a change, called the malignant transformation, may be triggered by some external physical agent, such as X-rays or excess ultraviolet irradiation from sunlight or by various carcinogenic chemical agents (Lehninger, 1978).

Cancer cells can arise in any body tissue and at any age. But the pattern of incidence and death rates of malignant diseases vary with sex, age, race and geographic location (Rubin, 1983). Cancer is not a single disease but a complex of many diseases (Powar, 1983).

Cancer, the second major cause of death in the west, the first being heart attack is the seventh in order of disease that causes death in India (AMPI Bulletin, 1982).

In the past, cancer of all sites combined occurred more frequently among females; now males have a higher rate than females of the same race (Devesa et al, 1978). Breast cancer in females and prostate cancer in males are the most common malignancies (Allen et al., 1986).

Breast cancer is the most common neoplasm in occidental women (Ganz et al., 1987). Because it affects a sexually important part of the body, its treatment may be physically and emotionally disfiguring (Keys et al., 1983).

The disease is not new. It has been cited even several 1000 years ago, in the Egyptian medical records in papyrus as mammary swellings or lumps (Farrow, 1971). Despite advances in diagnosis and treatment in the recent years, the death rate from breast cancer has not changed from fifty years ago (Ashikari et al., 1986).

In India, we find that the relative frequency of breast cancer differs region wise, religion wise and language wise. Thus, it is more common in Bombay, than in Madras or in Trivandrum. It is more common among Christians and Muslims than among Hindus and is more common in Gujarati, Urdu and English speaking women (NCR, 1983).

The striking differences in breast cancer rates and the large increase in the breast cancer rates among migrant populations have led to a wide spread belief that diet is an important determinant of this disease (Katsouyanni et al., 1988). The increase is more among populations migrating from low to high incidence areas (Buell, 1973).

Carcinogenesis occurs in several stages including carcinogen metabolism, initiation and promotion, cell differentiation and tumour cell progression, tumour growth and development and host defences. Each step can be affected by nutritional modification (Poirier, 1987). Thus coffee and caffeine intake were associated with breast cancer differentiation (Pozner et al., 1986). Alcohol consumption increased the risk of breast cancer (Graham, 1987) and smoking increased the incidence of breast cancer especially in pre-menopausal women (Brownson et al., 1988). Cancer itself affects the nutritional status of the individual (Thiele, 1980).

Breast cancer is positively correlated with total fat, animal protein and energy from animal foods (Hems, 1978). Fibre, trace elements, vitamins, food additives and certain foods also play an important role in carcinogenesis of breast (Vobecky et al., 1979).

The current interest in Vitamin A, is its association with the process of carcinogenesis. Vitamin A has a protective role against cancer (Bjelke, 1975).

In Vitamin A deficiency, there is a transition of the normal tissues to neoplastic tissues (Watson et al., 1987).

Vitamin C is primarily involved in collagen formation and also in the development of immunocompetence (Williams and Caliendo, 1984). Vitamin C reacts with tumour inducing compounds and thereby prevents cancer (Glatthaar et al., 1986).

The relationship of nutritional factors to resistance and susceptibility to infectious process has long been a matter of concern (Axelrod, 1971).

The development of breast cancer is probably related to female hormones since it occurs naturally most often in females than in males and can be preveented by ovarian castration at an early age (Mac Mohan et al., 1973).

In patients with secondary deposits in bone appreciable increase in Alkaline phosphatase activity may occur. A small increase is seen in breast cancer (Varley et al., 1980).

Despite the difficulties and disappointments of cancer research, each new bit of information is immeasurable help in the solution of man's most difficult disease problem. Much information is not available regarding the levels of vitamins, immunoglobulins and hormones in the breast cancer patients in Coimbatore district where there is markedly a high incidence of the disease.

Hence a study on 'Biochemical changes in Breast cancer' was undertaken to find out the vitamin status, hormonal status

and immunoglobulin pattern in premenopausal and postmenopausal breast cancer patients attending the Valavadi Narayanaswamy Cancer Centre, Coimbatore. Age matched healthy females were used as control subjects.

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INTRODUCTION

Not withstanding the artificial boundaries that man creates between him and his neighbours there are certain afflictions which threaten mankind as a whole. Cancer is one such danger confronting all of us (Tomatis, 1984). Cancer more frequently attacks middle-aged and older people than it does young people. Today the average span of life is increasing, hence more people reach the ages when cancer takes its greatest toll. Cancer as a cause of death for both sexes is exceeded only by heart disease (American Cancer Society, 1975).

A. CANCER IN GENERAL

1. HISTORY OF CANCER

Cancer was known in antiquity, being described in early writings of Greek and Romans. Tumours in Egyptian mummies dating back 5000 years represent the first known human malignant growths, although there is pathologic evidence of bone tumours occurring in dinosaurs and other prehistoric animals (Laszlo, 1985). Today, however, cancers are the cause of approximately 16% of all deaths in the United States and more in many European countries. This great increase, especially in the past few decades, is the result of improved detection procedures, environmental causes, and the increase in the human life span (Wistreich and Lechtman, 1984).

2. INCIDENCE OF CANCER

The incidence or frequency of cancer rises sharply from ages 35 to 75, the highest rate being between ages 50 and 75.

WORLD-WIDE DISTRIBUTION OF DIFFERENT TYPES OF CANCER

- URINARY TRACT CANCER
- BURKITT'S LYMPHOMA
- LUNG CANCER
- STOMACH CANCER
- MOUTH CANCER
- BREAST & INTESTINAL CANCER
- CANCER OF OESOPHAGUS
- MELANOMA

(TIME LIFE BOOKS, 1981)

Between the ages of 30 and 55, more females than males die of cancer, but both before and after this period cancer death in males exceeds those in females. One sees therefore that cancer is most likely to appear during the middle years, man's most active and most productive period, when his earning capacity should be greatest (Davis, 1954).

Cancer of the lungs, breast and colon are most common in western countries. In Asia and Africa liver cancer is more common (Watson et al., 1987). In Japan, stomach cancer predominates but colon and breast cancer are relatively rare (Schein, 1985). In the People's Republic of China, nasopharyngeal cancer, oesophageal cancer and hepatocellular carcinoma are most common (Miller, 1978).

Cancer is regarded as an emerging health problem in India. A steady trend of increasing mortality is being observed over the past few years. Jussawala (1973) observed that cancer is one of the 10 leading causes of death today in India and is advancing year by year. The incidence of cancer is about 100 per 1,00,000 population in India (Patel, 1979) as against 300 per 1,00,000 population in United States of America (National cancer institute, 1974).

Recent survey shows that the incidence of cancer is 86 per 100,000 a year in India. An estimated 3 lakhs persons die annually on account of cancer. In India, cancer of cervix among women is highest and is attributed to early marriage, frequent delivery and inadequate medical care besides unhygienic conditions. India records a national incidence of 67.9% of cancer

of cervix among females against the world incidence of 47.7% (AMPI Bulletin, 1982).

In India cancer is more frequent in urban areas than in rural areas. Cancer of the base of the tongue is most common in Gujarat. Cancer of the buccal mucosa and cancer of the lips are common in South India and Bihar respectively. Palate cancer is common in Coastal Andhra; oesophagus cancer is common in Gujarat. Stomach cancer and probably cervix cancer are most common in southern parts of the country while anal cancer is most common in the Northern States (Mehta, 1980).

3. HYPOTHESIS ABOUT CANCER

Several hypotheses explain why a cell becomes cancerous. The principle among them are somatic mutation hypothesis, the viral gene hypothesis and the defective immunity hypothesis.

According to the somatic mutation hypothesis, cancer is the result of somatic mutations (ie mutations not involving germ cells), without viral infection occuring in a cell. Such a mutation may alter the control mechanism of a cell, leading to unregulated division or cancer.

The viral genes hypothesis suggests that cancer is caused by the tumour producing viruses or the oncoviruses. There is however no clear cut evidence of malignant tumours being caused in humans by virus.

According to the defective immunity hypothesis the defence mechanism fails under certain conditions and tumours are formed.

This may be because the number of immune lymphocytes may not be large enough to block tumour development. The insufficient production of these lymphocytes may be due to mutation in the lymph cells, poor nutrition, emotional stress or other factors (Powar, 1983).

4. CLASSIFICATION OF NEOPLASIA

Neoplasia have been classfied by many methods in an attempt to understand its nature. The best available classification and that currently used is based upon:

Histogenesis : tissue of origin and cell type

Biologic behaviour : benign or malignant

Anatomic site

Degree of differentiation

Histogenesis

Tumours can arise from virtually all types of normal tissues. Usually they retain enough of the features of the normal cells and tissue pattern so that their origin can be determined. Using this concept benign tumours are usually named by adding the suffix - oma to the name of cell or tissue eg., neuroma, osteoma. Malignant tumours are divided into those of mesenchymal origin - sarcomas and those of endothelial origin - carcinomas.

Biologic Behaviour

Consideration of biologic behaviour results in the division of tumours into benign and malignant groups. The former are generally innocuous growths that do little harm to the host

while the latter are aggresive neoplasms which if untreated generally result in metastases and death.

In addition to these categories of tumours there remains another group, the so called preinvasive or " in situ " malignancies. These terms are generally limited to lesions of epithelial origin. These lesions have most of the cytomorphologic features of malignancy but lack one cardinal feature, invasiveness. They represent a stage in the development of cancer in which the tumour is confined to its epithelium.

Anatomic site of origin

The classification of neoplasms have never been a static list. Processes formerly thought to be malignant are now known to be benign; lesions that were formerly believed to be innocuous are now known to be malignant. Tumours once thought to be of one cell type of origin were later shown to originate from a totally different type of cell. It has resulted in the use in some cases of several different names for the same neoplasm. So this type of classification is a bit confusing (Florey, 1970).

Degree of differentiation.

This aspect of classification is of major importance in prognosis. In addition to the basic classification, malignant lesions are also divided according to grade and stage.

Grade is an evaluation of the degree of differentiation of the tumours and usually therefore of the degree of malignancy.

The stage of the tumour is simply an evaluation of the extent of the tumour at the time of diagnosis and is not necessarily related to its grade. Several different staging systems are used depending on tumour sites (AJC, 1979).

5. FORMS OF HUMAN CANCER

It is not yet clear whether the condition referred to as cancer consists of several diseases having a common pattern of general symptoms or whether it is a single disease that occurs in many forms depending upon the tissue from which it evolves. In any case more than 100 clinically distinct types of cancer are recognized. However the four major types are carcinomas, leukemias, lymphomas and sarcomas.

Carcinomas are solid tumours derived from epithelial tissues such as breasts, glands, skin, nerves and the linings of gastro intestinal, urinary and genital systems.

Leukemia also called cancer of the blood is characterised by the uncontrolled proliferation and accumulation of leukocytes most of which do not mature into functional cells.

In lymphomas abnormal number of lymphocytes are produced by the spleen andlymphnodes.

Sarcomas are characterised by tumours growing from bone, cartilage, connective tissue, fat and muscle (Wistreich and Lechtman, 1984).

6. PROPERTIES OF CANCER CELLS

Transformation is the process by which normal cells acquire

many of the morphological as well as growth properties of cancer cells. The characteristic properties of such cancer cells are:

(i) Immortalisation

Normal cell cultures do not survive indefinitely while transformed cell cultures are immortal and can grow indefinitely.

(ii) Loss of contact inhibition

Normal cells in a culture stop growing when their plasma membranes come into contact with one another. This inhibition of growth after contact is called contact inhibition. Transformed cells on the otherhand lack proper recognition and are unable to go into a quiescent stage and grow continuously until they kill themselves (Abrecrombie and Heaysman, 1954).

(iii) Reduced cellular adhesion

The stocky quality of normal cells that leads to adhesion displays considerable specificity. A given type of cell prefers to stick to others of its own kind and shows very little, if any, affinity for other types. But in cancer cells their normal cellular affinities no longer hold. The inability of cancer cells to form light adhesive junctions allows them to be adjacent to almost any cell type. This may be one reason why malignant cells invade a variety of normal organs.

(iv) Invasiveness

One of the most important characteristics of transformed

cells is their invasiveness, ie., the ability to invade other tissues which could be the result of changes in the plasma membrane and/or proteases released by the cells.

(v) Loss of anchorage dependence

Most normal cells must be attached to a rigid substratum (ie., they must be anchored) in order to grow. But transformed cells can grow even when they are not attached to the substratum. This characteristic feature is used to select transformed cells from a normal cell population (Powar, 1983).

(vi) Decreased serum requirements of cancer cells and secretion of growth factors.

Many cancer cells can grow in culture medium supplemented with much less serum than that required by the corresponding normal cells. Many years ago, it was found that some cells that require little or no serum to grow produce their own growth factors. This finding and the fact that cancer cells require low concentration of serum led to the notion that cancer may arise when cells begin to uncontrollably produce growth factors for which they also express the corresponding receptors (Sporn and Robert, 1985).

(vii) Selective agglutination by lectins

Lectins can bind to receptors on the cell membrane and cause agglutination of cells. In normal cells the receptors for lectins lie in a diffuse manner on the cell surface and are immobile and therefore agglutination is impossible; in

transformed cells, the receptors are mobile within the membrane resulting in local region of high binding site concentration and therfore cause agglutination (Powar, 1983).

(Viii) Molecular changes in cell membrance components

The cell membrane consits of four main types of phospholipids which forms the lipid bilayer with glycolipids and glycoproteins inserted into this bilayer. Cancerous transformation does not change the relative amount of the four main phospholipids. Differences however and sometimes seemingly quiet specific ones have been seen in both the nature and kind of the glycolipids and glycoproteins. The gangliosides are diminished in amount in all cancers and even the enzymes for their biosynthesis are reduced. Changes in glycoproteins are also seen, the major change being the slow disappearance of LETS or large, external transforming sensitive protein (Hakomori, 1986).

(ix) Alterations of the cytoskeleton of transformed cells

Normal fibroblasts contain large number of fibres, cables in which the various cytoskeletal proteins (eg., actin myosin and tropomyosin) are regularly arranged. In contrast transformed cells contain fewer such fibres . . Correspondingly the touching of the cancer cells does not lead to cessation of cell movement but instead frequently induces the uncoordinated generation and retraction of blebs, microvilli and ruffles from the cell surface. So tumour cells have a more

ruffled surface than normal cells with many more surface processes (Pollack et al., 1975).

(x) Increase in negative surface charge of cell membrane

Comparisons of surface membrane charges by microelectrophoresis in normal malignant cells showed that the anodic mobility in the latter was higher indicating an increase in negative surface charge.

(xi) Increased sugar transport.

Tumour cells consume much more glucose than normal cells because they have to grow and multiply. There is thus a greater increased rate of sugar transport across the surface cell membrane after transformation.

(xii) Appearance of virus-specific transplantation rejection antigen.

Plasma membranes of most transformed cells contain antigens which are not present in normal cells. These tumour antigens can elicit an immune response against themselves in genetically similar hosts. This recognition of antigens by the immune system results in destruction of the newly formed cells and their descendants.

(xiii) Defective electrical communication

The electrical communication normally seen between individual cells is defective in some cancer cells.

(xiv) Increased secretion of proteolytic enzymes.

Large amounts of proteolytic enzymes are secreted by all

types of cancer cells except those of blood forming tissues. The cancer cell secretes a protease called the cell factor which acts on the inert serum protein plasminogen to form plasmin a proteolytic enzyme. It has been suggested that plasmin removes many proteins projecting from the cell surface by enzymatic digestion and signals the cell division.

(xv) Aldolases.

In most mammalian tissue the enzyme aldolase exists in the form of three isozymes A, C and B. The first two predominate in embryonic tissues and the third in adult differentiated tissues. In some tumours especially in poorly differentiated and rapidly growing cancers like certain hepatomas isozyme B is replaced by isozyme A (Powar, 1983).

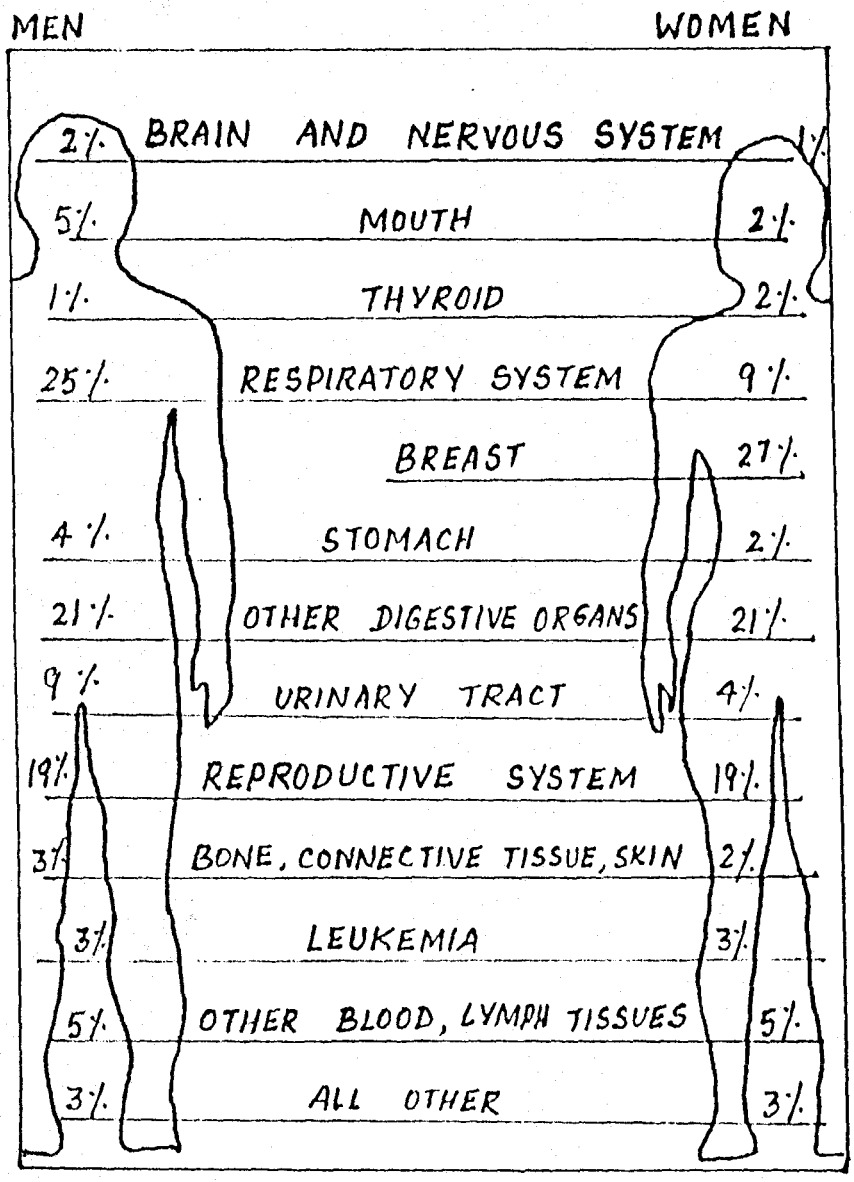
(xvi) Increased glycolysis.

The first convincing biochemical difference between normal cells and cancer cells was discovered over 60 years ago by the German biochemist Otto Warburg. He observed that virtually every type of cancer cell that forms a solid tumor excretes much larger quantities of lactic acid than does its normal counterpart. This lactic acid overproduction(often called the warburg effect) arises from glucose via the glycolytic pathway. This increase in fermentation is because cancer cells consume much more glucose than the normal cells (Watson et al., 1987).

(xvii) Mitosis in cancer cells

The chromosomes in the cancer cells vary in number and

HOW CANCER AFFECTS SEXES



(TIME LIFE BOOKS, 1981)

size. The separation of the chromatids may be delayed, thus resulting in unequal distribution of chromosomes in daughter cells. In some instances the nucleus divides but the cytoplasm does not thereby giving rise to a multinuclear cell. The quantity of DNA in the nucleus of the rapidly dividing cancerous cell is very great but whether this is a cause of the unlimited uncontrolled growth and division or nearly an accompanying characteristic is difficult to decide (Langley, 1961).

7. PREDISPOSING FACTORS OF CANCER

The essential cause of cancer is still unknown. Certain factors, however, are so frequently found associated with the development of malignant growths that they are referred to as predisposing causes (American Cancer Society, 1975).

(i) Sex

Important sex variations exist presumably due to endocrine factors and/or exposure to different external factors.

(ii) Age

Cancer is essentially an affliction of maturity. The prevalence increases with age, although in recent years more cases are recorded in children. Possibly the high maternal age at birth predisposes the individual to cancer (Davis, 1985).

(iii) Heredity

While there is no evidence that cancer is inherited, there is some evidence that susceptibility to cancer may be inherited. Studies showed that inbred laboratory mouse

strains had a high incidence of tumours (Gardner and Snustad, 1981)

(iv) Diet

Over 1000 years we have learned what things are immediately toxic and what things are seemingly safe to eat; only recently we have become aware of the potential danger of repeated exposures to small amounts of harmful substances in the diet (Lowenfels and Anderson, 1977). Nutrients may affect in either of these three ways (1) certain dietary constituents may themselves be carcinogenic in nature (2) whereas others may act as nutritional modifier in carcinogenic process or (3) as a vehicle for transportation of carcinogens (Bansal, 1986). A positive correlation has been shown between fat consumption and deaths from neoplasm of breast, ovaries and rectum.

(v) Hormones

Relation clearly exists between endocrine glands and development and/or progression of cancer. Thus estradiol is the central agent in the process of breast cancer appearance and progesterone does not seem to have an opposing action (Viko and Apter, 1986).

(vi) Race

Variation of cancer type and site with race is possibly due to genetic and/or exposure to varying exogenous factors. Thus the Black population in US have a higher incidence of cancer of prostate, uterine, cervix, lungs, oesophagus and oropharynx, and a lower incidence of breast cancer and cancer of the corpus

uteri than does the white population. This is because of the change in diet and environment (Schein, 1985).

(vii) Socio Economic Class

Studies have shown that colon cancer and endocrine related cancers were positively associated with socio economic class, while no such association was found for rectal cancer. Contrary to data from developed countries, all smoking-related cancers were positively associated with socioeconomic class (Cuello et al., 1982).

(Viii) Area

The excess of cancer cases in urban areas is due to a variety of factors eg., better diagnostic facilities and exposure to more exogenous factors than residents in rural areas.

(ix) Temperature

Lea, (1965), has shown a relationship between environmental temperature and death rate from neoplasm of breast, which increases with rising mean annual temperature.

(x) Infection

Clustering of acute leukemia cases, relation of Reovirus and lymphomas etc., indicate infective factors in some forms of cancer. Immune response is depressed by presence of cancer in the body.

(xi) Occupation

There is a proven association between cancer and certain

occupations due to exposure to specific external irritants in course of work. Thus exposure to asbestos causes lung cancer (ABC'S of the Human Body, 1986). Bladder cancer in males is predominant in areas of high industrialization and around oil-processing areas. Cancer of the nasal sinuses has been found among nickel refinery workers (Ultmann and Golomb, 1985).

(xii) External factors

A wide variety of irritants act as exogenous factors in cancer causation. In majority of cases long exposure is required before malignancy develops. Important irritants include (a) ionizing radiations. According to Burnet every type of ionizing radiation has a measurable mutagenic power, and in addition the effect is cumulative (b) Carcinogenic - hydrocarbons found in soot of town air, exhaust of petrol engines and inefficient diesel engines and in cigarette smoke.

8. DIAGNOSIS OF CANCER.

An early diagnosis of cancer is essential for curing the disease. While the growth is still limited to one area it is usually removed or destroyed. It will spread if unnoticed. Cancer would be easier to treat and cure if cells do not detach themselves from the original site and spread to distant sites. Metastatic cancer usually goes beyond the reach of the surgeon's knife.

A careful history, both personal and family, is taken as the first step in diagnosing possible cancer.

The complete medical examination by a doctor includes all the common sites and symptoms of cancer.

Laboratory tests of the blood and urine may be included in the routine examination.

A chest X-ray for all adults is advised as part of the annual check up. Special examination of other organs by X-rays or special instruments may be necessary.

Positive identification of cancerous tissue can be made only by the microscopic examination of suspected tissue by a pathologist. Surgical removal of suspect tissue is called a biopsy (American Cancer Society, 1975).

9. TREATMENT OF CANCER.

The standard cancer treatments are surgery, radiation and chemotherapy. One recent advance in surgery is the laser beam, which can be sharply focused to avoid damaging normal cells. Another new technique is cryosurgery, which uses extreme cold as a kind of knife. This bloodless surgery reduces the risk of spreading cancer throughthe bloodstream.

One of the paradoxes of cancer is that radiation can not only induce it but also cure it (Cohn, 1979). Technicians administer radiation both by machine and in needle or capsules of radioactive material implanted in the tumour.

Chemotherapy is the use of a combination of chemicals to destroy cancer cells. Among the newer drugs are antimetabolites, which resemble cell nutrients but actually interfere with nutri-

tion in the cells. New metabolites have been developed but they are so powerful that they are not used in treating infections. They interfere with the synthesis of DNA and so prevent reproduction of the tumour cell. In the past, researchers relied largely on trial and error to find cancer drugs. Nowadays computers are used to predict the effectiveness of many drugs in a short time. An exciting new development is "monoclonal antibodies" or human defence chemicals created for a specific tumour and injected into patient. This and other methods appear promising but their effectiveness remains to be proved (ABC'S of the Human body, 1986).

B. BREAST CANCER

Breast cancer is the most common and most dreadful malignancy in women. Not only does it strike in the prime of life, for no reason understood, there is no known method of prevention (Keys et al., 1983).

1. EPIDEMIOLOGY OF BREAST CANCER.

Breast cancer is slowly increasing in incidence and prevalence. It accounts for 18% of cancer deaths in females. Breast cancer is more frequent on the left than the right breast possibly because the left breast is usually larger than the right one.(Petrikis et al., 1982).

Cancer of the breast is most common in the western countries (Watson et al., 1987). Breast cancer accounted for 26% of all new cases of cancer in females in the United States of America, especially among the postmenopausal women. In Japan, the

incidence is lower in case of postmenopausal women, but more common among taller and heavier women (Bondanna and Carbone, 1985). They also have a long-term survival rate (Chabon et al., 1974).

Breast cancer is the second commonest cancer in women in India, after the cancer of cervix. About 20 per 100,000 women suffer from this cancer in India (Sen, 1987).

2. ETIOLOGY OF BREAST CANCER,

There is no known single cause of breast cancer. Genetic and/or hormonal factors play a role in some patients, while viral, dietary and immunologic process are potential contributing mechanisms. The common risk factors are:

(i) Nulliparity

Breast cancer is more common in single and nonparous women (Carter, 1967), especially those above 50 years (Simpson et al., 1988).

(ii) Pregnancy

Pregnancies must continue to term to offer protection against breast cancer (Ewertz and Duffy, 1988). Late first pregnancy also increases a woman's chance of developing breast cancer (James and Lloyd, 1988; Lane and Carpenter, 1987), while an early first pregnancy is known to protect against breast cancer. This effect is mediated by a long-term depression of prolactin secretion after pregnancy (Musey et al., 1987).

(iii) Abortion

A first trimester abortion, induced or spontaneous, before the full term pregnancy might elevate the risk of breast cancer (Hadjimichael et al., 1986).

(iv) Lactation

Kalache et al (1980), studied three main measures of lactation whether the women had breast fed at all, whether she had breast fed for more than 16 weeks and the mean duration of breast feeding. It was found that lactation is not in any way related to the risk of breast cancer.

(v) Family history of breast cancer.

The high risk group includes women with a strong family history of breast cancer (Rosemond, 1971). History of breast cancer increases the risk of cancer in the contra lateral breast (Fletcher and O' Malley, 1986; Anderson, 1974; Langlands et al., 1976).

(vi) Use of oral contraceptives.

The risk of breast cancer is unrelated to the age at first oral contraceptive use (Meirek et al., 1986). Among breast cancer women aged 45 years and more, there was no evidence of an association between oral contraceptive use and breast cancer. Under 45 years there was a significantly elevated risk associated with increasing duration of use before first full term pregnancy (Mc Pherson et al., 1987; Ross et al., 1980).

(vii) Cigarette smoking

Smokers had an earlier natural menopause than non smokers, so smoking may increase the incidence of breast cancer especially in premenopausal women (Brownson et al., 1988).

(viii) Alcohol

Alcohol increases the risk of breast cancer (Wynder and Harris, 1988). It is recommended that women at high risk for breast cancer should curtail their alcohol ingestion (Graham, 1987). The pathway linking alcohol to cancer is complex and is likely to be indirect. One possibility is that alcohol may be linked through alterations in the concentration of LDL cholesterol or that of certain vitamins (D'antonio et al., 1986).

(ix) Menopausal status

In Britain and other high risk countries about a third of patients with breast cancer are premenopausal at diagnosis. In the remainder initiation might have occurred in the premenopause, even though the clinical presentation was late in life. Carcinomas in the postmenopausal females might have resulted from random initiation with respect to age, alternatively they might have been initiated during the reproductive life span. So the hypothesis is that the genesis of breast cancer is in the premenopause (Simpson et al., 1988).

(x) Age

The role of age in breast cancer has been debated inconclusively for a long time. The incidence of breast cancer

rises progressively up to the age of 45 and then gradually slides down in the Asian countries while in the west it continues to rise with age (Pal and Sengupta, 1981). The rate of breast cancer is lower among the younger age groups (Bixby and Va'squez, 1987).

(xi) Radiation

Low dose radiation exposure has been reported to cause carcinogenesis in the postpuberty and prior to menarche (Land, 1984). Although exposure at any age increases the risk of breast cancer, radiation-induced tumours do not tend to occur before the age of 30 years. The minimum latency period is 5-10 years (Evans et al., 1986). The impact of radiation on development of breast cancer is well established for women who have had repeated fluoroscopies (Boice and Monson, 1977) and have been treated with x-rays for post partum mastitis (Shore et al., 1977).

(xii) Coffee and Caffeine intake

Coffee and caffeine intake in breast cancer has a significant association with tumour differentiation as females with moderately to well differentiated tumours have higher coffee and caffeine intake (Pozner et al., 1986).

(xiii) High fat diet.

Diet is an important factor in human breast cancer etiology (Mettlin, 1984). There is a positive correlation between fat consumption and breast cancer. A high fat diet increases

the incidence of breast tumours than a low fat diet (Lane and Carpenter, 1987). Within the USA age-adjusted breast cancer mortality is positively associated with demand for total energy, protein, fat, beef and table fats and negatively associated with egg demand (Gaskill et al., 1979).

(xiv) Race

The influence of ethnic origin on the prognosis of breast cancer has been a matter of some controversy. Data from USA shows that whereas Black women develop breast cancer less frequently than White women, those who do so are less likely to survive than their white counterparts. This difference is due to socioeconomic, cultural and behavioral factors (Dansey et al., 1988).

(xv) Cerumen

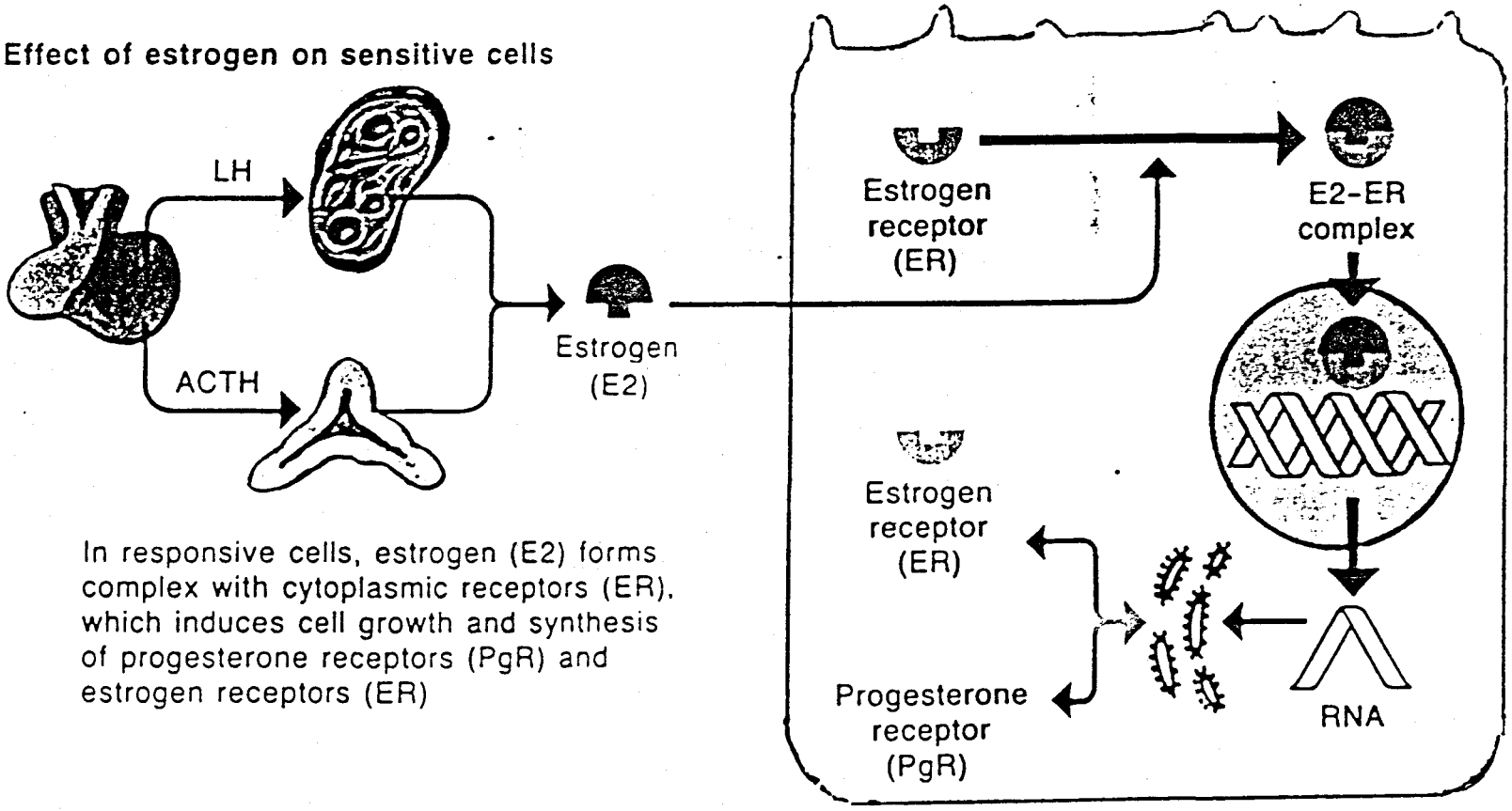
An association between earwax or wet type cerumen and breast cancer rates in diverse population is reported. It is plausible since the mammary and cerumenous glands are histologically of the apocrine type and have many similarities in their secretions. The allele for the wet type cerumen is dominant and predominant in west Europe, Caucasian Americans and Negro Americans. The allele for dry cerumen is recessive and dominant in mangoloid population (Petrakis, 1971).

(xvi) Blood Group

There is a positive correlation between breast cancer and the ABO blood group A (Hems, 1970).

Hormonal Influences in Breast Cancer

Effect of estrogen on sensitive cells



In responsive cells, estrogen (E2) forms complex with cytoplasmic receptors (ER), which induces cell growth and synthesis of progesterone receptors (PgR) and estrogen receptors (ER)

(xvii) Early menarche

Age at menarche is a risk factor for breast cancer, although the effect is relatively weak. Women with an early menarche especially prior to the age of 12 have the highest risk (Lane and Carpenter, 1987). Experimental studies support the hypothesis that a critical body composition of fatness is essential for estrus in the rat, as it appears to be for age at menarche in the human females. Hence the effect of diet and nutrition on breast cancer could be atleast partly through age at menarche (Grey et al., 1979; Kato et al., 1988; Henney and Devita, 1987).

(xviii) Hormones

The development of breast cancer is probably related to female hormones since it occurs naturally most often in females as compared to males and can be prevented by ovarian castration at an early age. (Mac Mohan et al., 1973; Sartwell et al., 1977).

3. DETECTION AND DIAGNOSIS OF BREAST CANCER

CLINICAL DETECTION

(i) Breast cancers are most often discovered as a painless lump or mass by the patient herself or on routine examination. As it enlarges additional signs of advancement include skin dimpling, nipple retraction, bleeding from the nipple, reddening of the skin, ulceration, pain, fixation of the chest wall and appearance of enlarged axillary nodes (Strax, 1980).

(ii) Early detection is vitally important because it improves prognosis. According to Haagensen et al., (1960) 98% of breast cancers are detected by the patient herself.

(iii) Location: 50% of breast cancers are in the upper outer quadrant, 20% are in the central portion of the breast, 20% are in the medial quadrant and 10% are in the lower outer quadrant.

(iv) Localised against regionalized presentations:

In women in general, only 45% of the breast cancers are found before axillary mode metastases have occured (ACS ,1983).

DIAGNOSTIC PROCEDURES

More people who have had a diagnosis of cancer are alive today than ever before (Dietz, 1985).

(i) To be adequate, physical examination must include careful inspection in more than one position.

(ii) Mammography is a radiodiagnostic method for discovering and conforming breast tumours (Gorisek et al., 1987).

(iii) Thermography is sometimes performed in conjunction with mammography but is too nonspecific to be of much value. Both false-negative and false-positive rates are unacceptably high. Ultrasonography has also not been of much value (Seventh Annual Seminar, 1969).

(iv) Percutaneous needle aspiration is widely used in some parts of the world in two clinical situations:cystic lesions and solid lesions (Zajicek et al., 1967).

(v) Excisional biopsy is recommended for smaller masses (Lewisons and Mantague, 1981), and incisional biopsy for larger

lesions. This can be done as an outpatient procedure under local anesthesia. There is no need to do masectomy during the time of biopsy.

(vi) Some authors have suggested contralateral breast biopsy (mirror image biopsy) because of an incidence of up to 10% of occult contralateral breast cancers. Careful breast examination and mammography are probably more effective for detecting the presence or absence of contralateral cancers (Urban, 1967).

4. BIOCHEMICAL CHANGES IN BREAST CANCER

(i) Vitamins

A number of micronutrients have been suggested as possible late-stage inhibitors of human cancer. Retrospective studies of serum vitamin A, measured as retinol , have demonstrated lower levels in patients with cancer than in controls without cancer (Wald et al., 1980). Retinoids act by inhibiting oncogenesis. They reduce the overproduction of mRNA, and act at the promotion stage of cancer (Patorino et al., 1984). A deficiency of ascorbate has been reported with dissolution of the intercellular matrix which might facilitate local infiltration and dissemination of neoplastic cells. Studies in laboratory animals have shown that ascorbate seems to concentrate in malignant tissue and thus deplete systemic reserves (Creagan et al., 1979).

(ii) Hormones

The decrease in breast cancer risk with oophorectomy

under the age 35 is atleast 70%, implying an association with ovarian hormones in two-thirds of the women (Bonadonna and Carbone, 1985). Estrogen can promote the growth of breast cancer, while it can protect against heart disease. The concern over the effect of estrogen has hightened as studies suggested that birth control pills, which contain estrogen might increase a woman's risk of developing breast cancer (Townsend, 1987). Estradiol is the central agent in the process of breast cancer appearance and progesterone does not seem to have an opposing action (Viko and Apter, 1986).

(iii) Immunoglobulins

The relationship of nutritional factors to resistance and susceptibility of infectious processes has long been a matter of concern to nutrition scientists and many efforts have been made to establish a relationship between nutritional factors and the development of antibody production (Axelrod, 1971). Retinoids and carotenoids enhance cellular immune function (Patorino et al., 1984). Ascorbic acid increases host resistance by promoting immune mechanisms (Hodges, 1982).

5. PREVENTION OF CANCER

The specific causes of cancer are still not fully understood. Scientists agree that most cancers are linked to how you live and where. To what you eat, drink, breath and smoke. The positive steps to prevent cancer, suggested by the Indian Cancer Society are:

(i) Eat more vegetables : Studies show that certain kinds of vegetables (green and leafy or deep yellow) can help protect against cancers of colon, rectum, prostate, breast, stomach, cervix and respiratory system.

(ii) Add more high-fibre foods : A diet rich in roughage is a safeguard against cancers of colon and rectum.

(iii) Choose foods with vitamin A : These may help protect against cancers of oesophagus, larynx, mouth, stomach, colon, rectum and cervix.

(iv) Choose foods with vitamin C : These may help ward off cancers of the oesophagus, mouth, colon, rectum, stomach and cervix.

(v) Do not forget weight control : Obesity is linked to cancers of the colon, uterus, gall bladder and breast. Exercise and low calorie intake help in keeping weight down.

Experimental Procedure

III EXPERIMENTAL PROCEDURE

The experimental procedure pertaining to the study "Biochemical changes in breast cancer" is presented in the following sequence:

1. Survey of breast cancer incidence as reported in the Valavadi Narayanaswamy Cancer Centre, Coimbatore.
2. Selection of participants.
3. Collection of Blood.
   (i) Separation of Serum
   (ii) Separation of Plasma
4. Collection of Urine
5. Biochemical analysis in blood.
6. Qualitative analysis in urine
7. Statistical analysis.

1. Survey of incidence of breast cancer as reported in the Valavadi Narayanaswamy Cancer Centre, Coimbatore.

Data regarding the number of cancer patients who have attended the cancer centre from 1986-1988, and the incidence of breast cancer among these patients were collected from the hospital register.

2. Selection of Participants

Thirty one breast cancer patients attending the Valavadi Narayanaswamy Cancer Centre, Coimbatore, for a period of three months were taken for the study. Fifteen age matched healthy

women were taken as controls. The distribution of participants according to age and menopausal status is presented in Table Iα and IIα.

Details regarding the age, marital status, diet, number of children, period of lactation, number of abortions and type of family planning adopted were collected from the participants of the study using the questionnaire, the details of which are given in Appendix I. A 24 hour dietary recall was also done in the participants.

TABLE - Ia

GROUPING OF PARTICIPANTS ACCORDING TO DIFFERENT AGE GROUPS

Participants	Age in years			
	36-45	46-55	56-65	66 and above
Breast Cancer	14	10	3	4
Control	9	6	-	-

TABLE - IIa

GROUPING OF PARTICIPANTS ACCORDING TO MENOPAUSAL STATUS

Participants	Premenopausal period	Postmenopausal period
Breast Cancer	22	9
Control	9	6

3. Collection of blood

The blood was collected as follows (Oser, 1976). Tied a tourniquet (of soft rubber tubing or a strip of bandage) tightly around the arm of the patient, a couple of inches above the elbow. Had the patient clench her fist firmly, washed the skin surface about the prominent vein on the inner surface of the elbow (usually the median basillii) with 70% alcohol, allowed to dry, held the vein immobile by pressing on it with the thumb below the elbow and into the vein inserted a sharp, sterile hypodermic needle (No.20) an inch and a half long which was attached to a dry sterile syringe of suitable capacity. The needle should penetrate the vein from the side and at an angle of 50°C with the surface of the arm, the level of opening of the needle being kept upward or to the side . As soon as the blood was seen to enter the syringe, retracted the plunger slowly until the desired amount of blood has entered the syringe. Before removing the needle from the vein, loosened the tourniquet, had the patient unclench her fist and on the skin, at the point of entrance of the needle held in place a small pad of folded guaze moistened with 70% alcohol, withdrew the needle, detached it from the syringe and then transferred the blood into a suitable container (not too vigorously, which might cause hemolysis). Pressure on the guaze pad will effectively prevent bleeding from the skin puncture. It is important that the pressure be maintained

for a minimum of five minutes, to prevent the formation of a painful hematoma at the site of puncture.

(i) Separation of Serum

The blood was transferred to a centrifuge tube and allowed to clot. The clot was carefully removed and centrifuged after which the supernatant was separated. The separated serum was frozen till used for analyses.

(ii) Separation of Plasma

The blood was transferred to a heparinised centrifuge tube. It was then centrifuged and the supernatant was carefully separated.

4. Collection of Urine

Fresh urine samples were collected from the participants and used immediately for the qualitative analysis using Multistix.

5. Biochemical analysis in blood

Estimation of Vitamin A

Vitamin A was estimated in serum by Carr-Price reaction (Varley et al., 1980). The details of the method are given in Appendix II.

Estimation of Vitamin C

Vitamin C in plasma was estimated titrimetrically using 2,6, dichlorophenol indophenol (Varley et al., 1980). The details of the method are presented in Appendix III.

Estimation of Cholesterol

Cholesterol level in serum was estimated by Zak's method, the procedure of which is given in Appendix IV

Estimation of Alkaline phosphatase

The Alkaline phosphatase activity of serum was determined by the method of King and Armstrong (Varley et al., 1980). The details are presented in Apprendix V.

Estimation of Immunoglobulins IgG, IgA, and IgM.

The serum Immunoglobulins, IgG, IgA and IgM were estimated using immunodiffusion plates (Behringwerke, 1988), the details of which are given in Apprendices VI and VII.

Estimation of Serum Hormones

The serum hormones, Follicle stimulating hormones, Luteinizing hormone, prolactin and estradiol were estimated using Radio immuno assay Kits (Diagnostic Products Corporation, 1988 and Isopharm Radio pharmaceutical Division, 1988).

RADIO-IMMUNOASSAY OF FOLLICLE STIMULATING HORMONE (FSH)
(Diagnositic Products Corporation, 1988)

PRINCIPLE:

Diagnostic products corporations FSH procedure is based on a highly specific anti-FSH antibody and 125I tracer. Sample and antibody are pre-incubated. Then radiolabeled FSH competes with FSH in the sample for antibody sites during a second incubation step. The antibody bound fraction is precipited and counted. Finally, patient sample concentrations are read from a calibration curve.

MATERIAL SUPPLIED:

1. FSH antiserum
2. (125I) FSH
3. FSH Calibrators seven in number labeled A through G
4. Precipitating solution

SPECIMEN COLLECTION:

The patient need not be fasting and no special precautions are necessary. Collected by veinpuncture into plain tubes and separated serum from cells by centrifugation. Heparinised plasma is also suitable.

Samples may be stored under refrigeration at 2-8°C for up to seven days, or upto three months frozen at -20°C. Prior to assay allowed the samples to come upto room temperature and mixed by gently swirling or inversion. Aliquot, if necessary

to avoid repeated freezing and thawing. High patient samples should be diluted in the Kit's zero calibrator.

PROCEDURE:

All components except the precipitating solution must be at normal temperature prior to use.

1. Labelled eighteen tubes in duplicates: T (total counts), NSB (nonspecific binding), A (maximum binding) and B through G. Labelled additional tubes, also in duplicate for serum samples and controls.

Calibrator	WHO 2nd IRP-HMG mIU/ml	WHO 2nd IRP78/549 mIU/ml
A (MB)	0	0
B	2	0.8
C	5	2.1
D	10	4.1
E	20	8.2
F	40	14.4
G	100	41.0

2. Pipetted 200µl of the zero calibrator A into the NSA and A tubes and 200µl of each of the remaining calibrators B through G into correspondingly labeled tubes. Pipetted 200µl of each patients serum or plasma samples and each control into the tubes prepared.

3. Added 100µl of FSH Antiserum to all tubes except the NSB and T tubes Vortex.

4. Incubated for 3 hrs at 37°C
5. Added 100µl of (125I) FSH to all tubes.Vortex
6. Covered the racks,incubated for 18hrs at room temperature.
7. Added 1.0ml of cold precipitating solution to all tubes. Vortex
8. Centrifuged for 15 minutes at 3000xg.
9. Using a foam decanting rack, decanted (or aspirated) the supernatant, retaining the precipitate for counting.
10. Counted each tube for 1 minute.

CALCULATION OF RESULTS:

To calculate FSH concentrations from a logit-log representation of the calibration curve first calculate for each pair of tubes the average blank-corrected counts per minute.

Net counts = Average CPM minus Average NSB CPM.

Then determine the binding of each pair of tubes as a percent of maximum binding (MB) with the NSB-corrected counts of the A tubes taken as 100%

$$\text{Percent Bound} = \frac{\text{Net counts}}{\text{Net MB counts}} \times 100$$

Using the logit-log graph paper provided with the kit, plot percent Bound on the vertical axis against concentration on the horizontal axis for each of the calibrators B through G and draw a straight line approximating the path of these six points FSH concentrations for the unknownw may then be estimated from the line of interpolation.

RADIO-IMMUNO ASSAY OF LUTEINIZING HORMONE (LH)
(ISOPHARM, 1988)

PRINCIPLE:

The radio-immunoassay method is based on the competition of unlabelled LH in the standards (or samples) and radio iodinated LH for the limited binding sites of the specific antibody. At the end of the incubation the antibody bound and free LH are separated by the addition of the precipitating reagent. LH concentrations of the samples are quantitated by measuring the radioactivity associated with the bound fraction of sample and standards.

REAGENTS PROVIDED IN THE KIT

Human LH standard	1 vial
125I labelled LH	2 vials
LH antibody	1 vial
Antirabbit gammaglobulin	1 vial
LH free serum	1 vial
Polyethyleneglycol (PEG)	1 vial
Assay buffer	1 vial
Control serum A	1 vial
Control serum B	1 vial

Reagents supplied in the lyophilised form are under vaccum sealing. Decap carefully so that the contents do not come out. Do not discharge or discard the rubber closures. Added the specified volume of buffer as per protocol.

Allowed to stand at room temperature for 5 minutes. Mixed gently. Avoid foaming. All the reconstituted reagents except PEG and the buffer must be stored at 5°C or below. Avoid repeated thawing and freezing.

SPECIMEN COLLECTION

Collected 5ml of blood without any anticoagutant in a glass vial or tube. Allowed the blood to clot at room temperature. Rimmed the clot, centrifuged and collected the serum. Stored the sample at 2°C to 80°C for assay on the same day or freezer at 20°C if the storage is expected to exceed 24 hours.

ASSAY

Radio immuno assay flow sheet is shown in Table

1. Numbered 16 assay tubes for the standard curve and two tubes for each clinical sample and control.

2. Added buffer and other reagents to tubes sequentially as shown in the flow sheet.

3. After the addition of the LH antiserum the contents of the tubes should be yellowish in colour. Mixed and incubated for 2 hours at room temperature.

4. Added 125I - LH. The tubes should now be brown in colour. Mixed and incubated for 2 hours at room temperature.

5. Added anti IgG and PEG.Mixed thoroughly. The contents should be light green in colour.

6. Incubated for 20 minutes at room temperature

7. Centrifuged all the tubes except 1 and 2 at 1500xg for 20 minutes or for longer time at lesser centrifugal force.

8. Discarded the supernatant, carefully blotted out the solution adhering to the rim of the tube and counted the precipitate.

9. Counted the tubes in a well type gamma scintillation counter. The optimum counting time is indicated by an accumulated counts of at least 20,000 in tubes 1 and 2. Counted all the tubes for the same time.

CALCULATION

(i) Substract the background from all the counts to get actual counts.

(ii) Average all duplicates

(iii) Average count of tubes 1 and 2 is called total count.

(iv) Average count of tubes 3 and 4 is called blank count

calculte % blank

$$\% \text{ blank} = \frac{\text{Blank count}}{\text{Total count}} \times 100$$

(v) Subtract blank count from the average of the all remaining duplicates. This is called corrected average counts.

(vi) Calculate zero standard binding (Bo) as follows.

$$\% \text{ Bo} = \frac{\text{corrected average count of tubes 5 \& 6}}{\text{Total count}} \times 100$$

$$\% \text{ B/Bo} = \frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of tubes 5 \& 6}} \times 100$$

(vii) Plot the standard curve in one of the following ways,

(a) Percentage B/Bo against concentration of LH (mlu/ml) on a line as graph sheet.

(b) Percentage B/Bo on logit scale and concentration of LH on the log scale of a logit-log graph sheet.

(viii) Read the sample value directly from the standard curve in mlu/ml of serum.

Note: If the sample value higher than 200mlu/ml is obtained the sample should be diluted in assay buffer and reassayed.

ASSAY PROTOCOL

No	ASSAY buffer (ml)	STANDARD/ sample (ml)	LH Free serum (ml)	Anti-LH (ml)		125I-LH (ml)		Anti rabbit	PEG (ml)	
1,2	-	-	-	-	Mix and incubate at room temperature for 2 hrs	0.1	Mix and incubate at room temperature for 2 hrs	-	-	Mix and incubate for 20min. at room temperature. Centrifuge at 1500 x g for 20 mins. Decant the supernatant and count the precipitate.
3,4	0.3	-	0.1	-		0.1		0.1	1.0	
5,6	0.2	-	0.1	0.1		0.1		0.1	1.0	
7,8	0.1	0.1E(5mlu/ml)	0.1	0.1		0.1		0.1	1.0	
9,10	0.1	0.1D(10mlu/ml)	0.1	0.1		0.1		0.1	1.0	
11,12	0.1	0.1C(20mlu/ml)	0.1	0.1		0.1		0.1	1.0	
13,14	0.1	0.1B(50mlu/ml)	0.1	0.1		0.1		0.1	1.0	
15,16	0.1	0.1A(100mlu/ml)	0.1	0.1		0.1		0.1	1.0	
17,18	0.1	0.1 sample 1	-	0.1		0.1		0.1	1.0	
19,20	0.1	0.1 sample 2	-	0.1		0.1		0.1	1.0	

RADIO IMMUNO ASSAY OF PROLACTIN (DIAGNOSTIC PRODUCTS CORPORATION, 1988)

PRINCIPLE

The coat-A-count procedure is a solid-phase radioimmunoassay wherein 125I-labeled prolactin competes for a fixed time with prolactin in the patient sample for sites on prolactin-specific antibody. The antibody being immobilised to the wall of a polypropylene tube, decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled prolactin-counting the tube in a gamma counter then yields a number which converts by way of a calibration curve to a measure of prolactin present in the patient sample.

MATERIAL SUPPLIED

1. Prolactin Antibody-coated tubes
2. Buffered (125I) prolactin
3. Prolactin calibrators, seven in number, labeled A through G

SPECIMEN COLLECTION

The patient need not be fasting and no special precautions are necessary. Collected blood by venipuncture into plain tubes avoiding hemolysis and separated the serum from the cells. The procedure cells for 200µl of serum per assay tube.

The samples may be stored under refrigeration at 2-8°C for up to seven days, or up to three months frozen at - 20°C.

Prior to assay allowed the samples to come up to room temperature and mixed by gentle swirling or inversion Aliquot, if necessary, to avoid repeated freezing and thawing.

PROCEDURE

1. Plain Tubes: Labeled four plain (uncoated) 12x75mm polypropylene tubes T (Total count) and NSB (non-specific binding) in duplicate. Coated Tubes: Labelled fourteen prolactin Antibody-coated tubes A(maximum binding) and B through G in duplicate. Labeled additional antibody-coated tubes also in duplicate for controls and patient samples.

CALIBRATOR	ng/ml	mlu/l
A (MB)	0	0
B	5	115
C	10	230
D	20	460
E	50	1,150
F	100	2,300
G	200	4,600

2. Pipetted 200µl of the zero calibrator A into the NSB and A tubes and 200µl of each remaining calibrator, control and patient samples into the tubes prepared. Pipetted directly to the bottom.

3. Added 1.0ml of Buffered (125I) Prolactin to each tube, vortex briefly and gently.

4. Incubated for 18 hours at room temperature.

5. Decanted thoroughly.

6. Counted for 1 minute in a gamma counter.

CALCULATION OF RESULTS

To calculate prolactin concentrations from a logit-log representation of the calibration curve, first calculate for each pair of tubes the average NSB- corrected counts per minute.

Net counts = Average CPM minus Average NSB CPM

Then determine the binding of each pair of tubes as a percent of maximum binding (MB) with the NSB-corrected counts of the A tubes taken as 100%

$$\text{PERCENT BOUND} = \frac{\text{Net counts}}{\text{Net MB counts}} \times 100$$

Using the logit-log graph paper provided with the kit, plot percent Bound on the vertical axis against concentration on the horizontal axis for each of the calibrators B through G and draw a straight line approximating the path of these six points. Prolactin concentrations for the unknown may then be estimated from the line of interpolation.

RADIOIMMUNOASSAY OF ESTRADIOL (Diagnostic Products Corporation,1988)

PRINCIPLE

The coat-A-Count estradiol procedure is based on antibody-coated tubes. 125I labeled estradiol competes with estradiol in the patients sample for antibody sites. After incubation separation of bound from free is achieved by simply decanting. The tube is then counted in a gamma counter, the counts being inversely related to the amount of estradiol present in the patient sample. The quantity of estradiol in the sample is determined by comparing the counts to a standard curve. There is no extraction in this assay.

MATERIAL SUPPLIED

1. Estradiol Antibody-coated tubes
2. Buffered (125I) Estradiol
3. Estradiol calibrators, labeled A through G.

SPECIMEN COLLECTION

The patient need not be fasting and no special preparations are necessary. Collected blood by venipuncture into plain tubes and separated the serum from the cells, noting the time of collection. The procedure calls for 100µl of serum per tube.

Samples may be stored under refrigeration for two days in glass tubes or for up to two months frozen at - 20°C. Prior to assay allowed the samples to come to room temperature and mixed by gentle swirling or inversion. Aliquot, if necessary to avoid repeated thawing and freezing. Do not attempt to thaw

frozen specimens by treating them in a water bath. Variations in albumin or IgG of up to 3 gm/dl may have an effect on the assay.

PROCEDURE

Estradiol has a strong tendency to absorb to untreated plastic surfaces. Accordingly it is important to use containers made of glass rather than plastic for both tracer and patient samples. All components must be at normal room temperature prior to use.

1. Plain Tubes: Labeled four plain (uncoated) 12x75mm polypropylene tubes T (total counts) and NSB (nonspecific binding) in duplicate. Coated Tubes: Labeled fourteen Estradiol Antibody-coated tubes A (maximum binding) and B through G in duplicate labeled additional antibody-coated tubes, also duplicate for controls and patient samples.

Calibrators	pg/ml	nmol/l
A (MB)	0	0
B	20	0.07
C	50	0.18
D	150	0.55
E	500	1.84
F	1800	6.61
G	3600	13.20

2. Pipetted 100µl of the zero calibrator A into the NSB and A tubes and 100µl of each remaining calibrator, control and patient sample into the tubes prepared. Pipetted directly into the bottom.

3. Added 1.0ml of Buffered (125I) Estradiol to every tube, vortex.

4. Incubated for 3 hours at room temperature

5. Decanted thoroughly

6. Counted for 1 minute in a gamma counter

CALCULATION OF RESULTS

To calculate estradiol concentrations from a logit-log representation of the calibration curve, first calculate for each pair of tubes the average NSB-corrected counts per minute.

Net counts = Average CPM minus Average NSB CPM

Then determine the binding of each pair of tubes as a percent of maximum binding (MB) with the NSB - corrected counts of the A tubes taken as 100%

$$\text{Percent Bound} = \frac{\text{Net counts}}{\text{Net MB counts}} \times 100$$

Using the logit-log graph paper provided with the kit, plot percent Bound on the vertical axis against concentration on the horizontal axis for each of the calibrators B through G and draw a straight line approximating the path of these six points. Estradiol concentrations for the unknowns may then be estimated from the line by interpolation.

6. Qualitative analysis of urine

Urine analysis was done qualitatively with the Multistix Ames Reagent strips (Henry, 1979). Biochemical parameters like Glucose, Bilirubin, Ketone, Specific gravity, Blood, pH, Protein, Urobilinogen, Nitrite and Leukocytes were analysed in the fresh urine samples of eight breast cancer patients and in two controls.

7. Statistical analysis

The levels of significance in the changes of blood constituents between the various groups of participants were determined by performing Student's 't' test.

$$\text{'t' value} = \frac{\overline{X1} - \overline{X2}}{\sqrt{\frac{n_1 S1^2 + n_2 S_2^2}{n_1 + n_2 - 2} \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

$\overline{X_1}$, $\overline{X_2}$ = mean of the two groups

n_1, n_2 = number of samples in each group

S_1, S_2 = Standard deviations of the two groups.

Results and Discussion

IV RESULTS AND DISCUSSION

The results of the study "Biochemical changes in Breast cancer" are discussed under the following headings:

1. Date regarding breast cancer patients undergoing treatment in the Valavadi Narayanaswamy Cancer Centre, Coimbatore from 1986 to 1988.
2. Biodata of breast cancer patients.
3. Vitamin A status in breast cancer patients and in controls.
4. Vitamin C status in breast cancer patients and in controls.
5. Cholesterol levels in breast cancer patients and in controls.
6. Alkaline phosphatase activity in breast cancer patients and in controls.
7. Serum Immunoglobulin levels in breast cancer patients and controls.
8. Hormonal status of breast cancer patients and controls.
9. Qualitative analysis of urine of breast cancer patients and controls.
10. Comparison of the blood constituents in the breast cancer patients and controls irrespective of age group and menopausal status.

1.Data regarding breast cancer patients undergoing treatment in the Valavadi Narayanaswamy Cancer Centre, Coimbatore from 1986 to 1988

Table I presents the report of cancer incidence in Coimbatore during the three years, 1986, 1987 and 1988. There is a steady increase in the number of patients attending the cancer centre. The percentage of breast cancer cases has increased over a period of three years while that of oral cancer and oesophageal cancer has not increased.

Out of the breast cancer cases that have attended the centre, 10 per cent had a family history of breast cancer. 10 per cent of the patients had not lactated. Only a small percent were on contraceptive pills. About 80 per cent of the patients were non-vegetarians. There were an equal number of pre menopausal (those who have the menstrual cycles), peri menopausal (those who are within five years of stopping of the cycle) and post menopausal (after five years of stopping of the menstrual cycle) patients.

TABLE I

REPORT OF CANCER INCIDENCE DURING THE YEARS 1986, 1987 and 1988

Year	Total number of cancer patients	Percentage of different types of cancer				
		Cervix	Oral	Oesophagus	Breast	Others *
1986	12,350	50	25	5	7.5	12.5
1987	12,913	36	23.5	7	10	23.5
1988	14,763	30	25	7	12	26

* Cancer of other organs

FIG.1. INCIDENCE OF CANCER IN THE YEARS 1986, '87 AND '88

[DATA COLLECTED FROM VALAVADI NARAYANASWAMY CANCER CENTRE, COIMBATORE]

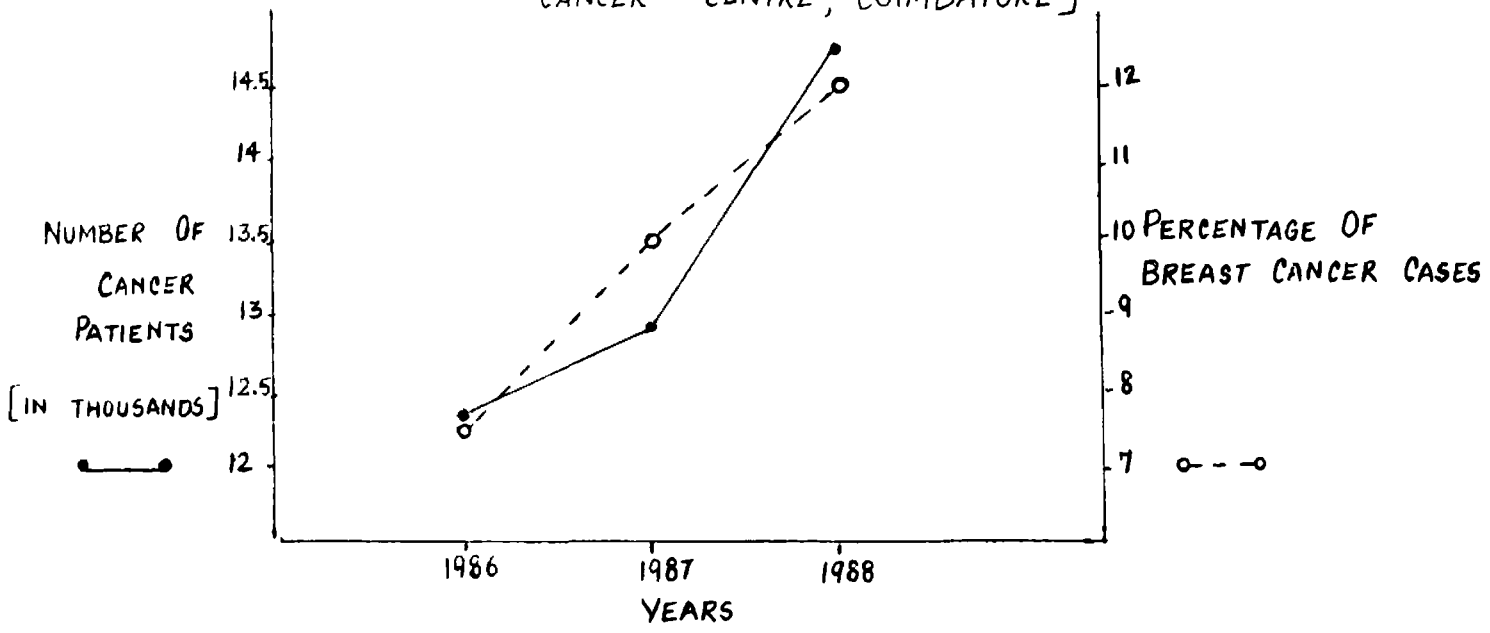
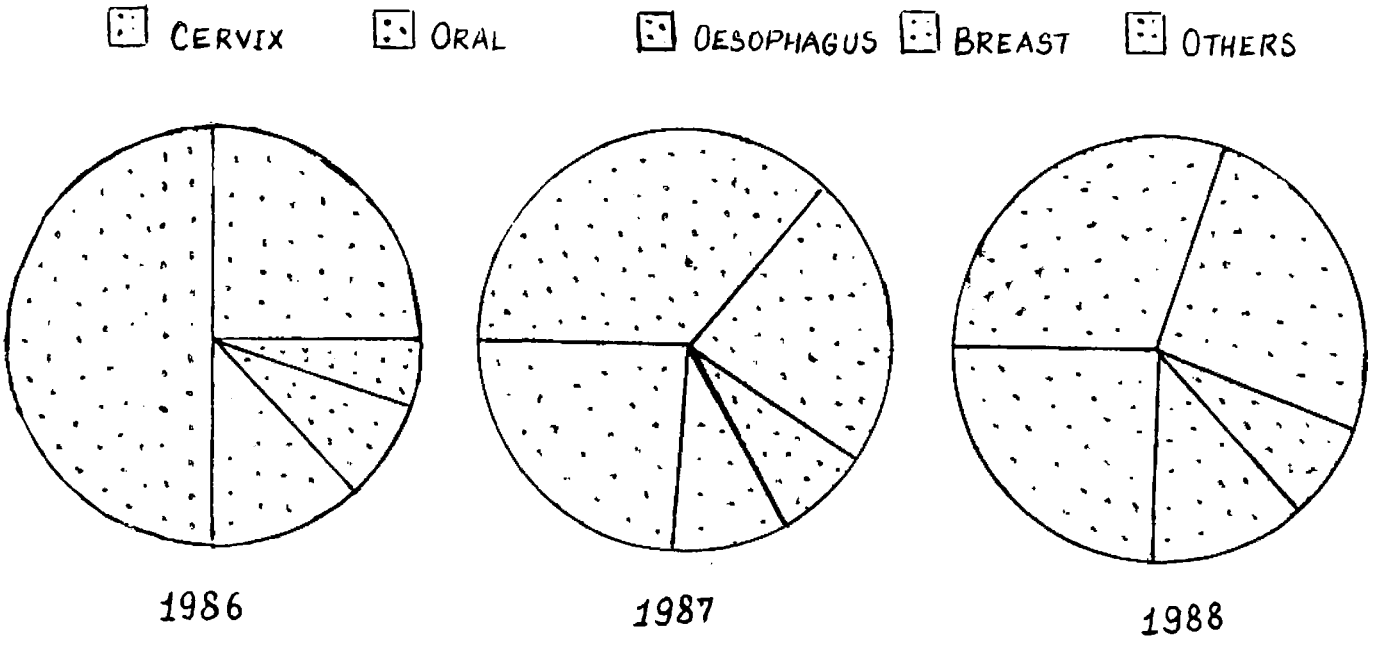


FIG.2. OCCURRENCE OF DIFFERENT TYPES OF CANCER IN THE YEARS 1986, '87 AND '88



2. Biodata of breast cancer patients:

About thirtyone breast cancer patients attending the cancer centre for the past three months were taken for the study. All the participants of the study belonged to the middle income group. All of them were in the advanced stage of breast cancer. They were either in the pre or post menopausal state. Most of them were married. Six of them had a past history of breast cancer while three of them had a family history of cancer. All of them were non-vegetarians. They were free from personal habits like chewing, smoking and alcohol. The age of menarche of these patients was 13-16 years. All of them, except 9 patients were menstruating regularly. The age at first child birth and the age at last child birth was 18-24 years and 30-45 years respectively. Twelve patients had no children, while the others had one or more children. They also reported poor breast feeding of less than six months. Three of the patients had spontaneous abortions while the others had no abortion at all. No patients had adopted family planning. Blood sample was collected from the participants before any treatment was started.

3. Vitamin A status in breast cancer patients and in controls

TableII presents the mean levels of vitamin A in breast cancer patients and controls in different age groups, namely 36-45, 46-55, 56-65, 66 and above.

TABLE II

MEAN LEVELS OF VITAMIN A IN BREAST CANCER PATIENTS AND CONTROLS IN DIFFERENT AGE GROUPS

(Mean ± S.D. mg/dl)

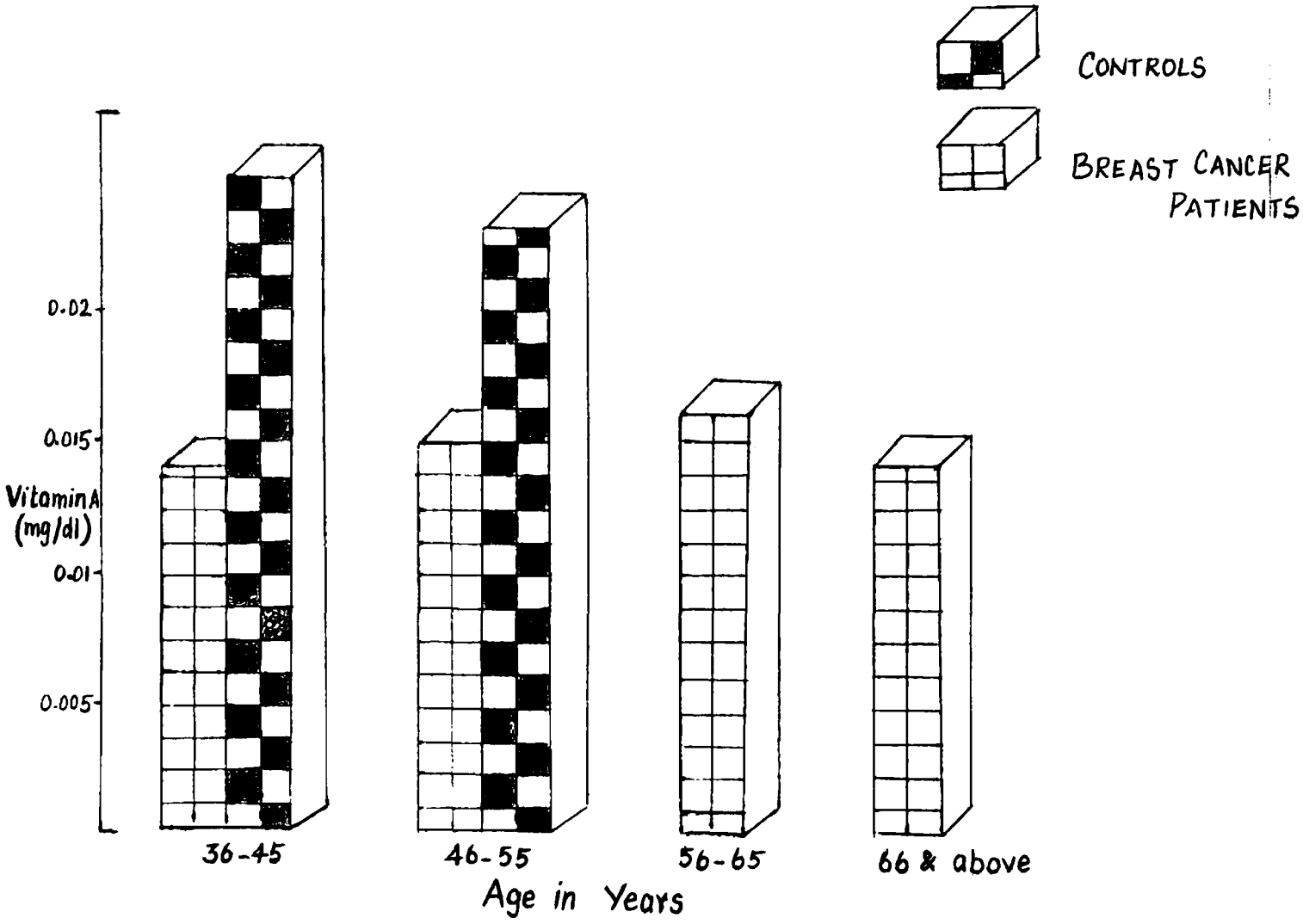
Participants	Age in years				Groups Compared
	36-45	46-55	56-65	66 & above	
Breast Cancer	0.014±0.005 A	0.015±0.004 B	0.016±0.002 C	0.014±0.006 D	A Vs B NS A Vs C NS A Vs D NS B Vs C NS B Vs D NS C Vs D NS
Control	0.025±0.007 E	0.023±0.008 F	-	-	E Vs F NS E Vs A ** F Vs B *

NS = Not Significant * = Significant at 5% level

** = Significant at 1% level.

It is seen that there is no significant difference in the mean levels of Vitamin A, between the different age groups, in breast cancer patients. There is no significant difference in the mean levels of Vitamin A between the different age groups

FIG.3. MEAN LEVELS OF VITAMIN A IN BREAST CANCER PATIENTS AND CONTROLS IN DIFFERENT AGE GROUPS



in controls also. Within the age group 36-45 years, the mean levels of Vitamin A is higher in the controls (0.025±0.007mg/dl) than in the breast cancer patients (0.014 ± 0.005mg/dl) ($p<0.01$). In the age group 46-55 years the mean levels of Vitamin A is higher in the control group (0.023 ± 0.008mg/dl) than in the breast cancer patients (0.015 ± 0.004mg/dl) ($p<0.05$).

Table III presents the mean levels of vitamin A in breast cancer patients and controls in pre and post menopausal states.

TABLE III

MEAN LEVELS OF VITAMIN A IN BREAST CANCER PATIENTS AND CONTROLS IN PRE AND POSTMENOPAUSAL STATES

(Mean ± S.D. mg/dl)

Participants	Premenopausal status	Postmenopausal status	Groups compared	
Breast cancer	0.015 ± 0.005 A	0.016 ± 0.004 B	A Vs B	NS
			C Vs D	NS
Control	0.025 ± 0.007 C	0.023 ± 0.008 D	C Vs A	**
			D Vs B	*

NS = Not significant

* = Significant at 5% level

** = Significant at 1% level.

There is no significant change in the mean Vitamin A levels between the two states in breast cancer patients and in controls also. The level of Vitamin A in the premenopausal control group is higher than that in premenopausal breast cancer patients in the study ($P < 0.01$). The level of Vitamin A in the post menopausal control group is also higher than that in the post menopausal breast cancer participants ($P < 0.05$).

This study agrees with that of Wald et al.(1980) who reported lower levels of Vitamin A , measured as retinol in patients with cancer, than in controls without cancer, A similar result was reported by Kark et al.(1981). A high total Vitamin A intake was inversely related to the risk of breast cancer (Katsouyanni et al, 1988). Tyler (1986) also reported a relationship between dietary Vitamin A and cancer, and indicated a lower level of serum retinol , its binding protein and carotenoids in cancer cases than in controls. There is thus an inverse relationship between serum levels of Vitamin A and subsequent risk of cancer in general (Cambien et al 1980 ; Kummet and Meysken 1983). Ramachandran and Mathew (1986) reported a lower dietary intake of carotenes in cancer patients. Consumption of vegetables has an inverse association with cancer of the breast (Graham et al, 1982). But the possible protective role of Vitamin A and its precursors in human tumours are still uncertain (Marubini et al, 1988). In the present study also the 24 hour dietary recall of the breast cancer patients

revealed that their Vitamin A intake was lower than the recommended dietary allowances (ICMR Report,1988).

4. Vitamin C status in breast cancer patients and in controls.

Table IV presents the mean levels of Vitamin C in breast cancer patients and controls in different age groups, namely 36-45, 46-55, 56-65, 66 and above.

TABLE IV

MEAN LEVELS OF VITAMIN C IN BREAST CANCER PATIENTS AND CONTROLS IN DIFFERENT AGE GROUPS.

(Mean ± S.D. mg/dl)

Participants	Age in years				Groups Compared
	36-45	46-55	56-65	66 and above	
Breast Cancer	0.36±0.09	0.40±0.07	0.32±0.05	0.37±0.09	A Vs B NS
	A	B	C	D	A Vs C NS
					A Vs D NS
					B Vs C *
					C Vs D NS
Control	0.81±0.06	0.67±0.15	--	--	E Vs F *
	E	F			E Vs A **
					F Vs B **

NS = Not Significant

* = Significant at 5% level

** = Significant at 1% level

It is seen that there is no significant difference in the mean levels of Vitamin C, among the breast cancer patients

FIG.4. MEAN LEVELS OF VITAMIN C IN BREAST CANCER PATIENTS AND CONTROLS IN DIFFERENT AGE GROUPS

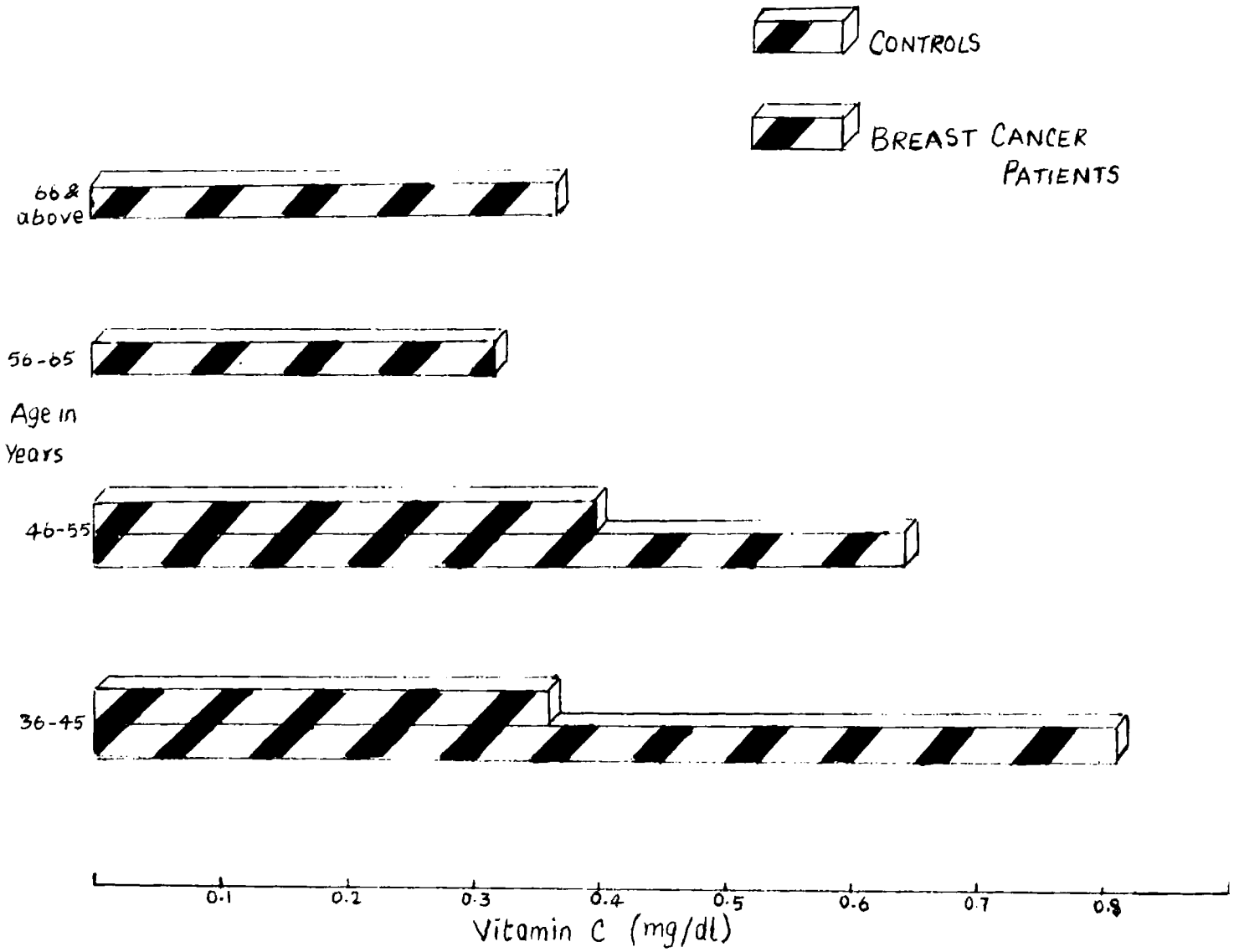
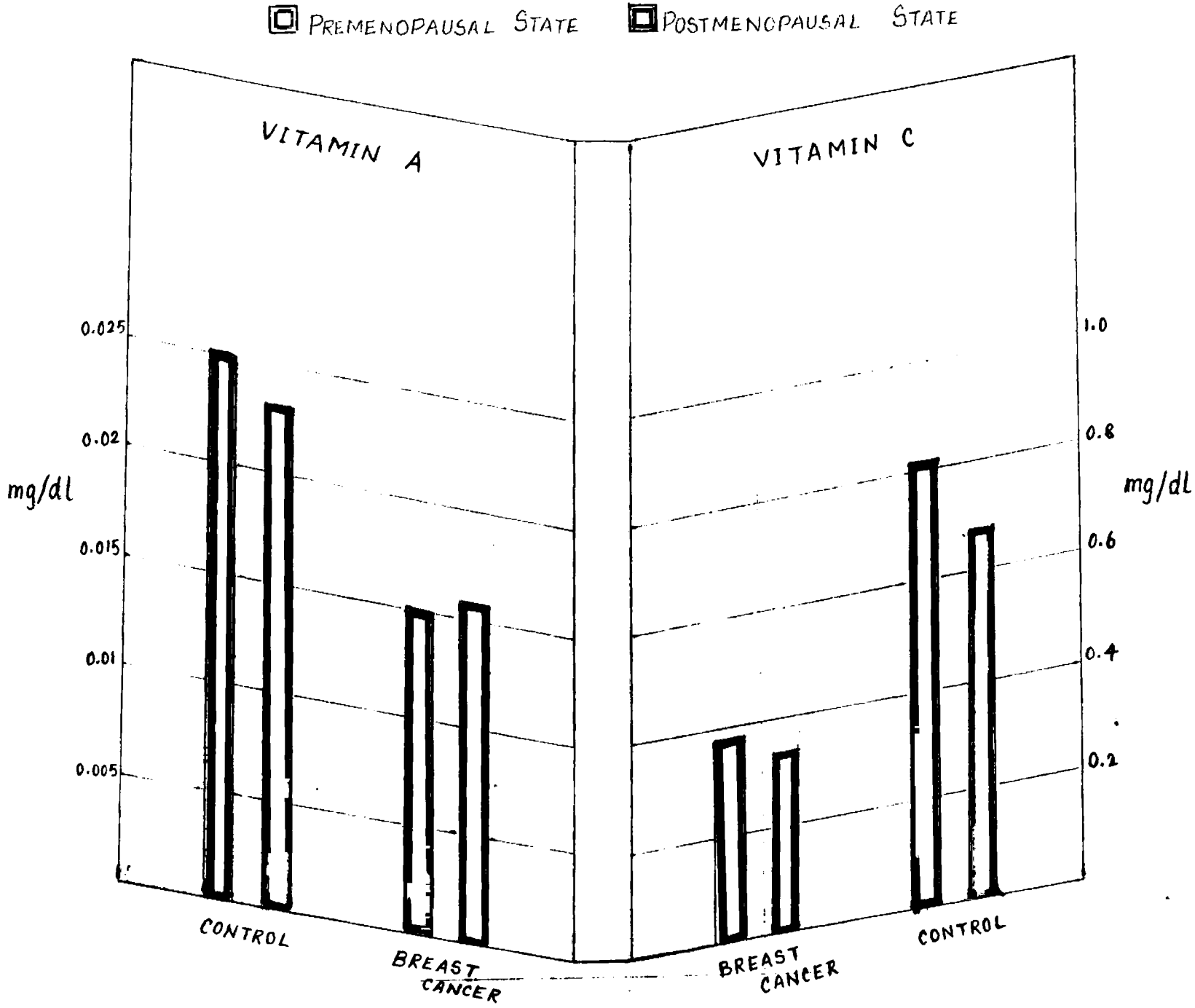


FIG. 5. MEAN LEVELS OF VITAMIN A AND VITAMIN C IN PRE AND POST MENOPAUSAL STATES



in the age group 36-45 and 66 and above. But there is a significant difference in the mean Vitamin C level among the breast cancer patients in the age groups 46-55 and 56-65. The mean Vitamin C levels are higher in the age group 46-55 (0.40 ± 0.07 mg/dl). In the control group, the mean levels of Vitamin C in the age group 36-45 is significantly higher (0.81±0.06 mg/dl) than that in the age group 46-55 (0.67±0.15 mg/dl). The mean Vitamin C levels of the controls, in the age groups 36-45, and 46-55 (0.81 ± 0.06 and 0.67 ± 0.15 mg/dl respectively) are significantly higher than the mean Vitamin C levels of the breast cancer patients belonging to the corresponding age groups (0.36 ± 0.09 and 0.40 ± 0.07 mg/dl respectively) ($p < 0.01$).

Table V represents the mean levels of Vitamin C in breast cancer patients and controls in pre and post menopausal states.

TABLE V
MEAN LEVELS OF VITAMIN C IN BREAST CANCER PATIENTS AND CONTROLS IN PRE AND POST MENOPAUSAL STATES
(Mean ± S.D.mg/dl)

Participants	Premenopausal state	Post Menopausal state	Groups compared	
Breast Cancer	0.38±0.09	0.34±0.09	A Vs B	NS
			C Vs D	*
	A	B	C Vs A	**
Control	0.81±0.06	0.67±0.15	D Vs B	**
	C	D		

NS = Not Significant
** = Significant at 1% level
* = Significant at 5% level.

There is no significant difference in the mean levels of Vitamin C in the two states of breast cancer patients. But in the control group, the mean levels of Vitamin C in the premenopausal state (0.81 ± 0.06 mg/dl) is higher than that in the postmenopausal state (0.67 ± 0.15 mg/dl) ($p < 0.05$). The mean levels of Vitamin C in pre and post menopausal states in the controls (0.81 ± 0.06 & 0.67 ± 0.15 mg/dl respectively) are also significantly higher than that in the pre and post menopausal states in breast cancer patients (0.38 ± 0.09 and 0.34 ± 0.09 mg/dl respectively) at 1 per cent level.

Vitamin C inhibits the formation of nitrosamines and thereby inhibit carcinogenesis (Wenck et al, 1983). Tumour tissues avidly take up Vitamin C from the blood, which results in lowered levels in the blood and white blood cells. Theoretically this depletion of Vitamin C in normal body tissues reduces the ability to combat cancer growth (Williams and Caliendo, 1984). That a deficiency of Vitamin C might facilitate infiltration and dissemmination of neoplastic cells was also shown by Creagan et al.(1979). Bansal(1986) also reported that a rich Vitamin C diet helps to reduce the incidence of tumours. Plasma ascorbic acid is lower in malignancy as compared to normal conditions. It further falls on radiotherapy (Gupta et al., 1986). Moderate doses of Vitamin C can significantly reduce gastric nitrosation in man (Krytopoulos, 1987). Vitamin C deficiency appears to affect migration of macrophages in Vitro (Ganguly et al.,

1976). Vitamin C stimulates the immune system and hence enhances immune surveillance of cancer development. (Creasy, 1984: Crary and Mc Carty, 1984).

In the present study, the decreased Vitamin C level in the breast cancer patients might be due to their reduced intake or poor absorption of Vitamin C.

5. Cholesterol levels in breast cancer patients and in controls

Table VI gives the mean levels of cholesterol in breast cancer patients and controls in different age groups, namely 36-45, 46-55, 56-65, 66 and above.

TABLE VI

MEAN LEVELS OF CHOLESTEROL IN BREAST CANCER PATIENTS AND CONTROLS IN DIFFERENT AGE GROUPS

(Mean ± S.D. mg/dl)

Participants	Age in Years				Groups Compared
	36-45	46-55	56-65	66 and above	
Breast Cancer	328.9±89.7 A	321±108.8 B	373.3±34.0 C	306.3±112.2 D	A Vs B NS A Vs C NS A Vs D NS B Vs C NS C Vs D NS
Control	171.1±20.3 E	166.4±36.7 F	-	-	E Vs F NS E Vs A ** F Vs B **

NS = Not significant ** = Significant at 1% level.

The table shows that there is no significant difference between the different age groups in breast cancer and in the

control also. Within the age group 36-45 years, the mean levels of cholesterol is higher in the breast cancer patients (328.9 ± 89.7 mg/dl) than in the controls (171.1 ± 20.3 mg/dl) of the same age group. In the age group 46-55 years, the mean levels of cholesterol in the controls is lower (166.4 ± 36.7 mg/dl) than that of the breast cancer patients (321 ± 108.8 mg/dl) belonging to the same group ($p < 0.01$).

Table VII shows the mean levels of cholesterol in breast cancer patients and controls in pre and post menopausal states.

TABLE VII

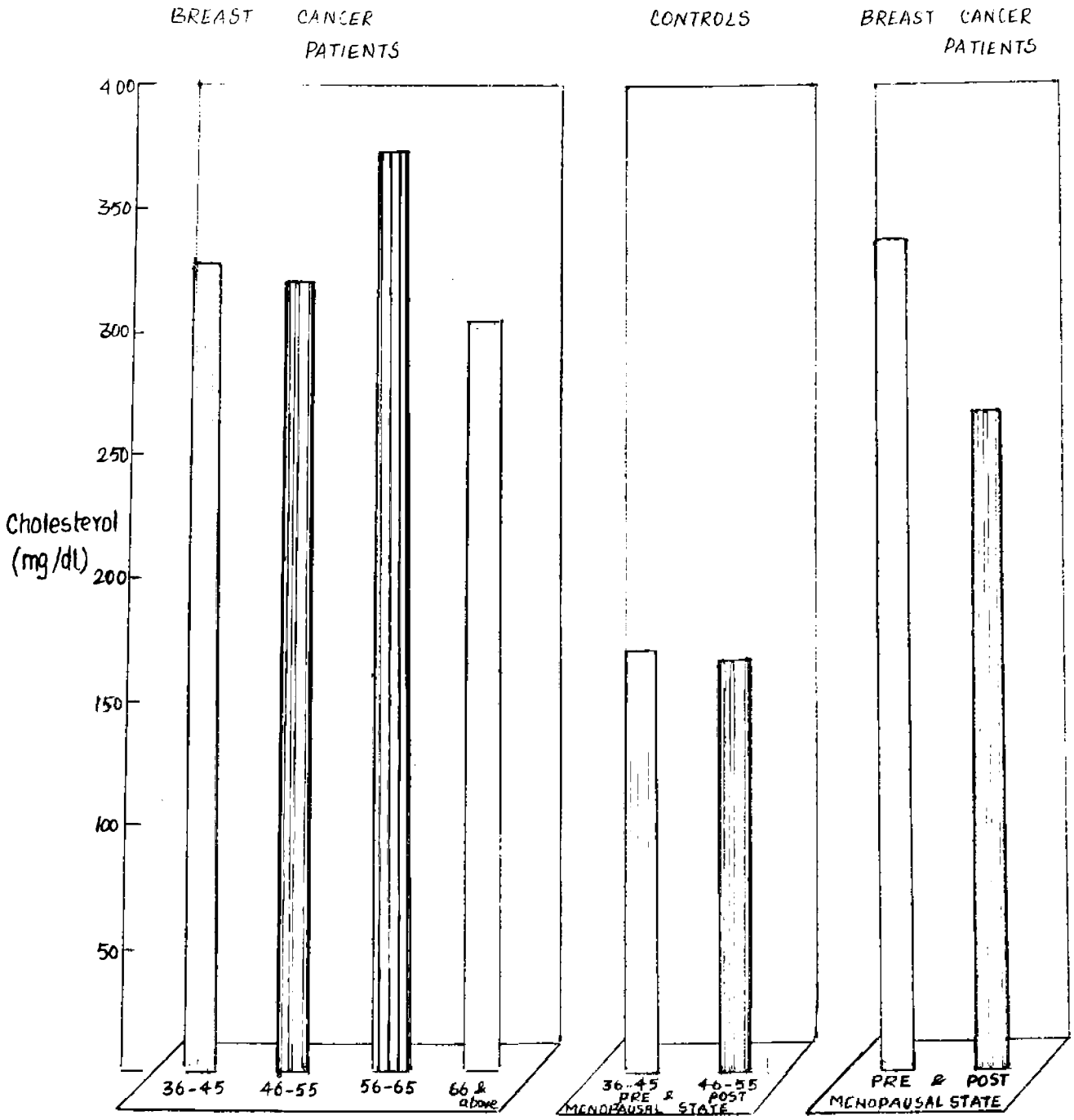
MEAN LEVELS OF CHOLESTEROL IN BREAST CANCER PATIENTS AND CONTROLS IN PRE AND POST MENOPAUSAL STATES

(Mean ± S.D. mg/dl)

Participants	Premenopausal state	Post Menopausal state	Groups compared	
Breast Cancer	337.3±91.1 A	268.0±105.6 B	A Vs B	NS
			C Vs D	NS
			C Vs A	**
Controls	171.1±20.3 C	166.4±36.7 D	D Vs B	*

NS = Not significant
* = Significant at 5% level
** = Significant at 1% level

FIG.6. MEAN LEVELS OF CHOLESTEROL IN BREAST CANCER PATIENTS AND CONTROLS



In the control group, those participants in the age group 36-45 belong to premenopausal state and those participants in the age group 46-55 belong to post menopausal state.

Among the control groups and among the breast cancer patients there is no significant difference between the mean levels of Cholesterol in the pre and post menopausal stages. In the pre menopausal stage, the controls have a lower mean level of Cholesterol (171.1 ± 20.3 mg/dl) than the breast cancer patients belonging to the same menopausal stage ($p < 0.01$). The post menopausal breast cancer patients have a higher level of Cholesterol (268 ± 105.6 mg/dl) than the controls in the same menopausal stage (166.4 ± 36.7 mg/dl) ($p < 0.05$).

Basu et al(1974) showed that females with breast cancer had a higher amount of Cholesterol, than females with tumour elsewhere. They also showed that plasma Cholesterol had a linear relation in all tumours. Studies have confirmed that the incidence of breast cancer is higher in those on a high fat diet (Sabine, 1983). Many cancers exhibit either markedly reduced or no feed back inhibition of Cholesterol synthesis by Cholesterol feeding, because of the failure of the ingested Cholesterol to inhibit HMG-COA reductase activity. The reason for this failure is unknown (Feingold et al.,1983). Beni et al (1986) have reported high values of Cholesterol and low values of HDL Cholesterol in breast cancer patients. Thomas et al (1982) have reported a positive correlation between high Cholesterol and total cancer mortality.

Experiments with animals show that the amount and type of dietary lipid influences the growth of mammary tumours (Tannenbaum and Silverstone, 1953; Hopkings and Carroll, 1979). Menopausal status is of particular interest since it has been postulated that dietary factors may influence the risk of breast cancer more strongly among postmenopausal women (dewaard, 1975). Dietary fat has been considered as a promoter of carcinogenesis (Carroll, 1980; Carroll and Khor, 1975). The exact mechanism of action is unclear (Hopkins and West, 1976; Welsch and Aylsworth, 1981). On the basis of the evidence available from experimental and epidemiological studies, it appears that a reduction in dietary fat intake would decrease the risk of developing cancer and might even help to delay or prevent development of metastases (Wynder and Cohen, 1982). The present study illustrates a positive correlation between serum cholesterol and incidence of breast cancer. The 24 hour dietary recall revealed that the consumption of fat in the breast cancer patients is higher than that in the control subjects.

6. Alkaline phosphatase activity in breast cancer patients and in controls.

Table VIII presents the mean levels of Alkaline phosphatase activity in breast cancer patients and controls in different age groups namely 36-45, 46-55, 56-65, 66 and above.

TABLE VIII

MEAN LEVELS OF ALKALINE PHOSPHATASE ACTIVITY IN BREAST CANCER PATIENTS AND CONTROLS IN DIFFERENT AGE GROUPS

(Mean ± S.D KA units/dl)

Participants	Age in years				Groups Compared	
	36-45	46-55	56-65	66 and above		
Breast Cancer	10.8±3.5	9.9±2.3	9.5±4.8	10.6±4.1	A Vs B	NS
					A Vs C	NS
					A Vs D	NS
	A	B	C	D	B Vs C	NS
					C Vs D	NS
Controls	10.6±3.1	11.5±1.5	-	-	E Vs F	NS
					E Vs A	NS
	E	F			F Vs B	*

NS = Not significant * = Significant at 5% level

There is no significant difference in the mean levels of Alkaline phosphatase activity in the different age groups in breast cancer patients and controls. The mean level of Alkaline phosphatase activity of the controls in the age group 46-55 years is higher (11.5 ± 1.5 KA units/dl) than that of the breast cancer patients in the same age group (9.9 ± 2.3 KA units/dl)

Table IX shows the mean levels of Alkaline phosphatase activity in breast cancer patients and controls in pre and post menopausal status.

TABLE IX

MEAN LEVELS OF ALKALINE PHOSPHATASE ACTIVITY IN BREAST CANCER PATIENTS AND CONTROLS IN PRE AND POST MENOPAUSAL STATES

(Mean + S.D KA units/dl)

Participants	Premenopausal state	Postmenopausal state	Groups compared	
Breast Cancer	10.6 ± 3.0	9.91 ± 3.5	A Vs B	NS
	A	B	C Vs D	NS
Control	10.6 ± 3.1	11.5 ± 1.5	C Vs A	NS
	C	D	D Vs B	NS

NS = Not significant

It is seen that there is no significant change in the in the enzyme activity amoung the controls and breast cancer patients both in premenopausal and post menopausal states.

There is an elevation of Alkaline phosphatase activity in cancers especially those of bone, liver, breast and blood (Moriyama et al., 1983). A reduced bone turnover is seen as a reduction in Alkaline phosphatase level, especially in post menopausal women who are prone to osteoporosis (Coe et al., 1986). Serum Alkaline phosphatase is increased during metastatic neoplasms (Murray et al., 1988). A reduction of dietary fat intake might help to delay or even prevent development of metastases (Wynder and Cohen, 1982). An increase in Alkaline phosphatase is seen in patients with secondary deposits in bone (Varley et al., 1980).

The results of the present study show no significant change in serum Alkaline phosphatase activity between breast cancer patients and the controls, suggesting that there has been no metastases of the cancer. That there was no metastases was also confirmed by the Hospital records.

7. Serum Immunoglobulin levels in breast cancer patients and controls.

Table X shows the mean levels of immunoglobulins in breast cancer patients and controls.

TABLE X

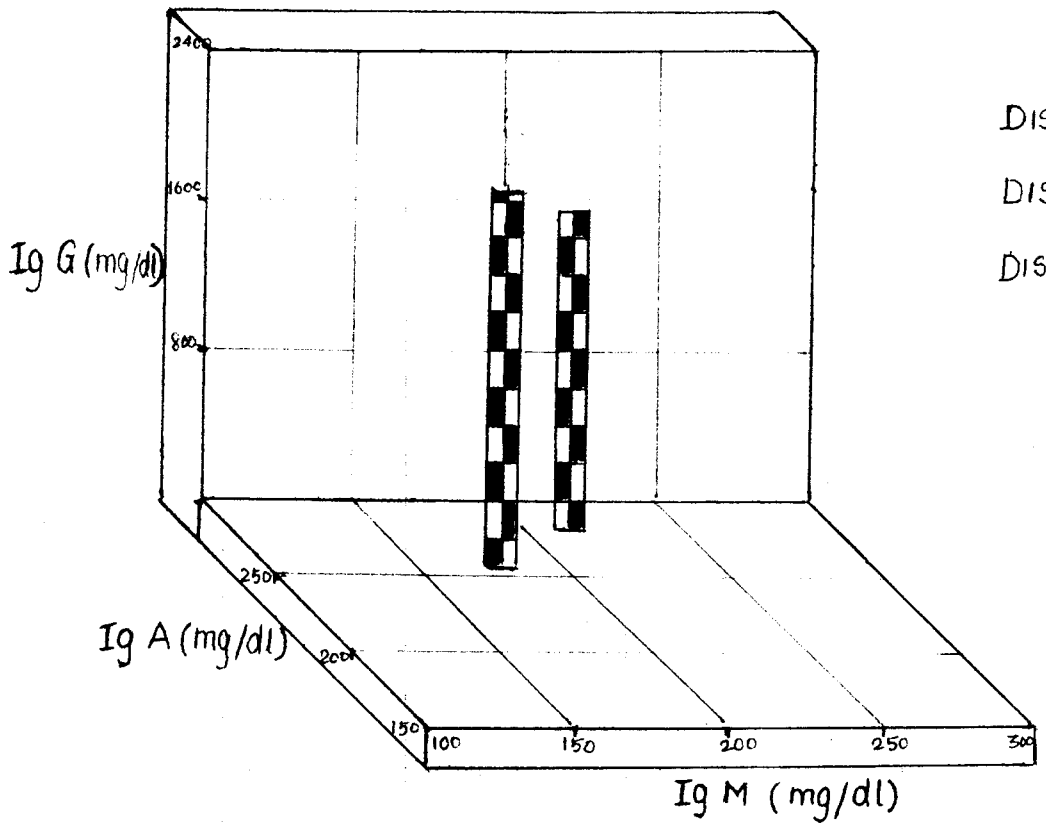
MEAN LEVELS OF IMMUNOGLOBULINS IN BREAST CANCER PATIENTS AND CONTROLS

(Mean mg/dl)

Participants	Immunoglobulins		
	Ig^M	Ig^A	Ig^G
Breast Cancer	213.4	281.2	1805.7
Controls	181.27	254.9	1975.6

In the breast cancer patients, the mean levels of IgM, IgA and IgG are 213.4 mg/dl, 281.2 mg/dl and 1805.7 mg/dl respectively, while in the control group the mean levels of these immunoglobulins are 181.27 mg/dl, 254.9 mg/dl and 1975.6 mg/dl respectively. Thus the mean levels of IgM and IgA are found to be higher in the breast cancer patients compared to the controls while the mean levels of IgG is found to be lower in breast cancer patients compared to the

FIG.7. MEAN LEVELS OF IMMUNOGLOBULINS IN BREAST CANCER PATIENTS AND IN CONTROLS



DISTANCE ALONG X AXIS DENOTES Ig M

DISTANCE ALONG Y AXIS DENOTES Ig A

DISTANCE ALONG Z AXIS DENOTES Ig G

CONTROL

BREAST CANCER PATIENTS

PLATE I

Due to high cost and unavailability, the Immunoglobulin plates were shared and only wells 1,2,9,10,11 and 12 were used for the study.

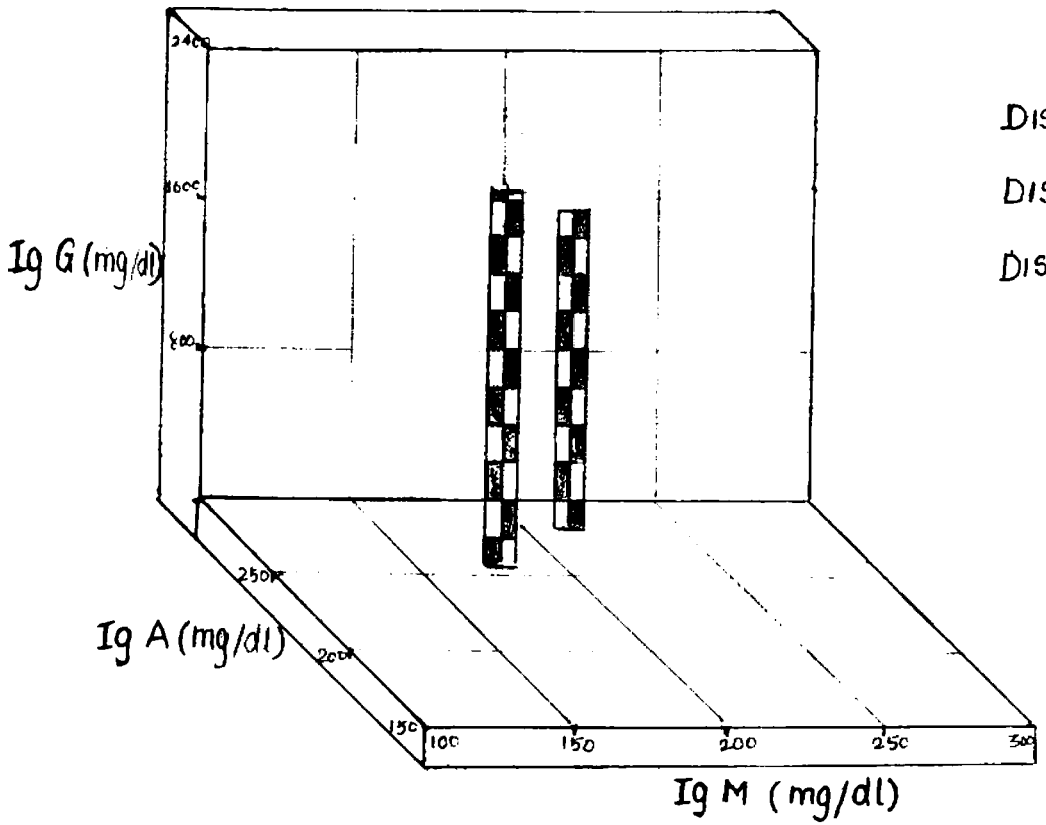
PLATE II

Due to high cost and unavailability, the Immunoglobulin plates were shared and only wells 1,2,9,10,11 and 12 were used for the study.

PLATE III

Due to high cost and unavailability, the Immunoglobulin plates were shared and only wells 1,2,9,10,11 and 12 were used for the study.

FIG.7. MEAN LEVELS OF IMMUNOGLOBULINS IN BREAST CANCER PATIENTS AND IN CONTROLS



DISTANCE ALONG X AXIS DENOTES Ig M

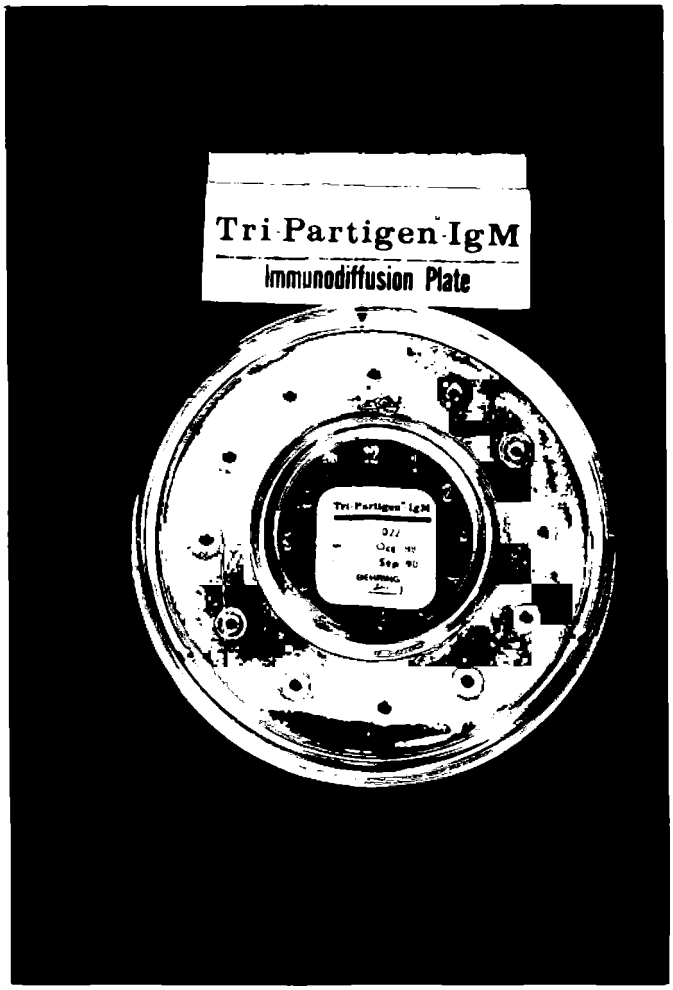
DISTANCE ALONG Y AXIS DENOTES Ig A

DISTANCE ALONG Z AXIS DENOTES Ig G

CONTROL

BREAST CANCER PATIENTS

PLATE I

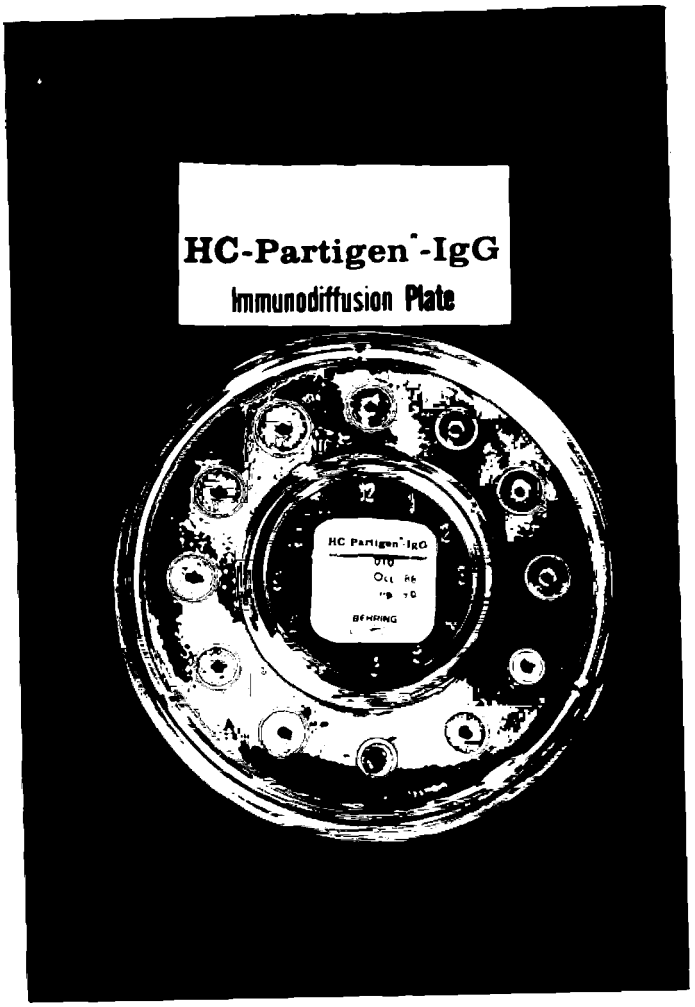


Due to high cost and unavailability, the Immunoglobulin plates were shared and only wells 1,2,9,10,11 and 12 were used for the study.

PLATE II

Due to high cost and unavailability, the Immunoglobulin plates were shared and only wells 1,2,9,10,11 and 12 were used for the study.

PLATE III



Due to high cost and unavailability, the Immunoglobulin plates were shared and only wells 1,2,9,10,11 and 12 were used for the study.

controls. IgM level ranges between 94.49 mg/dl and 287.28 mg/dl in the breast cancer patients and between 207.94 mg/dl and 224.32 mg/dl in the control group. IgM level ranges between 149.40 mg/dl and 338.40 mg/dl in the breast cancer patients and between 217.70 mg/dl and 338.40 mg/dl in the controls. IgG level ranges between 1500 mg/dl and 2125 mg/dl in the breast cancer patients and between 1618.3 mg/dl and 2058.8 mg/dl in the controls.

Initial immunocompetence determined by parameters of cell mediated immunity shows strong prognostic association with the subsequently observed course of breast cancer (Alder et al., 1980). Campbell et al (1986) reported no increased serum antibody titre in breast cancer relative to controls. The relationship between poor nutritional status and increased risk and morbidity from infectious diseases have been long recognised (Miller, 1987). A significant reduction of homologous tumour antigen recognition as measured by Leukocyte Adherence Inhibition-Cell Mediated immunity is seen in all advanced stage breast cancer patients. Patients with such serum blocking factors showed significant increase in IgG immune complexes, IgM, and IgA (Tsang et al., 1988). The altered immunoglobulin levels in breast cancer patients, as shown by the present study, may be due to altered immunity in these patients.

8. Hormonal status in breast cancer patients and controls

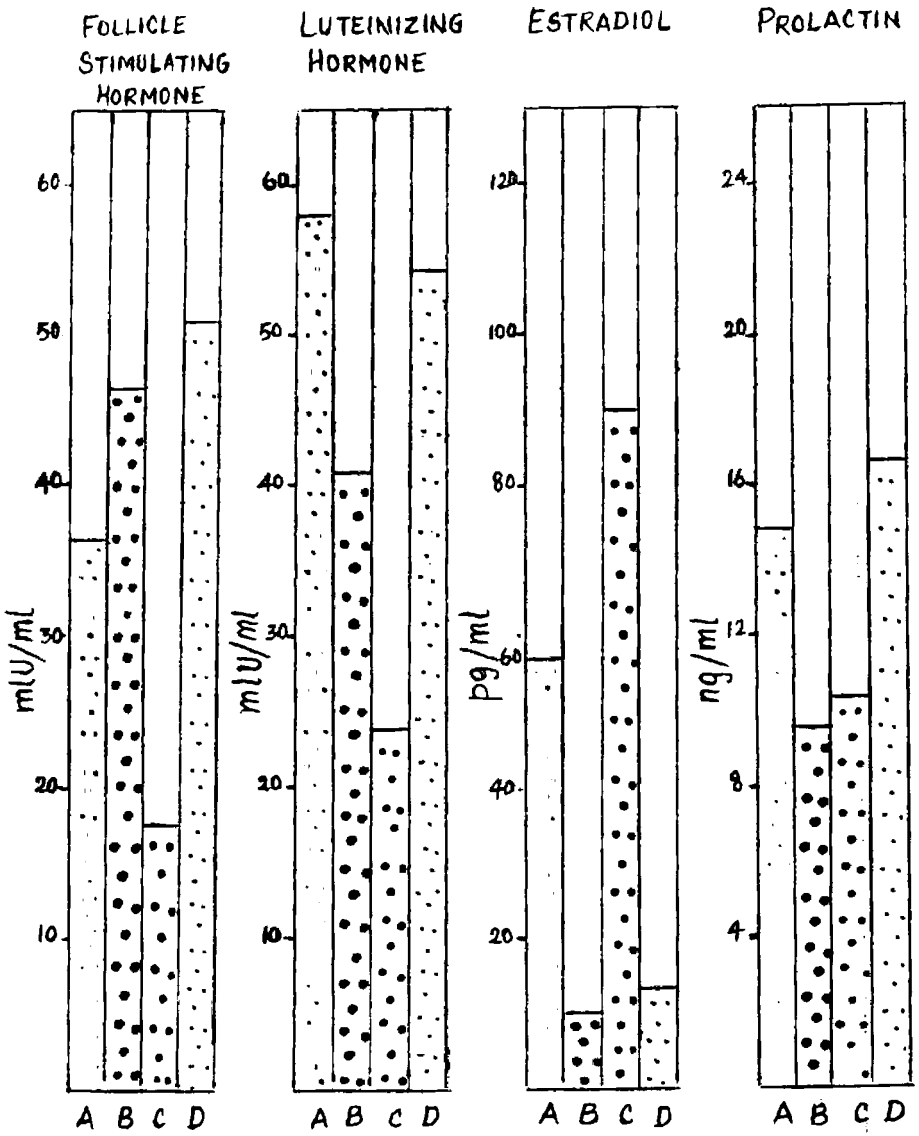
Table XI gives the levels of the Follicle stimulating hormone , Luteinizing hormone, Estradiol and Prolactin in breast cancer patients and in controls in pre and post menopausal states.

TABLE XI

MEAN LEVELS OF HORMONES IN BREAST CANCER PATIENTS AND CONTROLS

Hormones	Breast Cancer		Control	
	Premenopause	Post meno pause	Pre meno pause	Post meno pause
Follicle stimulating hormone (FSH) (mlu/ml)	36.64 (n=16)	46.81 (n=9)	17.5 (n=2)	51.33 (n=3)
Luteinizing hormone (LH) (mlu/ml)	57.9 (n=14)	41 (n=5)	27.7 (n=3)	54.5 (n=2)
Estradiol (pg/ml)	57.2 (n=5)	10.3 (n=3)	90 (n=3)	12.7 (n=3)
Prolactin (ng/ml)	14.8 (n=17)	9.63 (n=6)	10.3 (n=3)	16.5 (n=2)

FIG.8. MEAN LEVELS OF HORMONES IN BREAST CANCER PATIENTS AND CONTROLS IN PRE AND POST MENOPAUSAL STATES



A PREMENOPAUSAL BREAST CANCER PATIENTS

B POSTMENOPAUSAL BREAST CANCER PATIENTS

C PREMENOPAUSAL CONTROL

D POSTMENOPAUSAL CONTROL

Follicle stimulating hormone (FSH)

The level of FSH in the premenopausal breast cancer patients (36.64 mlu/ml) is less than the FSH level of the post menopausal breast cancer patients (46.81 mlu/ml). The level of FSH in the post menopausal control group (51.33 mlu/ml) is higher than the FSH level of pre menopausal controls (17.5 mlu/ml). The FSH level of the pre menopausal breast cancer patients (36.64 mlu/ml) is higher than that of the control group belonging to the same menopausal status (17.5 mlu/ml). The FSH level of the post menopausal controls (51.33 mlu/ml) is higher than that of the breast cancer patients belonging to the same menopausal status (46.81 mlu/ml).

Luteinizing hormone (LH)

The level of LH in the premenopausal breast cancer patients (57.9 mlu/ml) is higher than that of the controls belonging to the same menopausal status (27.7 mlu/ml). The level of LH in the post menopausal controls (54.5 mlu/ml) is higher than that of the post menopausal breast cancer patients (41 mlu/ml).

Within the breast cancer patients, those belonging to the pre menopausal status have a higher level of LH than those belonging to the post menopausal status (57.9 mlu/ml and 41 mlu/ml respectively). Among the controls, those belonging to the pre menopausal status show a lower level of LH than those belonging to the post meno-

pausal status (27.7 mlu/ml and 54.5 mlu/ml respectively).

Estradiol

The level of estradiol in the pre menopausal breast cancer patients (57.2 pg/ml) is higher than that of the post menopausal breast cancer patients (10.3 pg/ml). Pre menopausal control subjects showed a higher level of estradiol (90 pg/ml) when compared to the post menoapusal control subject (12.7 pg/ml). Pre menopausal controls have a higher level of estradiol (90 pg/ml) than the pre menopausal breast cancer patients (57.2 pg/ml). Post menopausal breast cancer patients showed a lesser amount of estradiol (10.3 pg/ml) when compared to the post menopausal control subject (12.7 pg/ml). Moore et al (1982) found that the percentage of free estradiol was increased in the serum of post menopausal breast cancer patients, while Adami et al., (1979) indicated that the plasma estrogen levels are higher in post menopausal breast cancer patients than in controls.

Prolactin

The prolactin level of pre menopausal breast cancer patients is higher (14.8 ng/ml) than that of the post menopausal breast cancer patients (9.63 ng/ml). The prolactin level of pre menopausal controls is less (10.3 ng/ml) than that of the post menopausal controls (16.5 ng/ml). The level of prolactin in the pre menopausal

breast cancer patients (14.8 ng/ml) is higher than that in the pre menopausal controls (10.3 ng/ml) and the level of prolactin in post menopausal controls (16.5 ng/ml) is higher than that of the post menopausal breast cancer patients (9.63 ng/ml). Prolactin has as important role in the development and growth of some experimental mammary carcinomas (Manni et al., 1977). Aldinger et al., (1978) reported an increased level of prolactin in breast cancer patients. Rose and Pruitt (1981) also reported a similar result.

The changes in hormone status may thus serve as indicators of risk or as stools to investigate the mechanism of carcinogenesis of the breast.

9. Qualitative analysis of urine of breast cancer patients and controls.

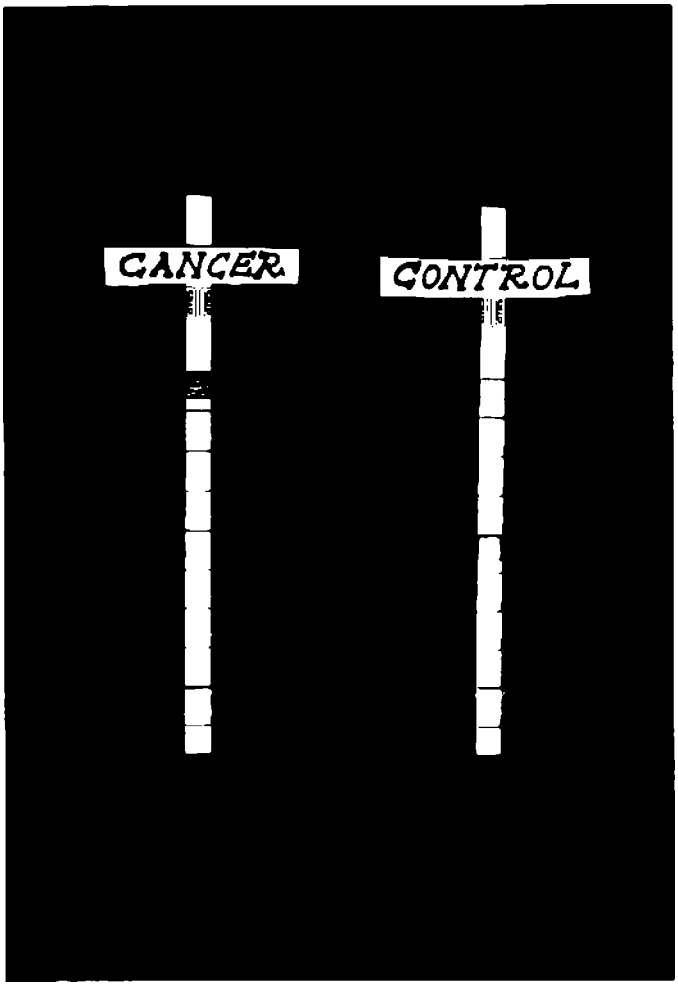
Table XII shows the results of the qualitative analysis of urine of breast cancer patients and controls.

TABLE XII RESULTS OF QUALITATIVE ANALYSIS OF URINE

	GLUCOSE	BILIRUBIN	KETONE	SPECIEIC GRAVITY	BLOOD	PH	PROTEIN	UROBILINOGEN	NITRITE	LEUKOCYTES
1	-	-	5mg/dl	1.025	-	6.0	Trace	0.2	+	Small +
2	-	Small +	5mg/dl	1.020	Non Hemolysed	6.0	Trace	0.2	++	Trace
3	-	-	-	1.020	Non Hemolysed trace	5.0	Trace	0.2	++	Small +
4	-	Small +	5mg/dl	1.015	-	6.0	Trace	0.2	++	Small +
5	-	-	-	1.02	-	5.0	Trace	0.2	-	Small +
6	-	-	5mg/dl	1.02	-	5.0	-	0.2	++	Small +
7	100mg/dl	Small+	-	1.02	Non Hemolysed	5.0	Trace	0.2	-	-
8.	100mg/dl	-	5mg/dl	1.02	-	5.0	-	0.2	+	Small +
9	-	-	-	1.015	-	6.0	-	0.2	+	Trace
10	-	-	-	1.015	-	6.0	-	0.2	+	Trace

1-8 are the results of breast cancer patients; 9 and 10 that of controls.

PLATE IV



MULTISTIX USED FOR QUALITATIVE ANALYSIS OF URINE

Except two breast cancer patients, all the other patients and the controls showed no glucose in the urine. The controls and five breast cancer patients showed no bilirubin in their urine, while two breast cancer patients showed the presence of bilirubin in urine.

Three breast cancer patients and the controls showed no ketone body in their urine, while five breast cancer patients showed the presence of ketone in their urine. The urine specific gravity of the two controls was 1.015, while that of the breast cancer patients was between 1.015 - 1.025. Only three breast cancer patients showed the presence of hemolysed blood while the other participants of the study showed the absence of blood in urine. The urine pH of the controls was 6.0. Three breast cancer patients showed a urine pH of 6.0, while four of them showed a urine pH of 5.0.

There was no protein in the urine of the controls and in the urine of one breast cancer patient, while all the other breast cancer patients showed traces of protein in their urine. All the urine samples showed the presence of urobilinogen. Except two breast cancer patients, all other breast cancer patients and controls showed the presence of nitrite in the urine. Leukocytes were present in the urine of all controls and all but one breast cancer patient.

10. Comparison of the blood constituents in the breast cancer patients and controls irrespective of age group and menopausal status.

Table XIII gives the comparison of the blood constituents in the breast cancer patients and controls irrespective of age group and menopausal status.

TABLE XIII

MEAN LEVELS OF BLOOD CONSTITUENTS IN THE BREAST CANCER PATIENTS AND CONTROLS

(Mean ± S. D)

Blood constituents	A Breast Cancer patients	B Controls	Groups compared	
Vitamin A (mg/dl)	0.015±0.0044	0.024±0.0076	A Vs B	**
Vitamin C (mg/dl)	0.368±0.086	0.756±0.122	A Vs B	**
Cholesterol (mg/dl)	327+94.9	169.24±26.85	A Vs B	**
Alkaline phosphatase (KA units/dl)	10.38±3.16	10.95±2.6	A Vs B	NS
Hormones				
Follicle stimulating hormone (mlu/ml)	30.54±43.12	37.8±18.62	A Vs B	NS
Luteinizing hormone (mlu/ml)	52.38±69.17	17.56±17.56	A Vs B	NS
Estradiol (pg/ml)	39.63±46.64	51.33±44.23	A Vs B	NS
Prolactin (ng/ml)	13.46±8.19	12.8±4.55	A Vs B	NS

NS = Not significant ** = Significant at 1% level

There is a significant increase at 1 per cent level in the mean levels of Vitamins A and C in the controls, compared to the breast cancer patients. The level of Cholesterol in the breast cancer patients is higher than that in the controls ($p < 0.01$). There is no significant difference in the Alkaline phosphatase activity in both controls and breast cancer patients. There is no statistically significant difference in the mean levels of the serum hormones in breast cancer patients and in controls.

To summarize, the low levels of the Vitamins in breast cancer patients may be due to a poor dietary intake of the Vitamins or due to poor absorption of these Vitamins in cancer. An increased fat intake increases serum Cholesterol level and this might increase the risk of breast cancer. There was no increase in Alkaline phosphatase activity in the breast cancer patients suggesting that there was no extensive metastases. This was clinically confirmed by the hospital records. An increased level of IgM and IgA and a decreased level of IgG in breast cancer patients compared to the normals may be due to an impaired immunity in the breast cancer patients. In pre menopausal breast cancer patients an increase in the mean levels of FSH, LH and prolactin and a decrease in the mean level of estradiol and a decrease in the mean levels of these hormones in post menopausal breast cancer patients reflect an association between carcinogenesis of the breast and the female sex hormones.

Summary and Conclusion

V SUMMARY AND CONCLUSION

The present study was an attempt to evaluate the biochemical changes in the blood constituents, especially, Vitamins A and C, Cholesterol, Alkaline phosphatase, Immunoglobulins IgM, IgA, IgG and hormones - Follicle stimulating hormone, Luteinizing hormone, Prolactin and Estradiol, in breast cancer. The effect of age and menopausal status on these constituents in breast cancer was studied and compared with those of normal healthy women taken as controls. The urine of the breast cancer patients was qualitatively analysed and the results were compared with those of normal healthy women. The results of the study are summarised as follows:

Thirty one breast cancer patients, all in the advanced cancer stage, were taken for the study. Age matched healthy females were taken as controls.

1. There was a significant decrease in the serum Vitamin A levels in the breast cancer patients when compared to healthy controls. Between the breast cancer patients there was no significant difference in the serum Vitamin A levels in the various age groups. Similarly, no significant change in serum Vitamin A levels was seen in the different age groups in the controls. A significant increase at 5 per cent level was seen in the age group 36-45 years where the serum Vitamin A of the controls (0.025 ± 0.007 mg/dl) was higher than that of the breast cancer patients (0.014 ± 0.005 mg/dl) belonging to the same age group. A significant increase at 1 per cent level was

seen in the age group 46-55 years, in which the serum Vitamin A level of the controls (0.023 ± 0.008 mg/dl) was higher than that of the breast cancer patients belonging to the same age group (0.015 ± 0.004 mg/dl). Menopausal status did not show any difference in the serum Vitamin A levels in breast cancer patients and in controls. But the mean serum Vitamin A levels of the pre menopausal controls (0.025 ± 0.007 mg/dl) was higher than that of the premenopausal breast cancer patients (0.016 ± 0.004 mg/dl) ($p < 0.01$) and the mean serum Vitamin A levels of the post menopausal controls (0.023 ± 0.008 mg/dl) was higher than that of the postmenopausal breast cancer patients (0.016 ± 0.004 mg/dl) ($p < 0.05$). This decrease in the mean serum Vitamin A levels in breast cancer patients might be mainly due to inadequate intake of the Vitamin.

2. There was a significant increase in the mean levels of serum Vitamin C in the breast cancer patients belonging to the age group 46-55 years (0.40 ± 0.07 mg/dl) than those belonging to the age group 56-65 years (0.32 ± 0.05 mg/dl) ($p < 0.05$). No significant increase was seen between the other age groups. There was a significant increase in the mean serum Vitamin C levels in the controls belonging to the age group 36-45 years (0.81 ± 0.06 mg/dl) than those belonging to the age group 46-55 years (0.67 ± 0.15 mg/dl) ($p < 0.05$). The mean levels of Vitamin C of the controls belonging to the age group 36-45 years (0.81 ± 0.06 mg/dl) was higher than that of the breast cancer patients (0.36 ± 0.09 mg/dl) belonging to the same age group ($p < 0.0$).

There was a similar increase in the mean levels of Vitamin C of the controls belonging to the age group 46-55 years (0.67 ± 0.15 mg/dl) than the breast cancer patients belonging to the same age group (0.40 ± 0.07 mg/dl) ($p < 0.01$). There was no change in the mean levels of Vitamin C between the two menopausal states of breast cancer patients. But the mean levels of Vitamin C of the premenopausal controls (0.81 ± 0.06 mg/dl) was higher than those of the breast cancer patients (0.38 ± 0.09 mg/dl) belonging to the same menopausal state ($p < 0.01$). The mean levels of Vitamin C of the post menopausal controls (0.67 ± 0.15 mg/dl) was higher than that in the post menopausal breast cancer patients (0.34 ± 0.09 mg/dl) ($p < 0.01$). But the mean levels of Vitamin C in premenopausal controls (0.81 ± 0.06 mg/dl) was higher than that of the post menopausal controls (0.67 ± 0.15 mg/dl) ($p < 0.01$). This decrease of mean serum Vitamin C levels in breast cancer patients might be due to poor dietary intake of the vitamin.

3. There was no significant change in the mean levels of Cholesterol between the different age groups of breast cancer patients and controls. But the mean levels of Cholesterol in the breast cancer patients belonging to the age group 36-45 years (328.9 ± 89.7 mg/dl) was higher than that of the controls belonging to the same age group (171.1 ± 20.3 mg/dl) ($p < 0.01$). Similarly in the age group 46-55 years the mean levels of Cholesterol in the breast cancer patients (321 ± 108.8 mg/dl) was higher than that in the controls

(166.4 ± 36.7 mg/dl) belonging to the same age group ($p < 0.01$). There was no significant difference in the mean Cholesterol levels of pre and post menopausal controls and in the mean Cholesterol levels of pre and post menopausal breast cancer patients. The mean levels of Cholesterol in pre menopausal breast cancer patients (337.3 ± 91.1 mg/dl) was higher than that of the pre menopausal controls (171.1 ± 20.3 mg/dl) ($p < 0.01$). Similarly, the mean levels of Cholesterol in post menopausal breast cancer patients was higher (268 ± 105.6 mg/dl) than that of the post menopausal controls (166.4 ± 36.7 mg/dl) ($p < 0.05$). This high level of Cholesterol in breast cancer patients might be due to their high fat diet.

4. There was no significant difference in the mean levels of Alkaline phosphatase activity in the different age groups in breast cancer and in the controls. But the mean levels of Alkaline phosphatase activity of the controls belonging to the age group 46-55 years (11.5 ± 1.5 KA units/dl) was higher than that in the breast cancer patients belonging to the same age group (9.9 ± 2.3 KA units/dl) ($p < 0.05$). There was no significant difference in the mean levels of Alkaline phosphatase activity in the pre and post menopausal states in breast cancer patients and in controls. This shows that there has been no metastases of the cancer.

5. The present study indicated that the serum level of IgM in breast cancer patients (213.4 mg/dl) was higher than that in the controls (181.27 mg/dl); the serum IgA levels in breast cancer patients (281.2 mg/dl) was higher than that in controls (254.9 mg/dl); but the serum IgG levels of the breast cancer patients (1805.7 mg/dl) was less than that in the controls (1975.6 mg/dl). This change in the immunoglobulin levels may be due to the altered immunity in the breast cancer patients.

6. In the present study the mean serum levels of hormones namely Follicle stimulating hormone, Luteinizing hormone, Prolactin and Estradiol in pre and post menopausal states were compered in breast cancer patients and in normal healthy controls. Only selected participants were taken for the hormonal study because of the unavailability of the Radio immuno assay kits and their high cost.

The FSH levels of premenopausal breast cancer patients (36.64 mlu/ml) was less than that of the post menopausal breast cancer patients (46.81 mlu/ml) but higher than that of the premenopausal controls (17.5 mlu/ml). Post menopausal controls showed a higher level of FSH (51.33 mlu/ml) when compared to premenopausal controls (17.5 mlu/ml) and post menopausal breast cancer patients (46.81 mlu/ml).

Premenopausal breast cancer patients had a higher level of LH (57.9 mlu/ml) than the post menopausal breast cancer patients (41 mlu/ml); while the pre menopausal controls had a lower level of LH (27.7 mlu/ml) than the post meno-

pausal controls (54.5 mlu/ml). The level of LH in pre menopausal breast cancer patients was higher (57.9 mlu/ml) than that of the premenopausal controls (27.7 mlu/ml) and the level of LH in the post menopausal breast cancer patients was lower (41 mlu/ml) than that of the post menopausal controls (54.5 mlu/ml).

Estradiol levels of premenopausal breast cancer patients (57.2 pg/ml) was lower than the estradiol level of premenopausal controls (90 pg/ml) but higher than that of the post menopausal breast cancer patients (10.3 pg/ml). Post menopausal controls showed a lower level of estradiol (12.7 pg/ml) when compared to thatof the premenopausal controls (90 pg/ml). But the level was greater than the estradiol level of post menopausal breast cancer patients (10.3 pg/ml).

The prolactin level of pre menopausal breast cancer patients was higher (14.8 ng/ml) than that of the post menopausal breast cancer patients and pre menopausal controls (9.63 ng/ml and 10.3 ng/ml respectively). The level of prolactin in post menopausal controls was higher (16.5 ng/ml) than that of the pre menopausal controls and post menopausal breast cancer patients (10.3 ng/ml and 9.63 ng/ml respectively).

The changes in hormone status may thus serve as indicators of risk or as tools to investigate the mechanism of carcinogenesis of breast.

7. Qualitative analysis of urine of breast cancer patients and controls showed that two patients had mild glucosuria. Two breast cancer patients showed the presence of bilirubin in their urine. Five breast cancer patients had mild ketonuria. Most of the breast cancer patients had a slight acidic urine. Three breast cancer patients showed blood in their urine. Traces of protein was found in the urine of seven breast cancer patients. All the urine samples analysed, including the controls, showed the presence of urobilinogen. Most of the urine samples had nitrite and leukocytes.

The results of the present study showed a poor Vitamin status, an increased Cholesterol level and an impaired immune profile in breast cancer patients. These results suggest that there is an influence of diet in the etiology of the carcinogenesis of the breast. Hence it may be suggested that by improving the nutritional status of the breast cancer patients, the severity of the disease can be checked. There was no increase in Alkaline phosphatase activity suggesting that there was no extensive metastases in the breast cancer patients. This was clinically confirmed by the hospital records. The pre menopausal breast cancer patients showed an increased level of the female sex hormones and the post menopausal breast cancer patients showed a decreased level of these hormones. The changes in the ovarian hormones in breast cancer reflect an association between carcinogenesis of breast and these hormones. It may be suggested that hormonal imbalance can act as a risk factor in promoting cancer.

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Appendix

APPENDIX - I

QUESTIONNAIRE

1. NAME
2. AGE
3. OCCUPATION
4. INCOME
5. RELIGION
6. MARITAL STATUS
7. PAST HISTORY OF BREAST CANCER
8. FAMILY HISTORY ABOUT BREAST CANCER
9. PERSONAL HABITS - Chewing/Smoking/Alcohol
10. FOOD HABITS - Vegetarian/Non-vegetarian
11. AGE AT MENARCHE
12. MENSTRUAL CYCLE
13. LAST MENSTRUAL CYCLE
14. NUMBER OF CHILDREN
15. AGE AT FIRST CHILD BIRTH
16. AGE AT LAST CHILD BIRTH
17. NUMBER OF ABORTIONS - Spontaneous/Induced
18. LAST ABORTION - Artificial/Natural
19. MENOPAUSE
20. DETAILS OF FAMILY PLANNING
21. HISTORY OF BREAST FEEDING
22. STAGE OF THE CANCER

APPENDIX II

DETERMINATION OF RETINOL IN SERUM USING THE CARR-PRICE REACTION (Varley et al., 1980).

Proteins are precipitated with ethanol and the retinol and carotenes extracted into light petroleum. After reading the intensity of the yellow colour due to the carotenes, the light petroleum is evaporated off and the residue dissolved in chloroform. Carr-Price reagent is added and the amount of blue colour produced is read. Since carotenes also give some colour a correction for this is made in order to obtain that due to retinol present.

REAGENTS:

1. Absolute Ethanol
2. Light petroleum bp 40°C - 60°C
3. A cylinder of carbondioxide
4. Chloroform
5. Acetic anhydride, use good quality analytical reagent.
6. Carr-Price reagent: Antimonyl chloride, 250g/l chloroform. Keep at room temperature in a tightly stoppered brown bottle filtering before use if necessary.

PROCEDURE:

Pipetted 3ml of serum into a stoppered centrifuge tube and added 3ml absolute ethanol, slowly drop by drop with shaking, in order to obtain a finely divided precipitate of protein. Added 6ml of light petroleum and shook vigorously for 10min, then centrifuged at a low speed for 1min.

Pipetted off as much as possible of the light petroleum layer taking care not to remove any of the watery layer with it.

DETERMINATION OF RETINOL:

Measured 4ml of the light petroleum solution, prepared as above into one of the colorimeter tubes and evaporated off the solvent by placing in a water bath at 40-50°C, preferably passing a stream of dry carbondioxide over the solvent surface. Dissolved the residue in 0.5ml of chloroform and added a drop of acetic anhydride to remove traces of water, which might cause cloudiness when the Carr-Price reagent is added. Zero the colorimeter with chloroform using an orange filter at transmission at 620nm, and with the tube in position, added quickly 3ml of Carr-Price reagent from a pipette with a wide tip. The colour developed rapidly reaching a clearly defined maximum in 5-15 seconds and then fades quickly. Noted this maximum reading of the colorimeter. A rather more stable colour is obtained if the Carr-Price reagent is cooled to 0-5°C before use.

It is very important that the glass wares used should be perfectly dry. This also applies to the chloroform and the Carr-Price reagent. When small amounts of water are present it may be impossible to read the colour.

Retinol standard curve

Prepare a solution containing 4.0mg retinol/l in Chloroform and set up a series of tubes:

Serum retinol (mg/l)	0	0.10	0.20	0.40	0.06	0.80	1.0
Retinol Solution (ml)	0	0.05	0.10	0.20	0.30	0.40	0.50
Chloroform	0.50	0.45	0.40	0.30	0.20	0.10	0

Add 3 ml of Carr-Price reagent and read as described.

APPENDIX III

DETERMINATION OF ASCORBIC ACID IN PLASMA BY 2,6 DICHLORDPHENOL INDOPHENOL TITRATION (Varley et al., 1980)

REAGENTS:

1. Trichloroacetic acid 100g/l (or) freshly prepared metaphosphoric acid 50g/l.

2. Solution of 2,6 dichlorophenol indophenol-weigh out accurately 40mg of the dye. 1.0ml of this solution is equivalent to 0.2mg ascorbic acid. Since the keeping qualities of the solution are poor, prepare freshly at frequent intervals. It should not be kept in use for more than a week.

The solution of dye can be checked against an ascorbic acid solution of known solution. Dissolve 40mg of ascorbic acid in 100ml of Acetic acid (100ml Glacial acetic acid diluted to one litre with water). Dilute 5ml of this to 100ml with acetic acid. Titrate 0.5ml of the dye with this solution. 5.0ml should be required to decolourise it.

Dilute 5.0ml of the dye to 25ml so that 1ml is equivalent to 40 g ascorbic acid.

PROCEDURE:

Mixed equal volumes (4ml is convenient) of plasma separated immediately after withdrawing the blood and of the TCA or metaphosphoric acid. Filtered or centrifuged;pipetted 0.2ml of the diluted dye solution into a testube and titrated with

the filtrate until the reddish colour has disappeared.

CALCULATION:

Since 0.2ml of the dye is equivalent to 8mg ascorbic acid

Plasma ascorbic acid (mg/l)

$$= \frac{1000}{\text{ml titration}} \times \frac{8}{1000}$$

$$= \frac{16}{\text{ml titration}}$$

NOTES:

1. Whilst metaphosphoric acid has advantage over TCA even in the solid form, it keeps poorly once the reagent bottle has been opened. So it is usually more convenient to use TCA.

2. If the plasma cannot be separated blood can be preserved by the method used by Mindin and Butler (1938). The blood is collected into a test tube containing one drop each of potassium cyanide (50g/l) and Potassium oxalate (200g/l) for 4 to 5ml of blood.

APPENDIX IV

ESTIMATION OF CHOLESTEROL

ZAK'S METHOD (Varley et al., 1980)

PRINCIPLE:

Cholesterol reacts with ferric chloride in the presence of concentrated sulphuric acid to give a pink colour. The intensity of the colour developed is directly proportional to the amount of cholesterol present and is read at 540mμ in a colourimeter.

REAGENTS:

1. Stock Ferric chloride reagent: 840mg of pure dry ferric chloride was weighed and dissolved in 100ml of Glacial acetic acid.

2. Ferric chloride precipitating reagent: 10ml of the stock ferric chloride reagent was made upto 100ml with glacial acetic acid.

3. Ferric chloride diluting reagent: 8.5ml of the stock ferric chloride reagent was diluted to 100ml with glacial acetic acid.

4. Standard cholesterol solution: 100mg of pure, dry, cholesterol was accurately weighed and dissolved in glacial acetic acid and made upto 100ml with the same.

5. Working standard: 10ml of the stock standard was placed in a 100ml standard flask containing 0.85ml of Ferric chloride stock reagent and made upto the mark with glacial acetic acid. 10ml of this solution contains 100 μg of cholesterol.

PROCEDURE:

0.5-2.5 ml of the working standard solution, corresponding to µg values of 50-250, were pipetted out into clean, dry test tubes. The volume of each tube was made upto 5.0ml with ferric chloride diluting reagent.

To 0.1ml of the serum added 4.9ml of ferric chloride precipitating reagent and mixed well. Allowed to stand for a while and centrifuged. Transferred 2.5ml of the clear supernatant to a dry test tube. Made up the volume to 5.0ml with the ferric chloride diluting reagent. Placed the tubes in cold water and added 4.0ml of concentrated sulphuric acid, drop by drop. The solutions were mixed well. The tubes were allowed to come to room temperature. A blank was also simultaneously prepared by taking 5.0ml of the diluting reagent and 4.0ml of concentrated sulphuric acid. After 30min, the intensity of the colour developed was read at 540mµ against the blank.

APPENDIX - V

DETERMINATION OF SERUM ALKALINE PHOSPHATASE METHOD OF KING AND ARMSTRONG (Varley et al., 1980)

In this method, disodium phenyl phosphate is hydrolysed with the liberation of phenol and formation of sodium phosphate. The amount of phenol so formed is estimated colorimetrically.

REAGENTS

1. Disodium phenyl phosphate, 0.01M. Dissolve 1.09g in water and make up to 500ml. Bring quickly to the boil. Cool, add a little chloroform and keep in the refrigerator.

2. Sodium carborate - Sodium bicarbonate buffer, 0.1M. Dissolve 3.18g of anhydrous sodium carbonate and 1.68g of sodium bicarbonate in water and make upto 500ml.

3. Buffered substrate for use. Prepare by mixing equal volumes of solutions 1 and 2. This has a pH of 10.

4. Phenol reagent of Folin and Ciocalteu. Dissolve 100g of sodium tungstate and 25g of sodium molybdate in about 700ml of water in a two litre round bottomed flask. Add 50ml of syrupy 85 percent phosphoric acid and 100ml of concentrated hydrochloric acid. Reflux for ten hours. Use all-glass apparatus if possible, otherwise wrap the stopper in tin foil. Add 150g of lithium sulphate, 50ml of water and a few drops of bromine. Boil without a condenser for about a quarter of an hour to remove the

excess of bromine. Cool, make up to a litre and filter. The solution should have no greenish tint. This is an improved Phenol reagent which reduces the possibility of turbidity due to insoluble urates.

Dilute this solution 1 in 3 for use in this determination, that is to 1 volume of Phenol reagent add 2 volumes of water.

5. 20% Sodium carbonate solution. Dissolve 20g of anhydrous sodium carbonate in water and make up to 100ml. This solution is almost saturated at room temperature and so should be kept in a warm place to prevent the salt from crystallizing out.

6. Standard Phenol solution. Stock solution. 100mg of phenol per 100ml of solution. Dissolve 1g of pure crystalline phenol in 0.1N hydrochloric acid and make up to 1 litre with the acid. This can be standardized as follows: Place 25ml into a 250ml flask. Add 50ml of 0.1N sodium hydroxide and heat to 65°C. To the hot solution add 25ml of 0.1N iodine. Stopper and allow to stand for thirty to fourty minutes. Add 5ml of concentrated hydrochloric acid and titrate the excess iodine with 0.1N sodium thiosulphate. Each ml of 0.1N iodine is equivalent to 1.57mg of phenol.

7. Diluted phenol standard for use. Dilute the stock standard 1 in 10 to obtain a standard solution containing 10mg phenol per 100ml of solution.

8. Standard phenol solution and reagent, containing 0.5mg

phenol per 100mg. Take 2.5ml of the dilute standard, add 15ml of the dilute phenol reagent and make up to 50ml with water. This is best prepared daily.

TECHNIQUE

Pipetted 6.0ml of the buffer substrate into a test tube and placed in a water bath at 37°C for a few minutes. Added 0.3ml of serum, preferably without removing from the bath. Mixed and corked and allowed to remain in the bath for exactly fifteen minutes. Then removed and immediately added 2.7ml of the diluted phenol reagent. At the same time set up a tube for control containing 6.0ml of substrate and 0.3ml of serum, to which is added immediately 2.7ml of diluted phenol reagent. Mixed well in both cases and centrifuged.

Took 4.0ml of supernatant fluid from each and added 1.0ml of 20% sodium carbonate. Put up a standard prepared by adding 1.0ml of sodium carbonate solution to 4.0ml of the standard phenol and reagent. Placed the three tubes in the 37°C water both for fifteen minutes and read in a colorimeter. As a blank took 2.8ml of water and added 1.2ml of diluted phenol reagent and 1.0ml of 20% sodium carbonate. A red filter is used, with transmission at 680nm.

APPENDIX - VI

QUANTITATIVE DETERMINATION OF THE IMMUNOGLOBULIN IgG. USING HC-PARTIGEN - IgG PLATE (Behringwarke, 1988)

COMPOSITION:

HC-partigen IgG Immunodiffusion plate contains a prepared agar gel in which H-chain specific antiserum to Human lgG is incorporated. The antiserum is produced by immunisation of sheep and goats.

PRESERVATIVES:

Sodium azide (1mg/ml)
Sodium P-ethyl-mercury-mercapto-benzene
Sulfonate (at most 0.1mg/ml)

PROCEDURE:

HC-partigen IgG is suitable for quantitative IgG determinations.

1. Opened the plate and left the opened plate to stand for about 5 minutes at room temperature to allow any condensation water that may have accumulated in the wells to evaporate.

2. Well 1 is filled with 5µl of control serum. Wells 2-12 are each filled with undiluted 5µl of the respective sera under test.

3. Closed the plate tightly and left it to stand at room temperature. Evaluation may be made after a minimum diffusion time of 50 hours.

4. At the end of the given diffusion time, the diameter D of the precipitin rings should be measured accurately to

0.1mm using a suitable calibrated instrument.

EVALUATION:

The immunoglobulin concentrations related to the measured diameters are read directly from the table of reference values. The results are reliable only when the value found for the control serum applied to well 1 lies within the confidence range taken from the table of values enclosed with each of the control serum. With Hoechst Behring control serum, the confidence range is ± 15% of the immunoglobulin concentration given with each pack.

If the protein concentrations of the serum samples diverge considerably from the normal value, this means that the resulting precipitin ring diameters will fall outside the assay range of the plate. In this case, the examination must be repeated using higher or lower dilutions of the serum sample.

APPENDIX -VII

QUANTITATIVE DETERMINATION OF IMMUNDGLOBULINS IgA and IgM USING TRI-PARTIGEN PLATES(Behringwerke,1988)

COMPOSITION:

Tri-Partigen-Immunodiffusion Plates contain a prepared agar gel in which H-chain specific antiserum to the respective immunoglobulin is incorporated. The antiserum is produced by immunization of sheep and goats.

PRESERVATIVES:

Sodium azide (1mg/ml)
Sodium P-ethyl-mercury-mercapto-benzene
Sulfonate (at most 0.1mg/ml)

PROCEDURE:

Tri-Partigen is suitable for quantitative immunoglobulin determinations.

1. Opened the plate and left the opened plate to stand for about 5 minutes at room temperature to allow any condensation water that may have accumulated in the wells to evaporate.

2. IgA and IgM are determined using undiluted serum.

3. Well 1 is filled with 5µl of control serum. Wells 2-12 are each filled with 5µl of the respective sera under test.

4. Closed the plate tightly and left it to stand at room temperature. Evaluation may be made after a minimum diffusion time of 50 hours (IgA) and 80 hours (IgM)

5. At the end of the given diffusion time , the diameter D of the precipitin rings should be measured accurately to 0.1mm using a suitable calibrated instrument.

EVALUATION:

The immunoglobulin concentrations related to the measured diameters are read directly from the table of reference values. The results are reliable only when the value found for the control serum applied to well 1 lies within the confidence range taken from the tables of values enclosed with each pack of Control Serum. With Hoechst Behring Control Serum, the confidence range is ± 15% of the immunoglobulin concentration given with each pack.

If the protein concentrations of the serum samples diverge considerably from the normal value, this means that the resulting precipitin ring diameters will fall outside the assay range of the plate. In this case, the examination must be repeated using higher or lower dilutions of the serum sample. For example in myeloma a dilution of 1:20 or 1:30 may be required Tri-Partigen IgM is not suited for IgM determination in seminal fluid.

APPENDIX - VIII

AMES REAGENT STRIPS - Tests for Glucose, Bilirubin, Ketone (Acetoacetic Acid), specific Gravity. Blood, pH. protein, Urobilinogen, Nitrite and Leukocytes in Urine (Henry, 1979).

Ames reagent strips for urine analysis are firm plastic strips to which are affixed several separate reagent areas. Test results may provide information regarding kidney and liver function acid-base balance and urinary tract infection.

PROCEDURE

1. Collected fresh urine specimen in a clean, dry container. Mixed well immediately before testing.

2. Removed one strip from bottle and replaced cap. Completely immersed reagent areas of the strip in fresh urine and removed immediately to avoid dissolving out reagents.

3. While removing, run the edge of the strip against the rim of the urine container to remove excess urine. Held the strip in a horizontal position to prevent possible mixing of chemicals from adjacent reagent areas and or contaminating the hands with urine.

4. Held strip close to colour chart on the bottle labels and compared reagent areas. Avoided laying the strip directly on the colour chart as this will result in the urine soiling the chart.