ANTI-p53 AND c-erbB2 AS PROGNOSTIC MARKERS IN SOUTH AFRICAN BREAST CANCER PATIENTS

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November 2000

ANTI-p53 AND c-erbB2 AS PROGNOSTIC MARKERS IN SOUTH AFRICAN BREAST CANCER PATIENTS

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DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree Doctor Technologiae in Medical Technology, to the Cape Technikon, Cape Town. It has not been submitted before for any diploma or examination at any other Technikon or tertiary institution. The work was undertaken at the breast clinic at King Edward VIII hospital and the Department of Immunology at the University of Natal Medical School.

The opinions and conclusions drawn are not necessarily those of the Cape Technikon

C.M. Winchester — November 2000

Carolyn Margaret Winchester — Date

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I dedicate this study to all women who die of breast cancer and to all those who fear it.

Thus the man will be more esteemed to be a good physician, for he will be the better able to treat those aright who can be saved, from having long anticipated everything; and by seeing and announcing beforehand those who will live and those who will die.

~ Hippocrates' Book of Prognosis ~

ABSTRACT

The diagnosis of breast cancer is not possible using currently available serological detection of cancer markers as these lack adequate sensitivity or specificity. This study investigates the prevalence and significance of anti-p53 antibody and c-erbB-2 protein in the post-surgical sera of South African breast cancer patients and correlates these features with the clinicopathological characteristics of breast cancer. Further, this study investigates the possibility of improving prognostic sensitivity by combining the two subject markers to monitor each patient. Further, this study will provide the opportunity to investigate whether only certain types of breast cancer can elicit an immunological response and at what stage and grade of tumour antibodies are present in the post-operative serum. The study also establishes a foundation for determining in South Africa whether there is a genetic influence in the response to p53 mutation and whther this response is higher in the indigenous African women compared to other South African women. The purpose of the study is to determine if the resulting findings can be used to enhance our ability to diagnose breast cancer and to identify node-negative breast cancer patients at high risk for early disease recurrence and or death, for whom adjuvant therapy is unequivocally justified.

The study accrued 92 South African breast cancer patients who were essentially women of colour 62 [67%] indigenous African women and 20 [22%] Caucasian of Indian descent, 6 [6%] of mixed [Coloured] background and only 4 [4%] Caucasian of White descent. A predominantly indigenous African population was chosen because they are the group most likely to benefit from an easily repeatable, affordable serological cancer marker. p53 and c-erb B-2 were reviewed, with emphasis on biochemical testing, relevant to clinical application, since improvement of analytical techniques and the establishment of clear cut-offs between benign and malignant disease has the potential to improve the diagnosis/prognosis of breast cancer. The literature review outlines breast cancer in South Africa together with prevailing knowledge of p53 and c-erb B-2. A review of existing and potential genetic cancer markers for breast cancer is outlined with the intention of establishing exactly what is currently known and or available to the oncologist.

ELISA methodology enabled the detection and quantification of serum auto-antibodies specific for p53 and c-erb B-2 immunoreactivity in patients with breast cancer. Commercially available kits [only available to research laboratories] were used, purchased from Oncogene Science (Calbiochem), Cambridge, USA, as the time-scale of this study was such that it was not practical nor prudent to raise an antiserum and then develop a working protocol. The lack of suitable standards would also have posed a particular problem. The processed data are exhibited and tables are presented at the conclusion of major issues, particularly pertaining to correlation with histological stage and grade of tumours.

The results of this study indicate an intrinsic variability that is unacceptably high in assays intended for clinical use. Findings of the study, supported by the literature indicate that the presence of antibodies to p53 [11% of women in the study were positive] is a complementary procedure in assessing the functional state of the p53 gene and seems to indicate a sub-group with poor prognosis. No elevated results were obtained for c-erb B-2 in the post-operative serum from breast cancer patients. The impact of surgery and medication on p53 antibodies and the c-erbB2 oncoprotein level needs to be further defined to determine its clinical utility. The study concludes with recommendations for a paradigm shift in our assessment and philosophy on breast cancer.

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Abbreviations

A-adenine	Alpha
AD4	Atypical ductal hyperplasia
ADEPT	Antibody directed enzyme pro-drug therapy
AEA	Antigen extraction agent
AIDS	Acquired Immune Deficiency Syndrome
ASIR	Age standardized incidence rates
AUC	Area under the curve
B	beta
bFGF	Basic fibroblast growth factor
BRCA	Breast cancer
BSA	Bovine Serum Albumin
c	Cytosine
C.V.	Co-efficient of variation
c-oncs	Cellular oncogenes
CA	Cancer antigens
Cath-D	Cathepsin-D
CD	Cluster Designation
Cdk	Cyclin dependent kinase
CEA	Carcinoembryonic antigen
cm	Centimetre
CpG	p represents the phosphate group connecting the C & G nucleotides
CR	Complete remission
DCIS	Ductal carcinoma *in situ*
DMBA	17,12-dimethylbenzanthracene
DMSO	Dimethyl sulphoxide $(CH_3)_2SO$
DNA	Deoxyribonucleic acid
dPCR	Differential polymerase chain reaction
EBM	Extracellular basement membrane
ECD	Extracellular domain
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assays
EMIT	Enzyme multiplied immunoassay
ER	Estrogen receptor
FBC	Full Blood Count
fmol/ml	Femtomoles per milligram
FN	False negative
FNA	Fine Needle Aspiration
FRD	Foundation for Research and Development
G	Guanine
GADD	Growth arrest DNA damage
Gp	Glycoprotein
H&E	Haematoxylin & eosin

NMS	Normal mouse serum
ns	not significant
OD	*Optical density*
OPD	o-phenylene diamine
PA	Plasminogen activator
PAP	Prostatic acid phosphatase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PdgF	Platelet derived growth factor
PF1	p53 factor 1
PHC	Primary Health Care
phe	Phenylalanine
PR	Progesterone receptor
Rb	Retinoblastoma
RI	Reference interval
RIA	Radioimunnoassay
RNA	Ribonucleic acid
ROC	Receiver Operator Characteristics
rpm	*revolutions per minute*
Rt^0	Room temperature
SEM	Standard error of the mean
SF	Scatter factor
Srp	Stress response protein
SSCP	Single strand conformational polymorphism
SV	Simian virus
T	Thymine
TAA	Tumour associated antigen
TPA	Tissue type plasminogen activator
TC	calibrated to contain
TGF	Transforming growth factor
TGFb	Transforming growth factor beta
TGFr	Transforming growth factor receptor
TIMP	Tissue inhibitors
TMB	Tetramethylbenzidene
TNM	Tumour Node Metastases : classification system
Tp	Tumour protein; True positive
tPA	Tissue plasminogen activator
TPA	Tissue polypeptide antigen
trp	Tryptophan
UK	United Kingdom
uPA	Urokinase Plasminogen Activator
USA	United States of America
UV	Ultra violet
v-oncs	Viral oncogenes
VAT	Value Added Tax
WAFI	Wild type p53 activated fragment
WHO	World Health Organization

HCI	Hydrochloric acid
HG	Histological grading
HIC	Hypermethylated in cancer
HIV	Human Immunodeficiency Virus
HNU	Human *neu* units
HPV	Human papilloma virus
HRP	Horseradish peroxidase
HRT	Hormonal replacement therapy
hsp	Heat shock protein
IARC	International Agency for Research on Cancer
IF	Intermediate filaments
IFCC	International Federation of Clinical Chemists
Ig	Immunoglobulin
IgG	Immunoglobulin Gamma
IgM	Immunoglobulin Mɲ
IHC	Immunohistochemistry
IL	Interleukin
IRMA	Immunoradiometric assay
IUCC	International Union Against Cancer
K-ras	Kirsten ras gene
kDa	Kilo dalton
LABC	Locally advanced breast cancer
LAK	Lymphocyte activated killer cell
Leu	Leucine
LFS	Li-Fraumeni Syndrome
LH	Luteinizing hormone
LR	Lifetime risk
M:	Metastasis : Molar
MAb	Monoclonal antibody
MASA	Mutant allele-specific amplification
MATK	Mitogen activated tyrosine kinase
MCA	Mucin-like carcinoma associated antigen
MDM2	Mouse double minute chromosome
MHC	Major histocompatibility complex
ml	Millilitre
MMP's	Matrix metalloproteases
mRNA	Messenger ribonucleic acid
MW	Molecular weight
N	Node
NaOH	Sodium hydroxide
NCI	National Cancer Institute
NDP	Nucleoside diphosphate
NF	Nuclear factor
ng	Nanogram
Ng	Nuclear grade
NK	Natural killer cells
nm	Nanometres

Glossary

Adjuvant chemotherapy	chemotherapy given at the time of surgery/radiotherapy.
Allele	gene on one chromosome that has a homologue on the other chromosome of a diploid cell.
Aneuploid	inexact multiple of normal DNA (chromosome) content.
Angiogenesis	growth of new blood vessels.
Antigen	molecule capable of generating an immune response.
Anti-oncogene	alternative term for a repressor gene.
Apoptosis	programmed cell death.
Benign	confined growth that is not malignant.
Cancer	an acceptable clinical definition would be 'a set of diseases characterized by unregulated cell growth leading to invasion of surrounding tissues and spread (metastasis) to other parts of the body'.
Carcinogen	agent capable of causing or promoting cancer.
Carcinogenesis	processes involved in the production of a cancer.
Carcinoma	epithelial cell cancer.
Carcinoma *in situ*	early stage of cancer that has not invaded its surroundings.
Case-control study	epidemiological method in which people with one characteristic are compared retrospectively with those without that feature.
Cell cycle	cycle of events required for cell multiplication.
Chromosome	structural unit that contains genetic material.
Codon	three-base sequence in DNA that codes for one amino acid.
Comedo	to eat up.
Comedocarcinoma	in which plugs of necrotic malignant cells may be expressed from the breast.
Cohort study	prospective epidemiological study in which the characteristics of a group of people are followed over a period of time.
Diploid	normal DNA content of cells with a double complement of each chromosome.
Domain	region of a protein serving one specific function.
Epitope	region of an antigen that is recognised by an antibody.
Exons	transcribed regions of a gene that are translated into protein.
G_0 G_1 G_2	phases of the cell cycle.
Gene	DNA containing regulatory and coding sequences for one protein.
Genotype	genetic (DNA) make-up of a cell.
Grade	histological classification of a cancer based on mitoses, nuclear shape and differentiation.
Growth factor	secreted polypeptide that regulates growth. Usually stimulatory but can be inhibitory.
Humoral	pertaining to the extracellular fluids, including serum and lymph.
Immune surveillance	processes by which the immune system monitors the body for foreign antigens such as cancer cells.

Incidence	number of new cancers developing in a defined population over a defined time.
Initiation	initial state of carcinogenesis.
Intron	gene sequences that are transcribed into RNA but removed before translation.
Ligand	agent that binds to a receptor.
Malignant	property of cancer cells to invade and metastasise.
Metastasis	process by which cancers spread to other parts of the body.
Mutation	a change in one or more bases in DNA.
Neoplasia	new growth of any type. Includes cancers and benign growths.
Oncogene	a gene whose protein product contributes to carcinogenesis. Normal cellular *oncogene is abbreviated to c-onc whilst viral oncogene is v-onc.*
Overall survival	period from first diagnosis to death.
Phenotype	characteristics of a cell.
Ploidy	DNA (chromosome) *content of a cell.*
Polyclonal	a term which describes the products of a number of different cell types.
Primary cancer	site of first formation of a cancer.
Prognosis	*future outlook for a patient with a cancer.*
Prognostic factor	factor that helps define prognosis.
Progression	changes that result in increased aggressiveness (dedifferentiation) of a cancer.
Promotion	*stage of carcinogenesis after initiation.*
Promotor	i) agent that promotes carcinogenesis; ii) regulatory region of a gene that initiates transcription.
Proto-oncogene	a gene that, as a result of mutation, can become an oncogene.
Recombinant	a process by which genes are rearranged during meiosis. The process also occurs during the somatic rearrangement of DNA which occur in the formation of genes encoding antibody molecules and T-cell antigen receptors.
Relapse	reappearance of a cancer.
Relapse-free survival	period between first diagnosis and appearance of secondary growths.
Relative risk	risk of developing a cancer in one group compared to that in a control group.
Remission	decline in cancer size as a result of treatment.
Repressor gene	gene whose protein product inhibits a cell function.
Retrovirus	RNA virus.
Somatic cell	not a germ cell so genetic complement cannot be passed to children.
S phase	phase of a cell cycle in which DNA is synthesized.
Stage	tumour classification based on size, nodal status and metastasis.
T-cell	class of lymphocytes with various subtypes.
Tumour	a growth that can be benign or malignant.
Tumour marker	antigen that provides prognostic information about a cancer.

CHAPTER ONE

INTRODUCTION

1.1 General introduction

Breast cancer is increasing in South Africa as more women adopt a Western lifestyle. In terms of mortality, breast cancer is the most common cancer in White South African women, (Sitas, 1998). In developed countries it is the most common cancer found in women and accounts for approximately 18% of all cancers world-wide (Teare, 1994). Breast cancer is a major health problem and a great deal of financial resources are directed towards research objectives. The reality of this diagnosis is hard to comprehend, for, despite tremendous efforts, there has been little improvement in the overall survival of patients with breast cancer over the past 40 years (Carney *et al.*, 1990). The technology has improved, but the percentage of women dying has not.

A women perceives her femininity and sexuality though her body image and her breasts form an integral part of this. The modern treatment of choice for cancer of the breast has been mastectomy, a treatment both physically and emotionally disfiguring; although there is currently a move for breast conservation (Bryant, 1995). Fear of mastectomy, with partner rejection, prevents even well-informed women from seeking timeous medical treatment. The result is late presentation with advanced or terminal disease and a disastrous outcome (Kessler, 1995). It is both ironic and tragic that a neoplasm arising in such an exposed organ, readily accessible to self-examination and clinical diagnosis, continues to exact such a heavy toll.

Clinicians come into contact with patients with breast cancer at a relatively late stage in its development and therefore deal with progression rather than carcinogenesis (Harnden,1992). Loss of tumour suppressor genes and activation of dominantly acting oncogenes and the proteins for which they code, describe the functional features that drive carcinogenesis at molecular level (Rosin, 1991; Hollywood *et al.*, 1995; Rozengurt, 1995; Zubay, 1998). New molecular understanding suggest that these genes and their products may become new biochemical tests for cancer (Diamandis, 1992).

This is based on changes in these genes being initiating and pathogenic events, not merely late sequelae (Angelopoulou *et al.*, 1994). However, despite provocative data additional studies are needed to determine the efficacy of their use in clinical practice.

Commercial kits are now available to research laboratories for the measurement of certain of these genetic markers and it may be feasible to consider adding such assays to the standard laboratory repertoire. The clinical laboratory has the potential to offer women a non-invasive procedure for the diagnosis of breast cancer yet there are a plethora of questions that demand answers before these markers are made available for routine use. Laboratory tests for primary cancer which would identify accurately the behaviour of the disease, the extent of its dissemination and the systemic therapy most likely to be appropriate, cannot elude the women of the world forever.

1.2 Background to the study

A clinical diagnosis of breast cancer represents one of the most important ways of reducing breast cancer mortality. The incidence of newly diagnosed breast cancer in South Africa is between 5000-6000 women per year and about 700 women succumb to the disease annually (Sitas, 1994). Less than half of all newly diagnosed cases will have early (stage I or II) breast cancer. South Africa is faced with a problem of increasing numbers of patients with locally advanced breast cancer for whom the optimum approaches have not yet been defined (Bezwoda *et al.*, 1994). A great deal of data indicates that Black women have lower survival rates at corresponding stages (Carbone *et al.*, 1993; Eley *et al.*, 1994; Walker *et al.*, 1995). Among Black breast cancer patients in Soweto, 50% mortality occurred in less than 18 months. The corresponding periods for White patients is 4-5 years (Walker, 1987). The difference persists even when allowances are made for delay in diagnosis, socio-economic status and a more advanced stage at diagnosis.

Breast self-examination is the most simple, repeatable and economic measure of cancer screening, yet there is a psychological barrier against this in many women and the percentage of women who perform self-examination varies from 15-40% of the population (Veronesi *et al.*, 1995). Clinical examination is not a very sensitive test - the sensitivity is approximately 50% (Veronesi *et al.*, 1995).

The most satisfactory clinical assessment of the primary tumour combines palpation with mammography (Carbone *et al.*, 1993). There is a significant difference between women of lower socio-economic groups and the remainder of the female population in respect of the utilization of mammography for screening and diagnosis (Harper *et al.*, 1996).

A significant proportion of women who have no recognizable breast disease have sufficient irregularities of the "normal" breast tissue to cause concern and necessitate clinical evaluation (Bibbo, 1991; Foreman, 1997). Benign conditions of the breast are more common than malignant ones and whether any are precancerous is controversial. However, the benign condition (fibrocystic changes are the dominant breast problem) creates a diagnostic problem. Although certain features of fibrocystic change tend to distinguish it from cancer, the only certain way of making the distinction is biopsy and histologic examination (Cotran *et al.*, 1992). Long established clinical and anatomic criteria usually predict which conditions are benign, but some defy easy characterization (Cotran *et al.*, 1992). Hormonal imbalances are considered to be basic to the development of fibrocystic changes (Cotran *et al.*, 1994).

The majority of breast lumps felt clinically prove to be benign. The prevalence varies according to the age of the patient, arising dramatically after the age of 40 years. About 80% of breast lumps in women under the age of 40 years are benign. In contrast, about 70% of breast lumps in women over the age of 70 years are found to be malignant. Failure to identify breast cancer has major consequences for both patient and physician. Usually, locally advanced cancers can be diagnosed fairly accurately on the basis of clinical features only, even by inexperienced clinicians (du Toit *et al.*, 1995). It is the early cancer that causes a diagnostic dilemma.

The notion exists that early detection of breast cancer leads to a cure or prolongation of life because patient survival is better when breast cancer is confined, as opposed to cancer that has spread at the time of diagnosis (Tarin, 1992; del Buono *et al.*, 1995; Chan①, 1996). The development of immunochemical tests on blood samples that would allow the early detection of breast cancer at a potentially curable stage is a promising new development. Many cancer markers have been proposed

but considerable experience in clinical trials is necessary to delineate their value. Rigorous analytical methodologies are required to validate which of the available markers should be used to permit appropriate surrogate determinations. Interpretation of cancer marker data is one of the most difficult tasks in a clinical laboratory. The identification of more accurate markers that are reproducible, easily accessible and independent in diagnosing breast cancer and/or clinical outcome would have a beneficial outcome on medical economy, mortality and treatment modalities.

1.2.1 Breast cancer genetics

The extent of the chromosome changes that may be present in a particular cancer may be enormous (Pitot, 1993; Tilton *et al.*, 1994; Evans, 1995; Negrini *et al.*, 1996). In general there is an association between chromosome breakpoints and known oncogenes. Deletion can result in the loss of tumour suppressor genes (O'Connor, 1995; Press, 1997). Translocation can result in deregulation of gene(s) adjacent to breakpoints or can fuse two genes, resulting in chimeric protein products with aberrant functions. Mutations or chromosomal rearrangements can also occur in somatic cells during the lifetime of an individual (Lewin, 1997). The generally accepted view is that most cancers, if not all, are both somatic and clonal in origin (Pinsky, 1997).

*** Tumour suppressor genes**

Observations suggest the existence of a substantial class of suppressor genes, the normal function of which is to govern cell proliferation (Knudson, 1993; Ory *et al.*, 1994). Supressor genes have proved to be of critical importance in human carcinogenesis and their limited numbers indicate a more general role than the more diverse oncogenes (Moshe, 1991). The basis for their importance is that in normal cells growth is restricted by inhibitory (repressed) proteins that must be reversibly inactivated for growth to occur (King, 1996; Okada *et al.*, 1997). The biological involvement of a repressor gene in cancer requires inactivation of both alleles (Figure 1) so that neither can make an inhibitor: however, inactivation of only one allele does not exclude a repressor mechanism [King,1996]. This is exemplified by p53 where presence of two mutant alleles generates an inactive product.

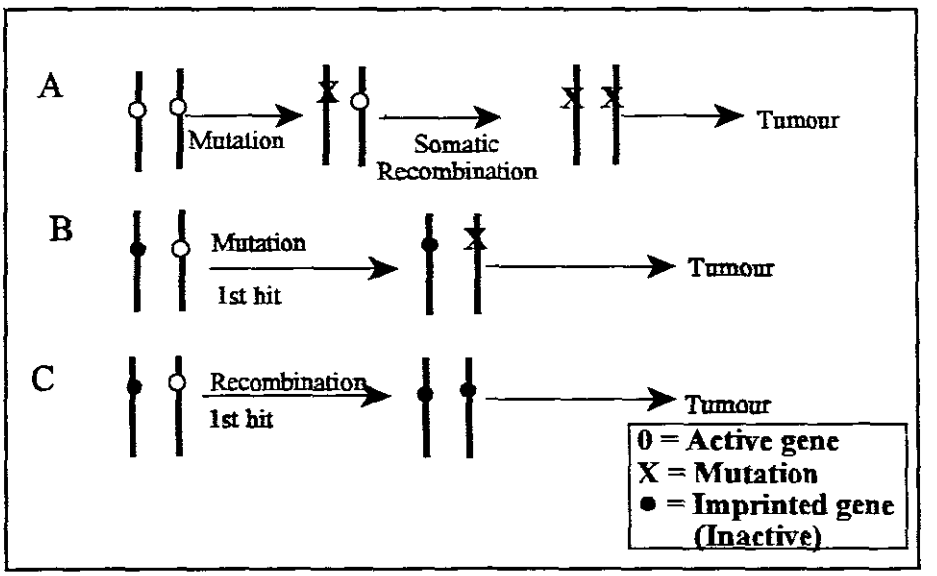


Figure 1 : Inactivation of tumour suppressor genes within an imprinted chromosomal region. Normally in a tumour suppressor gene where imprinting is *not* operating, two active alleles are present and two hits are required for gene inactivation (A). For an imprinted gene, if only one allele is expressed in an 'at risk' tissue, only one hit is required for gene inactivation and tumour development. This can occur via mutation (B) or somatic recombination (C) (Adapted from Squire, 1996).

Repressor proteins inhibit cell-function by complexing with other effector proteins and blocking their action. The two best understood suppressor genes are the retinoblastoma gene (RB1) and p53. When mutant p53 is overexpressed it can act in a dominant fashion by forming stable but inactive complexes with the normal protein (Semenza *et al.*, 1997). Its mechanism may be to inactivate the function of the active p53 protein. Nevertheless, when the normal wild-type allele is introduced into tumour cells that lack the p53 gene, tumourigenicity is supressed (Babu *et al.*, 1994).

Research demonstrates that mutant p53 proteins, arising from base-pair substitutions,(Lubin *et al.*, 1995; Peyrat *et al.*, 1995) can be considered as foreign and under certain circumstances, induce a specific humoral response in breast cancer patients. This raises an interesting possibility that p53 immune response, possibly due to p53 mutation, is a very early event in carcinogenesis. Some evidence supporting this possibility has been reported for liver [Trivers *et al.*, 1995; breast, lung and prostate [Trivers *et al.*, 1996]; and esophageal cancers [Cawley *et al.*, 1998] in which anti-p53

antibodies have been detected in some patients several years, 5-11 months and 1-2 months respectively, before a cancer diagnosis.

*** Oncogenes**

Oncogenes are a family of unique sequences of DNA whose abnormal expression is associated with the development of malignant cell behaviour (Vile, 1990; Gullick, 1991; Aznavoorain *et al.*, 1993; del Buono *et al.*, 1995). There are presently about 100 oncogenes, which under normal conditions can release cells from normal controls of growth, mortality and location, to cause neoplastic transformation (Kath *et al.*, 1991; Goynes *et al.*, 1995). Oncoproteins fall into groups of similar activity, each of which is presumed to act at the same point in a pathway (Nistico *et al.*, 1997). In the main, oncogene activation is the result of somatic events rather than genetic causes (Marks *et al.*, 1996). The evidence implicating oncoproteins in growth control [Figure 2] was strengthened by determination of the function of several oncogene products (Gullick, 1991; Anderson②, 1995). The c-erbB-2 gene [or *neu*] has been reported to encode the epidermal growth factor receptor [EGFR]. When EGFR binds to its receptor on the cell surface, it supposedly activates the protein kinase activity of the cytoplasmic domain of the molecule, so culminating in growth potentiation [Brandt-Rauf, 1997].

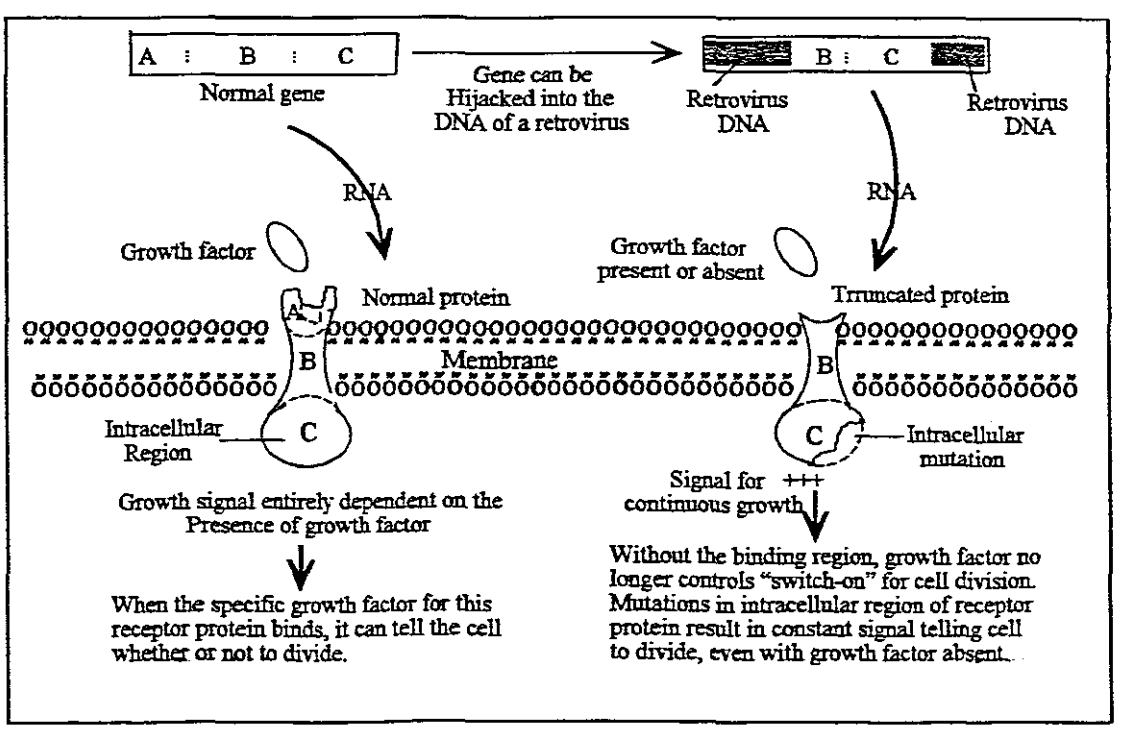


Figure 2 : Large-scale alterations in the structure of the protein product of the proto-oncogene may play a part in the development of a cancer. For example, several proto-oncogenes code for the rpotein receptors for growth factors. In this example, the proto-oncogene can convert to the oncogene. Such a conversion may involve loss of the section coding for the part of the receptor outside the cell. This loss may cause the receptor to transmit a continuous growth signal. (Vile, 1990).

Part of the EGFR is closely related in amino acid sequence to the product of the c-erb B-2 oncogene. Perhaps c-erb B-2 protein mimics the actions of EGFR but does so in an unregulated way (Figure 3).

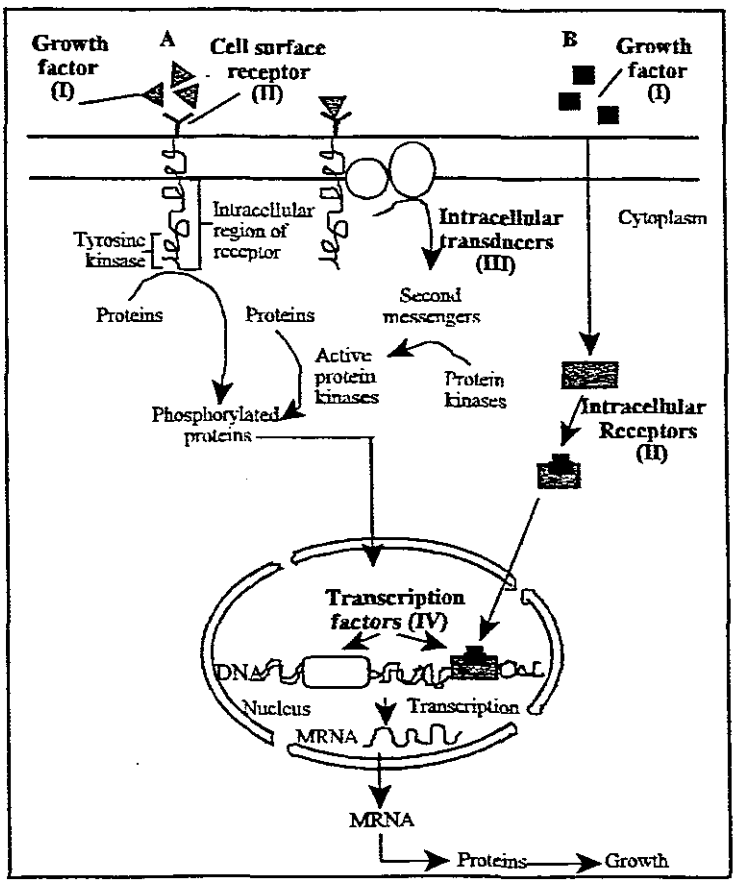


Figure 3 : Actions of growth factors. Growth factors bind to receptors that may be located on the cell surface or inside the cell. **A.** Cell surface receptors are proteins that often have intracellular domains with tyrosine kinase activity. When the growth factor binds to these receptors, they are activated and may directly phosphory late proteins. Other cell surface receptors act through transducers that cause the production of second messengers, compounds that activate protein kinases. These protein kinases phosphorylate proteins. Phosphorylated proteins, produced by either mechanism, activate transcription factors, causing genes to be transcribed. The protein products of these genes promote growth. **B.** Other types of growth factors enter the cell and bind to intracellular receptors. These growth factor-receptor complexes activate genes that promote growth. Proto-oncogenes produce growth factors (I), their receptors (II), transducers (III), and transcription factors (IV). Mutated proto-oncogenes (oncogenes) produce altered versions of I, II, III or IV that cause abnormal growth. Excessive expression or inappropriate expression of proto-oncogenes can also cause abnormal growth. (Marks *et al*, 1996)

There is a correlation between erb B-2 amplification, elevated erb B-2 expression and breast cancer (Babu *et al.*, 1994). Amplification of HER-2/*neu* (c-erb B-2) proto-oncogene at the DNA, mRNA and protein level has been implicated as prognostically important since it correlates with a shortened disease-free interval and decreased overall survival in breast cancer (Colomer *et al.*, 1994; Wiltschke *et al.*, 1994; Bieche *et al.*, 1996). Activation of an oncogene by translocation, point mutation or amplification is a dominant phenomenon because malfunction occurs if only one allele is involved in tumourigenesis (Becker *et al.*, 1995; Cooper *et al.*, 1992).

The assay for the *neu*-oncoprotein is presently based on IHC staining of tissue specimens (Somerville *et al.*, 1992). No serum method or reference interval (RI) have been satisfactorily validated and the marker is limited to research use only (Bond, 1994).

1.2.2 Laboratory diagnosis of cancer

The approach to the laboratory diagnosis of cancer is complex, specialized and sophisticated (Chan②, 1996). All currently known cancer markers are ineffectual because they suffer from relatively low specificity (Diamandis, 1992; Roulston *et al.*, 1993). It is necessary to establish combinations of markers that are capable of eliminating the inherent weakness associated with single markers if any real progress in breast cancer mortality is to be made.

Since there are no markers specific for breast cancer it is prudent to use a panel or profile. A panel of markers provides more information than a single marker and for this purpose it is essential that defined panels of antibodies are used with a standardized technique. This study investigated the possibility of improving sensitivity by combining two markers, p53 auto-antibodies and c-erb B-2. Apart from technical shortcomings, there was a major assumption that breast cancers produced some unique feature that non-neoplastic disease did not.

1.2.3 Clinical use of cancer markers

The diagnostic process for breast cancer has the potential of being transformed into a highly sophisticated technological procedure, allowing recognition of lesions which are often not palpable. There is evidence of a correlation between amplification and clinical outcome for some cancers.

Adjuvant therapy prolongs the life of many women with breast cancer and since oncologists are unaware which patients' cancer will recur, many women receive adjuvant treatment unnecessarily. The need to identify breast cancer patients who will benefit from adjuvant therapy and to spare others the side effects may improve by incorporating p53 auto-antibodies and/or c-erb B-2 into clinical decisions. It is hypothesized that the production rate of a marker varies, not only between patients with the same type of cancer, but also in the same patients over the course of their disease.

1.3 Motivation for the study

The motivation for this study stems from increasing concerns that, despite an enormous research effort, the overall mortality rate for breast cancer has not changed over the last 40 years. These poor results make the idea of early diagnosis *sine qua non*. The most important findings indicate that the role of markers in cancer diagnosis and prognosis, while of enormous potential, cannot currently be used as an aid or confirmatory method in either diagnosis or prognosis. This is due to lack of characteristics in an ideal cancer marker which satisfies both analytical and clinical requirements. Genetic markers, such as p53 and c-erb B-2, offer tremendous potential as early indicators of breast carcinogenesis, yet assessment of these markers have displayed a generation of disparate results.

The immune system is complex and its potential impact on a complicated process like carcinogenesis can be multifaceted (Green *et al.*, 1995; Ögmundsdöfter, 1995; King, 1996). Although the anti-p53 response and c-erb B-2 have the potential to identify patients with early carcinoma of the breast or to monitor patients with known carcinoma for recurrence, many of the results are still preliminary with conflicting conclusions.

From a clinical point of view it is necessary to define criteria of cancer marker increase indicative of disease. This is dependent on the analytical variability of the test system, within the reference interval (RI) and near the cut-off level, together with possible biological variation within patients. Diagnostic potential can only be utilized when there is a high degree of long term analytical performance, which

also has to cover the low concentration range of these analytes (Holzel *et al.*, 1995). These requirements are currently not met, so efforts for improvement of analytical performance are necessary to enable use of genetic cancer markers in clinical practice. It is difficult to correlate results from some of the studies, since a variety of procedures were used to measure anti-p53 and c-erb B-2. Several investigators used "in-house" developed protocols. Furthermore, no studies were found that evaluated an independent reference specimen, with known quantities of either anti-p53 or c-erb B-2, to ensure uniformity between sets of assays.

The role of assays for p53 mutation and p53 protein overexpression in the clinical management of cancer of the breast is still unclear (Wang *et al.*, 1993). Even in similar patient populations, different assay techniques produce alarmingly different results. Use of immunohistochemistry (IHC) as an adjunct to histological assessments is easy, yet the frequency of false-positive and false-negative interpretations remains unclear and data suggests that this method is not as sensitive as molecular genetic assays (Levine, 1994). Whilst IHC is relatively simple and inexpensive to perform, it is difficult to quantitate and interpretation is subjective. Previous studies have shown that ELISA and IHC show a reasonable correlation in assays of p53 and c-erb B-2 protein in breast cancer. However, unlike immunostaining, ELISA is quantitative and objective, moreover large numbers of samples can be handled simultaneously. Information contained in this study can enhance our knowledge and understanding of ELISA, which has the potential to facilitate the introduction of anti-p53 and c-erb B-2 into the routine laboratory repertoire.

Review of relevant literature and the research project could lead to the recommendation of two statistically significant predictors in the belief that implementing one or both could improve diagnostic potential ability. These assays may not only have the ability to distinguish between benign and malignant disease, but reviews indicate their potential ability to detect early malignant changes associated with the disease process (Brandt-Rauf, 1997). Since p53 is present in normal cells, there is no guarantee that increased levels of p53 will elicit an antibody response, so negative results would have to be interpreted with caution (Crawford *et al.*, 1982). The presence of anti-p53 activity in serum from breast cancer patients could be evidence of some change occurring in amount or type of

p53 in malignant transformation.

For anti-p53 and c-erb B-2 accumulation to be markers of value, it is important to standardize ELISA in relation to existing procedures and reagents. This study will investigate if only certain types (or cases) of breast cancer can elicit an immunological response against p53 and at what stage and grade these antibodies are present in measurable amounts in post-operative serum. The aim is to establish the prevalence and significance of anti-p53 and c-erb B-2 in the post-operative sera of breast cancer patients and to correlate this with established clinicopathological characteristics. The purpose in investigating the relationship between levels of mutant p53 protein product, c-erb B-2 and clinicopathological findings is to enhance our ability to diagnose breast cancer and to identify node-negative breast cancer patients at high risk for early disease recurrence and/or death, for whom adjuvant chemotherapy is unequivocally justified (Lonning, 1996).

Based on the above assumptions, this study, using a South African gene pool, is the product of an investigation to clarify if these markers are efficable for clinical use. Their potential application in the diagnosis of breast cancer, as well as any potential role they may have as prognostic or monitoring indicators, offers an increased perspective on the available decision-making modalities that could be offered by the laboratory for this patient group.

To accomplish these objectives the following was performed:

i) A questionnaire was completed by participants to determine which pre-analytical variables could impact on evaluation of the patient with breast disease.

ii) p53 auto-antibodies and c-erb B-2 were quantitated in post-operative serum using an automated ELISA method.

iii) Results were correlated with established clinicopathological indices.

1.4 Delimitations

- The research was undertaken in KwaZulu-Natal and included patients from 3 Provincial

hospitals in Durban, namely: Addington, King Edward VIII and RK Khan Hospitals.

- Case-inclusion criteria was restricted to women with microscopically confirmed breast cancer. Control subjects had no record of malignancy.
- The study did not interpret underlying molecular pathology of findings postulated.
- The research effort did not intrude upon the formulation of new methodology.
- The exact mechanisms by which regulatory control is lacking and causes malignant transformation was not elaborated upon.
- Finally, the study was not explanatory, but of an exploratory nature and investigated those genetic markers suggested by the literature to have diagnostic implications.

1.5. Directional hypothesis

Since malignant transformation results in unique biochemical components which can be quantitated, measurement of p53 auto-antibodies and c-erb B-2 appear to hold promise in the early diagnosis of breast malignancy; but the lack of finding a molecular marker in certain patients and therefore a negative result does not exclude a diagnosis of breast cancer.

1.6 Summary of Chapter One

The goal and objectives of this research project were developed in line with the present evaluation of the patient with breast cancer together with projections for the role of the laboratory for both diagnosis and prognosis. In Chapter One the background to the problem and the motivation for the study were addressed together with delimitations and directional hypothesis. Chapter Two consists of the theoretical basis of the study, namely a review of the foci which contribute to a laboratory diagnosis of cancer, together with existing breast cancer markers. The chapter concludes with discussion on existing knowledge pertaining to anti-p53, c-erb B-2 and established clinicopathological indices.

In Chapter Three the precise study objectives and all motivations for selection of specific areas are discussed. Chapter Four comprises the research methodolgy. All materials and methods used in the study are discussed and factors which influenced decision making are accounted for. Chapter Five describes results obtained from questionnaires, ELISA and clinicopathological indices of breast

tumours investigated in this study. Descriptive statistics were used to analyze the data so as to elicit answers to the problem addressed by this research. Chapter Six discusses findings and the significance of results. Separate facets are summarized and stated in accordance with the aims and objectives of the study. Chapter Seven concludes the study with a summary deduction supporting certain of the hypotheses together with recommendations for further investigations.

CHAPTER TWO

LITERATURE REVIEW

2.1 General introduction

Over the last decade there has been a shift in emphasis in clinical research. Academic clinical researchers have become consumers of industry-generated research products, rather than producers of such research products. This is inevitable, given the costs of developing new products, especially in a developing country. The amazing array of commercially produced MAb's which are potential biomarkers of gene expression are available as research products only. An advantage when using commercial kits, is that results can be compared with those of other researchers using the same assay kits.

2.2 Review of immunochemistry detection and diagnostic technology

Heterogeneity of antibodies (generated in response to a single antigen) accounts for antibodies not having an identical affinity for the antigen (Kemeny *et al.*, 1988). Scatchard or Langmuire equations characterize the affinity of the hapten for the specific antibody and in addition, the affinity of any ligand for its specific binding site, e.g. the binding of a low molecular weight substrate to an enzyme binding site. The enzyme-substrate interaction can be expressed by a straight line (Ferencik, 1993). During interactions of conventional antibodies with haptens, deviations from this linear relationship can be seen. These are caused by heterogeneity of antibodies and resulting heterogeneity of their binding sites: the resultant curve makes it impossible to determine K (where K is the equilibrium association constant). In this study it was desirable to have a homogenous population of identical antibodies, each with the same antigen-binding site.

2.2.1 Monoclonal antibodies (MAbs)

Earlier studies indicate that manufacturers supply MAbs which sometimes give non-specific results (Keren *et al.*, 1992). The precise reactivity of MAb's is a problem in breast cancer since portions of their structure may be deleted from the secreted product. If the reagent MAb happens to be directed against the deleted epitope, the amount of monoclonal protein present in sera may be grossly

underestimated. While MAbs have great specificity for a particular epitope, the biologic system often has genetic variants (Abbas *et al.*, 1994; Mach, 1995). If the MAb is raised against a determinant that is not present in South African breast cancer patients, variable results will be obtained from patient to patient. Potential solutions to these problems may require the use of polyclonal antibodies (PAb's) or cocktails of two or more MAbs to ensure that all epitopes are accounted for.

The usefulness of an antibody as a reagent in a laboratory assay *in vitro* depends on whether an antibody adheres strongly to the epitope (a high-affinity interaction) or weakly (a low-affinity interaction) (Keren *et al.*, 1992). Multivalency also dramatically affects the ability of antibody to associate with or disassociate from an antigen; (while hapten groups are monovalent, antigens are multivalent). Serum is highly heterogeneous with several different antibodies available to react with a particular epitope. Since most antigens are complex, having many epitopes, there are families of antibodies with different affinities available to react with each epitope.

2.2.2 Essential criteria for commutability

Commutability is defined as the ability of material to yield the same numerical relationship between results of measurements by a given set of measurement procedures, purporting to measure the same measurable quality as the expectation of the relationships obtained when the same procedures are applied to other relevant types of material (Moss *et al.*, 1995). Molecular heterogeneity is a feature of all proteins. This may be genetic, inherent in all individuals, or disease related. This gives rise to problems with commutability. In selecting immunoassays for exploring the features of commutability, only those in which the specificity of the antigen-antibody reaction are clearly defined, should be considered. This requires mapping of the relevant epitope on the peptide.

The problem of differing immunoassay method specificities may result in the measurement of entirely different analytes, for example c-erb B-2 assay, may measure the intact molecule or fragments of the oncoprotein, i.e. p120 and/or p185. Specificity is often ill-defined and the literature indicates that it frequently differs between different procedures that are nominally intended to measure the same analyte (Moss *et al.*, 1995).

2.3 Diagnostic application of tests

To determine whether these markers have clinical utility it is necessary to determine how well they comply and how that compliance compares with the performance of other approaches (Hamilton *et al* 1988; Peters, 1994; Koch *et al.*, 1994). One component of performance evaluation report requires definition of the clinical setting in which the study is undertaken. The clinical challenge was to interpret a series or combination of study results, given a number of diagnostic alternatives. Although laboratory results provide clues or patterns, oncologists need to recognize discordant data so as to avoid diagnostic error (International Clinical Chemistry Congress, 1996). Not all patients with breast cancer may have a positive test for a marker (they may have false negative results) and a marker may be positive in some women without breast cancer (they may have false positive results). A cancer marker is of value only to the extent that it alters or revises the probability estimate (Howanitz *et al.*, 1991; Henderson, 1993; Konishi *et al.*, 1993).

Laboratory tests are rarely used in isolation from a range of other information (Levesque *et al.*, 1994; Silvestrini *et al.*, 1994). When monitoring a patient by repeatedly performing a cancer marker, it is essential that oncologists recognize if a change in results indicates a change in status or if it merely reflects variability in marker measurement (Noe, 1994). In order to make this decision, the pattern of systematic and magnitude of random intra-individual variability of the analyte in the patient must be known. Knowledge of the intra-individual variation is required to (a) evaluate changes in serial results from patients with breast cancer, (b) judge the effectiveness of reference intervals (RI) and (c) set desirable standards of analytical performance (WHO, 1994).

2.3.1 Reference interval (RI)

Information regarding the RI is often the weakest link between the clinical laboratory and the oncologist. To improve decisions concerning the diagnosis of breast cancer, empirical data needs to be collected and interpreted using scientific knowledge and professional experience. This would be realized if mandatory accepted conditions of comparison of laboratory results with RI was undertaken in conjunction with more advanced techniques for decision making. These included:

1. If stages and grading in the pathogenesis of breast cancer were demarcated.
2 If the diagnostic sensitivity and specificity of the assay kits used were ascertained and comparisons made.

A major pre-requisite for transfer of RI's is that the population be homogeneous and there should be no major ethnic, social or environmental differences between them (Buttner, 1995). Since the gene pool investigated is South African, results generated by other research laboratories may show variation to those found in this study. Direct selection (defined as "individuals selected from a parent population using defined criteria") of reference individuals is the only method that agrees with the RI concept as recommended by the International Federation of Clinical Chemists (IFCC)(1992). For this study, the *a priori* strategy was used since it is best suited to smaller studies (Solberg, 1994; Ichihara *et al.*, 1996).

2.3.2 Cut-off values

In most cancer markers tests there is an overlap between healthy women and patients with breast disease, such that sensitivity and specificity can be reciprocally altered by selecting different normal cut-off values. Unfortunately, at least 5% of individuals are normally, at best, misclassified by a test (Walmsley, 1994). The Receiver Operator Characteristics (ROC) curves are an indirect measure of the overlapping zone between both populations (Huguet *et al.*, 1993). Visual examination of a ROC curve allows a quantitative evaluation of diagnostic accuracy. This index is equivalent to the probability that given two patients, one with breast cancer and one without, the study result is more suggestive of breast cancer in the women who has the disease.

2.4 Review of existing serological breast cancer markers

Cancer antigens (CA) can be detected in biopsied tissue samples using IHC and is useful for the analysis of differentiated antigen expression and the determination of histogenesis (McCarthy, 1995; Aziz, 1995). The detection of antigens in tissue sections by immunohistochemistry (IHC) is a routine procedure in diagnostic practice. The success of the technique is dependent both upon the preservation of the antigen and upon the type of antibody used (Tsuda *et al.*, 1990). Many antigens

are destroyed by endogenous proteases, fixatives and by processing procedures. Evaluation of tissue samples for the expression of markers in breast cancer has been undertaken since approximately 1980. Carcinoembryonic antigen (CEA), Tissue polypeptide antigen (TPA), ferritin, cancer antigen (CA) 15-3, Mucin-like carcinoma associated antigen (MCA) and CA 549 have all been measured in high speed cytosol using commercially available immunoradiometric assay (Gion *et al.*, 1993). CA 125 is not a useful marker in breast cancer (Seckl *et al.*, 1992). The ever increasing costs of health care make it mandatory that more studies are undertaken to demonstrate how the use of new markers can make an impact on clinical decision-making (Gion, 1992; Schwartz, 1995; Chan②, 1996).

Immunoassays (Table 1) tend to be cheaper and easier to perform and reproducibly between various batches or preparations is of a high order (Keren *et al.*, 1992). However, since immunoassays are binding assays they measure a concentration of a structure in the matrix (Goldberg *et al.*, 1996). They do not measure functionality of the analyte. Therefore substances detected by immunoassay are not necessarily biological active or even 'intact' molecular species. A comparison of commonly used techniques for *in vitro* studies of cancer is given in Table 1. Quantitation of breast cancer markers in serum provides an improved alternate method by detecting breast cancer which cannot be detected by other means (Bagshawe *et al.*, 1995; Chan②, 1996).

WESTERN BLOTTING	ELISA	IHC
Non-isotopic	Non-isotopic	Non-isotopic
Semi-quantitative	Quantitative	Qualitative
Whole population	Whole population	Individual cells
Cost effective	Cost may be issue	Cost effective
Sample defined	Sample defined	Samples restricted
Control over sample	Control over sample	No sample control
Semi-technique dependent	Technique dependent	Technique dependent
Archive results		Archive results

Table 1 : Comparison of used *in vitro* laboratory techniques for the study of cancer

A review of existing markers which have varying degrees of clinical utility are described below.

2.4.1 Carcinoembryonic antigen (CEA)

CEA is an oncofetal cellular adhesion molecule involved in invasion and metastatsis. The CEA level and the degree of elevation has been shown to correlate with pathologic stage of the cancer lesion.

Early or localized breast cancer does not show CEA elevation (Chan②, 1996). CEA has been found to be elevated in about 58% of patients with advanced disease (Tondini *et al.*, 1998; Colomer *et al.*, 1989), and is most useful in monitoring metastatic breast cancer during therapy and the development of bone or lung metastasis. Accumulated clinical data indicate that CEA levels are of limited use. For breast cancer diagnosis/prognosis, CEA is being replaced by other more specific markers, such as CA 15-3 (Chan *et al.*, 1994).

2.4.2 Carbohydrate markers

None of these markers are used to diagnose primary breast cancer, but they have adequate sensitivity and specificity in monitoring disease progression in metastatic breast cancer patients (Chan *et al.*, 1994).

CA 15-3 is a breast cancer associated high molecular mass glycoprotein that contains epitopes reactive with the MAb's 115D8 and DF3 (Van Dalen, 1995). These two antibodies are used in the immunoassay, one attached to a radiolabel (tracer-antibody) and the other to capture the antigen (Roulston *et al*,1993). Serum CA 15-3 levels correlate with tumour burden and is a more sensitive assay than CEA for detecting disease recurrence (Holzel *et al.*, 1995). Enzyme-Immunoassay (EIA) and Radio-Immunoassay (RIA) methods to measure CA 549 have been developed using antibodies against a human breast tumour cell line T4/7 and human milk fat globule membrane. CA 549 is detectable in the sera of healthy males and females (Butch *et al.*, 1996). Elevations in CA 549 are not normally observed in the early stages of breast cancer and levels may be high in benign disease, CA 549 has proved useful for monitoring treatment and disease progression.

2.4.3 Tissue polypeptide antigen (TPA)

Serum levels of TPA appear to correlate with tumour burden and may be clinically useful in breast cancer staging and prognosis (Gion *et al.*, 1993; Butch *et al*,1996). Considering that TPA is measurable with a simple, well-standardized and reproducible commercially available assay kit, its prognostic usefulness in breast cancer in association with other biochemical parameters should be further investigated (Chan②, 1996).

2.4.4 Mucin-like carcinoma - associated antigen (MCA)

MCA is a glycoprotein with a molecular mass of 350 kDA. The epitopes on this molecule are also recognized by DF3 and 115D8 antibodies of the CA 15-3 assay. MCA levels increase throughout pregnancy, while, CA 15-3 only increases slightly during pregnancy. Minimum elevation is observed in benign breast disease. MCA levels are elevated in 60% of metastatic breast cancer patients (Chan *et al.*, 1994). However, elevated levels are also found in ovarian, cervical, endometrial and prostate cancer patients (Devine *et al.*, 1993). MCA levels correlate with CA 15-3 levels but not with CEA levels. In monitoring metastatic breast cancer patients, changes in MCA levels parallel those of CA 15-3 levels.

2.4.5 Proteases

The protease, cathepsin-D, as produced by breast cancer cells *in vitro*, can exist in multiple molecular weight forms (Sloane *et al.*, 1994). The MW52,000 form of cathepsin-D has been reported to be an independent prognostic marker for disease-free interval and overall survival (Tandon *et al.*, 1990). Since these early findings, conflicting results have emerged on the value of cathepsin-D. It was shown by Duffy (1996) that when total populations are reviewed using the immunoradiometric assay (IRMA), most investigators found a relationship between high levels of this protease and poor prognosis. However, when subgroups were studied, different results were obtained. It is not easy to explain the divergent results obtained with IRMA, since all investigators used the same assays.

A possible explanation for the varying results may relate to differences in the patient populations. It is difficult to correlate results from some studies since a variety of procedures (Example EIA and IRMA) were used to measure cathepsin-D. Several investigators used "in-house" developed protocols (Shaheen *et al.*, 1995). Most of the investigations were retrospective studies performed on tissues or cytosols that had been stored frozen for years. Furthermore, none of the studies evaluated an independent reference specimen with known quantities of cathepsin-D to ensure uniformity between sets of assays (Shaheen *et al.*, 1995). This gives rise to some concern about the reproducibility of the assays in some reports (Duffy, 1996).

The most conflicting results, however, are found when cathepsin-D is detected using immunocytochemistry. An early publication described high levels of the protease to be positively associated with good outcome. More recently, high levels have been found to correlate with aggressive disease and show no relationship with patient outcome (Duffy, 1996). Differences in the specificity of antibodies may explain some of the conflicting results obtained with immunochemistry. In addition, different cut-off points for discriminating high from low values and different types of tissue (i.e. fresh versus formalin-fixed and paraffin embedded, may also be responsible). Finally, the ability of cathepsin-D to act as prognostic marker may depend on whether or not adjuvant therapy was administered. It has recently been shown by Duffy (1996), that cathepsin-D is of prognostic importance only in breast cancer patients with lymph node metastases not treated with tamoxifen.

- **Comparison of serum pro-cathepsin-D and CA 15-3**

In Brouilett *et al's* study (1997), no correlation was noted between these two markers. The reported total tumour cathepsin-D cut-off levels have varied depending on the assay methods used, from 20 to 70 p/mols per milligram protein. The reason for this wide variation is unknown. It probably reflects population differences, assay methods and statistical analyses.

- **Urokinase plasminogen activator (uPA)**

Urokinase plasminogen activator (uPA) appears to be one of the strongest prognostic markers described for breast cancer. uPA is as strong a marker as nodal status and stronger than other prognostic indexes such as tumour size, ER status and cathepsin-D levels (Duffy, 1996). Although uPA has consistently been shown to be a prognostic marker in breast cancer, different investigators use different cut-off points to discriminate high from low levels. The cut-off points vary from as low as 0.52 ng/mg protein to as high as 10 ng/mg protein (Duffy, 1996).

2.4.6 Differential biological significance of tissue-type and urokinase-type plasminogen activator in human breast cancer.

There are two main forms of PA: urokinase-type (u-PA) and tissue-type (t-PA). While both catalyze cleavage of the peptide bond between arginine-valine in plasminogen, thus converting the proenzyme to plasmin, they differ in many aspects such as their molecular weight, immunological reactivity and

amino acid sequence. A significant association between low levels of total PA activity and poor prognosis of breast cancer patients suggest the value of total PA assay as a prognostic indicator. Results demonstrate provocative evidence suggesting a differential biological significance of tPA and uPA co-expressed in human breast cancer (Yamashita *et al.*, 1993).

2.4.7 pS2

pS2 protein belongs to the family of estrogen-induced proteins in breast tumour cells (Masiakowski *et al.*, 1982) and is present in the cytosol as a small mature protein composed of 60 amino acids (Numez, *et al.*, 1987; Rio *et al.*, 1988). About 30% of breast carcinomas (ER and PR) do not respond positively to hormone therapy. pS2 expression is associated with estrogen receptors (ER) in 96% of cases, however, not all ER and PR positive breast cancers express pS2. A study performed on 205 breast cancer patients asserted the prognostic value of pS2 results showing a strong correlation between lack of pS2 and failure to respond to hormone therapy (Foekens *et al.*, 1990). pS2 is a factor of hormone dependence and good prognosis in breast cancer and identifies breast cancer responsiveness to hormone therapy.

2.4.8 Genetic markers

In the past 10 years researchers have made rapid advances in the understanding of cancer (Evans, 1995; Lugo, 1998) and a variety of possible cancer markers for the diagnosis of breast cancer have been proposed.

- **BRCA**

Although most breast cancers are sporadic, approximately 5% are familial (Xu *et al.*, 1996). There was great interest when the genetic basis of the majority of familial breast cancer was traced to a small locus in chromosome 17q21; namely BRCA-1 (Cotran *et al.*, 1994) and BRCA-2 on chromosome 13q (Vaughn *et al.*, 1996; Benjamin, 1997). About one in 300 women carry the gene, which is implicated in about 4% of breast cancers in all age groups but in close to 25% of those diagnosed before age 40 (Hulka② *et al.*, 1995). The cloning of the BRCA-1 and BRCA-2 gene, was a major breakthrough after years of research. However, considerable disappointment has followed

as there is a low frequency of mutation of BRCA-1 in non-familial breast cancer (Karp *et al.*, 1997).

BRCA-1 has many characteristics of a tumour suppressor gene (Hulka® *et al.*, 1995). In pedigree analysis it segregates as a genetic dominant trait, whereas in somatic cells it behaves like a recessive gene. It spans approximately 100 Kb of genomic sequence containg 22 coding and 2 non-coding exons. The 7.8 Kb messenger RNA encodes a 200 Kb protein of 1863 amino acids (Xu *et al.*, 1996), The distribution of the coding sequence in the BRCA-1 genomic region is uneven with the largest exon (exon 11) coding for 60% of the protein. Sixty three distinct germline mutations of BRCA-1 have now been identified in more than 100 patients with breast and\or ovarian cancer. These mutations are distributed across the entire coding region of the BRCA-1 gene and the majority (87%) are predicted to result in truncated proteins or loss of BRCA-1 transcript. No somatic mutations of the BRCA-1 gene have been identified in sporadic breast cancers (Xu *et al.*, 1996).

The advantage of genetic testing for cancer susceptibility appear obvious. However, cancer predisposition testing poses risks as well as benefits (Schneider *et al.*, 1995; Bilimoria, 1995). At risk individuals must decide whether the increased anxiety of a positive test result would outweigh the relief of a negative outcome (Karp *et al.*, 1997). Pre-disposition counselling for BRCA-1 and 2 is not economically feasible for the larger community in South Africa.

- **p16**

Cancer biology is now familiar with suppressor inactivation via large deletions, intragenic mutations, alternate splicing and promoter mutation, but new evidence shows that inactivation of the p16 gene by promoter methylation rather than DNA mutation is common in some types of cancer (Little *et al.*, 1995). Methylation inactivation of p16 (Figure 4) is akin to classical genetic imprinting and indicates that this may be a major mechanism for the inactivation of other tumour suppressor genes in neoplasias . p16 encodes a tumour suppressor which was found to be mutated by point mutation or deletion in cell lines derived from a wide variety of different tumours. Along with p53, it appears that p16 is a major player in regulating the progression of cells toward the tumourigenic phenotype. However, mutation of the p16 gene is not a common event in tumours as distinct from cell lines.

Following the p16 lead, it may be that BRCA-1 can also be switched off rather than genomically mutated.

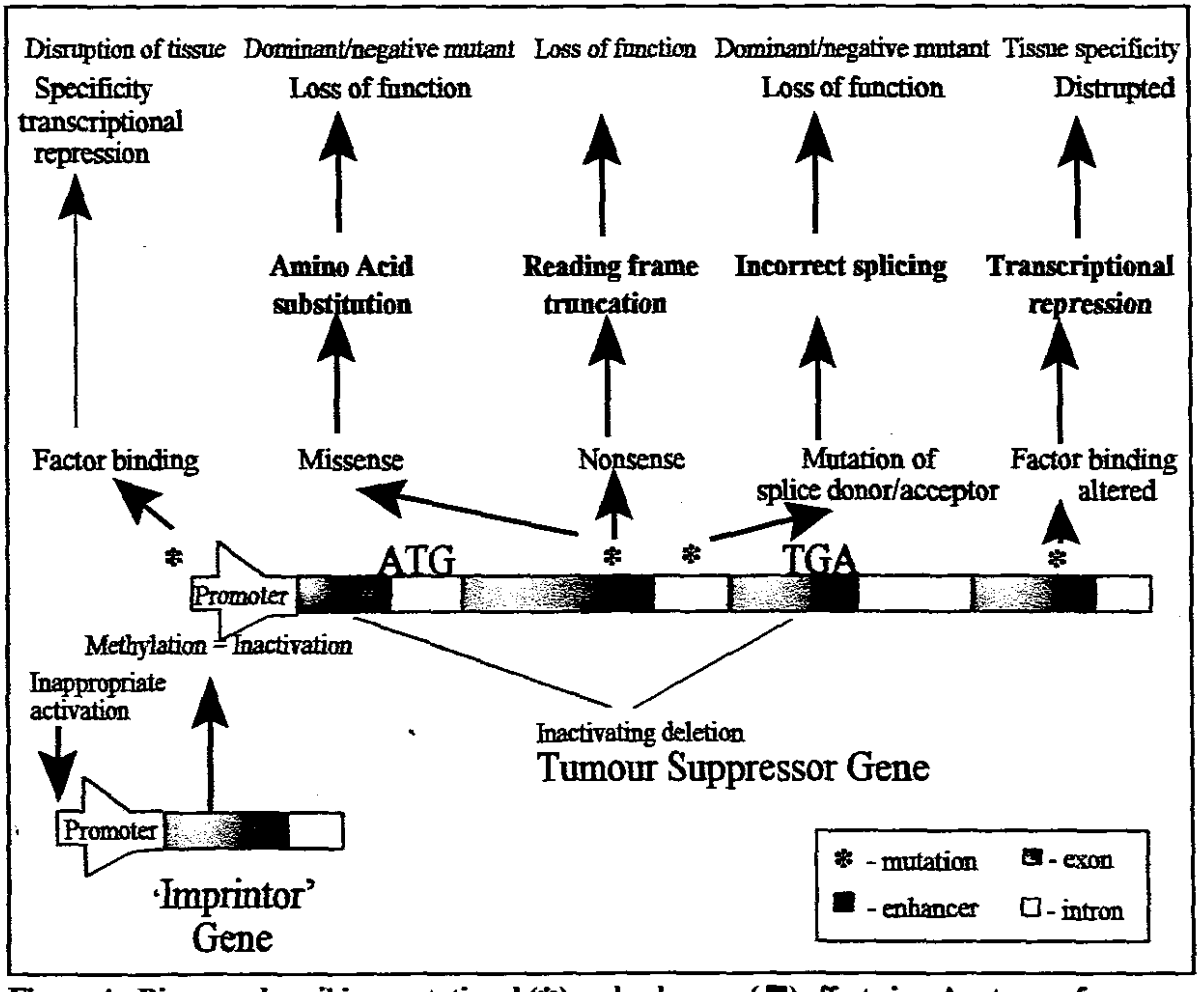


Figure 4 : Diagram describing mutational (*) and enhancer (■) effects in Anatomy of suppression (Adapted from Little *et al.*, 1995)

- **nm23**

A potentially important new prognostic factor in breast cancer is the reduced expression of a putative antimetastatic gene, called nm23 (Veronesi *et al.*, 1995; Amendola *et al.*, 1997). So far, the data on nm23 is confined to an analysis of a small group of patients, in whom a reduced level of nm23 gene expression is significantly correlated with positive node status and reduced survival (Hennessey *et al.*,

1991). It is likely that, if these preliminary reports are confirmed, that the cellular mechanisms whereby this altered gene affects cancer, progression will differ significantly from those of growth factor receptors, such as EGFR and HER-2/neu. nm23 gene family members have been proposed to play a role in cellular differentiation, as well as metastasis suppression. Expression has been shown to be inversely correlated with lymph node metastasis in breast cancer, i.e. lack of expression implies metastasis (Royds *et al.*, 1993). The human gene has three forms: nme1 and nme2 and DR-nm23, a recently identified third member of the family. (Toulas *et al.*, 1996[①]; Toulas *et al.*, 1996[②]; Duenas-Gonzalez *et al.*, 1996).

An investigation into the role of nm23-H1 in mammary development and differentiation undertaken by Howlett *et al* (1997) implies that whilst the basement membrane micro-environment is capable of directing the differentiation of normal human breast cells, neoplastic transformation abrogates this relationship, suggesting that intrinsic cellular events are also critical to this process. The data in Howlett *et al's* study identifies nmH2 gene expression as one of these events suggesting an important role in the modulation of cellular responsiveness to the micro-environment. The data also identified previously unknown growth inhibitory effects of nm23-H1 gene overexpression (Howlett *et al.*, 1997). Expression of nm23-H1 gene product in a number of tumours has been reported to correlate with low metastatic potential and therefore with less aggressive tumour types.

In breast cancer, the expression of nucleoside diphosphate (NDP) kinase/nm23 has been reported to correlate with good prognosis and a lack of nodal metastasis. A rabbit polyclonal antibody (Neomarkers LabVision: NDP kinase nm23 Ab-1) kit is available to research laboratories which has application for Western blotting, immunoprecipitation and IHC.

- **HIC-1**

HIC-1 (hypermethylated in cancer), is a new candidate tumour suppressor gene on 17q13.3. 60% of breast cancers lose 17q alleles, whereas only 30% of these tumours contain p53 mutations. These findings suggest that one or more tumour suppressor genes may reside telomeric to p53, within the 17q13.3 region. Furthermore, in one study of breast cancer the independent loss of 17q13.3

alleles was accompanied by increased levels of p53 mRNA, leading the investigators to suggest that 17q13.3 may regulate p53 expression (Wales *et al.*, 1995). The hypothesis was that the presence of a hypermethylated CpG (cytosine phosphate guanine) island in an area of frequent allelic loss in tumour DNA marks the presence of a candidate tumour suppressor gene.

This approach led to HIC-1, which appears to be an excellent candidate for a second tumour suppressor gene on the short arm of chromosome 17. This gene resides within an 11-kb region that is extremely CG rich and that is the site of both frequent aberrant methylation and allelic loss in most human tumour types. The HIC-1 gene contains a consensus p53 binding site 4kb upstream from the transcription start site and is activated by p53 in at least one human tumour cell line (Wales *et al.*, 1995). Thus, the gene joins others, including wild-type activated fragment 1 (WAFI), mouse double minutes (MDM2), growth-arrest DNA damage (GADD45) and BAX as a potential downstream target of p53 that may play a role in tumourigenesis. HIC-1 probably functions as a transcription factor. Identification of the precise p53 pathway in which HIC-1 is involved should clarify the role of this gene in normal and neoplastic cells.

- **CHK**

CHK is a gene that is "switched on" in breast cancer cells but remains "turned off" in normal breast tissue and is part of the all-important biochemical pathway controlling the development of breast cancer. The literature suggests that the CHK gene may limit growth of cancer cells by blocking the activities of oncogenes. This is a significant discovery that could result in new diagnostic, therapeutic, or prevention strategies for breast cancer. The CHK gene is a novel gene that is the cell's defense mechanism against breast cancer and that, it is thought, tries to fight the excessive growth stimulation that is involved in the erb-B pathway (Avraham *et al.*, 1997). There appears to be an association of Csk-homologous (CHK) formerly mitogen-activated tyrosine kinase (MATK) with HER-2/erb-2 in breast cancer cells.

Unlike many genes that are active in a variety of tissues, the CHK gene is switched on only in the brain and in blood cells. The initial studies of CHK in blood cells suggested it acts to oppose cell

growth. Groopman (1997) discovered that the CHK gene is active in breast cancer, but not in normal breast tissue. If the CHK gene is a defense mechanism, trying to limit the growth of cancer, it might be possible to treat breast cancer by manipulating levels of the CHK gene in malignant breast cells. The CHK gene may help improve early diagnosis of breast cancer, because by finding the CHK gene "switched on" in a breast biopsy, even in an early or *in situ* cancer, it may be possible to assume a malignancy is developing and develop strategies using CHK to benefit patients. The mechanism by which the CHK gene is functioning is not yet fully defined.

2.5 Suppressor gene p53

The 20-kilobase p53 tumour suppressor gene encodes a 393 amino acid nuclear phosphoprotein thought to regulate proliferation of normal cells (Allred[@] *et al.*, 1993) and is located in the short arm of chromosome 17 at position 17p13 (Figure 5). Two types of event can be triggered by activation of p53: growth arrest and apoptosis (Cohen, 1993; Canman *et al.*, 1994; Lewin, 1997). The outcome depends in part on which stage of the cell cycle has been reached (Suwa *et al.*, 1997).

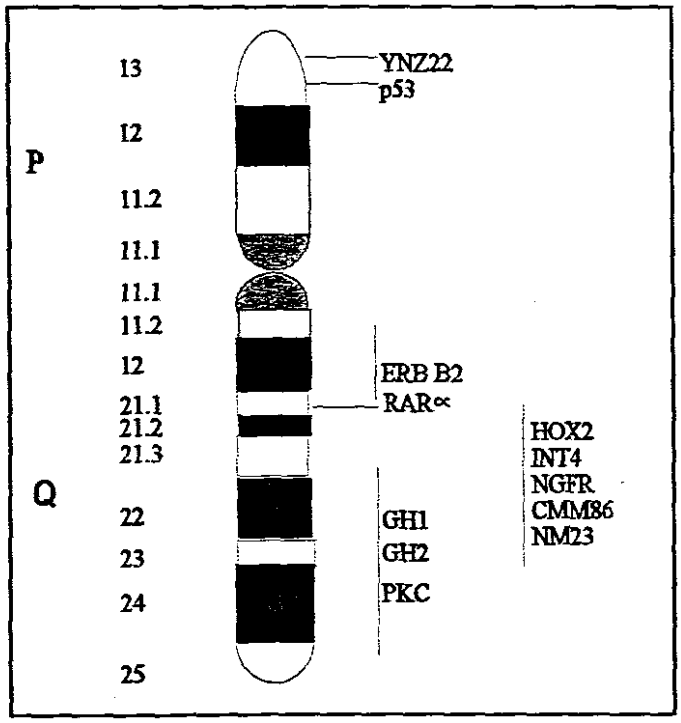


Figure 5: Chromosome 17 showing the location of p53 at position 17p13 (Adapted from Lane *et al.*,1997)

2.5.1 p53 gene structure

The p53 gene has 5 evolutionary well-conserved regions (Figure 6). Over 95% of the base substitution mutations in the p53 gene code for the internal and most conserved part of the p53 protein and occur in 4 well-conserved regions in exons 5,7 and 8 (Lane 1992; Lane 1996). Studies using monoclonal antibodies (MAb's) have shown that some mutant p53 proteins have an exposed epitope which is present but not exposed on wild type p53 (Labrecque *et al.*, 1993; Soussi *et al.*, 1994). The majority of p53 mutations (more than 80%) that have been detected have been found in other areas of the gene, mainly in exon 6 (Hernandez-Boussard *et al.*, 1998).

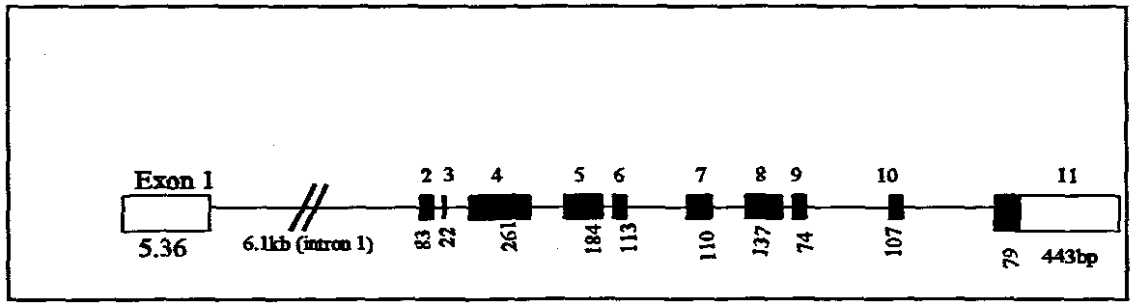


Figure 6 : p53 gene structure (Oncogene Science, 1999)

Although only 25% of breast cancers contain p53 mutations, 15% of these are G:T -> T:A transitions at non CpG sites and 11% are A:T -> G:C transitions with nontranscribed strands bias of 8, 9, 63 and 76% respectively, suggesting that exogeneous bulky carcinogens may play a role. The p53 promoter lacks a TATA or CAAT sequence but contains potential nuclear factor 1 (NFI) and p53 factor I (PFI) binding sites and a helix-loop-helix consensus binding sequence (Hesketh, 1995). A large number of p53 mutations lead to a single substitution of a nucleic base pair but 10% of human cancers are characterized by deletions or insertions in this gene. Normal and epidermal carcinoma cells express an alternatively spliced RNA at 30% of the level of the normal transcript that contains an additional 96 bases derived from intron 10 (Waterman *et al.*, 1996). Somatic mutation have been detected in most exons although the vast majority occur between exons 5 and 8.

A number of genes contain regulatory sequences capable binding p53 and whose activity is altered

by that interaction (Stuart *et al.*, 1995; Huws, 1997). Depending on the gene involved, both stimulating and inhibiting effects have been documented (King, 1996). Four cellular responses are influenced by these genes; cell proliferation, apoptotic death, DNA repair and angiogenesis (Figure 7).

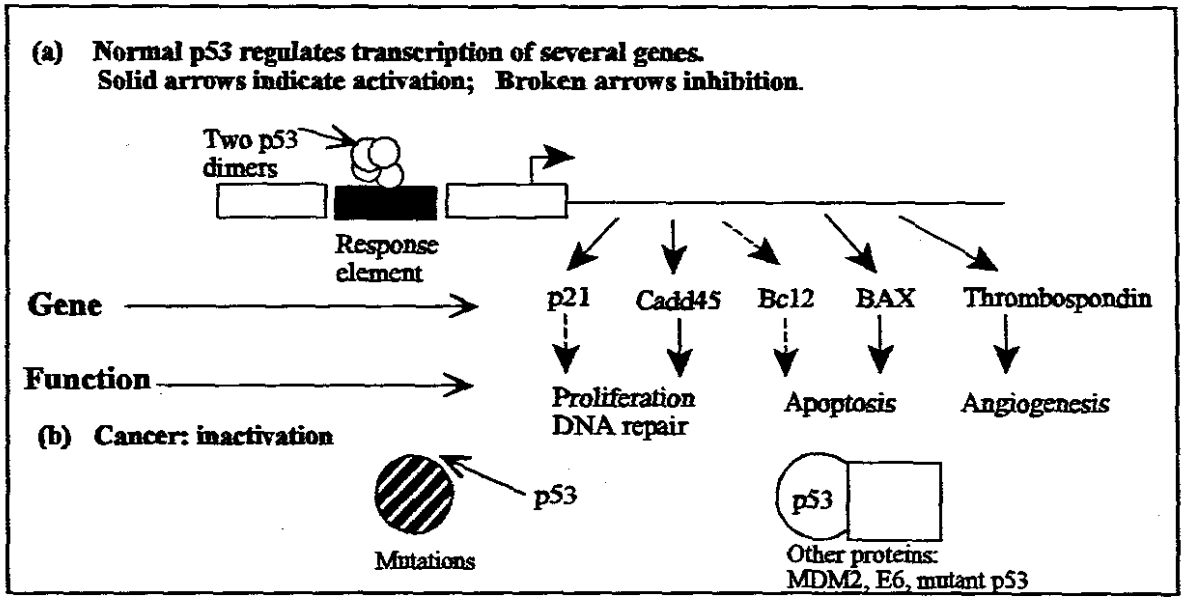


Figure 7: Actions of p53 (Adapted from King, 1996)

p53 activation leads to induction of wild-type p53 activated fragment 1 (WAFI), growth arrest DNA damage 45 (GADD45), and mouse double minute chromosome (MDM2) genes (Lonardo *et al.*, 1997). If DNA repair is unsuccessful p53 may trigger apoptosis p53 binds to specific sequences on double-stranded DNA (Voet *et al.*, 1995; Milner, 1995). All point-mutated form of p53 that are implicated in cancer have lost their sequence-specific DNA-binding properties (Voet *et al.*, 1995). The p53 gene contains the DNA for the transformation-associated protein p53. The normal role of p53 gene product may be to interact with either DNA or specific proteins in order to suppress neoplastic growth of certain epithelial cell lines (Bond, 1992).

Missense mutations frequently produce abnormal p53 proteins that are much more stable than normal p53 (Lane *et al.*, 1997; Lutz *et al.*, 1998) (Figure 8). Missense mutations within conserved regions II through V cause the p53 protein to lose its wild-type conformation (Jerry *et al*,1993; Brandt-Rauf,

1997). Additionally, it increases the half life of the protein from 20 minutes to several hours (Burns, 1993; Thorlacius *et al.*, 1993; Jerry *et al.*, 1993; Thomas *et al.*, 1997).

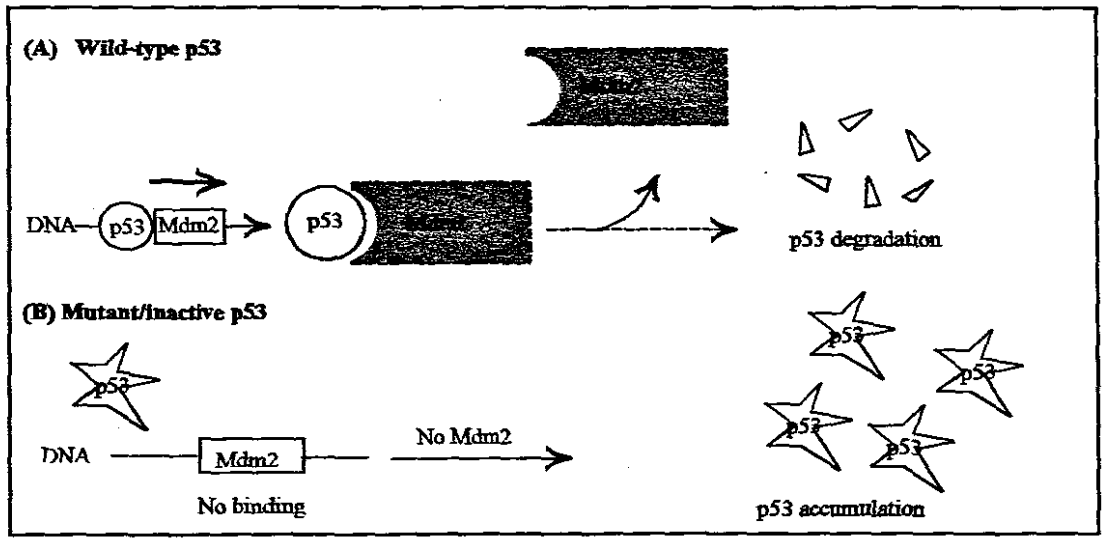


Figure 8: Why p53 is stable in tumour cells. (A) Wild-type p53 acts as a transcription factor for the MDM2 gene. In healthy cells, p53 induces the synthesis of MDM2, which then initiates p53's breakdown. (B) Accumulation of mutant p53 is not a result of the intrinsic stability of mutant protein, but rather its inability to activate MDM2 transcription. Consequently there is no MDM2-mediated p53 degradation, hence p53 protein accumulates. (Amended from Lane *et al.*, 1997)

The result of the mutant p53 proteins having longer half lives than the wild-type protein results in increased cellular concentration of p53 that is detectable (Pulkki *et al.*, 1996). The p53 protein is rendered unusually detectable by conventional Western blotting and immunocytochemistry techniques (Harris *et al.*, 1993).

2.5.2 p53 and heat shock protein (hsp)

Unlike wild type p53, some mutant p53 protein are able to complex with the M 70,000 heat shock protein (hsp 70). In addition to heat, the genes of this group are activated by other adverse environmental conditions, such as exposure to heavy metals and ethyl alcohol, oxygen deprivation, and in higher plants, water loss and wounding. The proteins encoded by the genes repair some of the damage to cellular proteins produced by the stress and protect cells against a second exposure (Wolfe, 1993). Heat shock proteins occur in several highly conserved families. The family which

complexes with the mutant p53 proteins has among other functions, the ability to induce other proteins to fold into conformations that facilitate movement through cellular membranes. The most important family in the heat shock response, is hsp90 (molecular weights averaging 90,000). These proteins bind to steroid receptors, protein kinases, actin, tubulin and other proteins important to cellular responses and stabilize them in an inactive state (Wolfe, 1993). Taken together, these observations demonstrate that subtle mutations in the p53 protein can affect the configuration of the entire protein.

2.5.3 p53 in breast cancer

Mutations in the p53 gene are common in breast cancer (Thorlacius *et al.*, 1993; Lasky *et al.*, 1996; Willsher *et al.*, 1996). In primary breast cancer about 20% of cases have a p53 gene mutation (Quesnel *et al.*, 1994). An association of p53 protein expression with tumour cell proliferation rate and clinical outcome in node-negative breast cancer was cited by Allred, *et al* (1993). Insights into the genetic basis of breast cancer have been afforded by the identification of frequent loss of heterozygosity of human chromosome 17q and the identification of p53, located on chromosome 17q, as the most commonly mutated cellular gene in human breast cancer (Jerry *et al.*, 1993; Ozbun *et al.*, 1995). Mutational Spectrum Analysis has indicated the pattern of change is different in breast, lung and colon cancers, suggesting that different carcinogenic events are involved in each case.

Missense mutations stabilize p53 protein, which accumulates, reaching levels detectable by IHC (Rosanelli *et al.*, 1993; Jacquemier *et al.*, 1994). Most breast carcinomas studied have been of a late stage, which precluded evaluation of the role of p53 alteration in the early stages of malignant transformation (Diamandis *et al.*, 1996). It is now widely accepted that most tumours are clonal, arising from a single altered cell. Studies on the clonality of breast cancer have shown p53 mutation patterns which suggest relatively early mutation preservation of this clonal alteration with metastasis. Mutant forms of the protein have transforming activity and interfere with the cell cycle regulatory function of the wild-type protein (Hollstein *et al.*, 1991; Thorlacius *et al.*, 1993).

Crawford *et al.*, (1982) reported that 12% of sera from breast cancer patients contained anti-p53

antibodies and that no such anti-p53 response was detected in individuals without cancer. Before any evidence of p53 mutations in cancer cells had occurred, anti-p53 activity in sera from breast cancer patients had been demonstrated (Labrecque *et al.*, 1993; Wild *et al.*, 1995). It remains unclear why some patients with tumours containing high levels of mutant p53 molecules do not produce anti-p53 antibodies.

In Labrecque *et al's* study (1993) there was no evidence that mutant p53 molecules contain dominant antigenic epitopes which are not present on the wild-type p53 protein, i.e. the anti-p53 response resulted in antibodies which recognized mutant and wild-type p53 proteins equally well. Data from Labrecque *et al's* study (1993) reveals that the anti-p53 response is unlikely due to the conformational change induced by the single amino acid changes associated with the mutant p53 proteins. It appears that the immune response against p53 results from loss of tolerance induced by accumulation of mutant p53 in the tumour cell (which may be released during tumour necrosis).

Based on the literature, p53 auto-antibody levels could be used with other established criteria to refine further the diagnostic and prognostic assessment of node-negative breast cancer (Peyrat *et al.*, 1995). Previous analysis of p53 status by molecular or IHC methods show that p53 alteration correlates with tumour aggression and patients with p53 alterations have shorter disease-free and overall survival rates (Peyrat *et al.*, 1995; Aas *et al.*, 1996). The importance of identifying high-risk patients at diagnosis is supported by evidence of an improved relapse-free survival after chemotherapy or hormone therapy for specific subsets of patients with lymph node-negative breast cancer. The production of p53 auto-antibodies in serum correlates closely with other factors that indicate poor prognosis (Schlichtholz *et al.*, 1992; Houbiers *et al.*, 1995). Alteration of p53 probably represents a relatively early event in cancer progression since it is evident in a small fraction of cases at the *in situ* phase of tumour growth (Silvestrini *et al.*, 1993).

Mutation of p53 has also been suggested to be a late event in breast cancer owing to the variety of overexpression of p53 in ductal carcinoma *in situ* and its association with some breast carcinomas (Rosen *et al.*, 1995; Rudas *et al.*, 1997). However, the absence of p53 mutations in early lesions has

not been demonstrated directly, but rather inferred by the lack of accumulation of p53 protein (Jerry *et al.*, 1993). In Lubin *et al's* study (1995) on lung cancer, p53 antibodies were always present at the time of diagnosis but never appeared during tumour development. This suggests that the humoral response can be used as a precocious marker of p53 alterations before clinical manifestation of disease (Lubin *et al.*, 1995).

2.6 c-erb B-2

There are four/five members of the c-erb B-2 family. Various groups have described a 185-kDa protein with tyrosine kinase activity (Figure 9) encoded by the c-erb B-2 (*neu*/HER-2) oncogene which is localized on chromosome 17q21-22 (Zoll *et al.*, 1992; Wright *et al.*, 1992; Wu *et al.*, 1995; Nistico *et al.*, 1997).

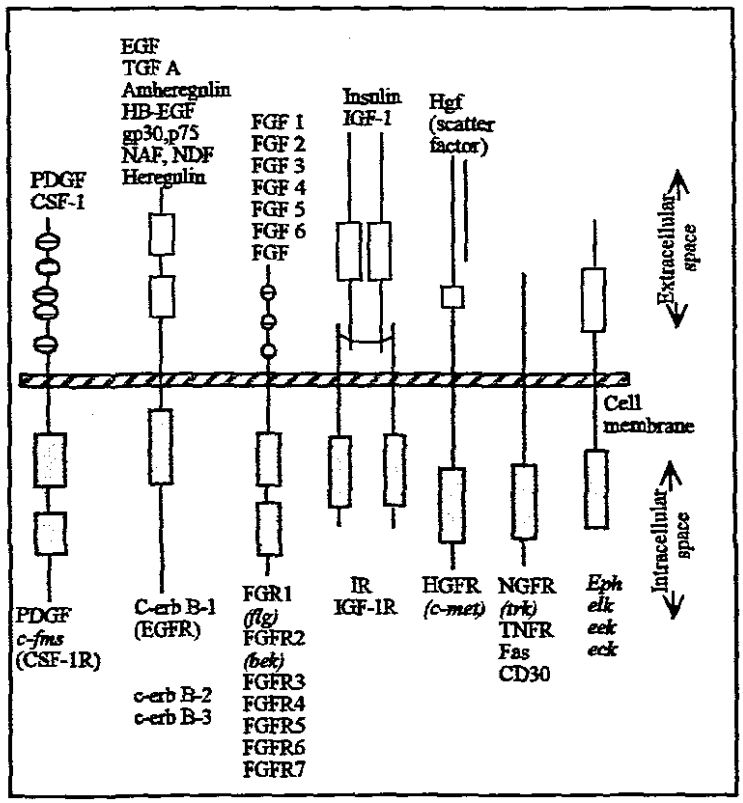


Figure 9: Transmembrane growth factor. Schematic representation of the different transmembrane protein tyrosine kinase receptor classes. Individual receptors belonging to each family are indicated in the lower portion of the diagram (for example, c-erb B-1, c-erb B-2 and c-erb B-3). Growth factors including putative ligands are indicated in the upper portion of the diagram (for example PDGF and CSF-1). (Hollywood, *et al.*, 1995)

c-erb B-2 has a cysteine rich extracellular presumably ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase domain, indicating that c-erb B-2 is a cellular receptor. It comprises both an extracellular and an intracellular domain. While the extracellular domain has ligand-binding activity, the ligand has yet to be clearly defined, but is thought to act as a growth factor (Kandl *et al.*, 1994).

2.6.1 Investigations into the role of amplification of the c-erb B-2 gene

Investigations into the role of amplification of the c-erb B-2 gene have appeared to give somewhat contradictory results.

- Antibodies to c-erb B-2 have been shown to inhibit both anchorage-dependent and anchorage-independent growth *in vivo* (Xu *et al.*, 1993).
- c-erb B-2 has been found to be amplified in 20-30% of primary breast cancers and gene amplification correlated with oncoprotein overexpression (Kandl *et al*,1994; Hubbard *et al*,1994).
- Findings tend to suggest that, whatever influence the presence of c-erb B-2 expression has on the biology of breast cancer (Niehans *et al.*, 1993), this effect is confined to the earlier clinical phases of the disease (Kandl *et al.*, 1994).
- There is also evidence that c-erb B-2 overexpression may be preferentially associated with certain histological sub-types of breast cancer (Kandl *et al.*, 1994; Lacroix *et al.*, 1989).
- In addition it has been suggested that c-erb B-2 amplification and protein expression correlate both with poor histological grade and lack of ER expression.

According to sequencing studies (Terrier *et al.*, 1996), the MW of the molecule should be 137,000 based on amino acid composition (Wu[2] et al., 1995). The structure of c-erb B-2 protein includes three domains:

- At the C-terminal end of the protein is a region containing 580 amino acids. This part of the molecule is intracellular (cytoplasmic) and has protein-tyrosine kinase activity (Figure 10) (Schroeter *et al.*, 1995) capable of autophosphorylation (Wu[3] et al., 1993).

The portion of the gene which encodes for the domain is 88% homologous to the corresponding part of the EGFr gene and is also closely related to other tyrosine genes.

- At the N-terminal end of the protein is a region containing 632 amino acids. This part of the molecule is extracellular, heavily glycosylated, and constitutes the ligand binding domain (*Wu et al*[@], 1995). The portion of the gene which codes for this domain has 44% homology with the corresponding part of the EGFr gene. The specific ligand for *neu* protein has been identified as a kDa glycoprotein, alternatively termed p44 and heregulin.
- the third domain consists of 22 amino acids and is a hydrophobic transmembrane anchor region. The human erb B-2 proto-oncogene is activated by gene amplification (Tsuda *et al.*, 1990; Kandl *et al.*, 1994) rather than by gene mutation (Anderson *et al.*, 1995). Point mutations leading to amino acid substitutions in this region have been shown to activate the rat *neu* protein for oncogenes.

2.6.2 c-erb B-2 oncoprotein in sera

The additional 48 kDa needed to reach 185 kDa (p185) is made up of glycosylation units, primarily mannose and glucosamine. While the intact native molecule has been widely reported to be 185 kDa, immunoblotting has shown that human serum and plasma specimens and supernatants from certain cultured cell lines contain another form of the protein which seems to be about 105 kDa. Since it reacts with antibodies to the cellular domain, this presumably represents the free extracellular domain (ECD). Any attempt to measure oncoprotein activity in serum from breast cancer patients must take into account that the ECD could be proteolytically separated from the intact oncoprotein, p185.

Wu *et al* [@]*.*,(1995) identified and characterized c-erb B-2 protein in serum and showed that the ECD, p120, was cleaved by the protease involved and then released. The protease responsible for release of p120 from p185 has not been identified. Measurement of protease activity could be an additional marker, since protease activity in serum may change with changes in tumour activity. This unknown protease cleaves the p185 molecules in the membrane, releasing p120 into the extracellular matrix and eventually into the blood circulation (Wu *et al*[@]*.*,1995). Almost all of the immunoreactivity measured in serum in the study by Wu *et al*[@]*.*, (1995) was associated with the p120

molecule (Figure 10). Overexpression of c-erb B-2 gene may occur without amplification (Slamon *et al.*, 1989). This implies that measurement of c-erb B-2 gene overexpression or the gene-encoded protein (p185) might be clinically more useful than determination of gene amplification (Wu *et al*[9]., 1995). Soluble erb B-2 protein levels in the serum can be used as a biomarker for spreading carcinomas that overexpress the c-erb B-2 protein. The release of this portion of transmembrane receptor may have implications for oncogenesis and its quantitation could have important prognostic value (Wu *et al*[9]., 1995).

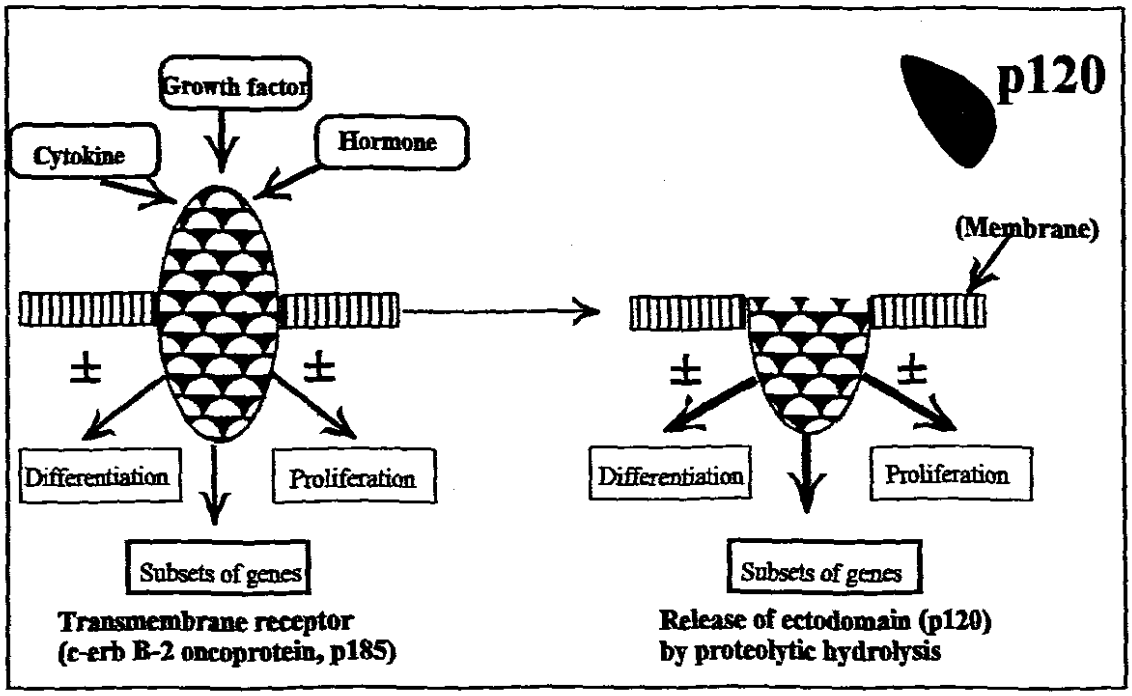


Figure 10: Intact c-erb B-2 oncoprotein (p185) is cleaved to produce the ectodomain (p120) by *unknown* protease(s). The figure also shows that the ectodomain is the portion of the molecule interacting with the growth factors, cytokines, and hormones, whereas the cytosolic domain containing the protein kinase is the portion of the receptor responsible to initiate various biological responses (Wu et al[9]., 1995).

2.6.3 c-erb B-2 and breast carcinoma

Increased expression of p185, often associated with gene amplification, is observed in a portion of human breast carcinomas. This may be a determinant of aggressive disease (Katsumata, 1995). The c-erb B-2 gene is reportedly amplified in 25-35% of human breast cancer. It was cited by Slamon

et al., (1989) and Zoll *et al.*, (1992) that this oncogene be a focus of attention for diagnosis and therapy in breast cancer. Many studies, (Narita *et al.*, 1992; Kynast *et al.*, 1993; Nugent *et al.*, 1994; Watanabe *et al.*, 1994; Wu *et al*[2]., 1995; Volas *et al.*, 1996) have reported elevated levels of the ECD of the p185 transmembrane growth factor receptor (encoded by the c-erb B-2 oncogene) in the sera of breast cancer patients, which suggests that this may be a biomarker of early malignant disease in certain cases of breast cancer.

In the context of a survival based analysis, genetic amplification of c-erb B-2 or expression of its protein were not found to be prognostically helpful in a study undertaken by Wolman (1992) and Tandon *et al.*, (1993). In breast cancer it appears to be as useful a prognostic indicator of overall survival as tumour size or estrogen and progeterone receptor expression (Chan, 1996). Since 1987 several studies have shown a relation between amplification of the translational product p185 (growth factor receptor) and disease-free and overall survival respectively (Kynast *et al.*, 1993). Taverna *et al.*, (1994) observed that high levels of c-erb B-2 protein are present in cells which have no apparent gene alteration, suggesting that other mechanisms exist to control the amount of erb B-2 RNA protein. Since high levels of protein generally correlate with poor patient prognosis (Hynes, 1993), a thorough understanding of the control of c-erb B-2 expression is important. One of these mechanisms may be the expression of specific transcriptional factors. The OB2.1 factor, which footprints the human c-erb B-2 promoter, is found in breast cancer cells that overexpress the c-erb B-2 gene (Antoniotti *et al.*, 1994).

Hormones also appear to play a role in the regulation of c-erb B-2 expression (Figure 11) and function (Davis *et al.*, 1995). Estrogen and EGF down-regulate c-erb B-2 oncogene protein expression by different mechanisms in breast cancer cells. The c-erb B-2 protein and mRNA content are controlled negatively and positively by estrogen and anti-estrogens, respectively (Taverna *et al.*, 1994). Estrogens have a positive effect on cell proliferation whilst anti-estrogens inhibit cell growth. Results from studies (Taverna *et al.*, 1994; Antoniotti *et al.*, 1994) suggest that there may be an inverse correlation between growth and erb B-2 expression. ER tumours are generally well differentiated and less invasive, whereas tumours overexpressing c-erb B-2 and/or EGFr are less

differentiated and more aggressive (Weigel *et al.*, 1993). 17β estradiol, which is strongly mitogenic for ER breast cancer cells, inhibits c-erb B-2 expression at both the mRNA and protein level (Antoniotti *et al.*, 1994). This may represent an important strategy in selecting anti-estrogenic adjuvant therapies wherever amplification of c-erb B-2 accompanies ER positivety (Wright *et al.*, 1992). c-erb B-2 gene amplification and protein expression have been implicated as prognostic markers for patients with recurrent progressive breast tumours (Rilke *et al.*, 1991) but recent studies indicate the role of c-erb B-2 as a diagnostic marker may far outweigh its usefulness as a prognostic marker (Underwood, 1995).

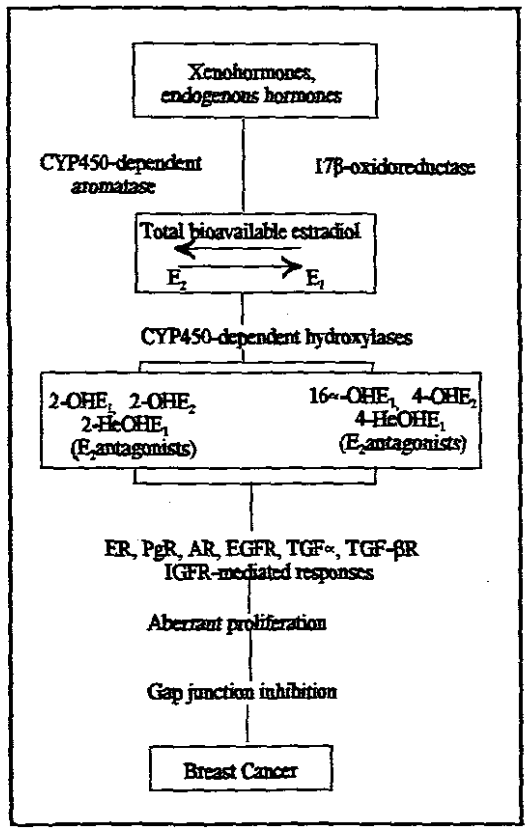


Figure 11: Hormones and breast cancer.
Steroid hormone metabolites having estrogenic or anti-estrogenic properties exert their growth modulatory effects indirectly via receptor-mediated mechanisms, leading to aberrant proliferation and breast cancer (Davis *et al.*, 1997)

The mean value and positive rate for this protein (assuming 20ml as the cut-off value) should be different in benign breast disease as compared to stages I, II and III in breast cancer. The very low levels of expression of p185, by normal adult tissues, makes the receptor an almost tumour-specific target. Amplification of the c-erb B-2 oncogene has been found to occur more frequently in p53

mutated tumours than controls (Thorlacius *et al.*, 1993).

2.7 Clinicopathological indices

A woman presenting with a lump in the breast needs a definite diagnosis as soon as possible as to its benign state or not; so as to relieve anxiety and to enable the physician to plan treatment strategies. Initial objectives are to exclude simple cysts by using fine needle aspiration (FNA) (Trott[①], 1993; Trott[②], 1993); solid tumours require open biopsy (with or without frozen section); Tru-cut or core needle biopsy, or the so-called triple assessment method (du Toit *et al.*, 1995). The latter method combines the results obtained by clinical examination, FNA cytology and mammography in order to make a definite diagnosis. Once sufficient tissue has been provided the clinical laboratory, especially pathologists, have the primary responsibility of establishing a histologic diagnosis of breast cancer, as well as its anatomic extent (Bloom *et al.*, 1957; Van Wyk *et al.*, 1995).

FNA biopsy of the breast has been shown to be a diagnostically accurate procedure with a rate ranging from 77%-99%. FNA is an invasive technique requiring highly skilled professionals to perform the procedure and experienced cytopathologists to interpret the aspirated material. These professionals are not easily available to women living in rural areas and the cost is a limiting factor to women of poor economic standing. Whilst the use of aspiration cytology to establish a diagnosis is time tested, its use to prove non-malignancy, is hazardous (Hutter, 1991).

* Accepted prognostic factors

The changing importance of prognostic factors in breast cancer during long term follow-up was documented by Lipponen *et al.*, (1992). Currently, the pathological features of lymph node status (presence or absence of axillary nodal metastatic involvement), tumour size, tumour type (for example, ductal carcinoma versus special histological types such as tubular, colloid, and papillary), grade of tumour (nuclear or histologic [or both]), and receptor status of tumour (estrogen-progesterone receptor positively or negatively) are the established prognostic factors for patients with breast carcinoma (Carter *et al.*, 1989; Elston *et al.*, 1991; Wold *et al.*, 1995; Silverstein *et al.*, 1997). The receptor status, a relatively weak prognostic factor, is generally used to determine

whether adjuvant hormonal therapy will be considered. Controversy continues regarding the value of these new prognostic factors, their interrelationships, and their advantages over better known features of prognosis such as tumour grade, axillary nodes with tumour deposits and tumour size (McGuire *et al.*, 1990; Perren , 1991; McGuckin *et al.*, 1995; Duffy, 1996).

2.8 Summary of Chapter Two

The 15 year history of p53 investigations is a paradigm in cancer research. It illustrates the convergence of previously parallel lines of laboratory, clinical and epidemiology investigation and the potential of rapid transfer of research findings from the laboratory to the oncology clinic. The literature indicates that current assay values obtained with different assay methods for breast cancer markers cannot be used interchangeably due to differences in assay methods and reagent specificity.. This is compounded since serial testing for cancer markers should only be used in conjunction with other clinical methods for monitoring breast cancer. This chapter investigated the current status regarding current and new genetic cancer markers in order to answer the important question, as to whether they have potential clinical utility in aiding diagnosis, assessing prognosis, predicting response to therapy and monitoring breast cancer patients.

CHAPTER THREE

AIMS:

3.1 Problem statement and objectives of the research

The purpose of this study was to determine if quantitative analysis of anti-p53 antibodies and c-erb B-2 in the sera of breast cancer patients could contribute to a profile which could be used in the diagnosis of primary, recurrent breast cancer or breast cancer management. These tests could be offered by the laboratory to assist in decision-making modalities for this patient group, without the need to perform painful, expensive tissue biopsies or FNA. These markers could perhaps also monitor the course of breast cancer (in order to assess the efficiency of therapy, to watch for drug resistance and/or to predict outcome). Lastly, the markers could be used to monitor patients in remission and to assess lead-time to relapse. The aims and objectives of this project were developed in accordance with existing regulations pertaining to the use of markers for breast cancer, the established laboratory criteria used for evaluation of new diagnostic tests and projections for technological and molecular advances in breast cancer.

3.2 *In vitro* studies of breast cancer

Previous research on p53 and c-erb B-2 predominantly involved Western women with breast cancer and the detection of these proteins in tissue sections using IHC. The success of this technique depends upon both the preservation of the antigen and upon the type of antibody used. Many antigens are destroyed by endogenous proteases, or by fixatives and processing procedures. IHC techniques are interpreted by subjective, semi-quantitative criteria and different antibodies are often used in these procedures. Results from different investigations vary (Levesque *et al.*, 1995) with the consequence that the accuracy of detecting p53 mutants and c-erb B-2 by IHC is questioned.

The advantages of quantitative immunologic assays versus IHC techniques have been summarized by Diamandis *et al.*, (in press). Despite advances made regarding characterization and structure of p53, the humoral immune response against mutant p53 remains to be clearly defined. These findings prompted this study to use an ELISA method to investigate if serological analysis of p53 auto-

antibodies and c-erb B-2 were effective as markers for breast cancer. ELISA is a cost-effective, non-invasive, easily repeatable, quantitative test which can be performed in most clinical laboratories in South Africa.

Earlier studies using ELISA also show inconclusive evidence of the efficacy of these markers. Most research studies used in-house protocols, while the literature indicates that certain research groups purchased kits (these kits are only available to research laboratories). This research project is a cross-sectional study of the prevalence of antibodies to mutant p53 and c-erb B-2 immunoreactivity in the sera of post-operative, pre-treatment predominantly indigenous South African women with breast cancer, followed by a retrospective observational study of the clinicopathological indices of the primary tumour. An observational cross-sectional study was chosen by the researcher as being the most effective means of achieving a satisfactory answer for a predominantly indigenous African population group. This study also establishes a foundation in South Africa for determining whether there is a genetic influence in the response to mutated p53, whether the response is higher in the indigenous African women compared to White, Indian and Coloured women.

3.3 *In vivo* studies of breast cancer

Despite considerable pathological uniformity, breast cancers appear to encompass a wide range of biologic behavior. After surgery, the use of adjuvant therapy, either hormonal or chemotherapeutic or both, is recommended by many oncologists. The current perception is that all women without involved nodes should receive adjuvant chemotherapy. It is hypothesized that a better stratification of patients could prevent many women from exposure to unnecessary discomfort and toxicity with the development of these new markers.

p53 tumour mutations may predict response to therapy (Fentiman, 1996) since Tamoxifen has been described to induce apoptosis. Potential explanations of the reduced effect of systematic adjuvant therapy for lymph node-positive breast cancer patients with p53 mutations might be that the mutant type of p53 is incapable of inducing apoptosis despite treatment with cytotoxic agents, including 5-fluorouracil. This observation resulted in studies to correlate p53 auto-antibody levels and established

clinicopathological characteristics of breast tumours. Evidence that some aspect of p53 auto-antibody may be preferentially associated with certain histological sub-types of cancer may be presented. Emphasis is placed on the possible lack of correlation between p53 and tumour stage suggesting that p53 protein overexpression and the subsequent immune response is an early event in breast cancer.

c-erb B-2 was investigated on account of its relative frequency (approximately 20%), which suggests it is an important predictor or disease recurrence and death. The validity of this assumption has been contested by certain research laboratories. This study attempted to test the hypothesis that amplification of c-erb B-2 and the subsequent presence of c-erb B-2 immunoreactivity in serum was either present early in breast carcinogenesis or an indicator of patient outcome. In order to achieve the aims of this study several issues needed to be clarified. The most important was that there must be the utmost confidence in the analytical accuracy and precision of the ELISA under investigation. Secondly, in order to translate the analytical data into clinically meaningful information, it was essential to determine what the RI was for a South African population group.

3.4 Sample size and laboratory studies

A problem in estimating the number of study cases which should be included (sample size) for this project was that there was insufficient information on these markers (particularly anti-p53). A sample was also being enrolled that had not been previously studied for cancer markers. The study involved 92 women of all race groups with microscopically confirmed breast cancer. Informed consent was obtained from all participants. Emphasis was placed predominantly on indigenous women because they are the population group most likely to benefit from the discovery of a reliable serological cancer marker. Influences of possible genetic differences between breast cancer population groups, with particular reference to p53 auto-antibody response and c-erb B-2 immunoreactivity, were taken into consideration. The "cut-off" level between the values obtained for women with breast cancer and those with benign inflammatory disease was established for different population groups.

When planning this research a small number of pre-analytical factors, which could possibility influence a diagnostic profile or which could substantiate any findings regarding current information on markers

for breast cancer were also included. To ensure the validity of the study, 42 control subjects were recruited at Natal Blood Transfusion Service clinics. This was an essential precaution due to the very high incidence of AIDS/ HIV in Kwa-Zulu Natal. Two control groups were identified - those women who had no evidence of breast disease (A) and those women who had benign breast disease (B). This was considered a prudent precaution as it could be established whether there were baseline differences between the levels of the markers in benign versus malignant breast disease.

It was hoped that serological analysis of anti-p53 and c-erb B-2 markers, using ELISA, may function as an additional approach to distinguish the functional state of these genes and may indicate a role which could have a positive outcome on breast cancer.

CHAPTER FOUR

MATERIALS AND METHODS

4.1 General Introduction

Recently there has been considerable improvement in the methodologies which can be employed for the diagnosis of cancer in research laboratories. The pace of scientific advance has generated numerous reagents and technical methods, several of which have emerged in tumour diagnosis (Stratton *et al.*, 1994).

Although IHC staining is a widely accepted method for "rough" quantitation, detection and localization of cellular protein expression, its sensitivity depends on the unpredictable effects that standard procedures for tissue harvest, fixation and processing exert on the preservation of the various epitopes of any antigen of interest (Kacinski, 1989). IHC methods, however, offer speed, simplicity and relatively low cost. The determination of anti-p53 and c-erb B-2 serum concentrations have two important advantages over tissue cytosols. Firstly, the use of serum specimens eliminates invasive tissue biopsy which has to be undertaken and assessed by competent medical practitioners and cytopathologists. Secondly, it allows for the easily repeated collection of specimens. This study focused on ELISA which is reproducible, cost-effective and is performed at most regional laboratories in South Africa.

4.1.1 Methodology used in evaluation of cancer markers

The clinical laboratory is faced with a series of practical problems before a cancer marker is used. Standardization assessment of sensitivity and specificity, determination of a cut-off, which context(s) a particular marker was suited for and whether the use of multiple markers (panel or profile) conferred any advantage over a single marker, were undertaken in accordance with clinical practice guidelines for the use of tumour markers in laboratory practice (American Society of Clinical Oncology, 1996).

The following were investigated to ensure reliability, validity and efficacy of data:

1. Pre analytical phase
   post-operative specimen collection
   physiological factors which affect results
2. Analytical phase
   choice of method
   standardization
   precision in performance
3. Post analytical phase
   presentation of results
   interpretation
   comparison with other data

4.2 Pre-analytical phase

- **Post-operative blood collection**

Since laboratory results are valueless if they are reported on the wrong patient, patients and controls were all verbally questioned for their name to minimize misidentification. Proper practices were followed in obtaining informed consent from all patients. Blood samples were drawn by forearm venepuncture (median cubital vein in the anticubital fossa) from selected individuals who had undergone surgery approximately 7 days previously. Veins on the back of the hand were used for 3 patients who had poor forearm veins.

In those women who had had a mastectomy the opposite forearm to the malignant breast was used since the surgery may have caused lymphostasis affecting blood composition. On the occasion of routine blood analysis at the Breast Clinic, an extra 5 milliliters (ml) of venous blood was taken from patients using an evacuated-tube system. Random blood samples were drawn from the normal control groups prior to blood donation at a Natal Blood Transfusion clinic. Collection of specimens for the establishment of RI and "cut-offs" from benign disease were collected identically to those from breast cancer patients.

Serum specimens were allowed to clot in an upright position for 30-60 minutes prior to centrifugation. If the samples were not given enough time to clot, latent fibrin formation could have occurred which could have caused problems during the analysis phase. After clotting, the blood was centrifuged (within 2 hours of collection) for 10 minutes at 3000 revolutions per minute (rpm) and the serum samples divided into aliquots using 0.5 ml -1.0 ml sterile tips and sterile microcentrifuge tubes. At least 2 microcentrifuge tubes per patient (minimum 0.5 ml each) were then immediately frozen at -70^{0}C until required. Different coloured tubes were used for each patient to distinguish aliquots in the event of contamination and/or the necessity of a repeat test. All sera used in the study were visibly free from haemolysis, lactescence or icterus. Sera were removed from the freezer as needed.and all samples were assayed in duplicate.

- **Physiological factors which influence results**

Factors, such as estrogen replacement therapy, which affect test results are addressed in questionnaires Addendum III and IV. Suspicion of an aetiological role for estrogens in the development of breast cancer stems primarily from epidemiological observations (Hilf, 1980; Howat, 1983; White *et al.*, 1994; Blaine, 1995; Adami *et al.*, 1995; Toniolo *et al.*, 1995; Hulke *et al.*, 1995; Wallis, 1996). Low risk has consistently been connected with high parity (Whitley, 1994; Hsieh *et al.*, 1994; Cotran *et al.*, 1994). Pre-analytical variables investigated which could influence a diagnosis of breast cancer were:

- Menstrual cycle (Simpson *et al*,1995; Chan *et al*,1994; Fentiman *et al*,1994; Bjarnason, 1996)
- Pregnancy (Stewart, 1994; Lambe *et al.*, 1994)
- Abortion (McCarthy, 1994)
- Hormonal replacement therapy (HRT)(Henderson *et al.*, 1991; Kessler, 1996)
- Obesity (Duffy *et al.*, 1991; Perry *et al.*, 1993)

The clinical significance of receptor assays is well recognized. Approximately half of the women whose tumours have detectable positive ER experience remission after some form of endocrine therapy. This number increases to three-quarters when progesterone receptor (PR) positive status, an estrogen-induced protein, is an additional feature of tumours (Walker, 1987).

4.3 Analytical phase

ELISA has become the most popular immunoassay used in research laboratories (Goers, 1993) and has 3 characteristics that made it the method of choice. Firstly, a solid-phase adsorbent allowed quick and thorough washing of unbound reagents. Secondly, the enzyme label provides a safe, stable and sensitive signal. Thirdly, ELISAs are relatively trouble free! In this study post-operative sera from diagnosed breast cancer patients and healthy control subjects were assayed (in batches) for antibodies to p53 and c-erb B-2 using ELISA. All analyses were done in duplicate. Patient and control subject's sera were analyzed in random order with the observer blind to patient/control data. None of the patients or controls were positive for antibodies to HIV.

4.3.1 ELISA

There are some common problems when performing ELISA. Reagents have to be well stored and protected from contamination by micro organisms or the introduction of unwanted reagents through the use of contaminated tips and troughs (reservoirs). Water is a major problem in standardization of assays between laboratories, even where identical reagents are used. The reasons why water affects ELISA have not been extensively examined. For this study, double distilled water was used (Aquation, Bibby). All laboratory glassware used was clean and well rinsed. This avoided the introduction of contaminants or adverse pH conditions into ELISA reagents, especially where initial dilutions of conjugate were concerned. The microtitre plates were kept separated and not stacked in order to avoid viscosity and edge effects when placed in the humidified incubator (set at 37^{0}C).

Materials required, but not provided by the kits:

- Pipettors: 2-20 μl, 20-200 μl and 200-1000 μl precision pipettors with disposable tips
- Precision repeating pipettor
- Wash bottle or multichannel dispenser for plate washing
- Microcentrifuge and tubes for sample preparation
- Vortex mixer
- Plate reader or spectrophotometer capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/595 nm or plate reader capable of measuring absorbance in 96-well

plate at a single wavelength of 450 nm

- Humidified incubator set at 37°C
- 500ml or 1000ml graduated cylinder
- Reagent reservoirs
- Deionized water of high quality
- Plastic wrap
- Liquid household bleach for inactivating clinical specimens and decontamination of plate washer
- Disposable paper towels

The micropipette tips used were Finn 60, which were the appropriate tips to fit the micropipettes used. Tips and troughs were purchased new for this project and tips checked to ensure that the ends were not damaged. New, clean tips were placed in tip boxes and autoclaved for 15 minutes at 121°C to ensure sterility. Specific troughs were used for conjugate and substrate to avoid cross-contamination. The tips, together with the troughs, were never re-used.

4.3.2 Method for standards and reference intervals (RI)

A pre-calibrated reliable standard and the practice of including standards in assays were essential for producing an accurate concentration estimate of the analyte in specimens using ELISA. The basic assumption underlying the use of a standard to confer a quantitative quality was that analytes in the standard and test specimens exhibit equivalent ability to displace the labelled ligand from solid phase receptor (competitive format) or bind to solid receptor (non-competitive format). The molecular configuration of the markers in the standard and test samples should ideally be identical. In addition, the dilution of the test matric should resemble that in the control matrix.

The level of antigen or antibodies in test samples from normal individuals may vary widely amongst control and test samples. This made it difficult to establish a common dilution to which all samples should be diluted. Variations in the matrix from patients with breast pathology may alter the kinetics of the antigen-antibody reactions and result in artefacts (negative or prozone reactions or non-specific binding or differential binding plateaux). Whilst it was not reasonable to have a reference dose curve

for each specimen's protein matrix, elevated immunoglobulin levels in samples may have produced a higher non-specific binding level in ELISA than would be seen in normal serum. This high non-specific binding would not be controlled for by the negative serum control and could lead to false positive results.

There are many instances where primary and secondary standards from international or national sources are not available for a given analyte. This is particularly true of antibody standards which are particularly important to ELISA for the setting of positive thresholds or the creation of standard curves from which quantitative measures are performed. This means that the relatively simple process of standardizing an in-house assay for routine use becomes an impossible task. In ELISA, calibration and interpolation of the data are the keys to calculation of results. Calibration requires the addition of various dilutions of the analyte (as standards) at known concentrations to the antibody-labelled antigen mixture. Most assay calibration curves exhibit roughly symmetric sigmoidal shapes when plotted against a log-transformed concentration axis. Once the standard curve was derived, the concentration of the marker in unknown specimens was determined.

Many factors were considered in determining the RI. They included different pre-analytical and analytical sources of variation as well as statistical variations attributable to sampling error and calculation procedures. As there is no standardized method to evaluate either anti-p53 or c-erb B-2, most laboratories undertaking research either calculate ranges without controlling potential sources of variations or simply import the intervals determined by other investigators without checking their validity (Ichihara, 1996). The determination of RI's for this study were according to the guideline described in the IFCC's document on RI's. Practical problems encountered included selection of women "with no evidence of breast disease" and issues concerning body weight, age, race, current and past medical histories and hormonal status. Excluded at initial selection were those who had a history of recent hospitalization or illness. No one was included who was pregnant, took chronic medication or exhibited overt obesity (greater than 20% of ideal body weight). 42 women, 35 - 70 years of age were chosen as the subjects for the RI.

4.3.3 Precision in performance

A key determinant of the sensitivity of the system was the quantity of reactant coated on the surface of the 96-well microtitre plate. The antigens were coated onto the solid surfaces by adsorption, a process that depends somewhat on hydrophobic interactions performed at alkaline pH. Partial denaturation of some proteins result in exposure of hydrophobic regions and ensures firmer interaction with the plastic. This was achieved by exposure to mild detergent. The assays used in this study were performed on kits that contained precoated solid surface. The adsorption process is non-specific and cognisance was taken that any substance could have adsorbed to plastic during the assay. After the antigen was coated onto the solid surface, individual serum were diluted in a buffer containing a detergent or large quantity of bovine serum albumin to prevent adsorption of non-specific antibodies.

Conjugate dilutions of 1:500 and 1:1000 should give good activity, low background and allow quantitation over the range of anti-p53 and c-erb B-2 quantitated. Higher concentrations increase non-specific readings and decrease the measurable analyte range. High-density binding of antigen may not have allowed antibody to bind through steric inhibition. Lower conjugate levels decrease specific absorbance measured but non-specific binding is unchanged. Serial dilution titration analyses were performed by Oncogene Science to determine optimal concentrations of reagents. The purpose of washing was to separate bound and unbound (free) reagents. This involved the emptying of plate wells of reagents followed by the addition of buffered PBS (0.1 M, pH 7.4) to maintain isotonicity. This process was performed at least 3 times for every well. Wells filled with washing solution were left for about 30 seconds before emptying (soak time). This procedure was accomplished using a specialist plate washer (Sanofi-Pasteur) - an apparatus that filled and emptied the wells automatically.

4.3.4 Precautions and recommendations in ELISA assays

All components were stored at 4°C , but brought to room temperature (RT°) prior to use. All individual steps were accurately timed. For a 1 hour incubation step, no more than 2 minutes either way was tolerated. The same procedure for addition of reagents was always used. The temperature of the substrate solution was important since this affected the rate of colour reaction. For this reason a range of 20 - 30°C was maintained. Great care was taken with conjugates since they were the

signal suppliers of the assay. . Reagents were not exposed to excessive light and the kit components were not used beyond the indicated kit expiration date.

The addition of conjugate and stopping solution was added accurately to ensure the same volume in each well and limit the effect of volume changes. (This also concerned the blotting of plates to remove residual washing solution). Substrate was always added immediately after the wash steps. All required strips were secured to the microtitre plate prior to commencing the ELISA. Precise, thorough washing of microplate wells was essential if the ELISA was to perform to specification. A typical ELISA requires two wash steps during processing; one following incubation of the sample in the coated plate and the other following incubation of the conjugate with the sensitised wells. In both wash steps, the wells needed to be filled to capacity to ensure complete washing of the well-wall surface. The first wash step ensured the removal of free, unbound antibody or antigen which was left in the microplate well and which could inactivate conjugated antibody at a later stage in the assay. This inactivation may be partial or complete and could lead to a false-negative result. The second wash step, to remove unbound conjugate prior to addition and incubation of the substrate, was equally critical. Any remaining unbound conjugate would have reacted with the substrate. A high background colour of the negative controls was an indicator of this event.

Soaking was the method used to achieve effective washing without dismantling the antibody- antigen complex, .

- The buffer and reagents used in this kit contained either sodium azide or chloroacetamide as preservatives.
- Unused samples and controls were properly disposed of as biohazard waste.
- Solutions were disposed of in compliance with local regulations. Disposable gloves were worn at all times in specimen and reagent handling. Reagents from different kits were never mixed

4.4 Instrument specifications

The ELISA was performed using the Diagnostics Pasteur LP400. This 9 channel filter photometer measures the light absorbance in 96 well microtitre plates and is combined with an optomechanical

system which has microcomputer electronics. This allowed the achievement of precision measurements with fast throughput. The measurement wavelength was chosen at the maximum absorbance of the sample. The reference wavelength was set outside the absorbance area of one sample, thus any influence of finger prints, scratches, dust on the plates was avoided and dual wavelength measures were performed to adjust any such problems. The first wavelength (measurement wavelength) was chosen as the maximum absorbance of the sample whilst the second wavelength (reference wavelength) was chosen where the sample had low absorbance.

It was important that the analyte be measured over the concentration range in which the ELISA had steep dose-response characteristics. In practice, this meant diluting the sample. To avoid high non-specific reactivity with sample components that stick directly to the coated plastic surface, a wetting agent (e.g. Tween 20) was used, also bovine serum albumin (BSA) was used as an additional blocking material in the sample diluent. The high sensitivity of ELISA implies a stringent limit on the acceptable background signal due to non-specifically bound reactants. Low background was achieved by thorough "blocking" of the test-wells with an inert irrelevant protein. Horseradish peroxidase, as the enzyme, was supplied in a purified form, and this was the enzyme, of choice in the new improved method for anti-p53 measurement used by Diamandis *et al., (1994)* which used time-resolved immunofluorometry.

4.5. Assessment of p53 alterations

p53 alterations can be assessed by three main approaches. The first is IHC, which is a qualitative procedure. The second approach is molecular analysis of the p53 gene in which PCR amplification and DNA sequencing lead to the specific identification of a mutation in the gene. The third approach consists of an assay of p53 antibodies found in the sera of breast cancer patients (based on the initial results of Crawford *et al.*, 1987].

The significance of cytoplasmic staining of p53 having been observed in a minority of breast carcinomas which may not be accompanied by a corresponding gene mutation is not clear (Moll *et al.*, 1992; Varley *et al.*, 1992; Walker *et al.*, 1991). The observation that certain breast cancer patients are serum positive for p53 auto-antibodies by ELISA and yet negative for IHC has fostered

a suggestion that in these cases immunogenic stimulus may have occurred at an earlier stage of tumour development (Mudenda *et al.*, 1994).

The techniques for detecting anti-p53 antibodies in patient sera are currently based on radioactive labelling, immunoprecipitation and immunoblotting. In a study undertaken by Lubin *et al* (1995), consistently good correlation was found between sensitive ELISA procedures and immunoblot and/or immunoprecipitation. Angelopoulou *et al.*, (1993) recently devised a quantitative immunological method for measuring p53 antibodies in serum using time-resolved immunofluorometry. Using an immunoblot assay, circulating antibodies to p53 protein were found in serum samples from patients with breast cancer. Western blot assay is not suitable for large scale diagnostic testing whereas ELISA techniques can be applied more widely in different laboratories.

4.5.1 p53 auto-antibody kit

The Oncogene Science Research Products p53-auto-antibody ELISA was designed to measure circulating antibodies to p53 in serum samples. p53 auto-antibody kit (Q1A 16) is a product of Dianova, Hamburg, Germany that had been licensed to Calbiochem Novabiochem, Corp. for world-wide distribution, excluding certain European countries and purchased in South Africa through AEC Amersham, United Kingdom.

The components in each kit were:

- one precoated microtitre plate containing 96 wells (12 strips of 8)
- standards (4 separate vials of human anti-p53 serum)
- sample dilution buffer
- horseradish peroxidase antibody conjugate
- conjugate dilution buffer : 1 bottle which was ready to use, containing 15 ml of protein containing buffer
- substrate buffer : 1 bottle, ready to use, containing 15 ml of acetate buffer, pH5.0
- stop solution : 1 bottle supplied, ready to use, containing 7.5 ml of 2 N HCl
- wash buffer : 1 x 100 ml bottle containing 50 ml concentrated phosphate buffered saline (PBS). This was diluted to 1 litre with distilled water prior to use
- substrate

- 2 vials of antibody conjugate were supplied, containing lyophilized peroxidase conjugated goat anti-human IgG polyclonal antibody. This needed to be reconstituted with conjugate dilution buffer prior to use
- TMB stock solution consisted of one vial containing 1 ml of the concentrated chromogenic substrate, tetramethylbenzidine (3,3,5,5-tetramethylbenzidine, TMBlue) which needed to be diluted with substrate buffer prior to use. This contained dimethyl sulfoxide (DMSO) which is a polar aprotic solvent for inorganic substances (including methyl cyanide)

4.5.2 Principle of the assay

The p53 auto-antibody ELISA is a sandwich-type immunoassay in which microtitre plates are precoated (bound to the solid phase) with p53 auto-antibodies to which human recombinant p53 protein is bound. The samples were diluted 1:100 in sample dilution buffer (provided with the kit) before analysis. Diluted test serum was added to excess antigen immobilized on the solid phase. The amount of specific antibody that bound (or was "captured") was then quantitated using labelled antibodies that specifically bind to the constant region of the immunoglobulin class of interest (secondary antibodies). The detector antibody (i.e. secondary antibody) is a horseradish peroxidase conjugated purified goat polyclonal antibody which recognized human IgG.

Performance of the test involved pipetting the sample and standard into wells and allowing the mixture to incubate for 1 hour at 37°C to allow the antibodies present to react (bind) with the immobilized p53 antigen. The wells were washed and the enzyme-labelled second antibody then added. The amount of antibody in the sample which was bound to the p53 antigen on the plate was measured by addition of peroxidase conjugated polyclonal antibody. This reacted with human IgG which then catalysed the conversion of the chromogenic substrate tetra-methylbenzidene (TMB) from a colourless solution to a blue solution (or yellow, after the addition of stopping reagents). The intensity of the colour reaction was then measured.

The colour was quantitated by spectrophotometry and reflected the relative amount of p53 antibody in the sample when compared to the supplied standards. Absorbance was read at dual wavelengths of 450 and 595 nm. Oncogene Research Products recommend using the 595 nm reference filter in

order to compensate for possible differences in the material of the microtitre plate. Readings may be performed at a single wavelength of 450 nm, however, backgrounds and readings may be higher due to plate contributions.

4.5.3 Reagent preparation

Before starting, the positions of the assay standards and samples were recorded. All reagents were allowed to be at room temperature for one hour before.

1. The wash solution was prepared by diluting the wash buffer to 1 litre with distilled water.
2. Preparation of samples: required volume was 100 μl per well. The samples were diluted 1:100 in sample dilution buffer and were assayed in duplicate.
3. Preparation of the detection antibody solution: The lyophilized antibody conjugate (which expired after 6 hours when diluted) was reconstituted with the volume of conjugate dilution buffer stated on the vial label. The solution was vortexed several times during the reconstitution time which lasted at least 30 minutes. 20 μl of reconstituted antibody conjugate in 1 ml conjugate was then diluted in 1 ml conjugate dilution buffer per required strip (Table 2).
4. The substrate solution was prepared just before use. The TMB stock solution (once opened and diluted only lasted 1 hour) was warmed to 37^{0}C and mixed thoroughly to dissolve TMB crystals. 20 μl of TMB stock solution was then diluted in 1 ml of substrate buffer per strip (Table 2). The substrate solution was stored at room temperature (RT) in the dark until use and was used within 30 minutes.

Table 2 : Preparation of the detection antibody solution and the substrate solution

Number of strips	1	2	3	4	5	6	7	8	9	10	11	12
I	201	402	603	804	1005	1206	1407	1608	1809	2001	2201	2401
II										0	1	2
I = *antibody conjugate or* TMB stock solution (μl) **II = conjugate dilution buffer or substrate butter (μl)**												

4.5.4 Detailed protocol:

Since conditions varied, both samples and standards were assayed in duplicate each time the assay was performed.

1. The p53 auto-antibody ELISA was provided with removable strips of wells so the assay could be carried out on separate occasions. The required strips were then secured to the microtitre plate. Unused strips were stored with the dessicant at 4°C in the self-lock bag provided. All wells were washed three times just before dispensing the standards and samples using an automatic microtitre plate washer and diluted wash solution. This was repeated four times for a total of five washes.
2. 100 μl per well of controls or diluted samples were added immediately after washing. One well was left empty for the assay blank. The microtitre plate was then incubated for one hour at 37°C in a humid chamber. The microtitre plate was then washed as described in step 1.
3. 100μl of the reconstituted antibody conjugate solution was added to each well except for the blank well. The microtitre plate was then incubated for 30 minutes at 37°C in a humid chamber. The microtitre plate was then washed again as described in step 1.
4. The substrate solution was then prepared by diluting TMB stock solution in substrate buffer immediately before used. The substrate solution was kept in the dark until ready for use. Immediately after the washing in step 3, 100 μl of prepared substrate solution was added to each well including blank well. The microtitre plate was then incubated for 30 minutes at RT (18° - 25°C) in the dark.
5. 50μl of stop solution was then added to each well, including the blank well, in order to stop the enzymatic reaction. To avoid assay drift it was important to add the stop solution in the same order and time intervals.
6. Absorbance was read at dual wavelengths of 450/595 nm using the Sanofi-Pasteur instrument.

4.6 c-erb B-2

There is a direct concordance between c-erb B-2 gene amplification and overexpression of the c-erb B-2 protein (Wu *et al*®., 1995; Nugent *et al.*, 1994; Anderson *et al.*, 1995). Lack of correlation

between seropositivity and tissue expression of c-erb B-2 (Table 2), was reported by Kandl *et al.*, (1994) in 24 patients.

Table 3 : Breast tumour tissue expression of c-erb B-2 protein and the presence of serum soluble c-erb B-2 fragment (Kandl *et al.*,1994).

N = 24	Serum soluble c-erb B-2 positive	Serum soluble c-erb B-2 negative
Tissue immunostaining c-erb B-2		
Positive	6	4
Negative	6	8

4.6.1. Assessment of c-erb B-2 alteration

In this study c-erb B-2 related protein was quantified in post-operative serum from patients who presented with primary, recurrent and non-recurrent breast cancer. The method measured a 100 kDa c-erb B-2 antigen fragment using a human *neu*/c-erb B-2 ELISA kit. According to the literature (Mori *et al.*, 1990 and Leitzel *et al.*, 1992) the detection in human serum of the external domain of c-erb B-2 can be measured by an automated chemiluminescent immunoassay or by ELISA using purchased commercial kits.

ELISA is more difficult than immunocytochemistry but is considerably simple and quicker (Vojtěšek *et al.*, 1993). Moreover, large numbers of samples can be handled simultaneously. ELISA has the potential to facilitate the introduction of the c-erb B-2 oncoprotein assay into the routine hospital laboratory (Terrier *et al.*, 1996).

4.6.2 Serum c-erb B-2 kit

The Oncogene Science ELISA kit was selected due to price constraints, availability and numerous publications which facilitated a comparitive study with previous work. The arbitrary human *neu* unit (HNU) was used to quantify c-erb B-2 related protein using the standards which were included in the

kits. The standards ranged from 0-100 HNU/ml.).

4.6.3 Principle of the assay for c-erb B-2

The Oncogene Science c-erb B-2 assay is a sandwich enzyme immunoassay, which utilizes a mouse MAb for capture and a rabbit polyclonal serum for detection of human *neu* protein. The capture antibody had been immobilized on the interior surface of the microplate wells. To perform the test an appropriate volume of specimen was incubated in the coated well to allow binding of the antigen by the capture antibody. A standard curve was simultaneously set up by adding standards as indicated in Table 4. The immobilized antigen was then reacted with the detector antiserum. The amount of detector antibody bound to antigen was measured by binding it with a goat-anti-rabbit IgG/horseradish peroxidase conjugate, which then catalyzed the conversion of the chromogenic substrate o-phenylenediamine into a coloured product. The plates were washed between each reaction step. The coloured reaction product was then quantitated by spectrophotometry (OD) and reflected the amount of *neu* protein in the sample.

4.6.4 Preparation of reagents

Table 4: Six standards HNU/ml were incorporated with each microtitre plate

Standard	HNU/mL	fm/mL	Volume
120	120	6	1 ml
90	90	4.5	1 ml
60	60	3	1 ml
30	30	1.5	1 ml
10	10	0.5	1 ml
0	0	0	2 ml

- One microplate was supplied, ready to use, with 96 wells (12 strips of 8) in a foil, zip-lock bag with a desiccant pack. Wells had been coated with monoclonal anti-*neu* protein antibody (mouse monoclonal anti-c-erb B-2 antibody).
- *Neu* Standards : (Table 4) 6 separate vials contained *neu* p185. Standards had been calibrated in arbitrary human *Neu* Units per ml HNU/ml) as well as in femtomole per ml (fm/ml) values.

Samples and standards were assayed in duplicate. A standard curve was performed each time samples were analyzed.

Table 5 : Human *neu* assay. Preparation of assay reagents

Strips used	Conjugate concentrate	Conjugate diluent	Substrate tablets	Substrate diluent
1	20 µl	0.98 ml	1	4 ml
2	40 µl	1.96 ml	1	4 ml
3	60 µl	2.94 ml	1	4 ml
4	80 µl	3.92 ml	1	4 ml
5	100 µl	4.90 ml	2	8 ml
6	120 µl	5.88 ml	2	8 ml
7	140 µl	6.86 ml	2	8 ml
8	160 µl	7.84 ml	2	8 ml
9	180 µl	8.82 ml	2	8 ml
10	200 µl	9.80 ml	3	12 ml
11	220 µl	10.78 ml	3	12 ml
12	240 µl	11.76 ml	3	12 ml

Preparation of assay reagents are shown in Table 5

- The sample diluent consisted of one bottle containing 100 ml buffer containing bovine serum albumin (BSA) and 0.1% sodium azide
- The detector antibody was supplied ready for use and contained 10 ml of rabbit anti-*neu* protein antiserum in 0.01 M PBS (pH 7.4), a protein stabilizer, and 0.1% sodium azide
- The conjugate diluent consisted of one bottle containing 12 ml of 0.01 M PBS (pH 7.4), BSA and 0.01% chloroacetamide
- The conjugate concentrate consisted of one vial containing 0.4 ml of 50x goat anti-rabbit IgG-horseradish peroxidase in buffer. This was diluted with conjugate diluent to make working conjugate
- The substrate diluent consisted of one bottle containing 18 ml of 0.1 M citrate buffer (pH 5.0) and 0.01% H_2O_2
- Four substrate tablets of o-phenylene diamine (OPD). These were dissolved in substrate diluent (1 tablet / 4ml) to make the working substrate
- One vial containing 3 ml of antigen extraction agent (AEA) sufficient for 18 ml of extract
- One 1 ml vial of lyophilized normal mouse serum (NMS) for use in assay of serum samples
- The stop solution consisted of one bottle supplied ready to use, containing 10 ml of 2.5 NH_2SO_4

- The plate wash concentrate consisted of one 100 ml bottle which was diluted with distilled water prior to use
- Receptor buffer was required but not provided with the kit. All other materials were identical to those stated for the p53 autoantibody kit. The microwell plates were read at a wavelength of 490 nm

* **Preparation for serum sample**

Use of the c-erb B-2 assay for analysis of high concentrations of serum was not recommended by Oncogene Science as heterophilic antibody, rheumatoid factor, and other interfering substances may have lead to false results. Serum is a complex biological fluid and the sample was diluted in sample diluent to which NMS (provided with the kit) had been added to a concentration of 10%. The addition of NMS to sample diluent served as a precaution against anti-mouse antibodies in the serum sample binding to the mouse capture antibody on the plate and causing false results. In addition, the initial concentration of the serum specimens examined did not exceed a concentration of 2% (a 1:50 dilution of specimen in NMS-treated sample diluent). The NMS was reconstituted with 1 ml deionized water, kept on ice, gently agitated until dissolved (30 minutes). The NMS slightly suppresses the overall signal, For absolute quantitation, the standard curve needed to be performed in the presence of 10% NMS. 10μl of NMS was added to each well of the standards.

4.6.5 Detailed protocol

- The microplate was removed from the bag. From the number of specimens to be tested, the number of strips required was calculated. Each specimen dilution or standard required 2 wells, and 4 wells were needed for the 0 HNU/ml standard
- The standard curve required 14 wells. Unused strips were stored in the zip-lock bag with desiccant at 2°-8°C
- Specimens were diluted (1:50) with sample diluent
- Standard and specimen dilutions were thoroughly vortexed and 100 μl of each were added to duplicate wells. 4 wells were set up with the 0 HNU/ml standard, 3 to measure the background absorbance and 1 to be used as the substrate blank well
- The microplate was covered with a piece of plastic wrap and incubated for 3 hours at 37°C.

- The plastic wrap was removed and the microplate washed with plate wash
- 100μl of detector antibody was added to all wells except the substrate blank well and then incubated at room temperature (15°-30°C) for two hours
- During the incubation with detector antibody, working conjugate was prepared by diluting the conjugate concentrate with conjugate diluent in a clean reagent reservoir
- The microplate was washed with plate wash
- 100μl of working conjugate was added to all wells except the substrate blank well and then incubated at RT (15°-30°C) for 30 minutes
- During the incubation with working conjugate, working substrate was prepared by dissolving substrate tablets in substrate diluent, vigorously vortexed to assure completed dissolution. Once prepared, working substrate was used within 30 minutes. Exposure to light was avoided
- The microplate was washed with plate wash
- Including the substrate blank well, 100μl of working substrate was added to all wells. The microplate was incubated in the dark at room temperature (15°-30°C) for 60 minutes
- 100μl of stop solution was added to each well to stop the reaction
- The absorbance was read at 490 nm (with a 620 nm reference filter) within 30 minutes. The substrate blank well was used to record zero the Sanofi-Pasteur reader

4.7 Method of capturing data

Planning data management began with developing rules for coding the variables for computer entry. In general, all coding systems were made prior to collecting the data. The use of spreadsheet software was the most common and easily accessible approach. Analytic statistics were used that allowed examination of (i) the patterns, (ii) the magnitude and (iii) the statistical significance of association among variables using various approaches.

4.7.1 Criteria for the admissibility of the data

- Only data from the ELISA's, completed under the researcher's auspices or those represented by her, were used. Results were double checked for errors to ensure the accuracy and validity

of the investigation

- Incomplete questionnaires (pre-analytical phase) were ignored at the discretion of the researcher as it was a possibility that they could prejudice or bias results
- Only data that complied with the strict compliances of the research protocol were used. Missing data meant that those patients results were not included in the study (The questionnaire forms were hand-edited while the study subject was still available. Omissions, illegible entries and gross errors were then timeously corrected.)

4.8 Statistical analysis

Statistical encounters in the medical laboratory are of fundamental importance and the introduction of any new test illustrates the extensive use of statistics undertaken prior to test use by the clinical laboratory. Some of the data in this research was gathered using questionnaires. Since every word in a question could have influenced the validity and reliability of the responses, an objective was to construct questions that were free of ambiguity and that would elicit accurate and honest responses. Collecting information about potentially sensitive areas like abortion or number of pregnancies was especially difficult. The data was recorded directly on the forms at the time the measurements were made to minimize the possibility of losses or transcription errors. As the data base was relatively small it was stored on a 20 mega-byte hard disc. This offered good speed of access and manipulation and was suitable for sophisticated data base management and statistical analysis programmes.

Descriptive statistics helped to summarize data in this study. It enabled examination of the internal consistency of the data, noting, for example whether the distribution of change in parity from one population group to another contained any unlikely values. Frequency distributions were examined for each variable collected. One reason for doing this was to complete the editing process, looking for outlying values that represented errors that survived previous efforts to edit the set data. Data were summarized by the mean, tables and graphs.

Analytic statistics looked at associations among two or more variables, for example by cross-tabulating, correlations and analysis of variance. The purpose was to estimate pattern and strength of associations among variables and to test the null hypothesis. Once the distribution of individual

variables were described, the researcher analyzed associations among predictor and outcome variables. This consisted of three steps: Firstly, inspection of the pattern of the association in the sample, secondly the computation of the magnitude of the association in the sample, and thirdly, estimation of the likelihood that the observation in the sample also existed in the population from which the sample was drawn.

The choice of the specific technique for each of the above depended on the type of variables examined. If both the variables were dichotomous, then the pattern of a possible association was revealed by cross-tabulation. It was important in this study to carry the analysis beyond an examination of the association between a single predictor and outcome variable. An experienced statistician was consulted to analyze multiple predictors, sequential outcomes and confounding variables using multivariate analytic techniques.

Univariate analysis was performed to determine the diagnostic role of the markers relative to the clinical and biological parameters of the disease. The diagnostic relevance of the following clinical parameters: age, menopausal state, number of children, HRT, etc. were evaluated. Among the biological parameters, the histological grade, the presence of ER, p53 auto-antibodies and c-erb B-2 immunoreactivity were selected. Multivariate methods are quite complex (Solberg, 1995), both in terms of their theoretical background and in terms of computational demands. They were performed using the Cox regression model. The relative risks and their confidence intervals which were statistically relevant are presented in Chapter Five.

Using as input, patient age, menopausal status, number of pregnancies and HRT, women with breast cancer participating in this study were divided into seven groups by decade (20-29, 30-39, 40-49, 50-59, 60-69, 70-79, 80-89 years) and categorical variables compared across groups. To ensure that women in one study were compared directly only with similar women in the same study, all analyses were stratified by study, as well as by other factors (Table 6).

Table 6 : Factors considered in statistical analysis in this study

Multivariant analysis to reveal:	Univariate analysis to reveal:
Lymph node involvement	% negative correlations] * c-erb B-2
tumour size (>2cm large)	
Histological grade	
Histological type	
c-erb B-2 protein immunoreactivity anti-p53	% positive correlations] * anti-p53
(Estrogen receptor status, where possible)	
Risk factors:	
- age at diagnosis	
- number of children	
- oral contraceptive use	
- menopausal state	

4.8.1 Method of data analysis

For each group or subgroup of tumours the anti-p53 and c-erb B-2 content values and the values of other parameters (means and standard errors of the mean [SME's]) were calculated. Clinical and biochemical characteristics of patients were expressed as a mean ± 2 SD or as a percentage. The diagnostic value of the markers anti-p53 and c-erb B-2 for breast cancer was assessed by cumulative frequency distributions and ROC curves. The cumulative frequency distributions displayed the cumulative percentage of breast cancer patients, patients with benign breast disease as well as women who have no evidence of breast disease (i.e.normal controls), against the serum marker concentration. An advantage of this format is that it showed the extent of possible overlap of the marker distribution of breast cancer patients with that of the patients with benign breast disease. The resulting figures allowed the reading of sensitivity and specificity at any requested cut-off level for test positivity. ROC curves plotted the sensitivity against one minus specificity at various cut-off levels of the diagnostic test.

An important aspect of this present study was to compare this research to other studies that included women with breast cancer. Information of the use of either anti-p53 and/or c-erb B-2 made the studies eligible for inclusion (post-analytical variables). Results of the markers studied were grouped into three broad categories depending on serum levels : negative, positive and critical areas. Studies were identified from review articles, from computer-aided literature searches and from discussions with colleagues. Special efforts were made to identify all studies that included relevant information.

Data for individual women were sought on sociodemographic factors, HRT, age at menarche (if applicable), menopausal status and age.

- ***Clinicopathologic patient data***

For each tissue sample, the following clinicopathologic data were obtained from the Department of Cellular Pathology (University of Natal Medical School, histologic type of tumour, primary tumour size, pathologic axillary lymph node status, ER values (if performed) and tumour grade (WHO, 1981). ER were quantitated by immunochemical analysis in the Department of Chemical Pathology at the University of Natal. Information on tumour spread was sought for patients with breast cancer. Information that permitted their classification into cancers that were localized to the breast and those that had spread was not always available. These were allocated to a separate stratum. Similar data were sought from TNM grading, ER status, axillary lymph node involvement and/or metastases to distant sites.

4.8.2 ELISA cut-off / threshold values

The ELISA method for each oncoprotein was validated by comparing the OD plot of the series with that of the negative control and the cut-off point was defined as 2-5 times the negative control. By fitting a curve of best fit to the observations of a bivariate distribution, estimates and predictions about the distribution of variables could be made. The factors included were menopausal status, ER status, number of children and HRT (if menopausal). In assays of antigen or hapten concentration by double antibody sandwich or inhibition assays, results are often obtained from interpolation from standard curves. In developing and validating quantitative ELISA's an important aspect was the choice of the positive/negative threshold value. This was done by allotting two or three times the mean response value (absorbency) of the negative control group as the minimum positive response value. Having established the positive threshold value, it was essential to prevalidate the immunoassay by quantifying the key assay parameters of sensitivity, specificity, cross reactivity, predictive value and precision. Provided that the relevant reference samples were assayed in sufficient number, each of these parameters should be readily determined.

4.8.3 Presentation of ELISA results

Anti-p53 results were interpreted from the OD readings of both low and high control values and were expressed as an immune index. The main deficiency of the absorbance value was that it was not linearly proportional to relative antibody activity as is the end-point titre of classical techniques (e.g. an ELISA absorbance value of 3.0 does not indicate 3 multiples of the antibody activity indicated by an ELISA value of 1.0). Various methods of mathematical transformation of absorbance values have been evaluated and a standard curve method was developed by Oncogene Science for c-erb B-2 to correct this deficiency. The results for each standard was interpolated as the dilution at which the serum dose-response curve intersected with position threshold level. End-points were re-plotted as a function of the ELISA value, obtained as the optimal serum working dilution, to produce a standard curve.

4.9 Summary of Chapter Four

This chapter outlined the rationale for both materials and methods used. The questions asked in the questionnaires were relevant to matters pertaining to factors affecting the diagnosis of breast cancer. Numerical data were used to provide a factual foundation as a method of summarizing, in a systematic manner, aspects of the variables and clinicopathological indices. Data capture methods are explained as well as criteria for admissibility of the data. Results of data processing that are relevant to the subproblems are discussed in Chapter Five.

CHAPTER FIVE

RESULTS

5.1 General introduction

Numerical data are presented in terms of the problem statement (discussed in Chapter Three). Relevant results that emerged for each subproblem and its hypothesis are documented.

5.2 Pre-analytical phase

Results of responses from the questionnaires were analysed on the number of respondents who had completed that particular question. The comments of the respondents are grouped under broad headings and, where warranted, are depicted in tables or figures.

Table 7: Sample realization of women participating in this study.

	Cancer patients	Control participants
Total number interviewed	127	112
Total number selected	92	92
Indigenous population group	62	10
Indian population group	20	10
Coloured population group	6	10
White population group	4	62

- **Sample realisation and population groups** (Table 7)

The 184 women comprised 92 breast cancer patients and 92 control participants. None of the control group had breast tumours. Indigenous African women comprised 67% of the breast cancer population, Indian women 22%, Coloured women 6% and White women 4%. There were very few indigenous women donating blood to the Natal Blood Transfusion Services. Indigenous African, Indian and Coloured women of the control group comprised 11% each and White women 67% (Figure 13).

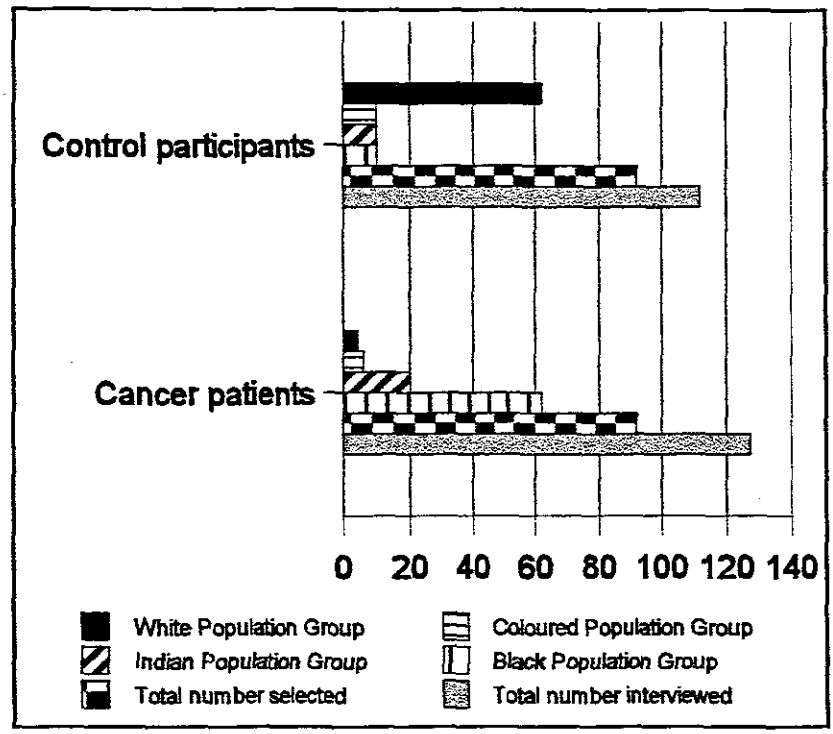


Figure 13 : Comparison of population groups participating in this study.

- **Mammograms versus income** (Table 8)

No indigenous participant had ever had a mammogram prior to discovery of a breast lump, pain or discharge.The average income for this same group indicates a below average income.

Table 8 : Representation of participants who utilized mammography

Mammogram	percentage
Women who had never had a mammogram	73
Women who had had mammograms	27

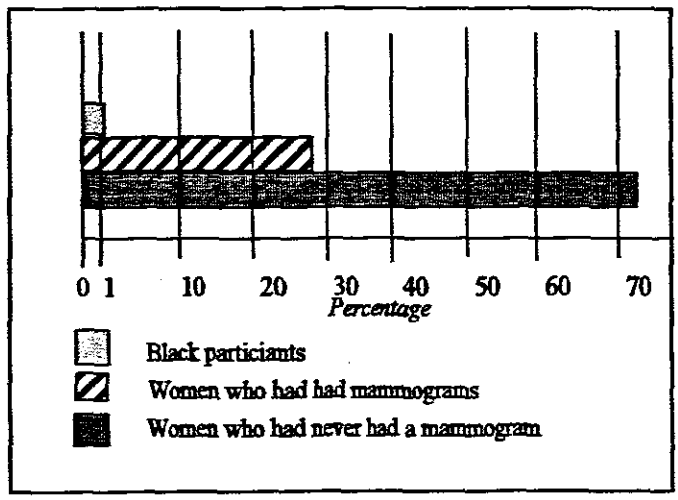


Figure 14 : Representation of participants vs mammograms

55% of women were neutral in their attitude towards the benefit of mammography (Figure 14). 73% of women in the control group had never had a mammogram. 27% had had mammograms; of these, nearly 17% had only sought help because of the discovery of a lump.

Table 9 : Reasons given as to why control participants had mammograms

Average age	49 years
Number of mammograms per patient (mean)	1.56
Number of children (mean)	1.8
Patients who had positive reasons for mammograms (%)	56%
Biopsies	30%
Mother with BRCA	13%
Relatives/friends with BRCA	13%
Participants who felt 'lumps'	16.6%

23 women in the control group (56%) had mammograms on the recommendation of a medical practitioner (Table 9). Pre-analytical variables that could influence results demanded that participants furnished details regarding their age, number of children, menopausal status and HRT (Figure 15).

Table 10 : Pre-analytical characteristics of control participants.

Total number of participants (n=92)	Breast cancer	Controls
Age (years)	44	49.5
Number of children (mean)	3	2
Menopausal	40%	37%
HRT	0%	11%
Weight (average kilograms)	85-95	65-75

Criteria for inclusion in the project for control participants required that their weight was not more than 20% above what was considered normal for their frame. The breast cancer patients had no such criteria and 84% showed overt gross obesity (Tables 10 and 11).

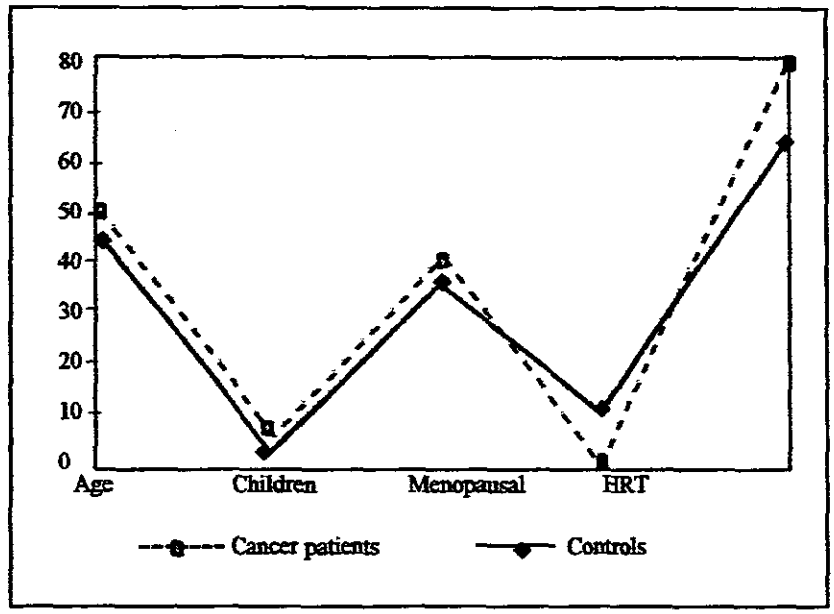
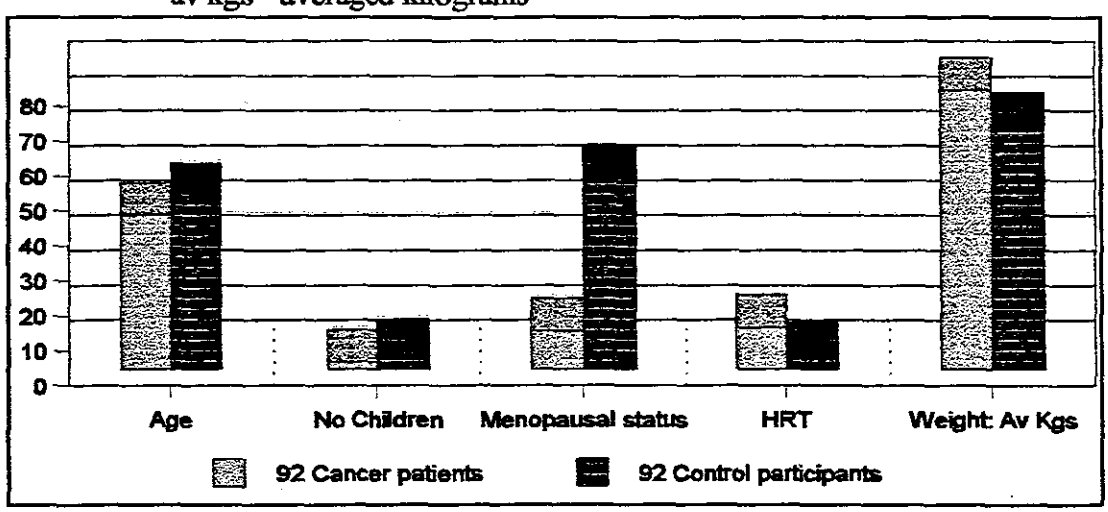


Figure 15 : *Graphic comparison of* breast cancer patients and control paticipants pre-analytical characteristics

Table 11 : Pre-analytical variables for breast cancer patients and control participants
av kgs= averaged kilograms



- Age (Table 12)

The clinical evaluation material comprised serum samples from breast cancer patients (n=92.), median age 44 years, range 20-89 years), patients with benign breast disease (n=10, median age 48 years, range 24-85 years) and normal controls (n=46, median age 49.5 years, range 20-69 years). Mean age at presentation in the study by Kandl *et al.*, (1994) in Johannesburg was 51.4± years for a similar South African population group.

Table.12: Age of cancer patients and controls participating in this study

Age (years)	Cancer patients (percentage)	Control group (percentage)
20-29	2	0.9
30-39	11	11.7
40-49	39	39
50-59	26	38
60-69	15	9.8
70-79	6	0
80-89	1	0
Mean (years)	44 years	49.5

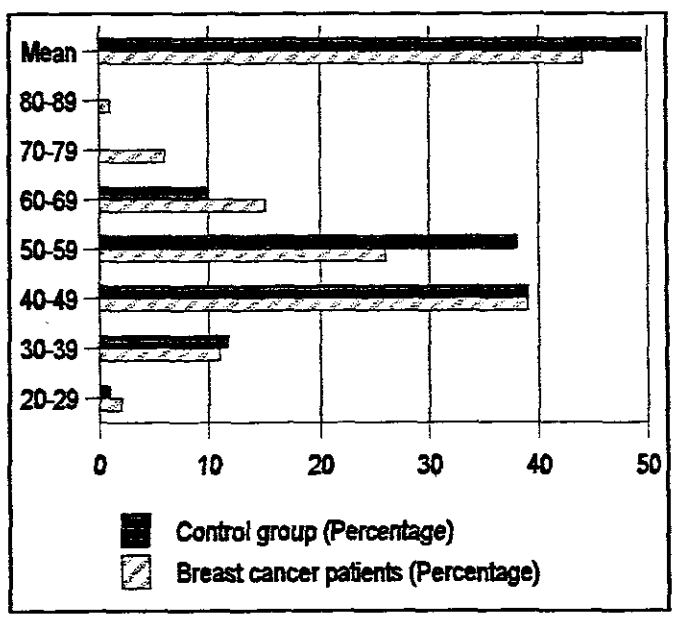


Figure 16: Histogram of age groups of women participating in the study

Approximately 39% of the newly diagnosed breast cancers found at the Breast Clinics were estimated to occur in women between the of 40-49 years ; mean 44 (Table 12). Cognisance of skewed results must be taken into account in all issues pertaining to pre-analytical indices due to the investigated group's lack of formal education (Figure 16); e.g..some patients were unaware of their correct age.

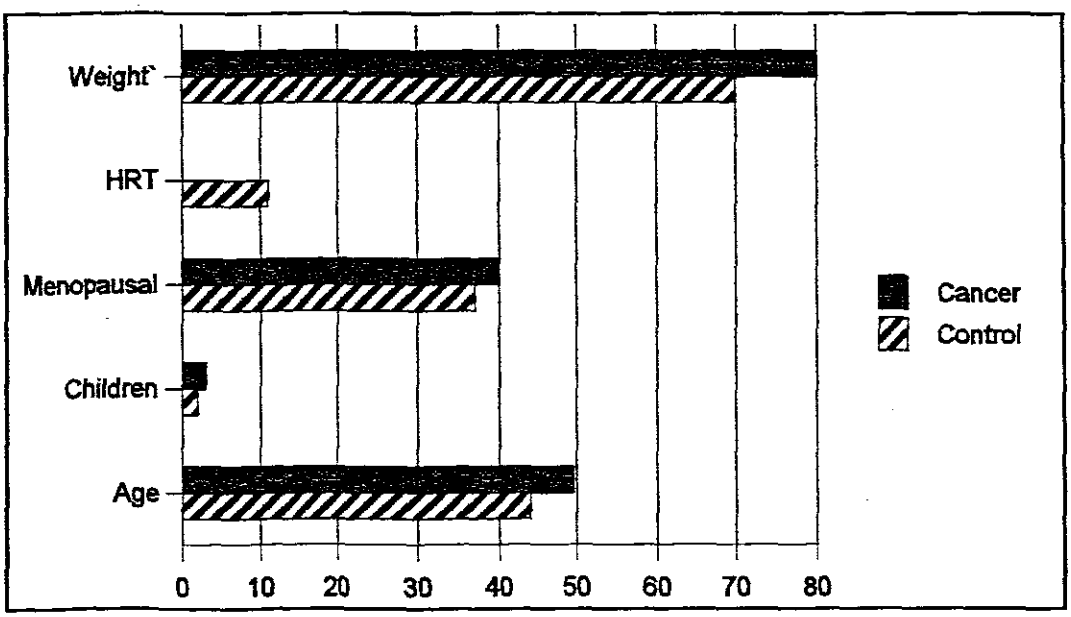


Figure 17 : Comparison of breast cancer patients and control group reproductive history

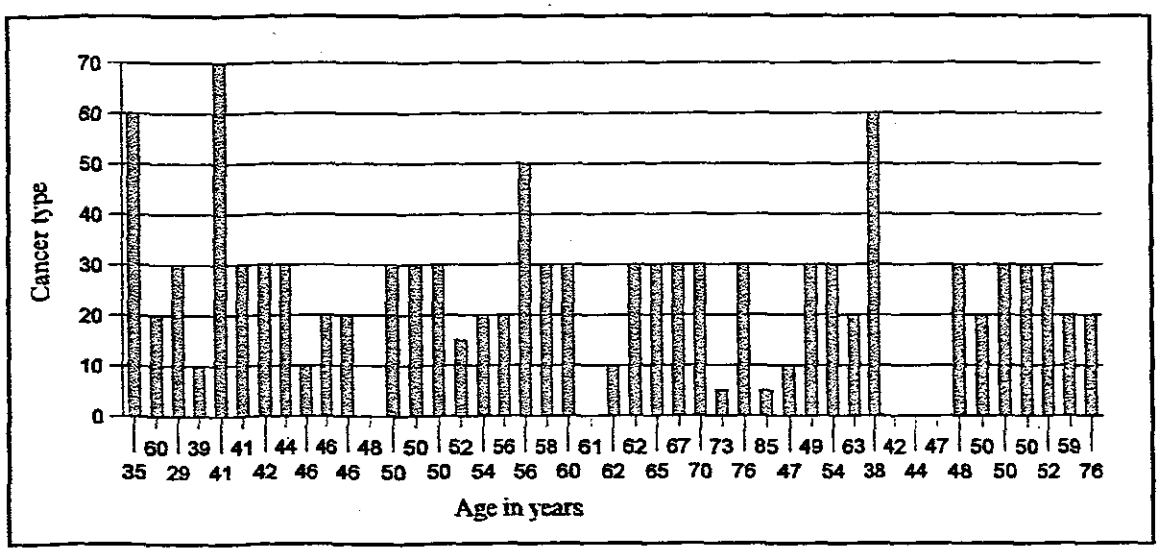


Figure 18: Type of breast cancer and age of patients

Cancer type representative values: 10=Invasive; 20=Invasive ductal; 30= Infiltrating ductal; 50=Squamous; 60=Medullary; 70=DCIS

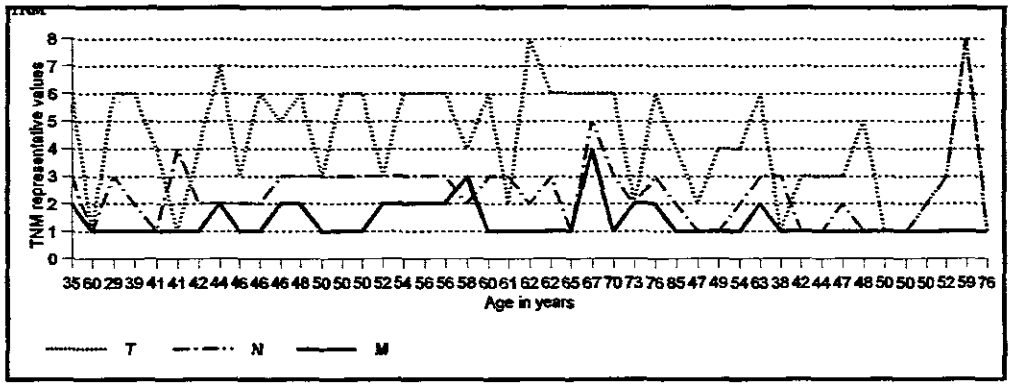


Figure 19: Age of breast cancer patients and TNM classification

Representative values:

T: 1=Nil; 2=T1; 3=T2; 4=T3; 5=T4; 6=T4b; 8=T3p

N: 1=Nil; 2=N1; 3=N2; 4=N3; 5=8.5

M:1=Nil; 2=M1; 2=M2/Pt3: 4=M4

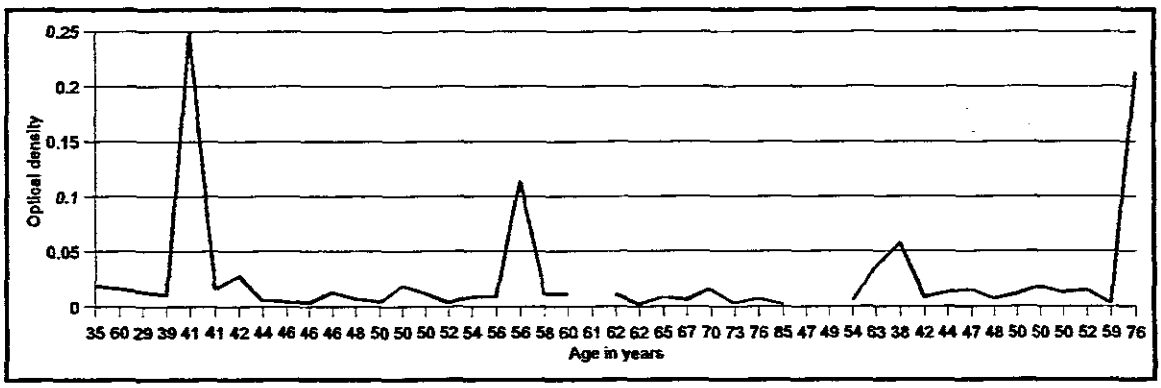


Figure 20: p53 auto-antibodies and age of patients with breast cancer

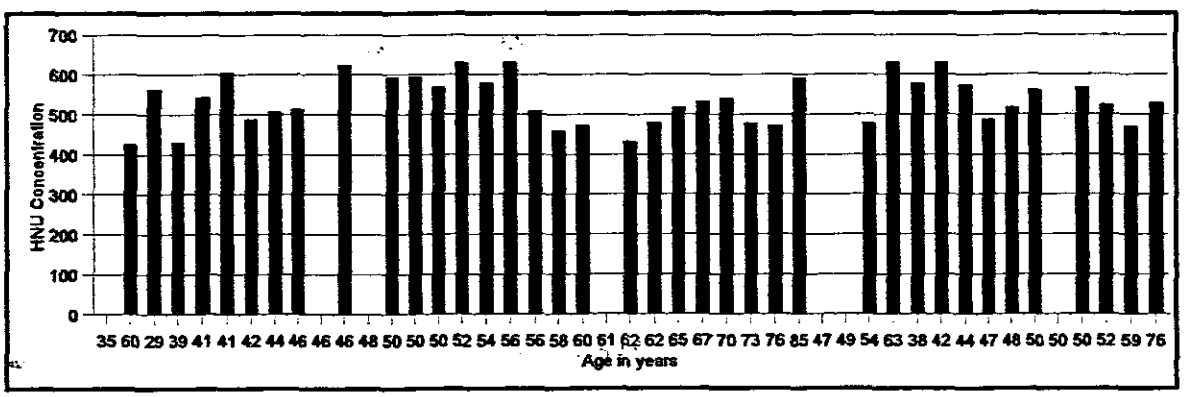


Figure 21: HNU/ml concentration and age of patients with breast cancer

Figure 22: A bar graph of type of cancer and breast (left and right) affected by cancer

Cancer type representative values:
10-Invasive; 20=Invasive ductal; 30=Infiltrating ductal; 50=Squamous; 60=Medullary; 70=DCIS

- **Hormones and breast cancer**

Much attention has focussed on a variety of unresolved effects of pregnancy, abortions and breast-feeding being associated with some increase in breast cancer (Table 13).

Table 13: Average number of children per control participant

	0 Children	1 Child	2 Children	3 Children	4 Children	5 Children
Control group	89%	21%	39%	25%	8%	0.8%
Cancer patients	.5%	1.6%	8.7%	42%	11.4%	17%

Only 2% of the breast cancer patients were childless. The mean number of children per patient was 5 for the same group (Figure 23). The hypothesis that pregnancy estrogens impart a transient increase of maternal breast cancer risk when full-term pregnancy occurs late in a woman's life, cannot be investigated due to the young age at first parity of the breast cancer patients randomly selected for inclusion in this study. There did not seem to be differences in selected responses between different breast cancer population groups.

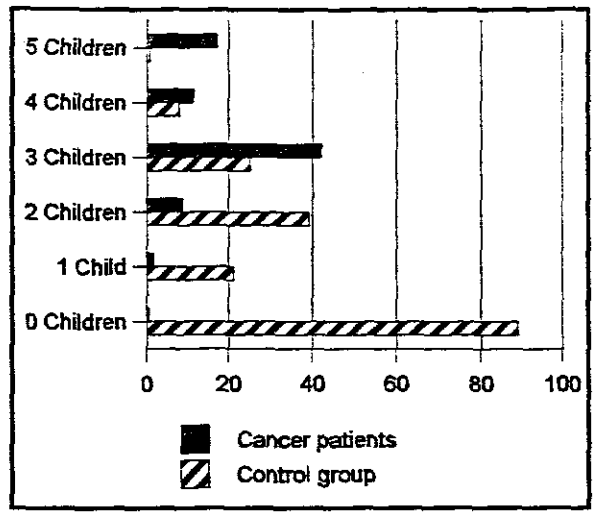


Figure 23: Average percentage of children for cancer patient and control group

- **Menopausal status** (Table 14)

The literature indicates that post-menopausal women show a limited increase in breast cancer with HRT on a short term whereas long-term use increases risk.

Table 14: Cancer patients and control patients' menopausal status

Menopausal Status	Cancer patients (%)	Control patients (%)
Pre	60	63
Post	40	37
Age	44	49.5

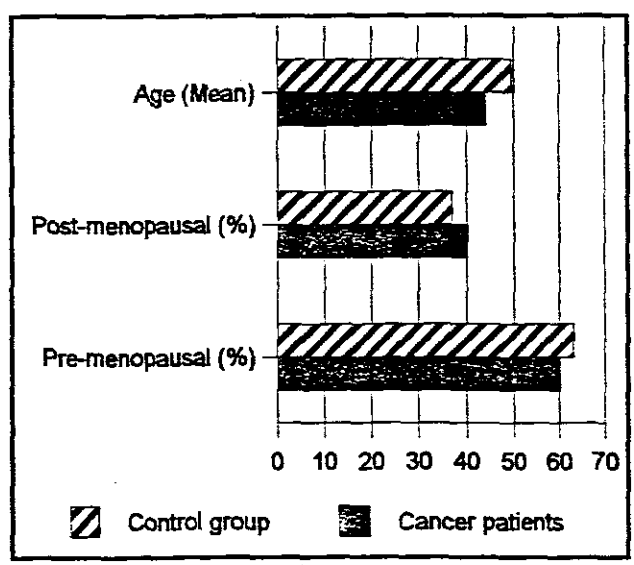


Figure 24: Histogram of cancer patients and control group's menopausal status

40% of the patients were post-menopausal (Figure 24). In a similar study undertaken in Johannesburg, 44% of the women were post-menopausal at the time of diagnosis. (Kandl *et al.*, 1994).

- **Breast cancer population** (Table 15)

The randomly selected study group consisted of 92 South African women who had primary (85%) and recurrent (16%) resectable breast cancers. 60% of these patients were pre-menopausal (i.e. were actively menstruating or were less than one year from the spontaneous cessation of menses). Only 4% had tumours under 2 cm in diameter at pathologic examination, and 25% of patients had histologically negative axillary lymph nodes (the median number of nodes examined was 4) and 90% of patients showed no clinical or radiologic evidence of distant metastases.

Table 15 : Clinical features of breast cancer patients participating in the study

	Number	Percentage
Number of breast cancer patients	92	100
Primary breast cancer	77	84
Stage I	11	12.0
Stage II	12	13.0
Stage III	19	21
Stage IV	38	41
Recurrent breast cancer	15	16
Local	6	6.5
Distant metastases	5	5.4
Bone (B)	2	2.2
Pulmonary (P)	0	0
Brain	0	0
Liver (L)	2	2.2
B+P	0	0
B+L	0	0
Multiple organs	3	3

Cancer was more common in the left breast (58%) than in the right (39%.) 3% of the women presented with bilateral breast cancer. According to the patient files one of the patients did not return for follow-up after a biopsy and one defaulted prior to radiotherapy and/or chemotherapy. Women younger than 35 years have a statistically significant increased risk of loco-regional recurrence. 16% of patients (n=15) participating in this project, median age 52 years, had evidence of recurrence (Table 16).

Table 16: Breast cancer recurrence.

Age	Stage/grade of tumour	Year previously diagnosed
35	$T_1 N_1 M_0$	1990 (lumpectomy 2.7.97)
38	$T_x N_2 M_0$	1991
39	$T_1 N_0 M_0$	information not available
40	$T_3 N_2 M_0$	1993
42	$T_2 N_0 M_0$	1991 $T_2 N_0 M_0$
42*②	$T_0 N_3 M_0$	information not available
43	$T_4 b\ pN_2 M_0$	1994
53	$T_4\ pN_1 M_0$	1987
54	$T_3 N_1 M_0$	1987
58*①	$T_3 N_1 M_0$	1993 $pT_2 N_1 M_0$
59	$pT_3\ pN_1 M_0$	1996 (diagnosed 04.11.96)
61	$T_1 N_2 M_0$	1980
62	$T_4 b\ N_2 M_0$	1996 - no evidence of recurrence: 1997 - recurrence
65	$T_x N_x M_0$	1990
85	$T_3 N_1 M?$	1995

*① *defaulted prior to radiotherapy*

*② refused surgery

- **Breast characteristics of control group** (Table 17)

The use of markers for the diagnosis of breast cancer is a relatively specific application which warranted benign breast conditions also being evaluated as a non-disease "healthy" group, as well as the upper limit of the normal population.

Table 17 : Breast characteristics of control participants

	Number	Percentage
Control participants	92	100
With no evidence of breast disease	82	89
With evidence of benign breast disease	8	8.7
Biopsy/biopsies with inconclusive evidence	2	2.17

Included in the number of responses from control participants were 9 women who had had biopsies (Figure 25). In this context, a breast biopsy referred to the surgical removal of a sample of breast tissue for the purpose of determining the presence or absence of cancer. (FNA was not considered as a breast biopsy). 2 women had inconclusive results, the remaining 8 were negative for breast cancer. They were included as the "benign breast disease" control group to facilitate a possible cut-off level.

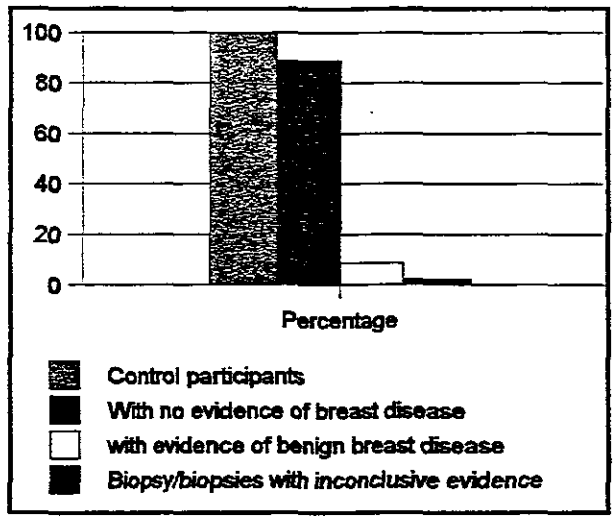


Figure 25 : Characteristics of control patients

5.3 Analytical phase

Assessment of the analytical performance of the ELISA kits for anti-p53 and c-erb B-2 was undertaken on the assumption that the assays tested measured the same analyte. Each component of the problem had been expressed in the form of appropriate sub-problems in order to facilitate the management of the problem as a whole, i.e. there were certain data relating to each sub-problem. These are exhibited in logical sequence within this chapter. Tables are drawn wherever applicable.

5.3.1 Acceptance criteria for anti-p53

The absorption values of the controls fulfilled the following criteria (Table 18).

Table 18 : Acceptance Criteria for anti-p53 ELISA:- as stated on the package insert (A) and obtained in the study (B)

A:

control	negative	low	medium	high
acceptance criteria (OD)	0,001-0,001	0,027-0.028	0,076-0,076	0,284-0,294

The absorption values of the controls for the ELISA are tabulated. The mean of the high control value was accepted as OD 0.290

B:

control	negative	low	medium	high
acceptance criteria (OD)	0,035-0,06	0,09-0,17	0,35-0,60	0,70-1,10

The results were interpreted by determining the relative p53-auto-immune index. The p53-auto-immune index for patients sera was calculated as follows:

$$\frac{E_{450}(sample) - E_{450}(low\ control)}{E_{450}(high\ control) - E_{450}(low\ control)} = p53\ auto\text{-}immune\ index$$

* **p53-auto-antibody negative samples:**

  1:100 diluted sera tested negative in western-blot for anti-p53 antibodies and had an absorption less than the low control.

* **p53-auto-antibody positive samples:**

  1:100 diluted sera tested positive in Western-blot for anti-53 antibodies and had an absorption value greater than or equal to the value of the low control. Positive control serum containing a constant amount of anti-p53 was obtained with the Oncogene Science kit. Samples in the study were considered positive at an OD above the positive control sample (Table 19).

Table 19: Example of samples interpretation as given in the Oncogene Science kit.

	OD (mean)	p53-autoimmune-index	Valuation
negative control	0.052	-0.093	
low-control I	0.108	0	
medium control II	0.314	0.340	
high-control III	0.713	1	
low-control + 10%	0.119	+0.008	upper limit of critical area
low-control - 10%	0.097	-0.008	lower limit of critical area
sample 1	0.078	-0.050	negative
sample 2	0.056	-0.086	negative
sample 3	0.141	+0.055	positive
sample 4	0.128	+0.033	positive
sample 5	0.116	+0.013	critical
sample 6	0.104	-0.007	critical

- **Critical area interpretation** (Table 20)

The sample intra-assay CV was 5%. An additional difference of 5% was assumed resulting from handling errors (e.g.pipetting). The following critical area was defined:

OD control I (low): Cut-off

OD control I (low) + 10% : upper limit of the critical area

OD control I (low) - 10% : lower limit of the critical area

The kit stated that samples with a p53-auto-immune index within the critical area should be repeated. A cut-off was established at OD 0.028; OD 0.030 upper limit of critical areas (+10%) and OD 0.025 upper limit of critical areas (-10%).

Table 20 : Cut-off for anti-p53 in breast cancer patients was established as 0.028

Control	OD (mean)	p53 autoimmune index	Valuation
Negative control	0.001	-0.103	
Low control	0.028	0.107	
Medium control	0.076	0.183	
High control	0.290	1.0	
Low control +10%	0.038	0.008	Upper limit of critical area
High control -10%	0.261	-0.008	Lower limit of critical area

- **Variability of method**

* **Precision:**

3 positive samples with different p53-autoimmune index were tested by Oncogene Science in 6 separate assays to determine inter-assay precision (Table 21).

Table 21: Inter-assay precision of p53 auto-immune index method

Sample	1	2	3
number	6	6	6
mean value of index	0.499	0.326	0.171
standard deviation	0.028	0.024	0.009
%CV	5.57	7.40	5.50

2 positive samples with different p53 auto-antibody concentration were tested in one assay in which 6 determinations were made per sample to determine intra-assay precision (Table 22).

Table 22 : Intra-assay precision of p53 auto-immune index method

Sample	1	2
number	6	6
mean value of OD	0.555	0.382
standard deviation	0.036	0.027
%CV	6.53	5.94

Intra-assay precision co-efficient of variation (CV) was less than 7%; inter-assay CV less than 7.5% per cent.

- **Criteria for positivity**

Positive control serum contained a constant amount of anti-p53 antibodies and was obtained with the kit. Negative control sera showed an absence of anti-p53 antibody. All samples were assayed in duplicate and considered positive at an OD above the positive control sample. No positive sera were detected among the reference control sera examined. A group of 42 normal serum samples were analyzed to determine a baseline ELISA value. The OD (ELISA value) at 450 nm of these samples ranged from 0.01 to 0.035. The ELISA value of none of the normal sera was above 0.06 (mean+3SD) of ELISA values of these sera. Therefore, in subsequent assays any serum giving a normalized ELISA value greater than 0.035 was considered positive for p53 auto-antibodies.

- **The anti-p53 standard and positive controls**

Index value defined as: $\frac{\text{E450 (sample)} - \text{E450 (low control)}}{\text{E450 (high control)} - \text{E450 (low control)}}$

Table 23: p53 auto index value indicating an absence of auto-p53 (negative)

Index value		Interpretation
Normal sera		Absence of auto-p53 antibody
1	0.028	negative
2	0.056	negative
3	0.042	negative
4	0.099	negative
5	0.056	negative
6	0.070	negative

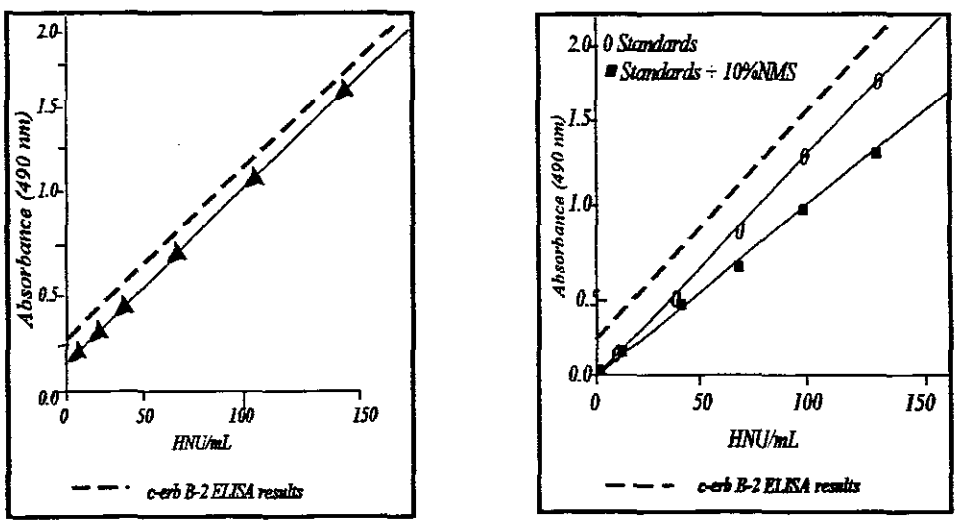
Negative controls (Table 23) comprised omission of serum samples (phosphate buffered saline control). Positive controls were serum samples containing known levels of anti-p53 antibody). All samples were run in duplicate and results are the mean value of the two readings.

5.3.2 Acceptance criteria for c-erb B-2

The HNU (Human *neu* Unit) is an arbitrary unit which quantitates *neu* antigen relative to the number of *neu* molecules having binding sites for both the capture antibody and the detector antibody, regardless of their molecular weight. Alternatively, since the standards are calibrated in femtomoles (10-15) of *neu* protein per ml, results may be expressed in fm/ml or fm/μg sample protein. The arbitrary HNU was used to quantify the c-erb B-2 antigen using the standards ranging from 0 to 100 HNU/ml included in the kit .

- **Concentration of unknowns**

– the concentration of c-erb B-2 protein for each specimen dilution was determined by interpolation from the standard curve. Overnight assay characteristics are shown in Figure 26



A: One day assay

B: Overnight assay

Figure 26: Assay characteristics of Oncogene Science Kit Q1A 10 compared to study results (dotted line) : slope (m) 0.637

– the absorbance values for each standard and specimen dilution (serum diluted 1:50) were averaged to obtain the mean absorbance

– the mean absorbance of the HNU/ml or fm/ml standard (background absorbance) was subtracted from the mean absorbance of each standard and sample dilution

– the substrate blank well, read against air, read less than or equal to 0.05 absorbance units with a 620nm reference filter

- the mean corrected absorbance of each standard was plotted on the y-axis versus the concentration of *neu* protein (in HNU/ml or fm/ml) on the x-axis
- if the protocol is strictly adhered to, the standard curve is nearly a straight line. Variations in the protocol, especially increased working conjugate incubation time, could have lead to non-linearity of the standard curve. It was suggested that for a non-linear curve, point to point or quadratic curve fit methods should be used

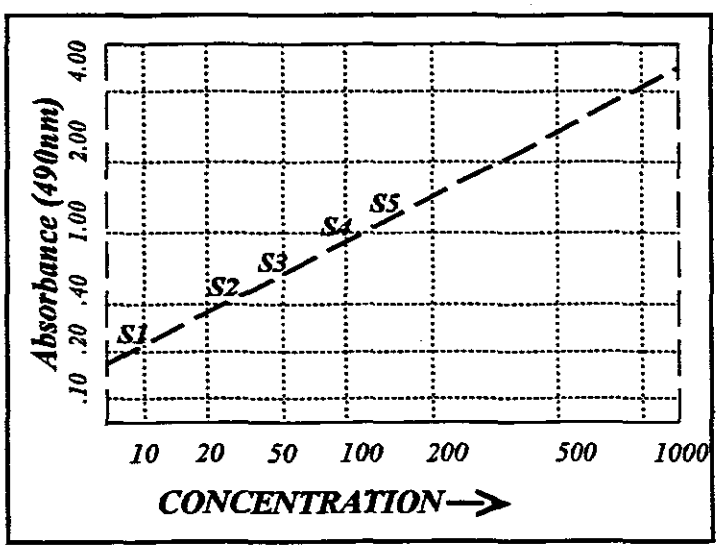


Figure 27: c-erb B-2 ELISA : Standard curve
(Microplate One)
Units of concentration : HNU/ML
Correlation coefficient : .992 Curve-fit mode: log/log
Offset (*y*-coefficient) : -1.316 Slope (m) : .637

Table 24: OD of c-erb B-2 standards (Microplate One)

STD	CONC.	ODs of individual wells				OD-Mean	CV percentage	Calculated concentration
S1	10.00	C01:	.224	D01:	.223	.223	.31	11.07
S2	30.00	A01:	.377	B01:	.369	.373	1.52	24.73
S3	60.00	G02:	.658	H02:	.680	.669	2.33	61.82
S4	90.00	E02:	.841	F02:	.837	.839	.33	88.18
S5	120.00	C02:	1.087	D02:	1.065	1.076	1.45	130.30

Figure 27 indicates results obtained in Microplate One. Table 24 displays OD values of c-erb B-2 standards for Microplate One. Figure 28 displays values obtained by Microplate Two. The OD's of individual wells for the 5 standards, S1 - S5 are indicated below in Table 25. Mean values are given for both microplates. They are to be interpreted in conjunction with Figure 28.

Table 25: OD of c-erb B-2 standards (Microplate Two)

Stan-dard	Concen-tration	ODs of individual wells				OD mean	CV percentage	Calculated concentration
S1	10.00	C01:	.224	D01:	.224	.224	.00	11.08
S2	30.00	A01:	.377	B01:	.370	.373	1.33	24.69
S3	60.00	G02:	.659	H02:	.683	.671	2.53	61.87
S4	90.00	E02:	.842	F02:	.840	.841	.16	88.15
S5	120.00	C02:	1.091	D02:	1.067	1.079	1.57	130.3

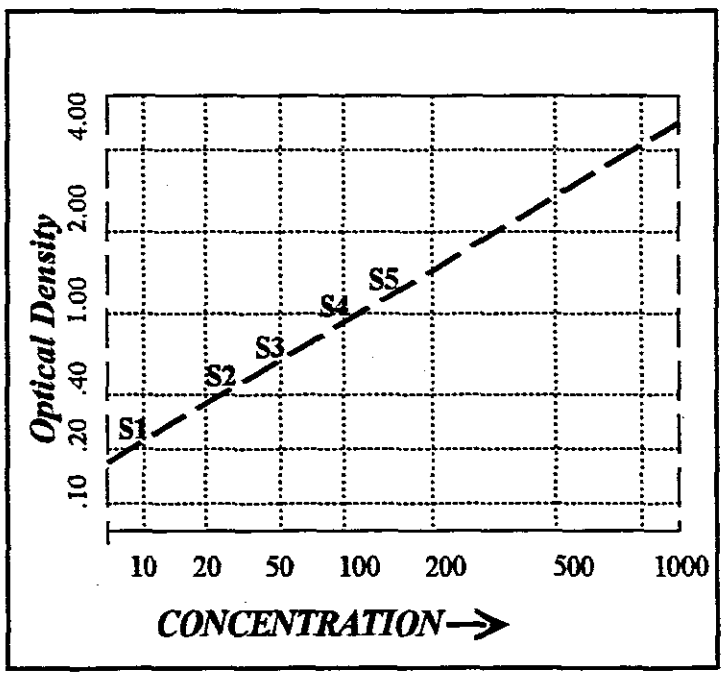


Figure 28: c-erb B-2 ELISA Standard curve (Microplate Two)
Units of concentration : HNU/ML
Correlation coefficient : .992 Curve-fit mode: log/log
Offset (y-coefficient) : -1.316 Slope (m) : .637

Samples containing lysates at 4 concentrations of p185 diluted in sample diluent were tested in both 4 & 8 separate assays (A and B), with 8 determinations per assay (Table 26[A] and [B]).

Table 26: c-erb B-2 Intra-assay precision for the method

(A)

Sample	1	2	3	4
number	32	32	32	32
mean (HNU/mL)	142	52	20	3.3
Standard deviation	5.1	3.2	1.0	0.89
%C.V.	3.6	6.1	5.0	26.8

(B)

Sample	1	2	3	4
number	8	8	8	8
mean (HNU/mL)	138	48	20	4
Standard deviation	2.3	2.0	1.2	0.83
% C.V.	1.6	4.2	5.9	21.5

5.3.3 Benign breast disease

The question as to whether p53 auto-antibodies and c-erb B-2 is elevated in benign breast disease was an important aspect of this study. During the same period of using the ELISA kits described, this study also evaluated 10 patients with non-malignant breast conditions. This was undertaken to establish the "cut-off" level between women with benign breast disease and breast cancer. These controls were age-matched with normal healthy women. The p53 auto-immune index was negative in women with benign breast disease. Values for c-erb B-2 are reported in 5.6. Patients with benign breast disease had undergone operative biopsy or FNA under local or general anaesthesia or had been diagnosed as benign breast disease by a surgeon, whereas breast cancer patients had undergone surgical resection or partial or total mastectomy.

5.4 Post-analytical phase

- **Diagnostic performance**

Diagnostic performance was evaluated by ROC analysis. Sensitivity versus specificity was plotted sequentially for the complete range of decision thresholds. The cut-off point was confirmed using ROC curves. Most ROC curves have a very steep section in which the sensitivity increases a great deal while the false positive rate hardly changes. The best cut-off point was selected where the ROC curves "turn the corner", for anti-p53 (Figure 29) and c-erb B-2 (Figure 30). An important advantage of ROC curves was that the curves for the different markers could be compared; the better the marker, the closer the curve was to the upper left hand corner.

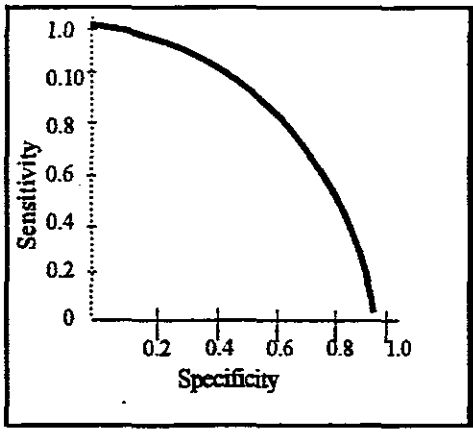


Figure 29: ROC curves for serum anti-p53

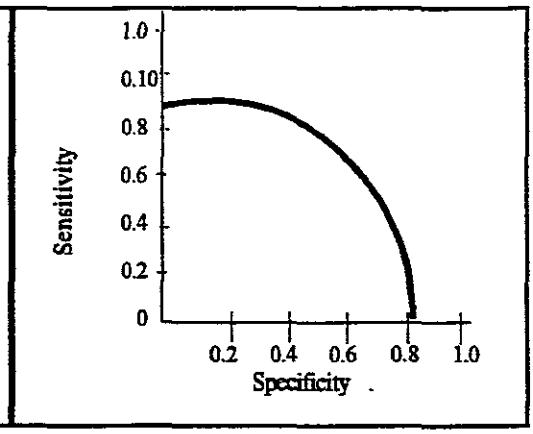


Figure 30: ROC curves for serum c-erb B-2

The *neu* assay detected 10 HNU (0.5 femtomoles) per ml of *neu* p185 or p105 in the sample tested. The signal of the 10 HNU standard was approximately twice the zero or background signal. The *neu* assay had been tested for specificity using purified *neu* proteins p185 and p105 (*neu* protein extracellular domain) versus EGFr p170 and p110 (EGFr extracellular domain).

5.4.1 The predictive value model

The CV is useful for the comparison of the precision of methods. This was used for the c ooparison of the precision of measurement of anti-p53 antibodies at widely different levels.

	Patients with positive result	Patients with negative result	Total
Patients with selected disease	TP	FN	TP + FN
Patients without selected disease	FP	TN	FP + TN
Total	TP + FP	FN + TN	TP+FP+TN+FN

Figure 31 : Predictive value model used to weigh the diagnostic value of the test results

FP= number of non-diseased patients with a positive test result
TN= number of non-diseased patients with negative test results
TP= denotes the number of diseased patients having a positive test result
FN= denotes the number of diseased patients with a negative tests result (Annesley, 1994)

Table 27: Predictive value model for anti-p53 (A) and c-erb B-2 (B) as distributed in test results for the two groups

(A)	Patients with positive result	Patients with negative result	Total
Patients with selected disease	10	82	92
Patients without selected disease	Nil	46	46
Total	10	128	138
(B)	Patients with positive result	Patients with negative result	Total
Patients with selected disease	Nil	46	46
Patients without selected disease	Nil	36	36
Total	Nil	82	82

By varying the decision level, sensitivity and specificity change in opposite directions. Using the formula given in Figure 31, the predictive value models for c-erb B-2 are given in Table 28.

Table 28: Sensitivity and specificity at different cut-off levels for discriminating between benign and malignant breast tumours (10 HNU is equivalent to 0.5 femtomoles/ml)

Cut-off	Femtomoles/ml	Specificity	Sensitivity	Negative predictive values	Positive predictive values
>0.5	10HNU	100	94	92	94
>0.3		83	94	77	87
>0.2		58	94	52	76
>0.18		50	100	31	74

- **Variability of method**

For both anti-p53 and c-erb B-2 measurements it was essential to assess the real variability of the method. This was performed by testing at least 3 dilutions of the appropriate reference to cover the upper, middle and bottom parts of the standard curve in triplicate, in several assays performed on different occasions. The anti–p53 intra-assay co-efficient of variation (CV) was less than 7%; the c-erb B-2 intra-assay CV was less than 6%; inter-assay CV was less than 12%. In order to negate the likelihood of known negative samples being scored as positive and known low positive samples scored as negative, extensive evaluation and definition of the control conditions within the assay were essential to prevent invalid data. In the study by Kandl *et al* (1994) intra- and inter-assay variation for c-erb B-2 was <2% (measured using an ELISA produced by Triton Diagnostics, Alameda, USA).

- **Sensitivity and specificity (as indicated in Table 27)**

Using the formula TP (TP+FN) for sensitivity, anti-p53 had a sensitivity of 98%, c-erb B-2 94%: (n=36). Specificity TN (TN+FP) indicated the proportion of subjects without the disease who had a negative test. (Anti-p53 100%; and c-erb B-2 100 % of women with benign disease). Specificity was calculated as the percentage of individuals in patients without breast cancer who had concentrations of these markers within the normal range. The sensitivity of p53 auto-antibody and c-erb B-2 immunoreactivity was not problematic; the area of clinical interest being well within the sensitivity of the immunoassay system.

Table 29 : Sensitivity and specificity of p53 auto-antibody and c-erb B-2

	Sensitivity per cent	Specificity per cent
p53 auto-antibody	100	100
c-erb B-2 (depends on cut-off level)	100-94	50-100

5.4.2 Reference range

The horizontal axis of each group represents the sample size (n=46). The vertical axis represents the position of the distribution. The two shaded areas delineated by the solid lines in the graph correspond to the 95% CI for the position of 50% (median). The determination of reference values for the markers evaluated in this study was established from a healthy female population (n≥46 subjects) using as many indices as possible to relate patient groups to reference groups. Indices included were age, race, number of children, menopausal status and HRT. All the p-values were two-sided and p-values of less than 5% were judged to be statistically significant. Serum antibodies to p53 were not detected in any of the control participants (p=0.0001), nor were elevated levels of c-erb B2 detected (p=0.002).

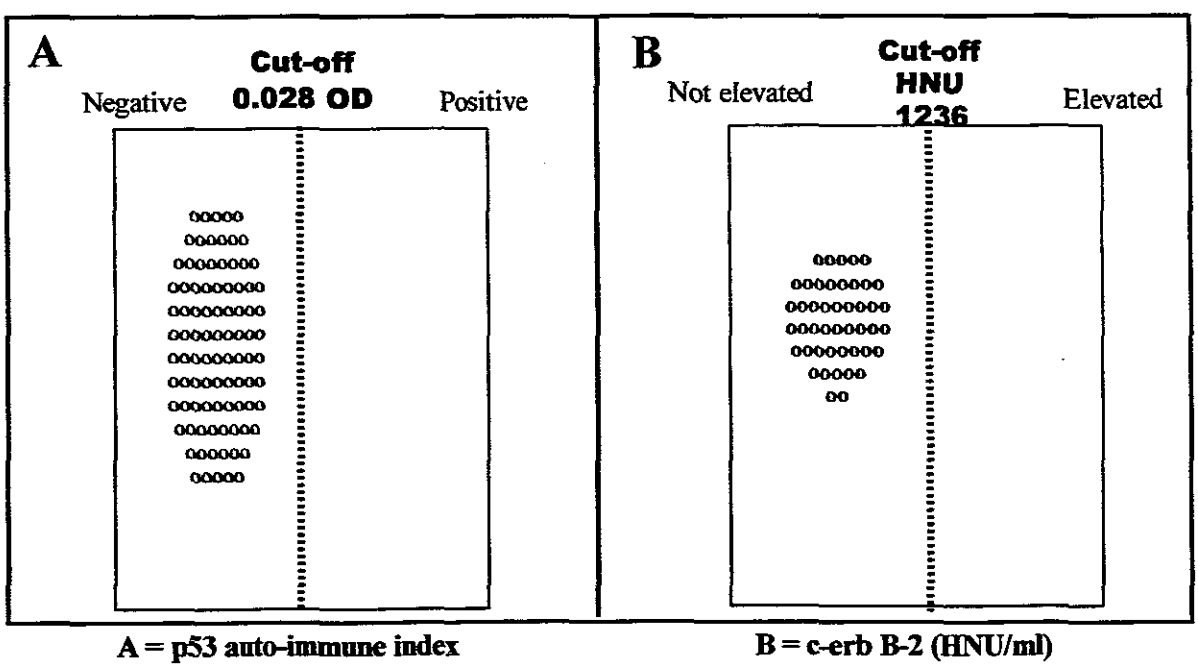


Figure 32: Variations in reference limits for Block A (p53 auto-immune index) and Block B (c-erb B-2 measured in HNU/mL)

5.5 anti-p53 results

This study detected anti-p53 antibodies in post-operative serum in 11% of breast cancer patients (Table 30) using ELISA kits purchased from Oncogene Research Products, USA (Catalogue QIA 16 Lot DO 3818 and Lot DO 2909, Expiry date - 17th February 1998).

Table 30 : Sample realization and test results of the study population of diseased and healthy individuals for anti-p53

Participants	Number	Percentage
Breast cancer patients	92	67
Controls	46	33
	138	100

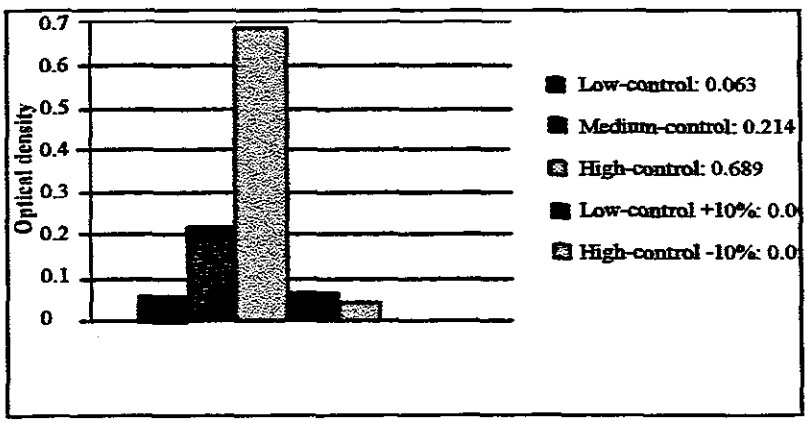


Figure 33 : Mean optical density for anti-p53 standards

Negative control samples comprised omission of serum samples (phosphate buffered saline control). Results used are the mean values of the two readings. Duplicate samples showed good concordance with only one specimen which did not pair equally. The remainder of paired samples did not have a different classification (high, medium, low levels) relative to the positive controls. The co-efficient of variation in a single serum sample with six duplicate measurements was 4.5%. Due to the enormously high cost of the kit, it was not economically viable to use a larger number of replicates.

Table 31: Results of anti-p53 ELISA for breast cancer patients

Test results	Disease status	
	Breast cancer	Benign nodule
Positive	11%	Nil
Negative	88%	100%

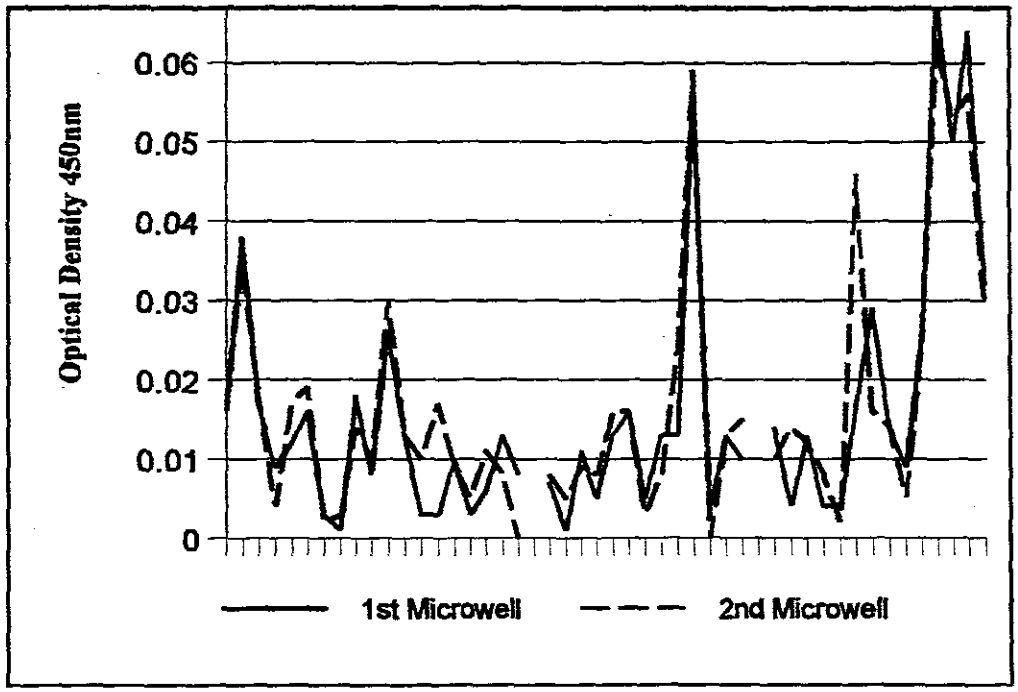


Figure 34 : Comparison between 1st and 2nd OD in microtitre plate for anti-p53 in breast cancer patients

Duplicate samples showed good concordance with no paired samples having a different classification (high, medium, low levels relative to the positive controls)

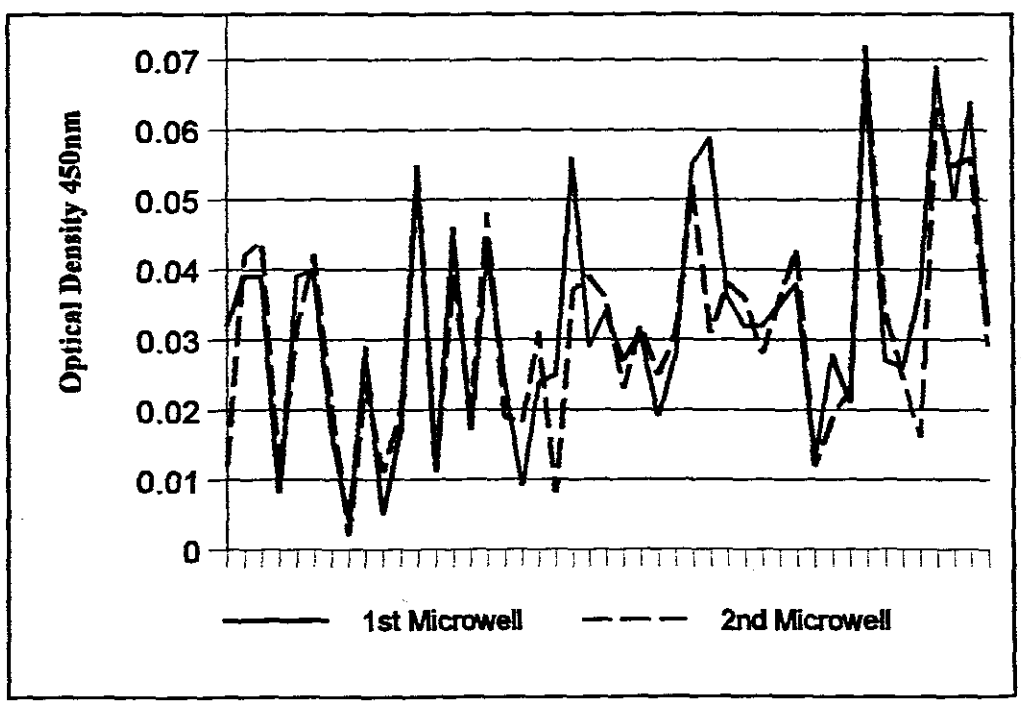


Figure 35 : Comparison between 1st and 2nd OD in microtitre plate for anti-p53 in control participants

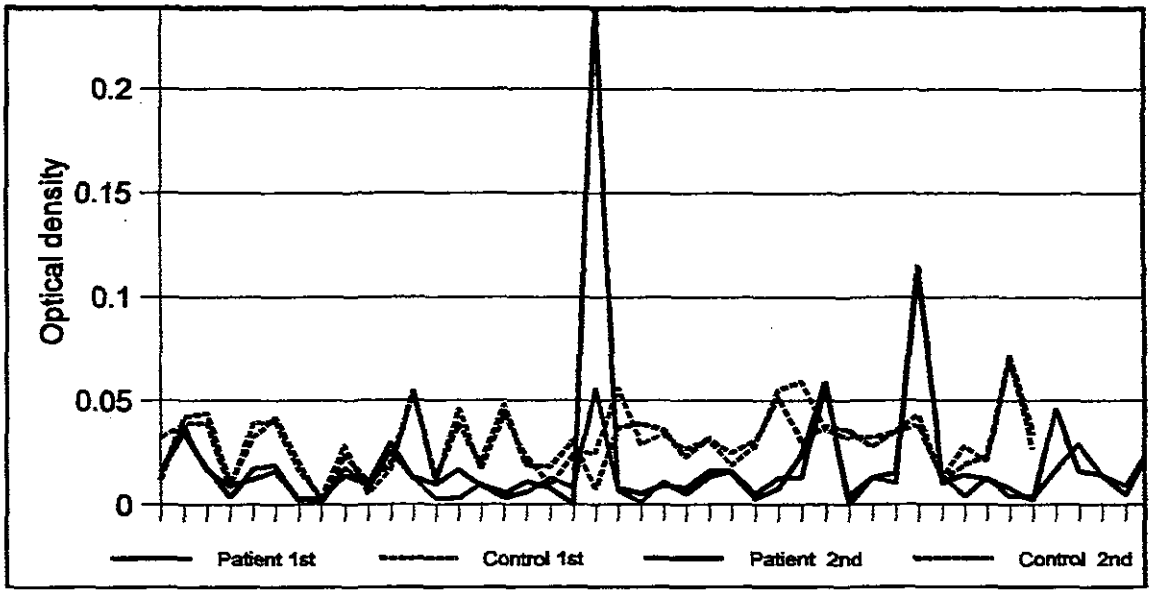


Figure 36: Comparison between OD in 1st and 2nd microtitre plates for breast cancer patients and control participants

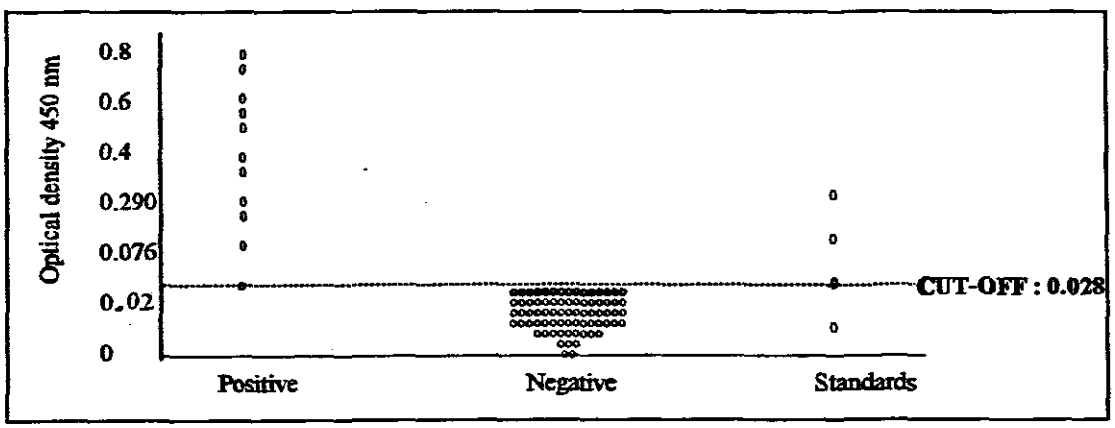


Figure 37 : Results of anti-p53 ELISA in serum samples from patients with breast cancer and the standards. The dotted line represents the cut-off value for positivity in the ELISA

10 patients were positive for anti-p53 antibodies. One patient showed a weak positive reaction and the auto-immune index was borderline. This was reported as negative

Raw data for anti-p53 and c-erb B-2 (both patients and controls) refer to Addendum VII.

Table 32: Characteristics of breast cancer patients positive for anti-p53 antibodies (Microplate One)

Name	HNU	Breast	Cancer type	Age	p53
Sample 1	529	Left	Invasive ductal	76	+ve
Sample 2	632	Left	Metastatic T4bN2M1	63	+ve
Sample 3	564	Left	Comedo-type growth - Invasive DCIS T4M8	41	+ve
Sample 4	577	Left	Medullary carcinoma TxN2M0	64	+ve
Sample 5	509	Left	Squamous carcinoma T4b N2 M1 Stage IV	56	+ve
Sample 6	488	Right	Invasive - T3N1M - Stage III - suggestive of ductal origin	42	Critical value for anti-p53

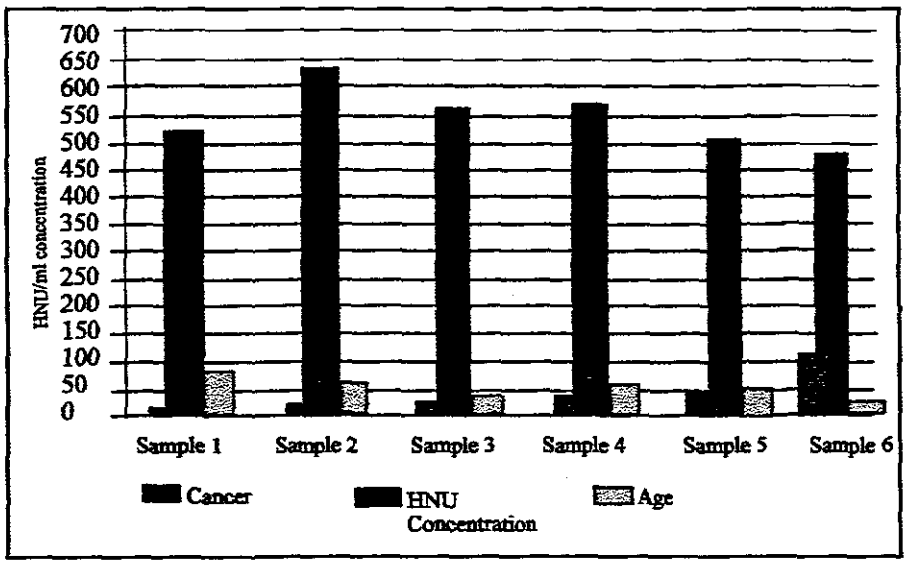


Figure 38: Characteristics of breast cancer patients positive for anti-p53 antibodies (Microplate One)

The number of positive sera (n=10) detected is too low to establish relative frequencies among the groups tested.

Table 33: Characteristics of breast cancer patients positive for anti-p53 antibodies (Microplate Two)

Name	HNU	Breast	Cancer type	Age	
Sample 1	577	All	Invasive ductal	75	+ve
Sample 2	560	Left	Infiltrating ductal	61	+ve
Sample 3	519	Left	Metastatic T4N2M1- invasive Carcinoma consistent with breast ductal origin	46	+ve
Sample 4	520	Right	pT3 pN1 M0 infiltratng ductal Carcinoma	41	+ve
Sample 5	568	Left	T3 N M0 invasive, suggestive of ductal origin	42	+ve

Sera from 5 of the 92 patients presented with primary tumours; 4 out of the 10 cases were metastatic at time of blood collection.

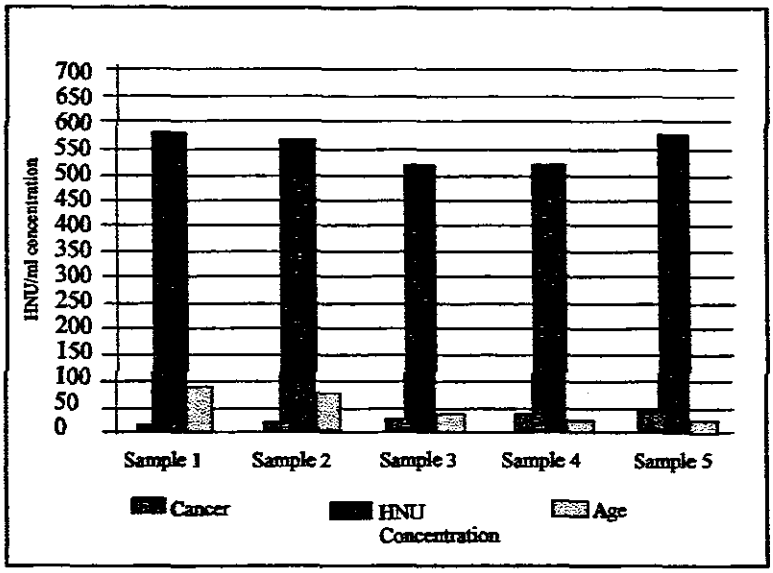


Figure 39: Characteristics of breast cancer patients positive for anti-p53 antibodies (Microplate Two)

The association of p53 auto-antibody with the characteristics of the breast cancer patients is shown in Table 32 and 33 respectively. There was no correlation with age or menopausal status. In a subset of women (i.e. less than 50 years of age), 5 of 10. patients (50 %) were found to be seropositive for auto-antibodies to p53 compared with 50% (5/10) of patients ages between 50 and 59. 50 % (5/10) of seropositive women were pre-menopausal and the majority (10/10) were symptomatic. Of the pre-menopausal seropositive women, 50% (5/10) seropositive patients were found to have grade III tumours. p53 auto antibodies and clinicopathological indices are reported on page 5.39.

5.5.1 Standards for p53 auto-antibodies and staging of breast cancer

To assess the sensitivity and validity of the ELISA systems investigated in this study, calibration curves with standards applicable to each system were investigated from the literature. In the studies by Vojtěšek (1993), 100μl aliquots of various concentrations of standard anti-p53 were assayed and the graph, shown in Figure 40 was obtained. The assay was standardized using pure soluble recombinant p53 isolated from baculovirus by immunoaffinity columns. The equation of the standard curve was solved by virtue of a curve fitting programme (Cricket Graph, Cricket

Software Inc., Malvern, PA19355, USA) and used to determine nanograms (ng's) of p53 per OD unit. From this conversion the concentration of p53 was calculated using the highest point from the linear range of the serial dilution series.

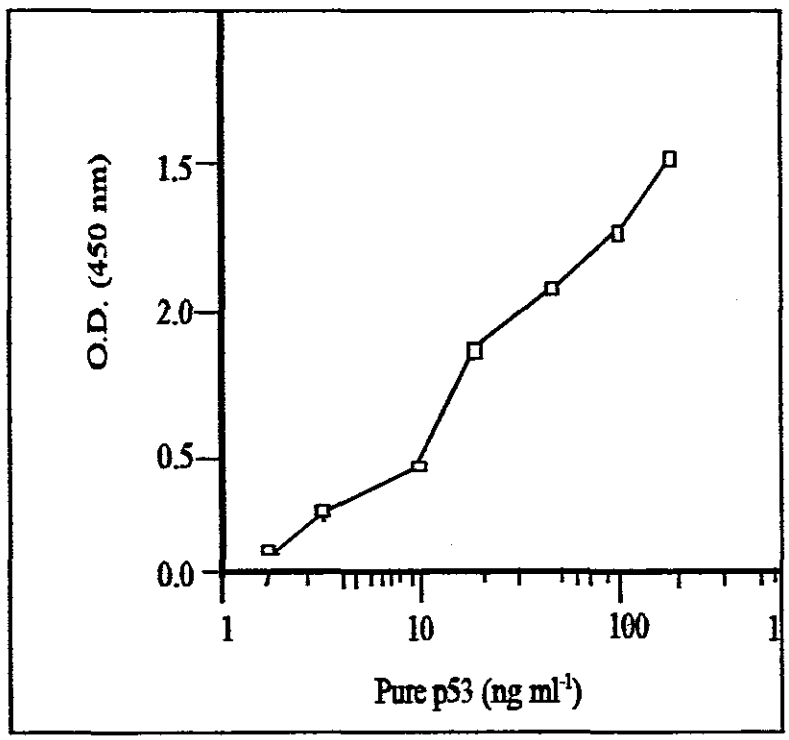


Figure 40: Two site immunoassay using known concentrations of soluble recombinant human p53 protein. Microtitre plates were coated with anti-p53 monoclonal antibody DO-1 and after incubation with soluble recombinant human p53 probed with anti-p53 polyclonal rabbit serum CM-1 (Vojtěšek, 1993)

Oncogene Science did not provide a calibration curve with the kit, but the following figure was provided on request (1999).

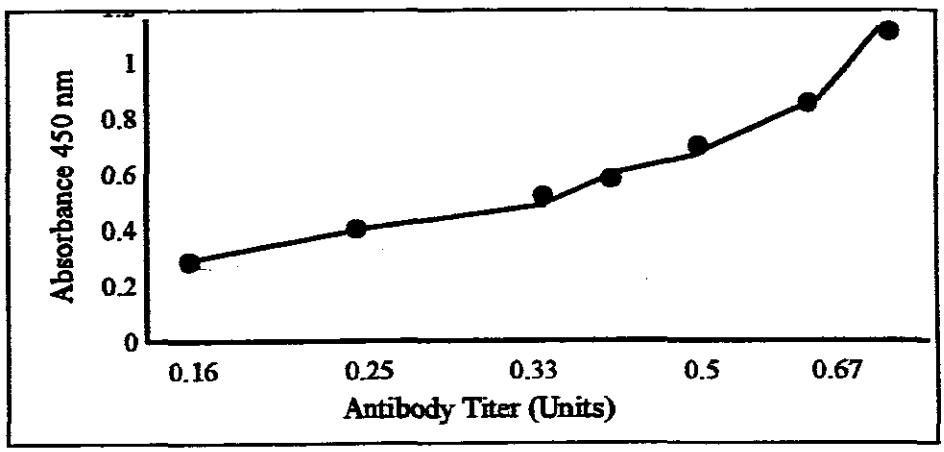


Figure 41: p53 Auto-antibody ELISA standard curve as provided by Oncogene Science on request.

There are no p53 standards available and standards are produced in cultured cell lines that are known to overexpress p53. The cell lysate is used (approximately 10^7 cells) and arbitrarily assigned a value to this solution. Table 34 gives an indication of the variety of p53 antigen used in assays to detect the p53 auto-antibody.

Table 34: Reactivity of human anti-p53 antibodies against various p53 mutant proteins and against wild-type recombinant p53 protein

	Source of p53 antigen				
Patient serum	**colo 320 HSR(+)**	**MIA PaCa-2**	**T-47D**	**OCIN2**	**recombinant p53 protein**
Breast ①	1.8 ②	1.9	1.4	1.2	1.3
Breast Ca	4.2	5.9	4.0	2.6	3.1
Breast Ca	4.0	5.3	2.9	2.0	2.2
Lung Ca	2.0	3.0	1.8	1.5	1.4
Lung Ca	2.8	2	1.7	1.3	1.3
Cell line/ mutations					
Codon	248 (CGG-TGG)	248 (CGG-TGG)	294 (CTT-TTT)	247 (CTT-GAT)	
Amino acid substitution	Arginine Tryptophan	Arginine Tryptophan	Leucine Phenylalanine	Valine Asparagine	
Malignancy	colon	pancreas	breast		

①: Ca = carcinoma

②: Data are expressed as the fluorescence ratio when the assay was performed with or without the presence of the lysate, respectively. 30 samples from normal volunteers tested negative (fluorescence ratio <1.7) when assayed with use of each one of the sources of the p53 antigen shown above. (Chan, 1996)

Table 34 could have been significantly improved if notations were supplied in the literature as to which sources of the data used p53 protein with and without lysate.. The higher values on p53 tested in cell lysates could provide an explanation for the higher values of cellular data when compared to the recombinant p53.

Table 35, displayed on the following page, is a summary, from the literature, of the methodology used to detect p53 antibody in sera. The various tumour locations are also displayed.

Table 35 : Summary of analyses of p53 antibodies in sera from the literature (1982-1996)

Authors	Year	Method	Site	Percentage positive
* Crawford LV *et al.*,	1982	immunoprecipitation	breast	9-14
* Crawford LV *et al.*,	1984	immunoprecipitation	breast	9-14
* de Fromental C *et al.*,	1987	EIA	childhood cancers B-cell lymphomas	12 20
* Hassapoglidou *et al.*,	1992		breast	9-14
* Davidoff AM *et al.*,	1992	EIA	breast	9-14
* Schlichtholz B *et al.*,	1992	ELISA	breast	9-14
* Mudenda B *et al.*,	1994	ELISA	breast	26
* Angelopoulou *K et al.*,	1994	New quantitative immuno-flurometric techniques	breast	5
* Peyrat Jean-P *et al.*,	1995	ELISA	breast	12
Winter S *et al.*,	1992	immunoblotting	lung	10
Winter S *et al.*,	1993	immunoblotting	small cell lung cancer	58
Volkmann *et al.*,	1993	ELISA	hepatocellular	25
Labrecque S *et al.*,	1993	immunoprecipitation	cancer patients	5.1
Lubin *R et al.*,	1993	ELISA	various cancers	
Angelopoulou *K et al.*,	1993	Time-resolved immunoassay	various cancers	9-14
Angelopoulou *K et al.*,	1994	New quantitative immuno-fluorometric techniques	ovarian and colon	15
Angelopoulou *K et al.*,	1994	New quantitative immuno-fluorometric techniques	lung	8
Marxsen *J et al.*,	1994	ELISA	pancreatic disease	6.4
Preudhomme *et al.*,	1994	ELISA	myelodysplastic	3.5
Schlichtholz B *et al.*,	1994	ELISA/immunoprecipitation	lung	24
Diamandis E *et al.*,	1995	fluorometric assay	various cancers	9-14
Lubin R *et al.*,	1995	ELISA	lung	30
Trivers G *et al.*,	1995	EIA (Modification)	angiosarcoma of liver	33
Raedle J *et al.*,	1995	ELISA	hepatocellular carcinoma	42
von Brevern C *et al.*,	1996	EIA	oesophagus	25
Ryder, SD	1996	ELISA	hepatocellular cancer	52
Pulkki K *et al.*,	1996	ELISA	multiple myeloma	15

* : Breast

* : Crawford : SV40 - transformed
human fibroblast cell line SV80 and
mammary carcinoma cell line MDA-MB-157

* : Angelopoulou : Method A - Mutant p53 antigen
Colo 320 HSR cells
Mouse MAb PAb 240 anti-p53
Method B - Mutant p53 antigen
Colo 320 HSR cells
Mouse MAb PAb 240 anti-p53

* : *p53 alterations can be assessed by three approaches. Tumour cells display either a mutation in the* p53 gene (detected by DNA sequencing) or an accumulation of the protein (detected by immunocytochemical analysis), or lastly, detection of the antibodies to p53 (ELISA).

Due to the lack of a suitable standard solution Angelopoulou *et al.*, (1993), using time-resolved immunoflurometry, devised an arbitrary system to calibrate methodology. Among the high p53 antibody-positive sera one was selected and its concentration arbitrarily defined to be 20.48 units/litre (u/l). This serum sample was then used in dilutions to construct calibration curves for both immunoassays (one 'competitive-type' and one 'non-competitive' type) from which the concentration of the sample was calculated.

5.6 c-erb B-2 results

The c-erb B-2 oncogene product in serum was measured by ELISA kits (Oncogene Science, QIA 10, Lot DO 3904-1: Expiry date 25th April 1998. The specificity of the c-erb B-2 (Oncogene Science kit) was for human *neu*/erb B-2 p185 and ECD p105 cleavage fragment. The ELISA's were performed on 9th December 1997). Due to the high cost of the c-erb B-2 kits, exacerbated by the Rand/Dollar exchange rate, only 45 breast cancer patients were analyzed for c-erb B-2

5.6.1 Serum c-erb B-2 in breast cancer

The post-operative serum levels in the 45 breast cancer patients ranged from 430-631 HNU/ml, respectively. Serum levels exceeding 1236 HNU/ml, 2SD above the mean of 2 positive controls (553+1236 HNU/ml) were considered elevated. The c-erb B-2 related protein levels ranged from 425 to 632 HNU/ml (mean 530) in the post-operative sera. No samples collected from patients who had loco-regional metatastic spread at the time of diagnosis (supraclavicular metastases) had elevated serum levels.

The patient sample 2, (refer Table 32), positive for anti-p53, had the highest concentration of HNU/ml (631), while another sample (a critical value for anti-p53) presented with the lower value for HNU/ml (488). This same sample was the only right -sided breast cancer among the women positive for anti-p53. There was no correlation between the presence of elevated serum c-erb B-2 and menstrual status (p=0.67).

	Number of patients (n=45)	Percentage	Range HNU/ml concentration
Benign breast disease	10	n/a	<400
Primary breast cancer Stage I & II	5	11	460-606
Recurrent breast cancer Stage III & IV	14	30	489-631
Non-recurrent breast cancer	26	58	425-632

Table 36: Post operative c-erb B-2 serum concentration in breast cancer patients.

The serum levels for c-erb B-2 were not normally distributed in patients with recurrence .

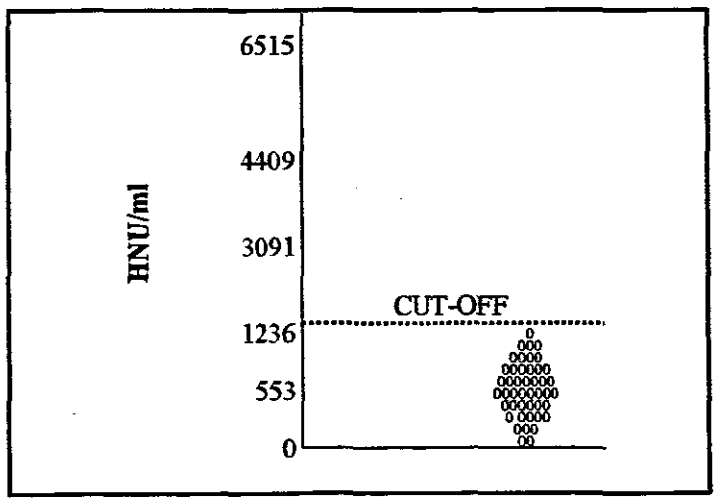


Figure 43: Post-operative c-erb B-2 in serum of breast cancer patients

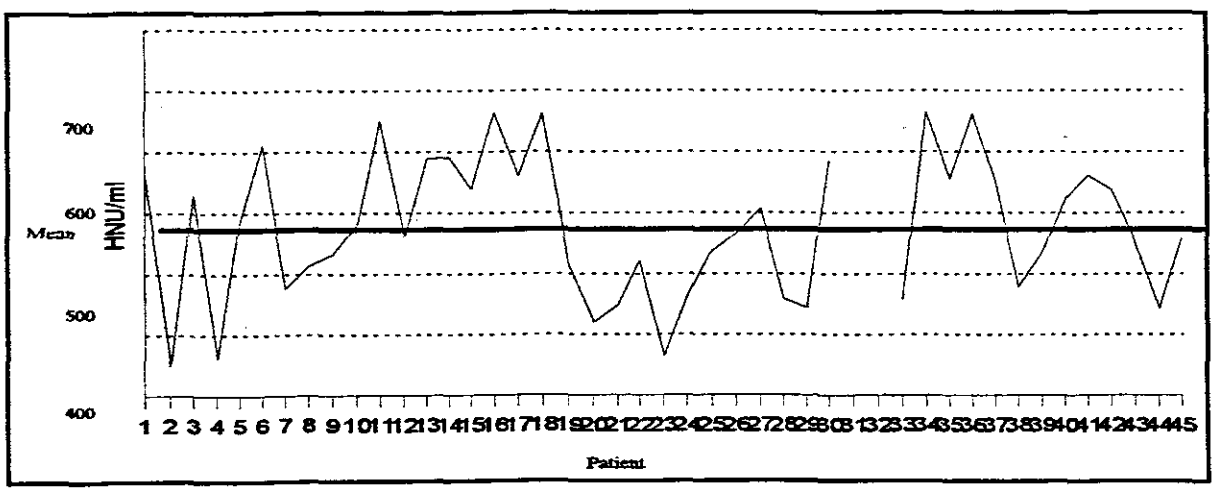


Figure 44: Individual c-erb B-2 results (HNU/ml) for patients with breast cancer (n=45)

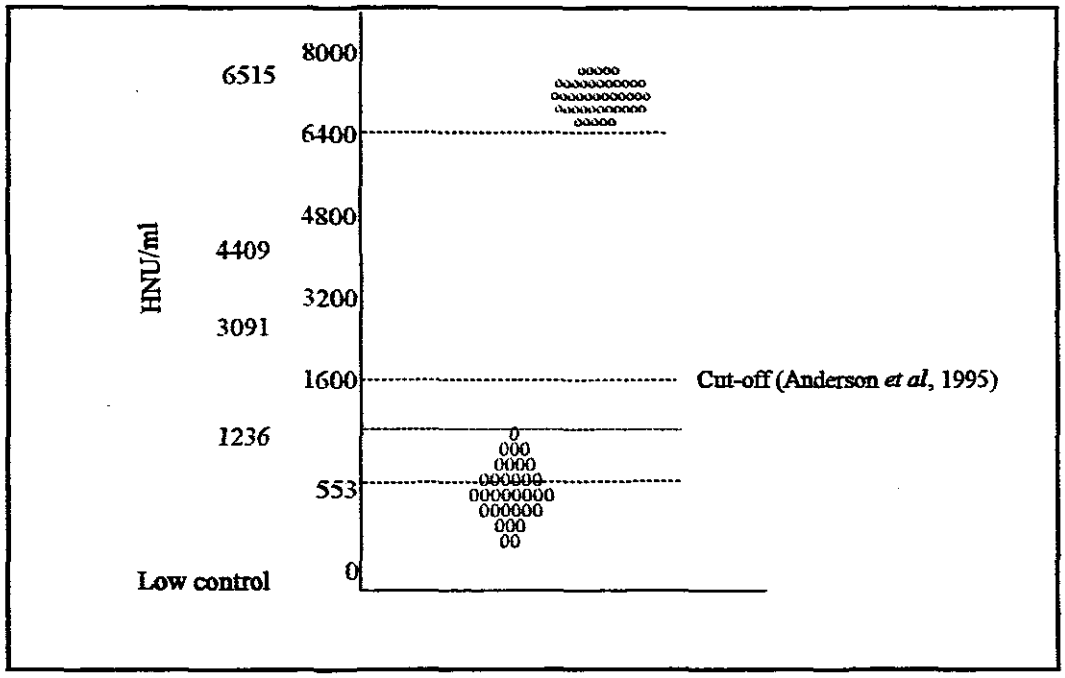


Figure 45 : Oncogene Science c-erb B-2 comparative results (Anderson *et al.*,, 1995 [red] with standards 0-100 HNU/ml. This study used standards 0-120 HNU/ml [blue]).

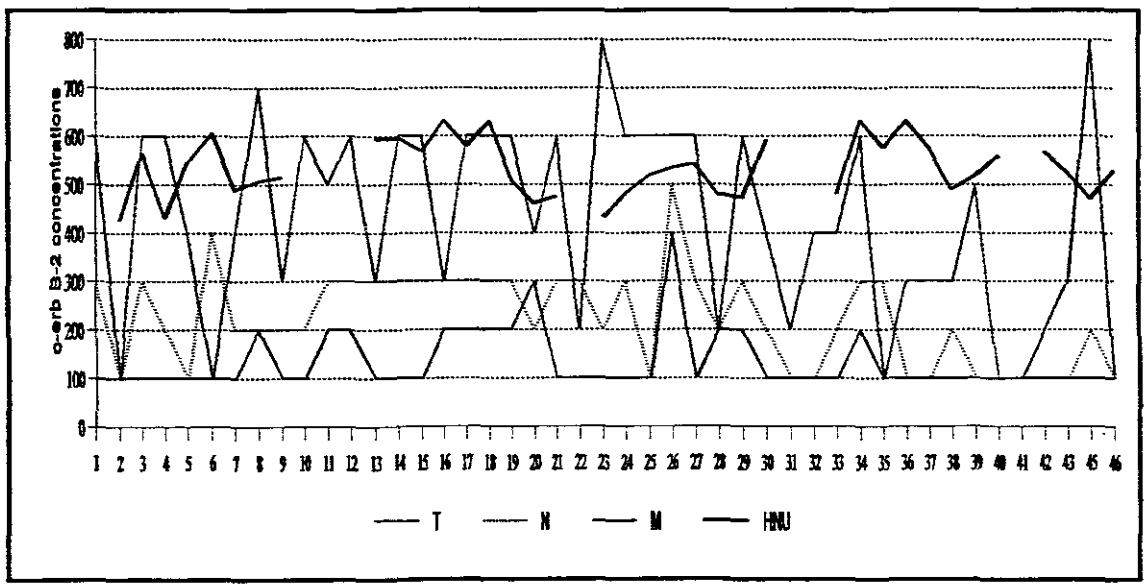


Figure 46: TNM results and c-erb B-2 concentration for breast cancer patients

Representative Values (HNU - as stated)
T : 1= Nil/unknown; 2= T2; 3= T2; 4=T3; 5= T4; 6= 4b; 8=p3
N: 1=Nil/unknown; 2= N1; 3= N3; 4= N4
M: 1=Nil/unknown; 2= M2; 3= pT2; 4= p11

Table 37: Post-operative serum levels for c-erb B-2 (HNU/ml) and TNM classification of breast cancer patients

Sample No	Age	T	N	M	HNU/ml
1	64	4b	2	1	580
2	60	T2	1	0	425
3	29	4b	2	0	564
4	39	4b	1	0	431
5	41	3	0	0	544
6	41	2	3	0	606
7	42	3	1	0	488
8	44	4b	1	1	506
9	46	32	1	0	515
10	46	4b	1	0	540
11	46	4	2	1	625
12	48	4b	2	1	530
13	50	2	2	0	594
14	50	4b	2	x	595
15	50	4b	2	x	569
16	52	2	2	1	632
17	54	4b	2	1	579
18	56	4b	2	1	631
19	56	4b	2	1	509
20	58	p2	1	0	460
21	60	4b	2	0	474
22	61	1	2	0	510
23	62	3p	1	0	432
24	62	4b	2	0	481
25	65	4b	0	0	518
26	67	4	M8.5	P11	533
27	70	4b	2	0	541
28	73	1	1	1	479
29	76	4b	2	1	472
30	85	3	1	0	593
31	47	1	0	0	error*
32	49	3	0	0	error*
33	54	3	1	0	479
34	63	T4b	2	1	632
35	38	x	2	0	577
36	42	2a	0	0	631
37	44	2FNA	0FNA	0FNA	574
38	47	2	1	0	489
39	48	4	0	0	517
40	50	4	1	1	561
41	50	3	1	1	580
42	50	1	1	1	568
43	52	2	1	1	525
44	59	p3	2	1	471
45	76		1	1	529

(Error* : OD readings not consistent)

Table 38 Post-operative serum levels for c-erb B-2 and patient characteristics

Patient characteristics	%	c-erb B-2 elevated %	c-erb B-2 not elevated %	p-value
Menopausal satus				
Pre	60	Nil	100	Not
Post	40	Nil	100	significant
State at presentation				
I	9	Nil		
II	13	Nil		
III	21	6	15	
IV	41	8	33	
Recurrent	15			

5.6.2 Serum c-erb B-2 protein in healthy control subjects

c-erb B-2 oncoprotein was not elevated in the serum of normal controls or in benign breast conditions. The distribution of serum c-erb B-2 levels in healthy control subjects were stratified for age (10 year intervals). The range was 400-580 HNU/ml (n=36). The mean levels were 5.024 ± 60 and 530 ±58 HNU/ml, respectively.

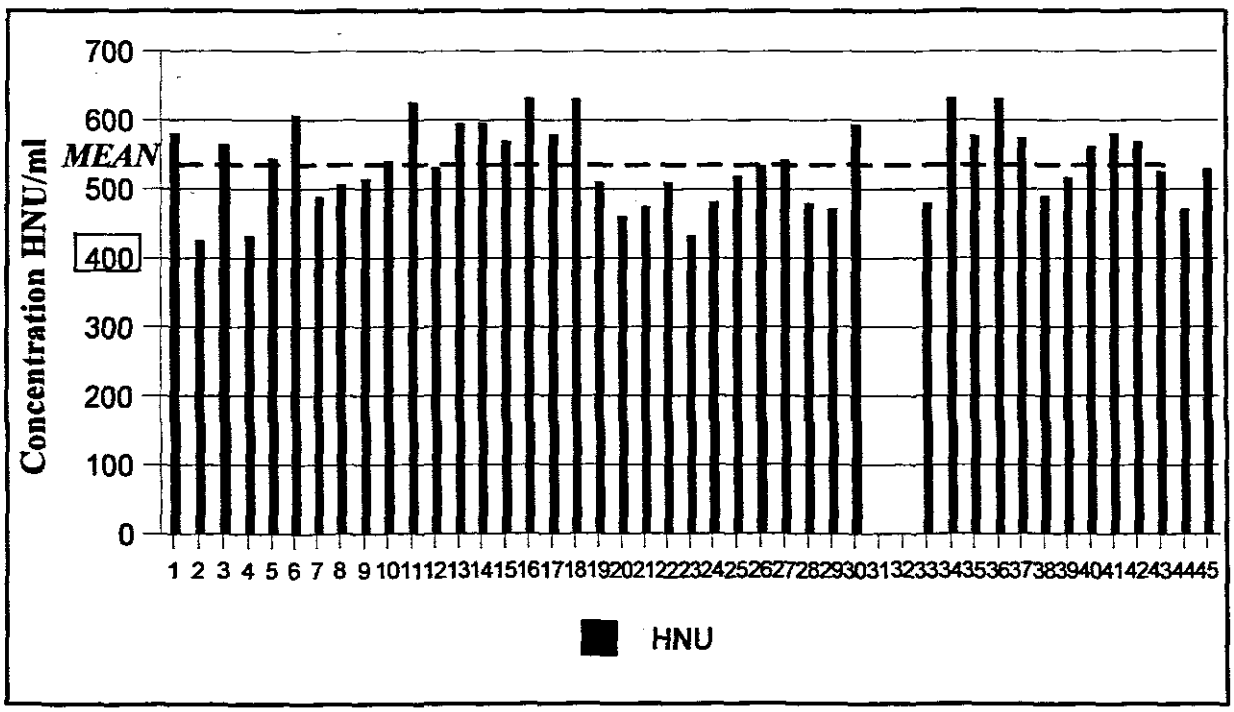


Figure 47 : Results of c-erb B-2 concentration (HNU/mL) as measured by ELISA 9th December 1999 for control group.

The control population ranged in age from 35 to 75 years and consisted of 45 women of different race groups. The mean level was 490 HNU/ml. The serum levels of c-erb B-2 were normally distributed in the 35 controls and the 10 women with benign breast disease. Serum levels ranged from 450 to 580 for the normal control group and from 400 to 580 for the benign breast disease group, HNU/ml respectively.

5.6.3 c-erb B-2 comparative results from the literature

c-erb B-2 gene product overexpression by IHC has consistently shown variation (Table 37). Results of serum analysis were also compared to validate results (Table 38). In the study by Watanabe *et al.*, (1995), the histogram of healthy women was observed as a log-normal

distribution which skewed slightly to the right. Watanabe *et al.*, used the kit produced by Triton Diagnostics.

Table 39: c-erb B-2 gene product overexpression observed by different groups with an immunohistochemical technique (Terrier *et al.*, 1996)

						Prognostic significance	
Authors	**Recognised domain**	**Number**	**(%) Over-expression**	**Nature of the antibody**	**Recognized sequence**	**Disease-free survival**	**Overall survival**
Thor *et al.*, (1992)	External	290	15	Monoclonal	-	p=0.0018 on T3-T4 patients	NS
Paik *et al.*, (1990)	External	292	21	Monoclonal	-	NS	p=0.0012
Kallioneni *et al.*, (1990)	External	319	23	Monoclonal	-	nd	p<0.001
Barnes *et al.*, (1988	Internal	195	9	Polyclonal	1243-1255	NS	NS
Walker *et al.*, (1989)	Internal	85	16.5	Polyclonal	1243-1255	p=0.0002	p=0.009
Wright *et al.*, (1992)	Internal	185	17	Polyclonal	1243-1255	p=0.025	p=0.04
McCann *et al.*, (1990)	Internal	314	17	Polyclonal	1243-1255	p=0.002	p=0.0001
Lovekin *et al.*, (1991)	Internal	602	15	Polyclonal	1243-1255	nd	p=0.0003O
O'Reilly *et al.*, (1991)	Internal	172	23	Polyclonal	1243-1255	p=0.016 on N+ patients	nd
Winstanley *et al.*, (1991)	Internal	465	22	Polyclonal	1243-1255	nd	p=0.05
Allred *et al.*, (1992)	Internal	613	14	Polyclonal	1243-1255	p=0.0001 in low-risk patients	p=0.0001 (ER+, T<3cm)
van de Vijver *et al.*, (1988)	Internal	189	14	Monoclonal	1242-1255	NS	p=0.04
De Potter *et al.*, (1989)	Internal	71	38	Monoclonal	1242-1255	p<0.001	nd
Tsuda *et al.*, (1990)	Internal	176	15	Polyclonal	1242-1255	p<0.01	P<0.001
Baak *et al.*, (1985)	Internal	96	14	Polyclonal	1242-1255	nd	p=0.07
Rilke *et al.*, (1991)	Internal	1210	23	Polyclonal	1242-1255	nd	p=3x10-5 on N+ patients

* Paraffin-embedded sections were used in all the studies
NS= not significant, nd= not determined; DFS: Disease-free survival; OS: Overall survival

Table 40: c-erb B-2 cut-off levels from similar studies in the literature

	Manufacturer	**c-erb B-2**	**Number of healthy women**
Narita *et al.*, (1992)	Triton Diagnostics (mean 18.37 u/ml)	20 u/ml	87
Kynast *et al.*, (1993)	Medac and Triton kit	30	19
Kandl *et al.*, (1994)	Triton Diagnostics	10 u/ml	24
Watanabe *et al.*, (1994)	Triton Diagnostics	12 u/ml	150
Anderson *et al.*, (1995)	Triton Diagnostics	>30 u/ml	not stated
Volas *et al.*, (1996)	Oncogene Science kit Q1A-04: (mean +2 SD)	450-1800 850-1600 HNU/ml	66 12 benign breast disease

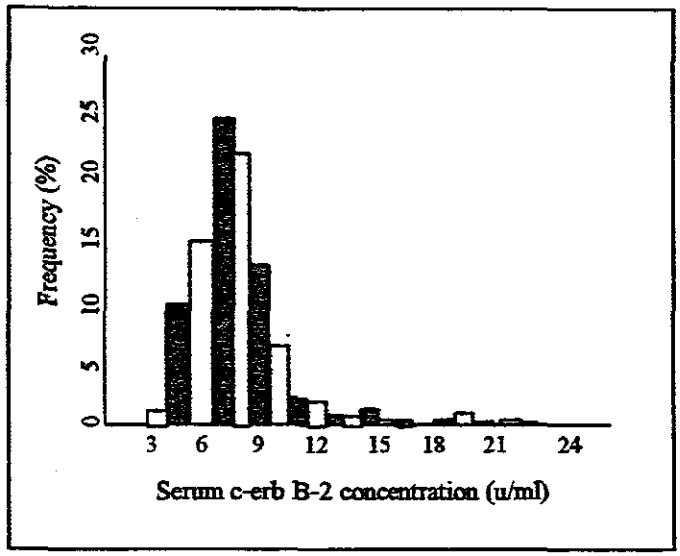


Figure 48: Histogram of serum c-erb B-2 levels in healthy women.
A log-normal distribution is shown with a skewness of 0.164 ($p<0.01$) and a kurtosis was 3.100 ($p<0.01$). The population consisted of 250 women. Nine with a serum c-erb B-2 value of 16 u/ml, were excluded from this histogram. (Watanabe *et al.*, 1995).

The cut-off level was set at 1600 HNU/ml, ±2SD from the log-normal distribution of the upper limit of the normal range in a healthy female population. Watanabe *et al.*, (1995) set the cut-off level as 12 u/ml for healthy women. The histogram results for this group are shown in Figure 48. Narita *et al.*, (1992), using a kit produced by Triton Diagnostics, (Ciba-Corning) defined the cut-off level as 20 u/ml[Table 41].

Table 41: c-erb B-2 results (Narita *et al.*, 1992) : serum level significantly higher in distant metastatic group

26u/ml	Percentage	Cancer type
11.8	0	benign breast disease
11.8	3.1	stage I/II primary breast cancer
38.2	29.4	stage III/IV
17.9	33.3	locally recurrent breast cancer
298.4	51	recurrent cancer distant metastases
12.9	0	no evidence of recurrence

Wu *et al.*, (1995) also estimated a 12 u/ml cut-off level. With this cut-off level, increased serum c-erb B-2 was found, in the literature, to be in approximately 12% of primary breast cancer patients, in 5% of non-recurrent breast cancer and in 39% of patients with recurrence. Anderson *et al.*, (1995) reflects the mean value and positive rate for this protein (assuming 1600 HNU/ml as the cut-off value in post-operative sera) as 850-1600 HNU/ml in benign breast disease (n=12);

in stages I and II primary breast cancer (n=13), 750-2000 HNU/ml, in stages III and IV recurrent breast cancer (n=93), 750-120000 HNU/ml (59%); in locally recurrent breast cancer 800-3550 HNU/ml (19%); 59% in recurrent breast cancer with distant metastases. 750-120,000 HNU/ml (68%), mean 14650 HNU/ml levels were not normally distributed in the study by Anderson *et al.*, (1995). Intra-assay precision (CV) was less than 6%; inter-assay CV less than 12% in Anderson et al,s study.

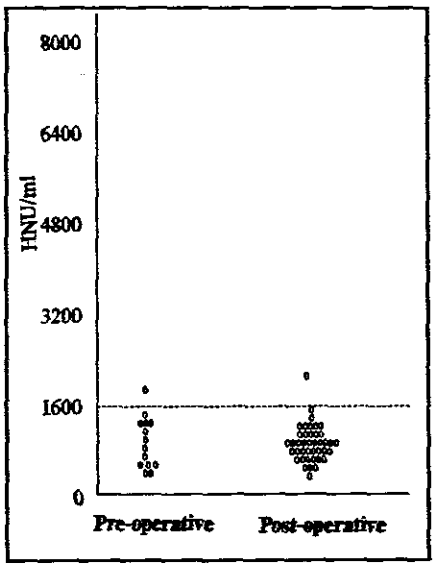


Figure 49 : Distribution of the c-erb B-2 related protein levels in sera from 13 pre-operative breast cancer patients and 62 post-operative breast cancer patients without recurrent disease. (Anderson *et al.*, 1995)

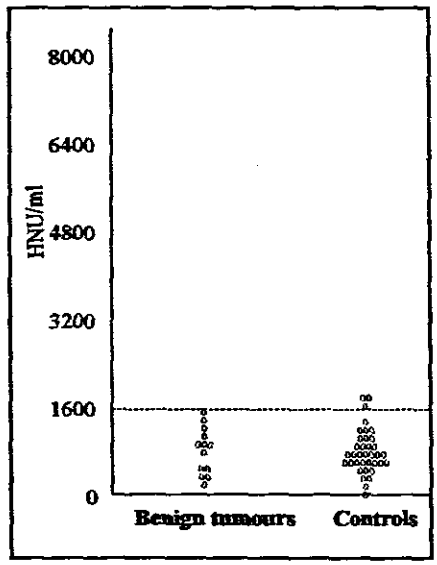


Figure 50 : Distribution of the c-erb B-2 related protein levels in sera from 12 females with benign breast tumours and 66 female controls. (Anderson *et al.*, 1995)

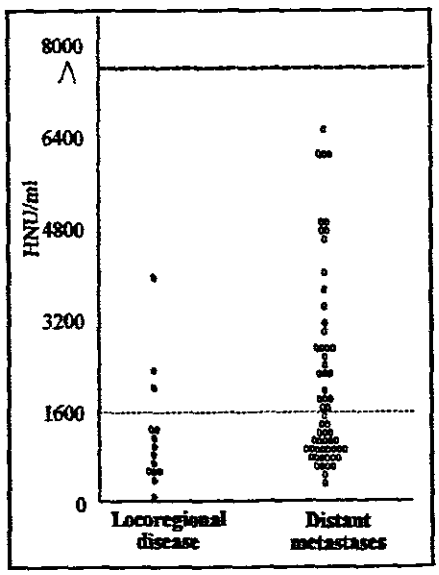


Figure 51 : Distribution of the c-erb B-2 related protein levels in sera from 16 breast cancer patients with loco-regional disease and 77 breast cancer patients with distant metastases. (Anderson *et al.*, 1995)

Anderson *et al.*, (1995) used an Oncogene Science kit (0-100 HNU/ml) but the standards differed from the kits used in this study (0-120 HNU/ml).

5.7 Breast cancer : A multivariate analysis of prognostic factors

Breast cancer displays great variation in biological behaviour (Allred *et al.*, 1993). Any parameter that reflects malignant potential of these tumours would be of enormous decisive benefit to the oncologist. The aim of this section of the study was to correlate the significance of p53 auto-antibodies and c-erb B-2 immunoreactivity together with pathological characteristics of the primary tumour (size, histologic type, nuclear grade and lymphatic vascular invasion). Tumours were categorized using the TNM system. Invasive carcinomas were classified using the largest dimension of the invasive component to determine size: ≤ 5mm or less = t1a; 6-10 mm= T1b; 11-20 mm= T1c. The lesion was measured macroscopically to the nearest mm.

5.7.1 Clinicopathological indices

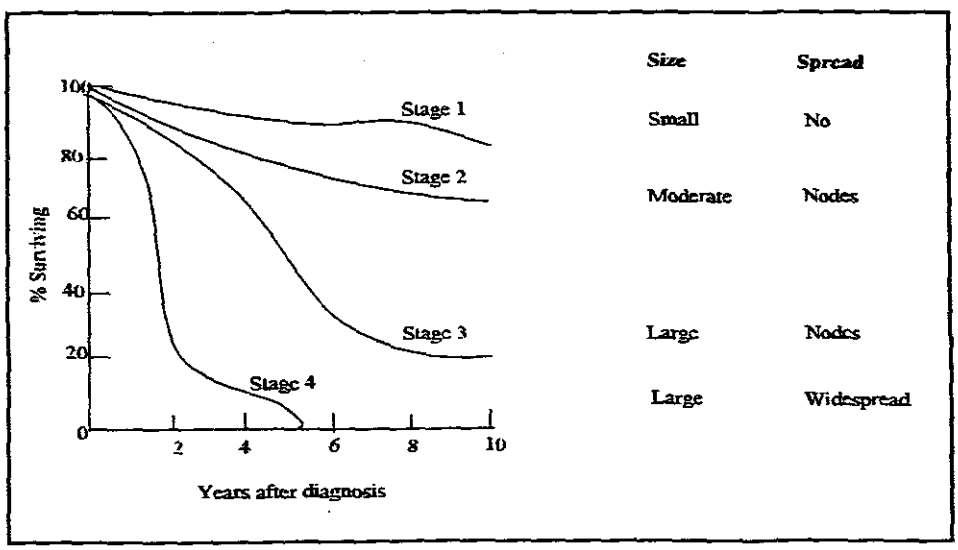


Figure 52: Overall survival of women according to the stage at which their breast cancer was diagnosed (King, 1996)

For each serum sample used in this study, the following clinicopathologic data were obtained from reports emanating from the Departments of Cellular Pathology at King Edward VIII hospital and Regional Laboratory Services, Durban; histologic type of tumour (both primary tumour size, pathologic axillary lymph node status, hormone receptor (ER) values (when available) and tumour

grade. ER values were quantitated by immunochemical analysis by the Department of Chemical Pathology, University of Natal Medical School. These were not always available and therefore comments are not statistically relevant. The tissue sections of tumours were staged using the International Union Against Cancer (UICC) TNM convention (UICC, 1987). Staging refers to the grouping of patients according to the anatomical extent of their disease and has vast implications for survival (Figure 52). The most widely used staging system is based on tumour size, axillary lymph node involvement and the presence or absence of distant metastasis (TNM system).

Histopathological features of tumours are shown in Tables 42 and 43. All the tumours described were assessed and confirmed as being breast carcinomas by the Histopathology Departments at King Edward VIII Hospital or at Regional Laboratory Services, Durban. All of the patients with invasive breast carcinoma underwent axillary lymph node dissection.

Table 42: All tumours : Invasive adeno-carcinomas

Ductal tumours	60 %
Lobular tumours	5%
Other tumours	35%

Table 43: Histoprognostic grading

Grade I	24 %
Grade II	37 %
Grade III	17 %
(13% not available)	

Permission to access results was kindly given by Professor R Chetty (University of Natal Medical School) and Dr B Akerman (Regional Laboratory Services, KwaZulu-Natal). A comparison of study populations regarding lymph node involvement is shown in Table 44. The importance of staging patients with breast cancer lies in its ability to stratify patients into different prognostic groups.

Table 44: Comparison of study populations (Axelsson *et al.*, 1995).

	Axelsson *et al.*, (1995)	Fox (1993)	Gasparini *et al.*, (1993)
Median time of follow-up, y	11.5	2.1	5.2
No. of node-negative patients	111	109	254
No. of node-positive patients	110	0	0
Node negative with metastasis at 2.1 y, %	9	16	-
Node negative with metastasis at 5.2 y, %	21	-	18
Node negative with metastasis at 11.5 y, %	21	-	-
Node positive with metastasis, %	54	-	-
Total recurrences, %	40	16	18
Node-negative deaths at 2.1 y, %	5	6	-
Node-negative deaths at 5.2 y, %	15	-	12
Node-negative deaths at 11.5 y, %	25	-	-
Node-positive deaths, %	50	-	-
Total deaths, %	39	6	12
Characteristics of node-negative patients			
Age <50 y, %	28	38	27
ER-negative, %	47	48	24
Histology: ductal tumour type, %	100	74	77
Tumour size, cm, %			
< 2	39	40	64
2-5	50		
> 5	11		
Tumour grade, %			
1	5*	22+	15#
2	55*	34+	56#
3	40*	35+	29#
Lymphatic invasion, %			
Treatment			
Surgery, MRM, %^	79	82	52
Surgery, lumpectomy, %	21	18	48
Adjuvant chemotherapy, %	11		0
Tamoxifen therapy, %	1		

* Modified criteria of Black *et al.*,
+ Modified criteria of Bloom and Richardson
# Criteria of Bloom and Richardson
^ MRM = modified radical mastectomy

All specimens in this study were graded by pathologists employed at Regional Laboratory Services and at the Department of Cellular Pathology, University of Natal, Durban, KwaZulu-Natal. The specimens were investigated without knowledge of the clinical and laboratory data. In breast cancer nuclear p53 protein accumulation has been correlated with histological grade and tumour progression.. Breast carcinoma overexpression is found in 25 to 60% of invasive tumours and is associated with a high histological grade and poor disease outcome. There is also an inverse relationship with hormone receptor expression (Happerfield *et al.*, 1995). Axillary lymph node metastases (ALNM) is the most important predictor of survival in patients with T_1 breast carcinoma. The incidence of ALNM increases as a function of primary tumour size (≤ 2 cm).

92 samples from 92 patients met the criteria stated in Chapter Three. The characteristics of the patients with clinical parameters for primary tumour carcinoma of the breast are summarized in Table 45. The median tumour size was 18 mm. in all tumours, 13% were T1 tumours. The majority of tumours (60%) were infiltrating ductal carcinoma and 5% were of the infiltrating

lobular type . 66 of 92 patients (72%) had ALNM.. About half of all breast cancer patients have metastatic disease in axillary lymph node when they are first seen by a physician (Allred *et al.*,[1], 1993). In this study tumours were selected which were either confined to the breast (Mo) or had distant metastases (M1).

Table 45: Clinical parameters for primary tumours

Tumour type	per cent
Infiltrating ductal	60
Lobular	5
Other	30
Unknown	5
Node status	
Negative	23
Positive	77
Unknown	Nil
Tumour size	
⟨2 cm	44
2.5 cm	17
⟩2 cm	25
Unknown	15
Age (years)	
Mean	44
Range	24-75

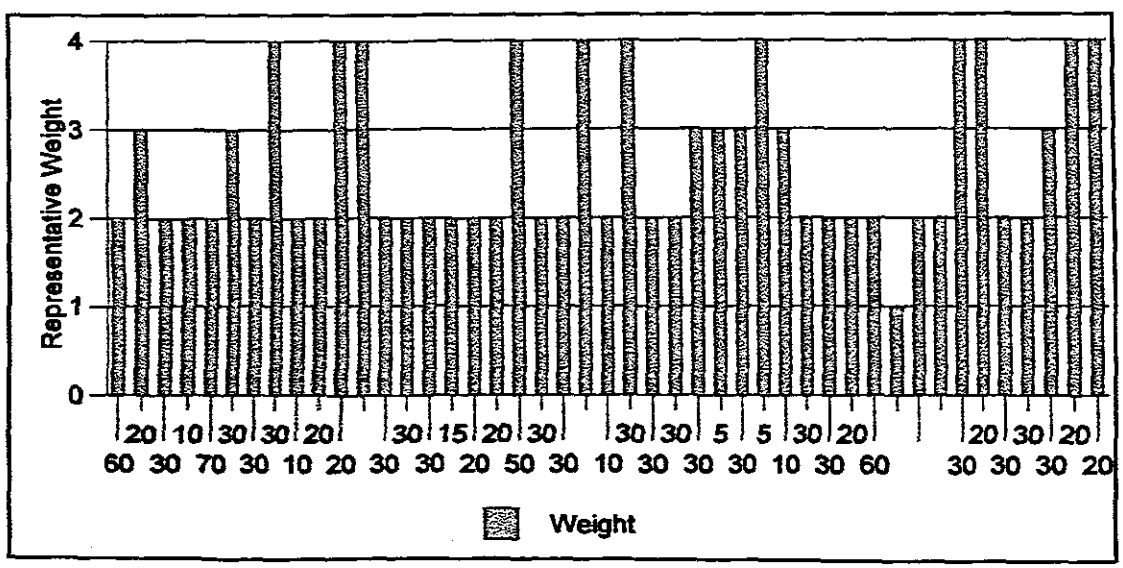


Figure 53 : Type of cancer plotted against weight of patients

Weight: 1=Thin; 2=Normal; 3=Fat; 4=Obese

Cancer type: 10=Invasive; 20=Invasive ductal; 30=Infiltrating ductal; 50=Squamous; 60=Medullary; 70= DCIS

Table 46: Distribution of selected characteristics by race among women participating in this study

Characteristic (n=92)	White 4%	4(n)	Black 67%	62(n)	Coloured 6%	6(n)	Indian 22%	20(n)
Body Mass index†								
Low normal	4	4	-	-	1	1	9	2
High normal	-	-	16	10	4	4	32	7
High	-	-	51	52	1	1	45	10
Unknown	-	-	-	-	-	-	-	-
		Per cent		Per cent		Per cent		Per cent
Tumour stage								
T1 12%	2		2		4		4	
T2 13%	2		4		1		5	
T3 21%	-		15		1		5	
T4 41%	-		36		-		5	
Positive nodes, No. (77%)	1		3		4		14	
0 22%	3		46		2		5	
1-3 60%	-		17		-		-	
4-9 17%	-		1		-		-	
>10	-		-		-		-	
Unknown Nil%								
Pathological grade								
Well differentiated 26%	2		10		2		12	
Moderately differentiated 44%	2		30		4		8	
Poorly differentiated 19%	5		12		-		2	
Unknown 13%	-		-		-		-	

The anti-p53 and c-erb B-2 values in serum in different lesion types is shown in Table 47. For purposes of statistical analysis, the benign and DCIS groups were pooled and compared with the invasive breast carcinoma group.

Table 47: Analysis of anti-p53 and c-erb B-2 in different breast lesion types

Breast lesion type	number	anti-p53	c-erb B-2 (Mean)
Benign	10	negative	420
DCIS	2	positive	431
Benign and DCIS	12	ratio 10:2	430
Invasive	14	3/10 positive	631

In this study the difference between the invasive breast carcinomas versus DCIS is significant ($p < 0.0001$). There was no significant change in serum titre p53 auto-antibodies in patients with *in situ* disease in whom the tumour had been surgically removed at least 6 months prior, according to Mudenda *et al.*, (1994). Patients with high titres and high OD absorbency at the beginning of the study continued to exhibit high levels in subsequent sera, reported Mudenda. This collaborated with the finding of Schlichtholz *et al.*, (1994).

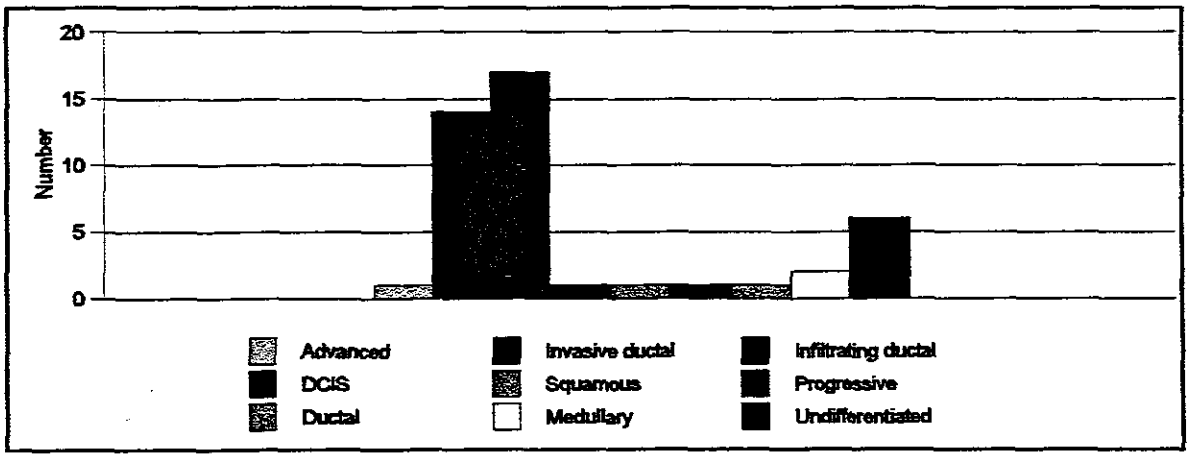


Figure 54: Cancer types found in this study

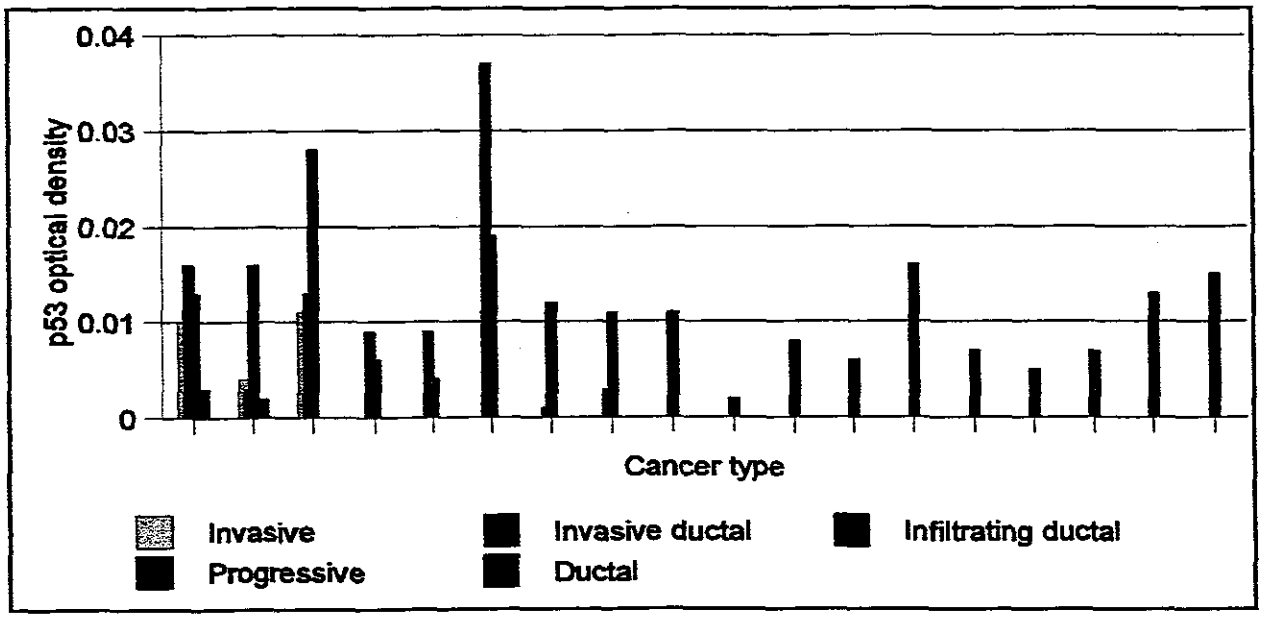


Figure 55: Type of cancer and p53 autoantibody levels
(1xSquamous with anti-p53 of OD 114; 1xDCIS with anti-p53 of OD 250 excluded)

- **Anti-p53 and c-erb B-2 content and axillary lymph node status**

In this series approximately 98% of patients underwent axillary lymph node dissection or sampling. Performance of these procedures is at the discretion of the surgeon and is performed mainly for the purpose of making decisions regarding adjuvant therapy. Table 48 compares the anti-p53 and c-erb B-2 values with pathologically negative, versus positive, axillary lymph nodes. None of these values are significantly different in lymph node negative versus lymph node positive carcinoma.

Table 48: Relationship of anti-p53 and c-erb B-2 with axillary lymph node status in invasive breast carcinoma

Parameter	Lymph node negative	Lymph node positive	Comparison
positive anti-p53 (n=10)	n=4	n=6	p=0.6
c-erb B-2 (n=45)	ns	ns	p=0.3
ns: not significant; n=number			

- **Anti-p53 and c-erb B-2 content and primary tumour size (T)**

Of the 92 breast carcinomas 79/92 (86%) had measurements of the primary tumour size recorded in the pathology reports. The reasons for lack of information on pathologic T-size included the following:

1) The primary tumour was excised at an institution other than King Edward VIII, RK Khan or Addington hospitals.
2) Only an incisional biopsy of a large tumour mass was performed.

Statistical analysis only included these cases in which the equivocal pathologic measurements of T-size were available. T was divided into 3 ranges 0-1.0 cm, 1.0 - 2.0 cm, and > 2.0 cm. Significant difference in the presence of positive anti-p53 were noted (Table 49).

Table 49 : Relationship of anti-p53 and c-erb B-2 with primary tumour size (T)

Parameter	T size range (cm)		
	0.0 - 1.0	1.0 - 2.0	2.0 - up
anti-p53	2/10 (20%)	2/10 (20%)	6/10 (60%)
c-erb B-2	461-568 HNU/ml	425-632 HNU/ml	560-632 HNU/ml

20 patients (n=45) had advanced cases with T4 primary tumours

- **anti-p53 and c-erb B-2 as a function of tumour grade in breast cancer**

Data on the architectural grade of the tumour was a criteria for selection into this study as a breast carcinoma patient. Patients with grade III were under-represented in this study (17% of the series). Table 50 shows the trend in this regard.

Grade	anti-p53 positivity	c-erb B-2 (Mean)
I : 24%	2%	570 HNU/ml
II : 37%	7%	580 HNU/ml
III : 17%	2%	620 HNU/ml
Not available : 13%	-	-

Table 50 : anti-p53 and c-erb B-2 content as a function of tumour grade in breast cancer

p53 antibodies were detectable in 7% of Grade II tumours. No significant relation was observed between the presence of anti-p53 antibodies and tumour size.

- **Correlations among the two markers and clinicopathological indices in breast cancer patients.**

There were no significant positive correlations of serum concentrations between p53 auto-antibody and c-erb B-2. c-erb B-2 does not show clear relationship with the grade of differentiation of the tumours.

Table 51 shows the median and maximum serum concentrations of the 2 individual cancer markers for breast cancer patients for the different stages of disease.

Table 51 : Median and maximum levels of anti-p53 and c-erb B-2 for patients (n=45) with breast carcinomas, for various stages of disease, according to TNM classification.

TNM Stage	Number of patients (%)	Numer positive serum anti-p53	Number elevated serum c-erb B-2 HNU/ml
I	9	Nil	Nil
II	13	Nil	Nil
III	21	3	Nil
IV	41	4	Nil
Unknown	15	3	
Total	100%		

To improve the clarity of the tables, minimum levels of marker concentration are not displayed, nor are the means, due to the skew distribution of the two markers. The minima approximate,

the lowest detectable concentration for all disease stages, locations and grades of differentiation. No increasing trend could be observed in the minima. 31 patients had c-erb B-2 serum levels below the cut-off of 553 HNU/ml but 14 had levels in the 600 to 630 HNU/ml range (30,000-31,550 corrected values). These patients had evidence of distant metastases.

5.7.2 Anti-p53 clinicopathologic correlation of tumour data

Serum antibodies to p53 were detected in 11% (n 10/92) of the sera of breast cancer patients, compared with none (0/46) of the control patients (p=0.001). Of the 92 tumours graded according to the TNM classification, a significant positive correlation was found between seropositivity and poor tumour differentiation (p<0.0012). In the advanced cases with anti-p53 antibodies present in their sera, 4 (n=10) patients had evidence of metastasis. The relationship between positive p53 autoantibody status and the clinicopathological parameters of breast cancer is shown in Table 52..

Table 52 : Association between presence of anti-p53 antibody and clinicopathological features of tumours

	p53 antibody (n=92)	
	Negative	**Positive (n=10)**
Lymph node		
< 0	22%	0
1-3	53%	7
> 3	14%	3
Size (diameter cm)		
< 2	39	Not
2-5	11	known
> 5	-	
Histoprognostic grading		
I	24%	2%
II	37%	7%
III	17%	2%
Not available	13%	

In 39 patients the tumours were smaller than 2cm or more; 11 patients 2-5cm. In the remainder (42 patients), no tumour measurements were available. No obvious association could be made between p53 host antibody status and either size of tumour (p=0.42), or axillary lymph node

metastases (p=0.435). 22% of patients were free of axillary lymph node metastases. All the tumours were characterized and typed according to the WHO (1981) and Bloom *et al* (1957) and 9% of patients were classified as having ductal carcinoma of no specific type, 2% as having lobular carcinoma and 5% as having ductal carcinoma *in situ* (DCIS), 11% had invasive ductal cancer and one patient had Paget's disease.

Table 53: Anti-p53 seropositivity rate and and characteristics of tumours (stage at presentation)

	Patient (n=92) characteristics		Anti-p53			
			Positive		Negative	
Stage at presentation	Number	Percentage	Number	Percentage	Number	Percentage
I	11	12	2	2	9	10
II	12	13	0	0	12	13
III	19	21	4	4	15	16
IV	38	41	4	4	34	37

Some of the subsets of metastatic and locally advanced breast cancer patients (Table 53) showed a high prevalence of seropositivity (2/10 with bone metastases, 2/10 with liver and 6/10 locally advanced disease) and of a total of 54 advanced breast cancer patients, 8 (15%) were found to be seropositive. There was no significant association with tumour stage and p53 autoantibody positivity.

Table 54: Correlation between p53 host antibody and histological grade of breast cancer

Grade (n=92)	Negative (n=82)	Positive (n=10)	Total
I	24%	2%	26%
II	37%	7%	44%
III	17%	2%	19%
Not available	13%	Nil	13%

The patients investigated in the study were not a representative sample of breast cancer patients since there was late stage at presentation among the indigenous African group. Grade 11 tumours comprised the greater histological grade [44%] of tumours in the breast cancer patients [Table 54].

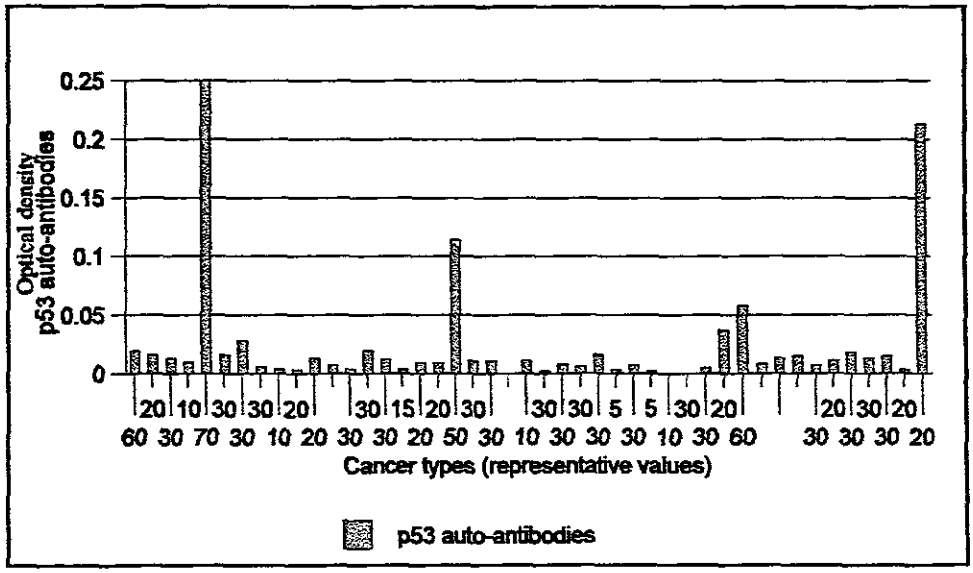


Figure 56: Breast cancer tumours and OD of p53 auto-antibodies

Cancer Type representative values: 10= Invasive; 20= Invasive ductal;
30= Infiltrating ductal; 50= Squamous; 60= Medullary; 70= DCIS

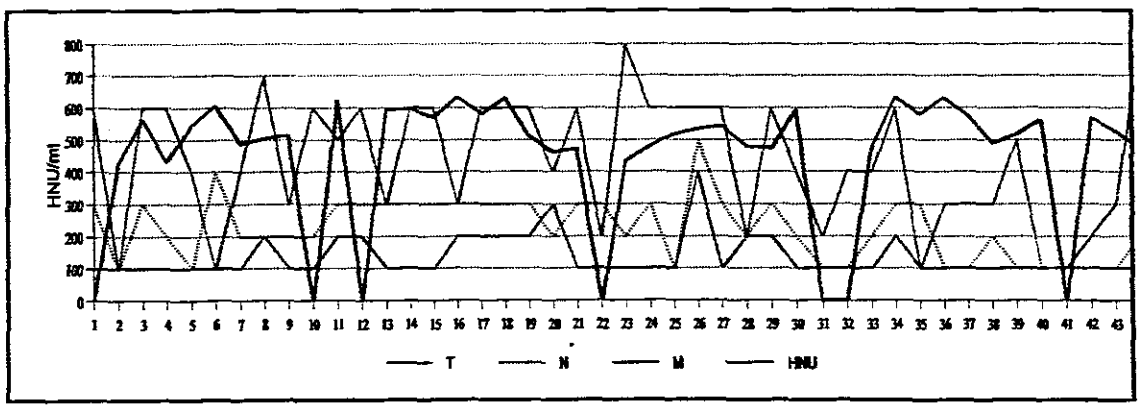


Figure 57: TNM and erb B-2/HNU

Representative Values (HNU - as stated)

T : 1= Nil/unknown; 2= T2; 3= T2; 4=T3; 5= T4; 6= 4b; 8=p3

N: 1=Nil/unknown; 2= N1; 3= N3; 4= N4

M: 1=Nil/unknown; 2= M2; 3= pT2; 4= p11

Table 55: c-erb B-2 HNU/ml using the first standard and which reflects the metastatic / recucurrent group to have slightly elevated values.

HNU/ml	Number	First standard 553 HNU/ml	
Benign breast disease (mean)	6	<400	1600
Primary breast disease	22	460	606
Recurrent breast disease	14	489	631

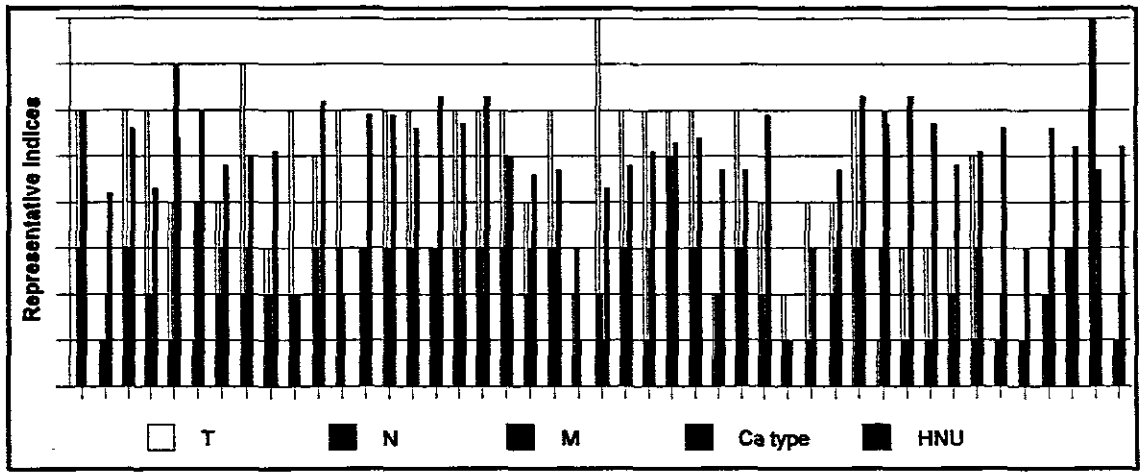


Figure 58 : TNM classification, the type of breast cancer and c-erb B-2 results

Representative indices:-
T: 10=Nil/unknown; 20=T1; 30=T2; 40=T3; 50=T4; 60= T4b; 70=Tp2; 80=Tp3
N: 10=Nil/unknown; 20=N1; 30=N2; 40= N3
M:10=Nil/unknown; 20=M1; 30=M2; 40=p11; 50=pT2
Cancer type: 10=Invasive; 20=Invasive ductal; 30=Infiltrating ductal; 40=Progressive; 50=Squamous 60=Advanced; 70=DCIS
HNU: as per indices x 10

The distribution of serum c-erb B-2 levels in healthy control subjects were stratified for age (10 year intervals). The mean level (and range) was 450-1800 HNU/ml in all healthy women (n=36). The mean levels were 1.048 ± 240 and 1.270 ± 204.

5.7.3 Post-operative serum c-erb B-2 results and clinicopathologic correlation of tumours

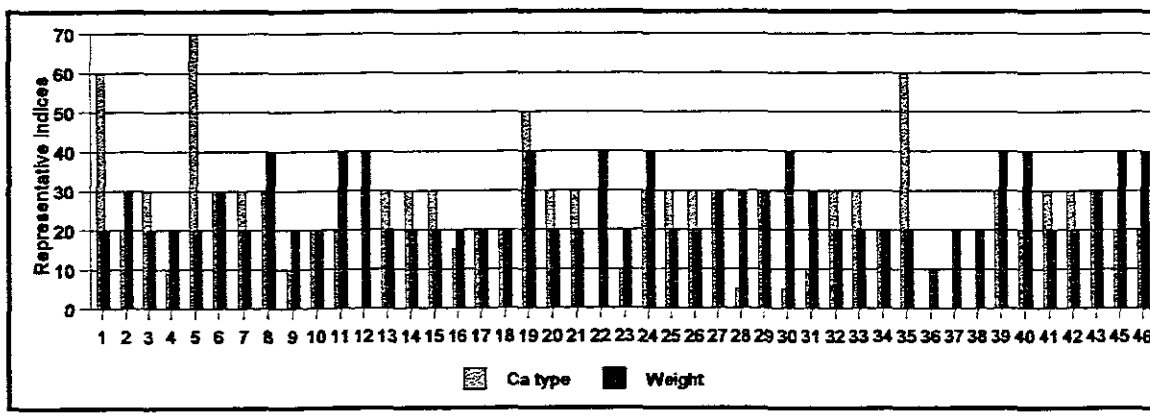


Figure 59: Comparison between weight and cancer type

Representative values
Cancer type: 10= Invasive; 20=Invasive ductal; 30=Infiltrating; 40=Progressive; 50=Squamous; 60=Advanced; 70=DCIS
Weight: 10=Thin; 20=Normal; 30=Fat; 40=Obese; 50=Exceptionally obese

5.7.3 Post-operative serum c-erb B-2 results and clinicopathologic correlation of tumours

Table 56: c-erb B-2 ELISA results correlated with TNM from breast cancer patients

Sample No	Age	T	N	M	c-erb B-2/ HNU/ml
1	64	4b	2	1	580
2	60	T2	1	0	425
3	29	4b	2	0	564
4	39	4b	1	0	431
5	41	3	0	0	544
6	41	0	3	0	606
7	42	3	1	0	488
8	44	4b	1	1	506
9	46	3b	1	0	515
10	46	4b	1	0	540
11	46	4	2	1	625
12	48	4b	2	1	530
13	50	2	2	0	594
14	50	4b	2	x	595
15	50	4b	2	x	569
16	52	2	2	1	632
17	54	4b	2	1	579
18	56	4b	2	1	631
19	56	4b	2	1	509
20	58	3	1	0pT2	460
21	60	4b	2	0	474
22	61	1	2	0	510
23	62	3p	1	0	432
24	62	4b	2	0	481

Sample No	Age	T	N	M	c-erb B-2/ HNU/ml
25	65	4b	0	0	518
26	67	4	M8.5	P11	533
27	70	4b	2	0	541
28	73	1	1	1	479
29	76	4b	2	1	472
30	85	3	1	0	593
31	47	1	0	0	error*
32	49	3	0	0	error*
33	54	3	1	0	479
34	63	T4b	2	1	632
35	38	x	2	0	577
36	42	2a	0	0	631
37	44	2FNA	0FNA	0FNA	574
38	47	2	1	0	489
39	48	4	0	0	517
40	50	4	1	1	561
41	50	3	1	1	580
42	50	1	1	1	568
43	52	2	1	1	525
44	59	p3	2	1	471
45	76		1	1	529

There was no correlation between either weight elevated or any specific site of relapse in the levels of post-operative c-erb B-2

Figure 60 : Comparison between Weight and Ca Type

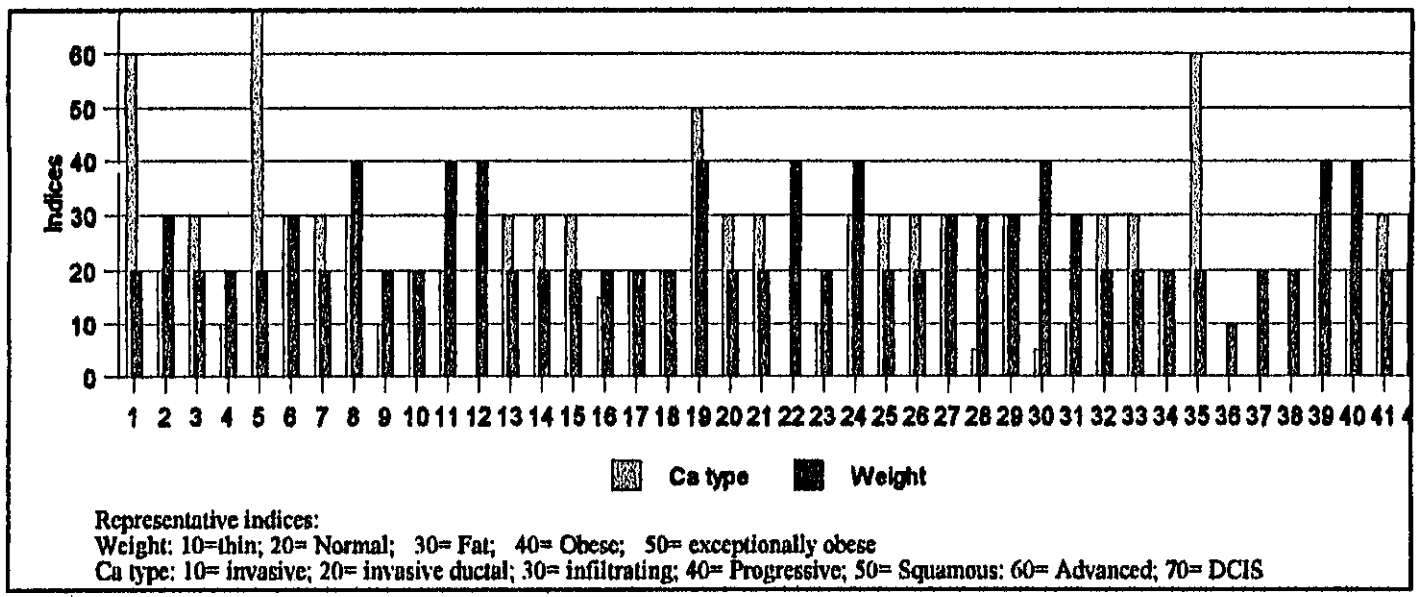
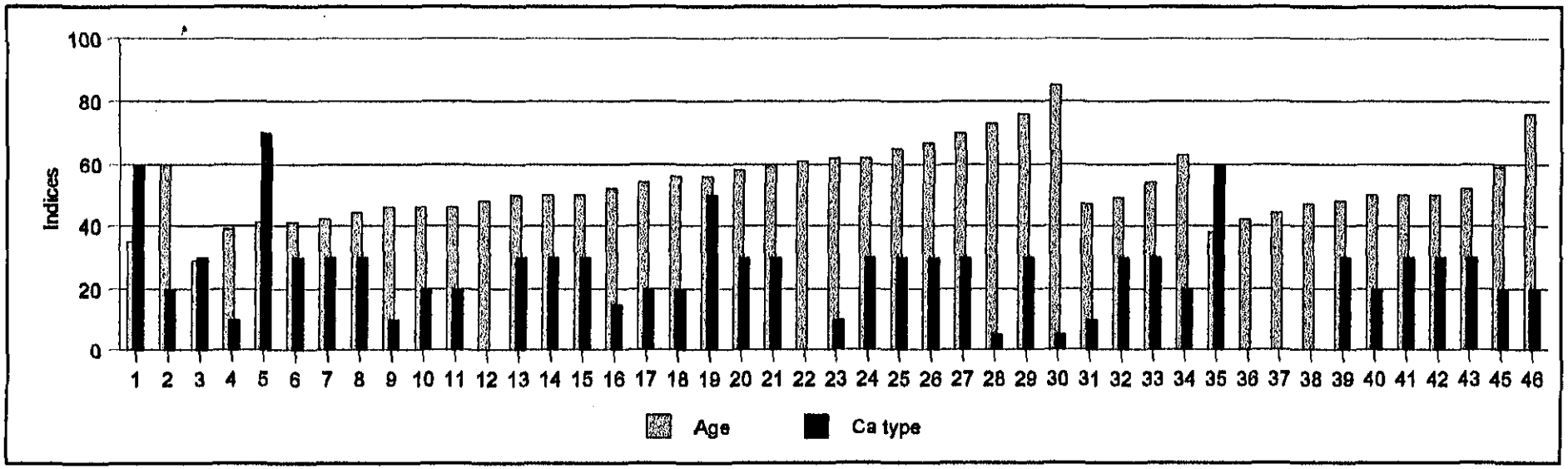
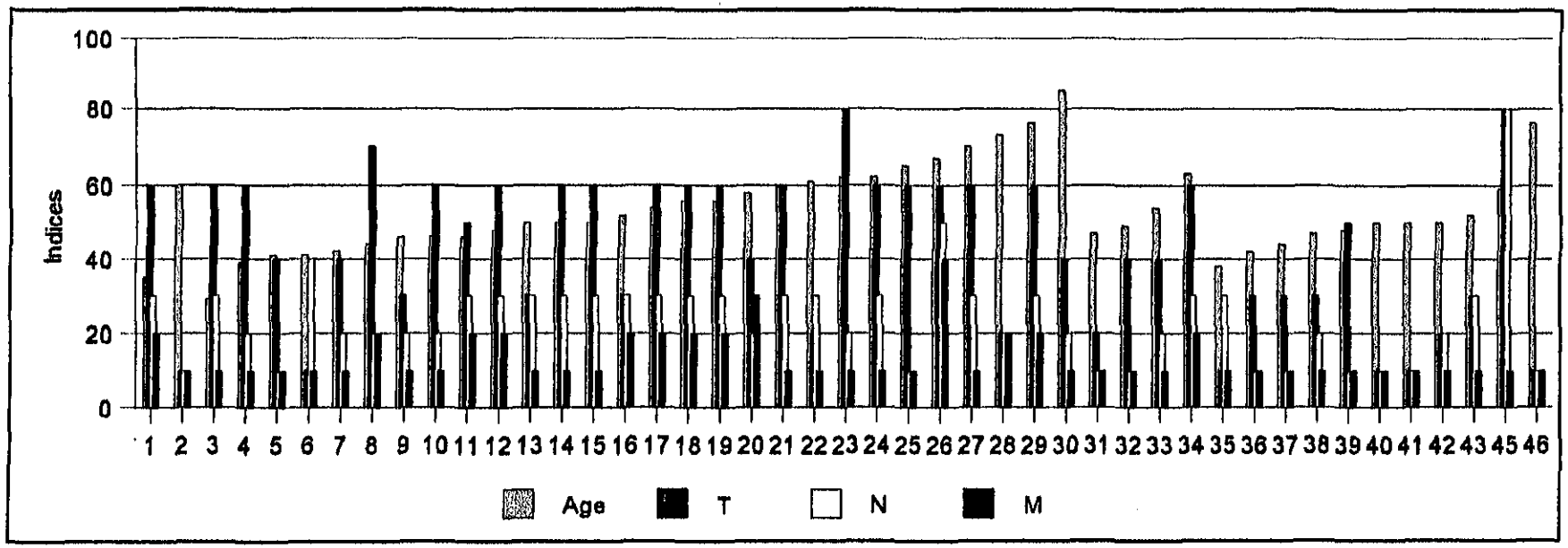


Figure 61 : Comparison between age and cancer type



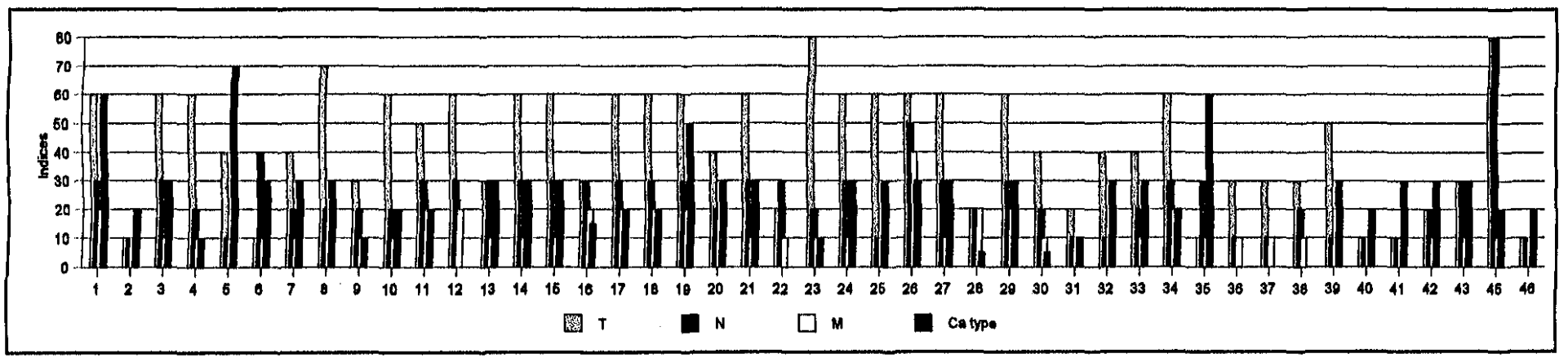
Representative indices
Age: as per indices value
Ca-type: 10=Invasive; 20=Invasive ductal; 30=Infiltrating; 40=Progressive; 50=Squamous; 60=Advanced; 70=DCIS

Figure 62 : Comparison between TNM and AGE



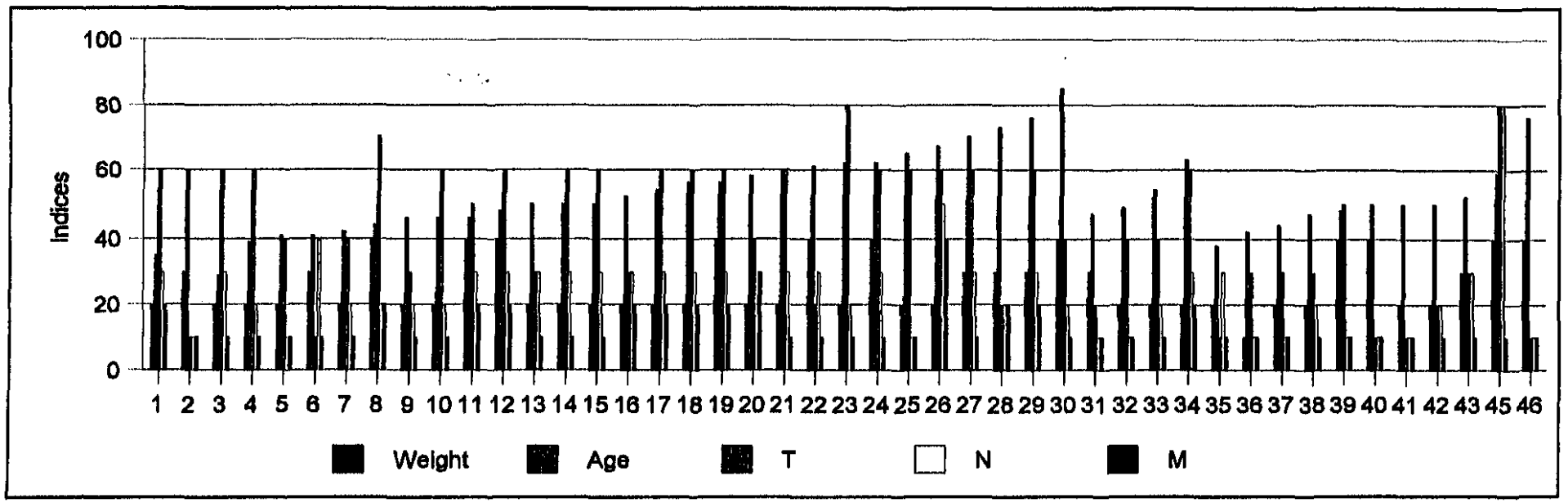
Representative indices:
Age: equivalent to indices
T: 10=Nil/unknown; 20= T1; 30= T2; 40= T3; 50= T4; 60= T4b; 70=Tp2; 80=Tp3
N: 10=Nil/unknown; 20=N1; 30= N2; 40= N3
M:10=Nil/unknown; 20=M1; 30= M2; 40= p11; 50= pT2

Figure 63: Comparison between cancer type and TNM



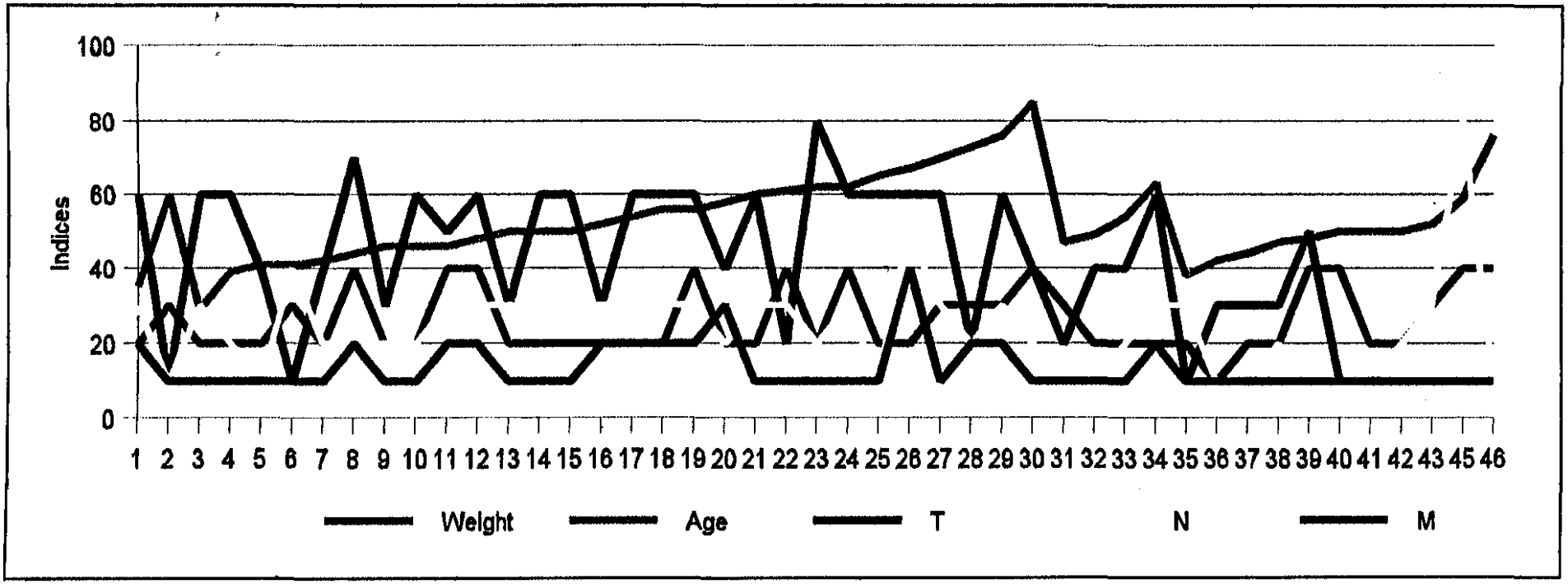
Representative indices:
Ca-type: 10=Invasive; 20=Invasive ductal; 30=Infiltrating ductal; 40=Progressive; 50=Squamous; 60=Advanced; 70=DCIS
T: 10=Nil/unknown; 20= T1; 30= T2; 40= T3; 50= T4; 60= T4b; 70=Tp2; 80=Tp3
N: 10=Nil/unknown; 20=N1; 30= N2; 40= N3
M:10=Nil/unknown; 20=M1; 30= M2; 40= p11; 50= pT2

Figure 64 : Comparison between WEIGHT, AGE and TNM



Representative indices:
Age: equivalent to indices
Weight: 10= Thin; 20= Normal; 30= Fat; 40= Obese; 50= Exceptionally obese
T: 10=Nil/unknown; 20= T1; 30= T2; 40= T3; 50= T4; 60= T4b; 70=Tp2; 80=Tp3
N: 10=Nil/unknown; 20=N1; 30= N2; 40= N3
M:10=Nil/unknown; 20=M1; 30= M2; 40= p11; 50= pT2

Figure 65: Comparison between WEIGHT, AGE and TNM



Representative indices:
Age: equivalent to indices
Weight: 10= Thin; 20= Normal; 30= Fat; 40= Obese; 50= Exceptionally obese
T: 10=Nil/unknown; 20= T1; 30= T2; 40= T3; 50= T4; 60= T4b; 70=Tp2; 80=Tp3
N: 10=Nil/unknown; 20=N1; 30= N2; 40= N3
M:10=Nil/unknown; 20=M1; 30= M2; 40= p11; 50= pT2

5.7.4 Non-invasive and minimal invasive breast cancer

Previous studies have thoroughly described and correlated prognostic factors in invasive breast cancer (Elston *et al.*, 1991; Lipponen *et al.*, 1992; Silverstein® *et al.*, 1995; Silverstein® *et al.*, 1995; Wold *et al.*, 1995. Due to the previous rarity of intraductal breast carcinomas (DCIS), there exist few reports on prognostic parameters (Table 57).

Table 57: Association of HER-2/*neu* expression with clinicopathological features known to have prognostic significance in human breast cancer. (Leong *et al.*, 1995)

Characteristics	"Early" IDC[a] + DCIS[b]	"Late" pure IDC
Number of patients	221	408
Age	p = (young)	not significant
Menopause	p = 0.02 (pre)	not significant
Tumour size	not significant	not significant
Estrogen receptors (ER)	p = 0.002 (neg)	p = 0.01 (neg)
Progesterone receptors (PR)	p = 0.003 (neg)	not significant
Nuclear grade	p = 0.005 (high)	not significant
Histologic grade	not significant	not significant
Ploidy	not significant	not significant
S phase	not significant	not significant

[a] Infiltrating ductal carcinoma

[b] Ductal carcinoma *in situ*

DCIS is not a single entity, but rather a spectrum of diseases. The spectrum concept is based on clinical evidence as well as on molecular and cytogenetic findings. p53 protein accumulation is also observed in DCIS (Walker *et al.*, 1991; Thor *et al.*, 1992). The results for DCIS in this study are in accordance with the literature. Several investigators have reported a proportion of p53 positive DCIS, ranging between 13-25%, using IHC on paraffin embedded sections (Davidoff *et al.*, 1992; Poller *et al.*, 1992; Rudas *et al.*, 1997). Concordant expression is often seen between the *in situ* and invasive components and between the primary tumour and metastasis (Leong *et al.*, 1995).

5.7.5 The literature indicates that size, nuclear grade, and HER-2/*neu* immuno-staining were shown to be statistically different; differences for p53 were not significant.

In the study of Silverstein *et al.*, (1995) immunostaining for HER-2/*neu* and p53 was performed on 4µm sections with the R-60 anti-HER2/*neu* antibody or DO-1 anti-p53 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) by an avidin biotin complex procedure scoring for HER-2/*neu* overexpression was either negative or positive immunostaining. Scoring for p53 was negative or positive based on nuclear staining of more than 50% of the tumour cells in the section. Patient and tumour characteristics for the groups are shown in Table 58.

Table 58: Tumour characteristics, recurrences, and breast cancer deaths by Van Nuys classification (Silverstein *et al.*, 1995)

	Group		
	1	2	3
Number of patients	139	157	129
Average size (mm)	20	24	37
Average nuclear grade	1.60	1.90	3.00
HER-2/*neu*positive (n = 144)	4/14 (10%)	15/53 (28%)	22/50 (44%)
p53 positive (n = 147)	3/42 (7%)	7/57 (12%)	8/48 (17%)
Recurrences (n = 38)	3/139 (2%)	10/157 (6%)	20/129 (16%)
Breast cancer deaths	0	1	1

Summary of a literature review regarding prognostically important clinicopathological features and c-erb B-2 are listed in Table 59.

Table 59 : Summary of literature review regarding prognostically important clinicopathological features that are significantly associated with amplification and / or expression of HER-2/*neu* human breast cancers. (Allred *et al.*, 1991)

Summary of literature review		
Association with negative estrogen receptors		
Reference	**Number of patients**	**Significance**
Slamon *et al.*, (1987)	189	0.05c
Berger *et al.*, (1988)	51	0.01c
Wright *et al.*, (1989)	18	0.03d
Tandon *et al.*, (1989)	728	0.02d
Zeillinger *et al.*, (1989)	291	0.02c
de Potter *et al.*, (1989a)	67	0.01d
Adnane *et al.*, (1989)	219	0.003c
Garcia *et al.*, (1989)	125	0.04c
Marx *et al.*, (1990)	163	0.001d
Kommoss *et al.*, (1990)	50	0.005d
Borg *et al.*, (1990)	300	0.001c,d
Allred *et al.*, (1991a)	736	0.002d
Association with negative progesterone receptors		
Reference	**Number of patients**	**Significance**
Tandon *et al.*, (1989)	728	0.0003d
Zeillinger *et al.*, (1989)	291	0.01c
Adnane *et al.*, (1989)	219	0.004c
Garcia *et al.*, (1989)	125	0.04c
Marx *et al.*, (1990)	163	0.05d
Kommoss *et al.*, (1990)	50	0.01d
Borg *et al.*, (1990)	300	0.001c,d
Allred *et al.*, (1991a)	736	0.003d
Association with poor histological grade		
Reference	**Number of patients**	**Significance**
Wright *et al.*, (1989)	198	0.04d
Walker *et al.*, (1989)	85	0.02d
Garcia *et al.*, (1989)	125	0.005c
Marx *et al.*, (1990)	163	0.05d
Tsuda *et al.*, (1990)	176	0.001c
Association with poor nuclear grade		
Reference	**Number of patients**	**Significance**
Berger *et al.*, (1988)	51	0.0002c
Barnes *et al.*, (1988)	195	0.04d
Tsuda *et al.*, (1990)	176	0.001c
Allred *et al.*, (1991a)	736	0.005d
Association with positive axillary nodes		
Reference	**Number of patients**	**Significance**
Cline *et al.*, (1987)	53	0.05c
Berger *et al.*, (1988)	51	0.02d
Tavassoli *et al.*, (1989)	52	0.03c
Tandon *et al.*, (1989)	728	0.04d
Borg *et al.*, (1990)	300	0.04c,d

References are listed by first author in chronological order.
Level of significance is indicated by *p*-value rounded to one significant figure.
c : Significance relative to HER-2/*neu* amplification.
d : Significance relative to HER-2/*neu* expression.

5.7.6 Racial difference in breast cancer

The results of this study indicated that levels of anti-p53 was elevated in 6.4% (4/62) of specimens from indigenous women, nil% (0/4) from White women , 21% (4/19) from Indian women and 16% (1/6) from Coloured women. The difference in the surface ratios in the 4 groups was also statistically significant (p0.002-0.004) with a higher OD of anti-p53 in Indian women. Unfortunately, due to the small number of women positive for anti-p53 in this study, relative frequencies among the 4 population groups is not statistically viable. Skewed distributions were also obtained within the populations as the Black and Indian women comprised the larger groups.

The distribution of selected characteristics by race among women participating in the study is given in Table 60. 97% White women and 93% Black women had their pathology specimens centrally reviewed. ER and PR receptor status was abstracted from the medical record and categorized as either negative/borderline (<10fmol/mg) or positive (>10 fmol/mg). Due to economic difficulties in KwaZulu-Natal receptor status was not requested on enough patients and therefore results are not statistically relevant.

Table 60: Racial differences in breast cancer : A multivariable model indicating different pre-analytical variables.

Factors	White	Black	Coloured	Indian
- Body weight (obesity)	17%	84%	16%	22%
- tumour characteristics (stage)	II (mean)	III (mean)	II (mean)	II (mean)
- socio-economic standing	good	poor	moderate	moderate
- occupation (employment)	yes	no	no	no
- age at time of diagnosis (mean)	44	44	44	41
- age at first pregnancy (mean)	21	15	17	17
- number of children	2	5	3	3
- access to mammography	83%	11%	32%	34%
- number of mammograms	2	0	0	0
- usual source of health care	Provincial/ private	Traditional Healer/ Provincial	Provincial/ private	Provincial/ private

5.8 Summary of Chapter Five

Inferences drawn from these data are limited by the data quality and by the lack of crucial information, particularly regarding survival of the patients investigated in this study. 7 patients who were positive for p53 autoantibodies had died within a year of blood collection. The remaining 3 could not be contacted and no evidence of their whereabouts or survival could be established. If overexpression of p53 is a common feature in higher grade tumours (Jerry *et al.*, 1993) it does appear to be an indicator of poor prognosis as indicated by van der Berg *et al.*, (1995); Sjögren[®] *et al.*, 1996; Sjögren[®] *et al.*, 1996). The data collected as pre-analytical variables was also limited by the absence or misinformation of data describing personal issues such as number of pregnancies/abortions and financial constraints which prohibited ER status being evaluated for most patients who attended King Edward VIII hospita

CHAPTER SIX

DISCUSSION

6.1 General introduction

The scenarios under which new cancer markers are introduced in the literature show much discordance. Similar cancer markers are used to investigate many different changes. Since these changes may be very different it is difficult to compare these results. The lack of interchangeability of results of different research measurements, of what is presumed to be the same analyte, has several practical disadvantages. Most obviously and potentially serious is the danger of misinterpretation as there is no established common quantitative unit and range of expression established for normal and benign tumours. The restriction in using research data, as recorded in the literature, where results are often expressed in arbitrary units without referral to the expression level for malignant and benign tumours, further complicates interpretation.

Findings of this study indicate that the presence of 11% positivity for anti-p53 are a complementary procedure for assessing the functional state of the p53 gene and seems to indicate a sub-group with poor prognosis. No elevated results were obtained for c-erbB-2 in the post-surgical serum specimens. No correlation with clinicopathological indices was noted.

6.2 General observations pertaining to study

Several observations were made during this study.

- **Mammography and self-breast examination aim at detecting "early" breast cancer, yet there are a number of inconsistencies between clinical observation and the contemporary model of the disease.**

It was noted in this study and from the literature (Baum, 1996) that recurrences often occurred at almost defined post-operative intervals. Relapse was common within the first 3 years after surgery (Beitz, 1993; Badwe, 1994), escalating again 7-8 years after surgery. Some women seem to have extremely long latent intervals (15 years) which might equate with cure, whilst others suddenly

experience a local and distant recurrences. The distribution of metastasis was often bizarre with lung secondaries being rare and skeletal and liver secondaries being common. It was anticipated that risk of relapse following local therapy would be time dependent, based on mathematical prediction of the growth rate of microscopic clusters of cancer cells, but this was simply not true.

- **Communication across a language and cultural gap has the most unfortunate potential for disastrous consequences in not successfully conveying information. This was a problem for gathering data on the clinical background of the patient.**

This problem presented itself more especially when health professionals are educated in a language which is not common to an appreciable proportion of the population. There was not only a language barrier but also a great cultural gap experienced by the researcher, which at times took on gulf-like dimensions. It was generally assumed at the commencement of the study that a nurse would/could act as an interpreter. This assumption was found to be incorrect or only partially correct at the Breast Clinic at King Edward VIII Hospital. Firstly, the researcher was never sure if information was being interpreted or translated accurately. This was especially evident when lengthy exchanges occurred between the nurse and the patient and the researcher was rewarded with a reductive summary.

Secondly, the sheer workload at the Breast Clinic did not facilitate good communication and nurses were not always available to interpret. Lastly, harsh attitudes of some nurses towards patients, particularly when venepuncture was a problem, or the patients were difficult, proved a further obstacle. The lack of the researcher's skills in communicating across cultural divides leads to the conclusion that this clinical research was hindered by language differences and differences in traditional background.

- **Selection of patients for this study were complicated by the fact that many of the patients failed to return to hospital to complete their treatment.**

This applied to Black women interviewed for this study and does not necessarily apply to all other Black breast cancer patients at King Edward VIII Hospital. The majority of Black patients generally lack the resources to continue medical treatment and did not return. Indian and Coloured women who failed to return for therapy usually opted for treatment in the private sector. Whilst several factors contribute to the high incidence of indigenous African breast cancer patients not returning

for treatment, Wright's study (1997) indicates that this is or may have been because many indigenous African breast cancer patients have a preference for alternative (indigenous African) healing options. The use of traditional medicines is widespread, mainly in rural areas because of the lack of modern health care facilities, but the present study was undertaken in an urban setting where patients elected to undergo non-traditional therapies. This strongly suggests that the use of herbal medicines identifies women who may forego available conventional therapy in preference to traditional therapies.

Wright's findings did not explain the difference in rates of non-representation for treatment for indigenous African cervical (30%) and breast cancer (80%) patients. Although the two patient groups shared social, economic and cultural backgrounds, their commitment to further supportive treatment programmes were remarkably different. A major research problem occurred in trying to locate non-returning patients in order to ascertain if any of the patients had died and if so from what cause. The fluid nature of many indigenous African women's lives regarding their place of residence made telephonic contact impractical, coupled to the problem of reluctance and/or inability to correctly reveal a patient's condition or possible cause of death. This made completion of patient's records extremely difficult.

- **Cancer of the breast appears to have overtaken cervical cancer and is now the most common cancer in South African women (16½ %), White (nearly 18%); Asian (24%); Coloured (18%) and Black (13%).**

Although White women are diagnosed more frequently with breast cancer than indigenous African women, mortality rates are higher among the latter (Sitas, 1998). While there has been little to no increase in breast cancer mortality among Whites, indigenous women have experienced a 20% increase from 1989 to 1998 (Sitas *et al.*, 1994). On average, Black women are diagnosed in later stages than other population groups and results indicate that they have less improvement over time which indicates a poor prognosis for these patients. Over two thirds of the patients have clinical lesions at stages III and IV at diagnosis. Attempts to learn more of the reasons for widely disseminated disease at presentation need to be investigated as there was only a low incidence of participants who utilized mammography (Table 8 and Figure 14). Education of rural women in breast self-examination is urgently required.

6.3. Specific observations pertaining to study

At the time this study were undertaken there appeared to be no *a priori* reason why an oncoprotein should not be viable as a useful marker. Now it is evident that more knowledge needs to be accrued regarding the timing of release of the oncoproteins into the circulation as a function of stage of malignant progression and the effects of treatment on serum levels. Assay format and antibodies used to date differ, in particular differences exist between assay kits in their ability to detect two different forms of c-erb B-2. Information regarding epitopes targeted was also seldom given in publications. Clinical laboratory utilization of anti-p53 and c-erb B-2 will only be usefully undertaken when there is a high degree of global comparability between assay results emerging from different researchers or when any one system gains the confidence of the medical oncologist. Nevertheless, although these differences limited the extent to which direct comparisons of results with those of previous studies could be made, several similar findings were observed.

Also, it may be important to emphasize that comparatively speaking, the attempt to examine the clinical usefulness of these assays has only been recently initiated, i.e. with the availability of the commercial kits to researchers. This allows fundamental research endeavours to connect these and other biomarkers to the disease of cancer. With respect to p53-Abs, there is only the probability of a given breast cancer patient having p53 antibodies. Generally, for those researchers who believe that the induction of antibodies depends predominantly on the presence of p53 mutations, the number of antibody positive patients is a portion of the incidence of p53 mutations, which is, for example, approximately 22% for breast cancer, 50% for lung cancer [Greenblatt *et al.*, 1995], and 50% for esophageal cancer [Holstein *et al.*, 1997].

However, for reasons that have been discussed in this thesis, the incidence of p53-Ab has been found to be lower than the mutation rate: about 12%- 15% for breast, 20% - 30% for lung, 20% - 25% for esophagus. Given these facts, a general application of assays to detect p53-Ab does not benefit clinically, even with a flawless assay system being made available. This makes the current study even more relevant and important.

- **While ELISA's are relatively common for measuring anti p53 immune response, many researchers used modified variations or different techniques. The majority of ELISAs**

use in-house MAbs with different standards and units making comparison of results difficult.

The literature shows no conformity with regard to MAb's, procedures or units and detection of anti-p53 activity can be performed by various immunological procedures (Diamandis *et al.*, 1993; Angelopolou® *et al.*, 1994). In the historical study cited by Crawford *et al.*, 1982 and prior to the advent of ELISAs, sera were screened in two stages by immunoprecipitation of 32p-labeled cell extracts using fixed staphylococci as immunoadsorbent to collect the immune complexes. In the first stage, extracts of the SV40-transformed cell line SV80 were used. Crawford selected these cells as an acceptable source of radioactive p53 and increased the assay sensitivity to recover serum activity in microliter volumes. Since some of the SV40 large-T antigen is associated with p53, the co-precipitation of large-T with p53 was an indication that the correct protein was being tracked (Crawford *et al.*, 1982). Although the screen using SV80 cell extract is a sensitive test for antibodies reacting with p53 associated with SV40 large-T, false positives were possibly generated by sera which contained anti-SV40 large-T (de Savigny *et al.*, 1996).

Another approach was that of Labrecque *et al.*, (1993) analyzing anti-p53 antibody response in cancer patients, immunoprecipitation analysis was performed to examine conformational epitopes rather than denaturation resistant epitopes. Two different p53 mutants (p53 serine 135 and p53 valine 246) and wild type p53 were synthesized in an *in vitro* transcription/translation coupled rabbit reticulocyte lysate system. Labrecque *et al* (1993) used this system because it synthesized the same levels of mutant and wild type p53 proteins in a nondenatured form. This was important to compare antibody reactivity against equal levels of different target antigens. The mutants employed by Labrecque *et al.*, (1993) fell within 2 of the 4 hot spot regions for missense changes and were representative of mutant p53 conformation. Winter *et al.*, (1993) used antibodies against tumour cell proteins. However, for each sera Winter *et al., (1993)* had continuous cultures of corresponding autologous tumour cell lines which were used to make protein extracts for immunoblot analysis. These cell lines (31-5B-5 and 31-5B-7) were derived by transfection with a cDNA expression constructs containing mutant human p53, into the non-small-cell lung cancer which contained a homozygous deletion of the p53 gene (Winter *et al.*, 1992). This is indicative of the variety of testing procedures.

- **The performance of accurate and precise ELISA's requires standardization of techniques and components together with practical experience in immunochemical**

techniques (Ferencik, 1993; Gion *et al.*, 1993; Goers, 1993).

Intra-laboratory inconsistencies in potential marker assays have prompted controversy regarding their use in routine clinical laboratory testing. The observed inter-laboratory variation in ELISA may be due to a lack of standardization of the methodology since there are numerous variations in the assays performed by different laboratories. Some of these variations may be due to the selection of microtitre plates and the conditions under which the plates are stored. The selection of microtitre plates, amount of antibody coating each well, solvent, blocking agent, dilution of patient sera and choice of the serum diluent all influence the outcome of ELISA. Some kits, such as the c-erb B-2 kit from Oncogene Science, were not "user friendly" and only well trained personnel, who are proficient with ELISA, could satisfactorily perform the assays. At this stage in the biomarker experience it is important to accept that good assays will evolve from good designs, reagents and demonstrated efficiency.

Many technical considerations are important in using biomarkers. Enzymes used in ELISA should have a low relative molecular weight, high stability and enzyme activity, and they should bind covalently to antibodies and to various functional groups of antigens in an active form. The chief disadvantage of enzyme labels is steric hindrance of ligand-binding reagent interactions imposed by the bulkiness of enzymes. Steric hindrance can render enzyme-labelling systems useless or at least insensitive. A variety of enzyme-antigen (or antibody) cross-linking reactions have been successfully utilized (e.g. glutaraldehyde). The major problem is that covalent linkage reactions frequently damage proteins rendering enzymes less active than normal.

In earlier studies, many false-positive reactions were noted (Crawford *et al.*, 1987) which may have been due to poor blocking reagents. Also, impure antigen preparations were often used. Rheumatoid factor, IgM anti-IgG, in a patient's serum creates problems with ligand immunoassays, giving false-positive test results. This can be countered in several ways. Coating the surface with a specific capture antibody (anti-IgM), only IgM molecules attach, the specific antigen is then added with a label (enzyme and others) that only attach to the specific IgM antibodies. A false-positive assay result from rheumatoid factor can also be avoided by using avian antibodies as one of the antibodies in the sandwich technique. This modification has been successful in several applications (Kemeny *et al.*, 1988; Glover, 1996).

Data has shown that short wave (254 nm) UV exposure reduces binding to polystyrene microtitre plates, thereby reducing the amount bound to coated ELISA plates. Thus by using UV exposed microtitre plates, decreased or false-negative ELISA results may be obtained for positive plasma. The percentage of research laboratories using ELISA increased from 25% to 60% over a 4 year period (from 1994 to 1998) (Duffy, 1999). While technology for cancer markers has improved, the precision of ELISA is dependent on a number of parameters including the concentration of the marker being assayed. .

- **Samples for ELISA assay usually require dilution to obviate non-specific interactions due to heterophile or inappropriate antibodies also present in the sera. Dilution of test samples necessary to decrease non-specific background in ELISA assays to acceptable levels of some samples is important, since this may require sample dilution which may result in higher minimum detectable concentration.**

This necessity may result in dilution of p53 protein to a level below detection limited, however, the literature indicates that such heterophile antibodies are a potential problem for anti-p53 assays (Ryder *et al.*, 1996). The importance of such a procedure is investigated in the study of Levesque *et al.*, (1995) where p53 protein levels above the detection limit of 0.04 ng/ml were only found in two sera from lung cancer patients. By pre-treating sera with an anti-p53 antibody linked to solid phase and by the addition of mouse serum to neutralize possible heterophilic antibodies, however, the signals arising from the sera were shown to be non-specific and possibly caused by heterophilic antibodies. Levesque *et al's* (1995) data did not support previous reports of p53 protein in the sera of lung cancer patients. Since immunoassays are subject to numerous sources of interference in serum, including heterophilic antibodies, results of p53 antibody analysis of serum specimens should be interpreted with caution (Diamandis® *et al.*, 1993). Nonspecifically aggregated IgG (which can also be found in poorly stored samples or, occasionally, after heat inactivation of complement) is more likely to stick to solid surfaces than monomeric IgG. This is also a problem with two-site immunoassays where mouse MAb's are used as the reagent. Here the aggregated antibodies can bind non-specifically and produce a signal. Such interference can be reduced by adding excess non-immune mouse immunoglobulin.

Initial ELISA experiments for detection of p53 antibodies in sera undertaken by Lubin and Soussi (manuscript in preparation) showed insufficient dilution and that different human sera leads to variable

background levels (Schlichtholz *et al.*, 1994). Lubin and Soussi (1993) therefore devised a highly specific ELISA by including an internal control. The antibody was tested with the relevant antigen, i.e. p53, and a second preparation, where this antigen was omitted and substituted with a non-p53 antigen. All results were expressed as the ratio between the value of the wells with p53 and corresponding wells without p53 (irrelevant antigen). In the study on sera from blood donors and patients with various carcinomas (Schlichtholz *et al.*,1994), a ratio higher than 2 was taken as the ratio which confirmed the presence of p53 antibodies.

- **There is an urgent need for standardization of reference intervals and ways of expressing results of current anti-p53 and c-erb B-2 assay technologies.**

Assay kits are difficult to standardize because of inherent variability in the MAb's produced by manufacturers. Each research group introduced some variation and since many used "in-house" protocols, it required subjective judgement to select what is apparently the most suitable kit, methodology and/or Mab (Hassapoglidou *et al.*, 1992; Hassapoglidou *et al.*, 1993). This study could not evaluate whether any variations, materially and practically affect assay results, but clearly demonstrated that assays are by no means standardized. Prior to use of these markers in a South African population group the RI needed to be established. In the control group none were positive for p53 auto-antibodies or had elevated levels of c-erb B-2. Interpretation of laboratory test results was more complex than simply comparing ELISA results against the RI and then associating results with patterns indicative of breast disease. Factors which possibly affected interpretation include variation in the concentration of a marker between patients for a given tumour burden and substances which modified their rate of synthesis or secretion by cells.

RI's are of limited use for markers that have low biological variation within an individual. Defining reference change limits or changes in assay values for a particular patient may be an improved and simple way to provide information which defines normal or disease progression. It offers the most reliable approach in retrospective data analysis. In the present study, the RI supplied by manufacturers of test kits, rather frequently (and sometimes inexplicably) did not correspond to results obtained on a local population by a local laboratory. A similar problem was encountered with the literature where RI's for anti-p53 and c-erb B-2 showed discordant results.

- **Considering that cut-off values used in this and other studies are obtained by very**

diverse criteria, results show homogeneity in the quantities studied and globally coincide with results published by other authors.

No improvement was observed in diagnostic accuracy of anti-p53 determined by the modified procedure performed by Diamandis *et al.*, (1994). If RI's are established for a particular patient, decisions regarding criteria for the cut-off value should be taken in close co-operation with surgeons and oncologists, because they may determine diagnostic/therapeutic procedures from laboratory results. If the concentration established on subsequent tests for the particular patient is outside the RI or upper level of normal it should be taken to indicate that a change in tumour size or recurrence has occurred.

- **Sources of p53 antigen, and measurements with the same method, furnished substantially variable measurement values.**

Considerable variations were seen in results obtained from samples subjected to tests with various kits even when using the same method, together with the application of the same method to different analytical instruments. In Peyrat *et al's* study on breast cancer (1995), a highly specific ELISA was devised where all plasma was tested with two antigen preparations, the first contained the relevant p53 antigen and in the second preparation this antigen was omitted. Results were expressed as the ratio between the value of the wells with p53 and the corresponding wells without p53. The researcher wrote to Dr Peyrat in France requesting information, but no response was forthcoming whereas Dr Diamandis in Toronto kindly provided information on p53 and its phenomenal antibody (Angelopoulou *et al.*, 1993; Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1995).

Angelopoulou *et al.*, (1993) devised a competitive and a non-competitive type of immunofluorometry to improve the method for measuring anti-p53 in the sera of cancer patients. For the new methodology, a mouse auto-p53 MAb PAb 240 was produced in a tissue culture supernatant from a cell line of sensitized lymphocytes. Its antibody concentration was approximately 30µg/ml. The rabbit polyclonal anti-p53 antibody CM-1, the goat anti-rabbit and goat anti-human antibodies conjugated to alkaline phosphatase and the goat anti-mouse antibody Fc fragment specific (EAM Ig) were approximately 1 mg/ml. Both methodologies, cited by Angelopoulou *et al.*, (1993), were based on different principles and are among the most sensitive reported in the literature since they are suitable for measuring analytes at atto mole levels.

A comparison of anti-p53 results was possible with the study of Marxsen *et al.*, (1994) who detected the anti-p53 response using a sandwich ELISA purchased from Dianova. To further substantiate results all sera were analyzed by Western blot technique using a cell lysate of Panc Tu-1 cells (p53 mutation at codon 176) as the antigen source. Ryder *et al* (1996) obtained p53 protein by amplifying the p53 gene from a Hep G2 cell line using PCR. The amplified product was cloned in the vecter pQE-8 and expressed in *Escherichia coli*. In the study undertaken by Mudenda *et al.*, (1994), soluble p53 protein with deletion of 132 amino acids from the N-terminals was produced by PCR and cloned in the pDS/RSB bacterial expression plasmid.

This study did not intend to compare the antigens/antibodies, methods and standards used by other researchers to quantitate p53 antibodies but the above citations are examples of the variability in analytical methods to measure what is purported to be the same analyte. Cancer marker tests currently in research use, where results from one laboratory are compared with those of others, must be of such reproducibility that additional variances, introduced by different working detail and physical conducts of each laboratory, are encompassed within the overall acceptable variability of results. Achievement of good precision demands that all reactants in an assay are highly reproducible and stable (within the limits of the stated shelf-life). The CV for these assays (mean 8%) was not acceptable for clinical tests. Previous studies using the same ELISA methodology were compared. When the CV of the scores were considered across all standards used, the average of the CV was unacceptably high (23%).

The ELISA kits used gave no indication as to the frame of reference, i.e., within total batch or total random error, nor to the level of the analyte to which the CV might apply. There is furthermore a very real danger of overlooking the direct connection between the CV and the error SD, i.e. a CV of 8 does not imply a 95% error range (approximately 8), but approximately 16%. The interpretation of the CV is further confused since some assay systems exhibits a non-constant error variance that is proportionally related to the analyte level, e.g. Oncogene Science Lot No. D03904. This particular situation admits a single figure summary of the within-batch and total random error characteristics of the c-erb B-2 assay. The CV is a popular term, but it must be used with caution and with appropriate qualification.

- **No statistically significant differences concerning sensitivity, specificity and accuracy were found between markers in a comparison study undertaken by Watanabe *et al.*, (1994) where serum from breast cancer patients was assayed concurrently for c-erb B-2, CEA and CA 15-3.**

CA 15-3 is currently the most widely used marker for breast cancer and remains the "gold standard" for circulating markers in breast cancer and is the assay against which anti-p53 and c-erb B-2 should be judged. Despite its use, CA 15-3 is a test not approved by the Food and Drug Administration in the USA and is considered as being only for research and experimental use. It was reported in Chapter 2 that while CA 15-3 concentrations are seldom elevated in early breast cancer, increased values are found in metastatic breast cancer. When paired data were tested to evaluate the relationship between c-erb B-2 and CEA and between c-erb B-2 and CA 15-3, sensitivity was significantly increased for CEA from 25% to 38% and from 32% for CA 15-3 alone to 43%.

One must further question the justification for routine use of the markers investigated in this study Breuer *et al.*, 1994; Bergh *et al.*, 1995). Results indicate that they are not warranted for three reasons. Firstly, the low incidence (12%) of positive values for anti-p53 (Vojtěsěk *et al.*, 1995) which, in turn, suggests that information regarding the immune response to mutant p53 needs to be firmly explained. Secondly, the question then arises as to whether serial determination of c-erb B-2 concentration can provide a lead time for the detection of metastatic disease. The literature indicates that serial concentrations have the potential to detect recurrences pre-clinically. This application, with regard to metastatic breast cancer, is of limited value since there is presently an absence of effective therapy for this patient group, and thirdly, c-erb B-2 appears to lack sensitivity in decreased concentrations.

6.4 p53 auto-antibodies

- **This study demonstrated the same prevalence (12%) of serum auto-antibodies to p53 protein as demonstrated by immunoblotting techniques, which have shown prevalence rates between 11-15% (Crawford *et al.*, 1982; Caron de Fromentel *et al.*, 1987; Davidoff *et al.*, 1992; Winter *et al.*, 1992).**

It is unlikely that this test in isolation would be suitable as a primary diagnostic or screening test for breast cancer. A summary of positive sera identified in this study is shown in Tables 32 and 33. Observations (Caron de Fromental *et al.*, 1992; Diamandis 1994) suggest that serological analysis provides an assessment of the functional state of the p53 gene in breast cancer patients and may prove to be a useful adjunct to molecular and IHC methods of tumour characterization which have, until now, concentrated on allele loss, gene mutation and protein expression. ELISA is a convenient and specific test for detection of humoral response to alteration in p53 gene expression..

p53 antibodies in serum were first reported in 9% of breast cancer patients in the historical study undertaken by Crawford *et al.*, in 1982. Davidoff *et al.*, (1992) found p53 auto-antibodies in the sera of 11% patients with breast cancer compared to 0 of 15 (0%) controls. All 7 positive cases had p53 gene mutations and increased p53 protein in their tumours (Davidoff *et al.*, 1992). Elevated serum levels of mutant p53 by ELISA were found in 15 of 82 (18%) of breast cancer patients compared to 0 of 20 (0%) of normal controls (Micelli *et al.*, 1992). Schlichtholz *et al.*, (1993) reported p53 antibodies in 15% of sera from patients with breast cancer whereas Lubin *et al.*, (1993), detected 12 of 93 (13%) of breast cancer patients. Angelopolou *et al.*, (1993) reported p53 antibodies in serum determined by two different methods, in 3% of breast cancer patients.

In a large study of p53 antibodies in serum with various malignancies determined by 2 methods, the highest prevalence of antibodies was found in ovarian and colon cancers (15%), lung cancers (8%) (Lubin *et al.*, 1995) and breast cancers (5%) with lower prevalences in other malignancies (<4%) and controls (>1-2%) (Angelopoulou *et al.*, 1994).

6.4.1 Does the humoral response to mutant p53 precede detection of the tumour?

Evidence suggests that the immune response to p53 is strongly related to the presence of missense mutations (Winter *et al.*, 1992; Greenblatt *et al.*, 1994; Preudhomme *et al.*, 1994) rather than stop, splice/stop, splice or frameshift mutations (Ryder *et al.*, 1996). Missense mutations are clustered in the evolutionary conserved regions, namely, exons 5 and 8, between codons 120 and 290 out of 393 amino acid residues which are usually directed against epitopes in the amino and carboxy terminals and not the conformational determining mid-region. In breast cancer no tumour specific "hot-spots" have been reported (Harris *et al.*, 1993). About 25% of mutations are detected in codons 175, 194, 273, and 280 (Thorlacius *et al.*, 1993).

It is unlikely that the amount of mutant p53 is the only factor determining immune response (Tilkin *et al.*, 1995; Vojtéšek *et al.*, 1993). Ryder *et al.*, (1995) states that the immunogenicity of p53 protein may be enhanced by complexing with oncogene proteins. This has been demonstrated in mice with the SV40 (Simian virus) large T-antigen. An alternate protein p53 immune presentation may also explain results. The role of hsp 70 and the immune response to p53 was been postulated on the basis that anti-p53 is associated with specific mutations in exons 5 and 6 which produce proteins known to associate with hsp 70 and to have a greater transforming potential. Unlike wild type p53, some mutant p53 protein are able to complex with the MW 70,000 heat shock protein (hsp 70). In addition to heat, the genes of this group are activated by other adverse environmental conditions, such as exposure to heavy metals, ethyl alcohol, oxygen deprivation and in higher plants, water loss and wounding.

Hsp's occur in several highly conserved families of polypeptide chain binding proteins or molecular chaperones (Zubay, 1998). The hsp family which complexes with the mutant p53 proteins, has among other functions, the ability to induce other proteins to fold into conformations that facilitate movement through cellular membranes. The most important family in the heat shock response, is hsp90 (MW averaging 90,000). These proteins bind to steroid receptors, protein kinases, actin, tubulin and other proteins important to cellular responses and stabilize them in an inactive state (Wolfe, 1993). Taken together, these observations demonstrate that subtle mutations in the p53 protein can affect the configuration of the entire protein. Ryder *et al's* data (1995) indicated that mutations in exon 5 and 6 are more likely to produce an immunogenic protein than those in exons 7 and 8. High levels of anti-p53 activity in mutations affecting codons 257 (exon 7) and 273 (exon 8) have been found. This is strong evidence against the hypothesis of hsp being the only required complexing protein as both of these mutants are unlikely to bind hsp 70.

- **It is probable that the immune response and factors that contribute to it, do not stay constant for different types of breast cancer.**

Several instances have now been described in which an asymptomatic individual has been shown to have had circulating anti-p53 antibodies in serum collected months to years before clinical onset of cancer (Trivers *et al.*, 1995; Lubin *et al.*, 1995). This supports the hypothesis that immune response to genetic alterations of the p53 gene can be a very early event. All mutations are not immunogenic in all patients [Wild *et al.*, 1995; von Brevern *et al.*, 1996], and the antibody production level of an

antigen sensitive patient may vary to undetectable levels in periods of their disease [Trivers *et al.*, 1995; 1996]. Chemotherapeutic compounds can be notoriously immunosuppressive and surgical trauma in some patients [e.g. those that have nutritional deficits] may impact on the p53 antibody production and the titre levels necessary for secure detection. This does not promote realistic patterns of serum protein events using one serum sample from patients who have been subjected to a therapeutic procedure. Few investigations were found which studied multiple serum samples over time.

Data from several studies on breast cancer in which tumours and sera from the same patients have been examined, still do not provide clarification whether or how tumour p53 status impacts on the likelihood that the patient will be serapositive (von Brevern *et al.*, 1996). In the study involving p53 antibodies in lung cancer patients, five patients were tested on several occasions during their treatment (Schlichtholz *et al.*, 1994). They maintained a high level of p53 antibodies in their sera. Peptide-scanning experiments have shown that the epitopes recognized by these sera did not change suggesting that the p53 presentation is similar during the course of cancer. Unfortunately, due to the small sample size, aggravated by absconding patients and budget constraints, this study did not permit a correlation between progression of disease and the level of p53 auto-antibodies. It did however indicate that the frequency of sera positive for anti-p53 antibodies did not increase significantly as the disease progressed (refer Chapter 5.5).

6.4.2 Most studies report no evidence to support a particular mechanism for the difference in patterns of acquired p53 mutations in breast cancer [refer Table 64].

The literature indicates that breast cancer in Black American women has a worse prognosis than that of White women (Hunter *et al.*, 1993; Eley *et al.*, 1994; Walker *et al.*, 1995). While continual lactation (Blaine 1995; Michels *et al.*, 1996) and multiparity (Walker *et al.*, 1987; Guinee *et al.*, 1994; Collaborative group on hormonal factors in breast cancer, 1996) contributes to late presentation, there is the possibility that a factor contributes to mutagenesis which is unique or more prevalent in Black women (Swanson *et al.*, 1993). Black women had the highest average annual age-adjusted mortality in 1984 and 1988 (Blaszyk *et al.*, 1994). The frequencies of specific p53 mutations in urban Black American women indicated a three-fold excess of A:T -> G:C transition in the p53 gene as compared to a rural United States' White population and a Scottish population (Blaszyk *et al.*, 1994).

6.5 c-erb B-2

The ELISA kits investigated did not detect c-erb B-2 in the serum of normal controls or in patients with benign breast disease, nor did it detect elevated levels in post-operative serum from breast cancer patients (Figures 43 and 44). While questioning its usefulness in post-operative prediction of the presence of micrometastases, there was no evidence to suggest that post-operative c-erb B-2, at primary surgery, was of any benefit in predicting survival after relapse in metastatic breast cancer Ideally, significant correlation between c-erb B-2 serum levels and clinical severity needs to be evident to justify c-erb B-2 as a cancer marker.

Breuer *et al.*, (1994) found 1 of 25 (4%) matched controls had elevated serum erb B-2 ECD by ELISA compared to 9 of 36 (25%) cases with newly diagnosed primary breast cancer ($p=0.03$); 2 cases with elevated serum levels had tumour tissue overexpression and 2 cases with elevated preoperative levels had normal postoperative levels (Breuer *et al.*, 1994; Breuer *et al.*, 1996). Elevated serum erb B-2 ECD was found in 3 of 60 (5%) controls, 0 of 12 (0%) cases of benign breast disease, 1 of 13 preoperative breast cancer cases, 2 of 62 (3%) post-operative cases without recurrent disease (Anderson *et al.*, 1995). Kynast *et al.*, (1993) reported serum erb B-2 ECD levels by ELISA in 0 of 10 (0%) controls, 0 of 35 (0%) patients without metastatic disease following removal of the primary breast tumour.

The absence of elevated levels of c-erb b-2 in the serum of normal controls or patients with benign breast disease is well documented (Kreipe *et al.*, 1993). Initial studies reported elevated serum erb B-2 ECD levels by ELISA (40- to 190- fold higher than controls) in 3 of 12 (25%) breast cancer patients compared to 35 controls (Mori *et al.*, 1990). Narita *et al.*, (1992) detected elevated serum erb B-2 ECD using an ELISA assay in 0 of 30 (0%) cases of benign breast disease.

Pupa *et al.*, (1993) reported elevated serum erb B-2 ECD levels by RIA in 0 of 50 (0%) healthy controls and 0 of 25 (0%) breast cancer cases with stage I/II disease compared to 6 of 40 (15%) cases with stage III/IV disease. The correlation between tumour overexpression and serum elevation (Pupa *et al.*, 1993) was statistically significant ($p<0.01$). A similar finding by Narita *et al.*, (1992) cites 2 of 64 (3%) cases of stage I/II primary breast cancer, 5 of 17 (29%) cases of stage III/IV primary

breast cancer. A close association between serum elevation and tissue overexpression has been reported, together with changes in serum levels reflecting clinical status. Most of the c-erb B-2 immunoassays reviewed in this study used antibodies prepared against a peptide or the ECD of the intact oncoprotein. The raising of a MAb against the serum dimer of c-erb B-2 would improve sensitivity of serum assays because the antibody so prepared will have a higher affinity for the dimer of the ECD of the oncoprotein. The results of different prognostic factor studies using serum were found to be inconsistent or contradictory. Currently, they are often found to be unreliable.

- **The literature indicates that c-erb B-2 protein level is elevated in patients with distant metastases.**

A correlation between expression of protein in cancer tissue and the serum level of the protein has been established. In IHC, the rate of expression of c-erb B-2 protein in breast cancer tissue has been reported to be 20-40% (Narita *et al.*, 1992; Narita *et al.*, 1994). Elevated serum c-erb B-2 protein levels were seldom detected in early stage cancer even though the primary cancer tissue was c-erb B-2 protein positive (Narita *et al.*, 1993). Elevation of serum levels of protein was seen in 51% of patients with distant metastases.

The detection of elevated serum levels has been reported significantly more often in patients with distant metastases (68%) than in patients with recurrent disease restricted to loco-regional areas (19%). No patients who had normal c-erb B-2 gene copy number in tumour had elevated serum levels (Anderson *et al.*, 1995). Another study reported elevated serum erb B-2 ECD by ELISA (defined as greater than 2 SD above the mean of normals) in 3 of 42 (7%) normal women, 5 of 33 (15%) women with untreated primary breast cancer, and 24 of 105 (33%) women with metastatic breast cancer (Carney *et al.*, 1991); 3 of 12 (33%) cases of locally recurrent breast cancer, 26 of 51 (51%) cases of recurrent metastatic disease, but 0 of 57 (0%) cases with no evidence of recurrence (Narita *et al.*, 1992).

Kath *et al.*, (1992) reported elevated serum erb B-2 ECD levels by ELISA in 26 of 61 (43%) patients with metastatic breast cancer and cited reasonably good correlation between serum and tissue levels of expression with clinical course of disease (Kath *et al.*, 1992; Kath *et al.*, 1993). Twelve of 53 (23%) patients with metastatic or locally advanced breast cancer were reported to have elevated levels of serum c-erb B-2 ECD by RIA compared to 0 of 69 (0%) controls; in two cases changes in serum

correlated with disease status during therapy (Hosono *et al.*, 1993); 9 of 26 (35%) patients with residual metastatic disease, 3 of whom had correspondingly elevated tumour tissue expression (Kynast *et al.*, 1993). The frequency (6%) of elevated levels of c-erb B-2 was found mainly in patients with aggressive (advanced) breast cancer.

There were seven cases of *in situ* carcinoma without invasion, in the study undertaken by Anderson *et al.*, (1995) and three (43%) had elevated serum levels, suggesting that this may be a biomarker of early malignant disease in certain cases of breast cancer.

- **No correlation was found between seropositivity and menopausal status.**

A trend for an inverse correlation was found between c-erb B-2 positive tumours and ER status in studies by Thor *et al.*, (1992). c-erb B-2 expression is under hormonal regulation and estrogens are a controlling factor. c-erb B-2 expression is inhibited under the influence of estrogen (Schroeter *et al.*, 1992). ER levels were not statistically feasible in this study as there were many patients without results. No association was found between ER status and c-erb B-2 levels in those patients (11%) who had ER results. This is in accordance with results obtained by Gotteland *et al.*, (1994) who found no association between ER, PR or c-erb B-2 and pS2 expression (measured by Northern blot). There was no significant correlation ($p=0.6$) between ER expression among 37 patients of known receptor status and serum c-erb B-2 in the study undertaken by Kandl *et al.*, (1994).

Leitzel *et al.*, (1995) determined the effect of overexpression of HER-2/*neu* on response to hormone therapy. An EIA specific for the ECD of the c-erb B-2 oncogene product was used to detect serum levels and indicated that 19% of patients had elevated serum c-erb B-2 protein levels, using a selected cut-off of 30 u/ml. Leitzel *et al.*, (1995) reported that patients with ER positive, c-erb B-2 negative tumours had shorter survival.

6.6 Clinicopathological indices

- **The selection of patients for this study (Addendum V) was subject to a variety of real-life forces that influence clinicopathological studies.**

Practical considerations, such as the availability of tumour tissue for histological assessment may have resulted in a relative under-representation of very small tumours and a predominance of moderately-

sized carcinomas in this study. The tumours analyzed do not fully represent the spectrum of all breast cancers. Realistic clinical populations were studied and emphasis was placed on using patients with less advanced breast disease.

- **Specific histologic types and sub-types of breast carcinoma that were absent or in small numbers in small tumours (<2cm) are markedly under-represented in this study and may well be those that give statistical power to marker studies.**

The study was not limited to patients with invasive ductal carcinoma as sub-types were also specified. Histologic type proved a confounding factor. Invasive breast carcinoma represented the majority (65-80%) of all breast tumours.

- **The most significant observation was the lack of an association of anti-p53 with poor histologic grade.**

The patients used in the study had a median follow-up time of 10 months, with a maximum of 14 months. This study was unable to indicate that anti-p53 antibodies was an independent prognostic factor for patient survival due to lack of information of the cause of death of some patients and the tendency of African women to abscond from western medicine. The relationship to metastatic disease in the present study was not clear, with a trend in favour of a higher prevalence in more advanced disease, as was originally noted in the historical study by Crawford *et al.*, (1982). Schlichtholz *et al.*, (1992) found no association with metastases and therefore suggested that the appearance of these antibodies was an early event in breast cancer. The demographics of the South African study population may be the significant factor in the determination of differences when compared to other studies.

Reports on three studies (Tsuda *et al.*, 1989; Elston *et al.*, 1991; Lipponen *et al.*, 1992) involving stage I and II breast cancer patients indicated that recurrences were more common in younger women and that patients younger than 35 years have a statistically significant increase risk of loco-regional recurrence. 15% of patients participating in this project, median age 52 years, had evidence of recurrence. Locally advanced stage III and IV breast cancers have a high likelihood of distant metastases at diagnosis or more often at recurrence and are associated with poor survival even when treated with neoadjuvant chemotherapy (Faille *et al.*, 1994). The mutational pattern of p53 in a

homogenous group of aggressive tumours (stage III and IV) were determined by sequence analysis in a study undertaken by Faille *et al.*, (1994). Mutations, except for one in exon 10, were found exclusively in exons 5 - 8, which are regions highly conserved between species and which contain the ususal mutational "hot-spots" as reported by others.

- **Co-expression of p53 and c-erb B-2 is associated with a shorter disease-free survival in node-positive breast cancer patients** (Poller *et al.*, 1992).

Complete sequencing of the p53 gene provides prognostic information in breast cancer, particularly in relation to adjuvant systemic therapy (Bergh *et al.*, 1995). Particularly intriguing is the potential use of anti-p53 and c-erb B-2 analyses in treatment decisions for lymph node negative breast cancers (Eccles *et al.*, 1994). The accumulation of p53 protein is suggested to be an independent prognostic marker of reduced survival, whilst the effect of chemotherapy on disease-free survival has been found to be greater in node-negative patients without c-erb B-2 overexpression. The majority of cancer markers in clinical use show a substantial degree of correlation between the disease course and the level of marker in serum, although variability of markers in follow-up of patients radically resected for breast cancer was cited by Gion *et al.*, (1993). Good correlation between CA 15-3 levels and tumour burden, as well as response to therapy in metastatic breast cancer cases, have been cited (Duffy, 1999).

6.6.1 Histological patient data

Identification of biological and molecular parameters, allowing discrimination between subsets of breast cancers, is an important challenge for improved management of the disease (Carter *et al.*, 1989).

- **Most data reports p53 auto-antibodies in sera but cannot relate the anti-p53 level status of the primary tumour or tumour stage, grade or histological type.**

The literature indicates that patients with p53 antibodies have decreased survival and decreased disease-free survival (van der Burg *et al.*, 1995). Silvestrini *et al.*, (1993) reported p53 as an independent prognostic marker in lymph node negative breast cancer patients. In patients with cancer stage A and B1 the presence of p53 antibodies indicated a subgroup with poor prognosis.

- **c-erb B-2's impact on prognosis is somewhat controversial**

Kreipe *et al.*, (1993) states that amplification of c-myc, but not c-erb B-2, is associated with high

proliferative capacity in breast cancer. The literature indicates that whilst some investigators reported a correlation between c-erb B-2 amplification and survival in node positive primary breast cancer (Tandon *et al.*, 1989; Wiltschke *et al.*, 1994;Terrier *et al.*, 1996) others found no such correlation (Zhou *et al.*, 1989). Allred *et al.*, (1992) found a highly significant correlation between disease-free survival and c-erb B-2 expression in "low risk patients". Poller *et al* (1991) suggested that c-erb B-2 amplification and protein expression correlate with poor histological grade. Histological grade is known to be a predictor of aggressive biological behaviour of invasive breast cancer (Tsuda *et al.*, 1990; Umekita *et al.*, 1994). There is also evidence that c-erb B-2 overexpression may be preferentially associated with certain histological subtypes of cancer (Maguire *et al.*, 1992). Immunostaining for c-erb B-2 occurs mainly in large cell DCIS and infiltrating ductal carcinoma, not of significance, especially those with an extratumoral DCIS component (Somerville *et al.*, 1992) which indicates that there is a low incidence in other types of breast cancer.

6.7 Summary of Chapter Six

Breast cancer has an enormous impact on society since it is the major cause of death from any cause in women aged 35 - 54 years (Duffy, 1999). In theory, measuring tumour-associated antigens or antibodies in patients should be a simple and non-invasive way of detecting and assessing breast cancer, but controversy regarding their value, their inter-relationships and their advantages over better known features, continues. Next we might ask how important early detection is for successful treatment. This is very unclear from the literature. Even if markers lead to an accurate diagnosis of breast cancer, this will not indicate how the cancer should be treated.

A fully developed cancer requires mutations in several genes but it is not yet evident whether there is a preferred progression in which these mutations are likely to occur. A full molecular description of both normal and neoplastic growth will enable medical scientists to discover if the enormous amount of literature on genes such as p53 and c-erb B-2 can translate into an application that will significantly decrease this scourge that destroys so many women. Advances in the human genome project and in molecular oncology will transform the way we understand and treat this mysterious disease we call breast cancer.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

7.1 General introduction

The results of this study indicate an intrinsic variability that is unacceptably high in assays intended for clinical use in the detection of cancer markers.. Variability is primarily reflected in the inescapable heterogeneity of tumours which accordingly elicit a wide variety of responses. The intrinsic variability in results was so large that it greatly compromised the ability to stratify subsets of breast cancer patients into groups. These assays will only have practical utility when studied under similarly controlled conditions. The researcher was not aware at the outset of this project and is now concerned that the design of the kits purchased from Oncogene Science may have effected the data produced in this study.

The design of the p53-Ab assay does not include any value for the measure of the non-specific binding inherent within each persons' sera. The test for specificity of the binding is a test that compares the binding of the human serum to the p53 antigen to the binding of previously determined antip53 preparations; the species of origin of which is not provided with the package insert given in the kit. An unidentified anti-p53 antibody was included as a positive control on each microtitre plate supplied with the kit. The study concludes that, in its present form, the assays investigated are variable in their methodology and while they are potentially beneficial, they are not presently suitable for general application in the clinical assessment of breast cancer.

Since the majority of discordant data have reasonable explanations, the present results are consistent with those in the literature. Given tumour variability and it's effects on the immune response, together with the so-called "publication bids" that leads researchers to publish positive findings rather than negative findings, it was not surprising that there are more published studies supporting efficacy of marker assays than denying them. Results demonstrate mixed appraisals of the clinical utility of cancer markers assays as reported in the literature. Despite moderate success with tissue-based markers,

attempts to find a sensitive and specific circulating breast cancer marker have not yet been successful..Genetic markers may prove useful with improved methodology.

As the government sets economic constraints on health care, the cost and consequences of both late stage presentation and patients who abscond from treatment protocols becomes more pressing. The availability and use of medical and laboratory services still vary regionally in South Africa. For women with breast cancer it is like Russian roulette! A consensus concerning the criteria to be used to ensure timely referral for breast cancer patients, particularly in rural areas, is urgently needed. Depending on where a woman lives, or her socio-economic standing, could cost her her life.

Despite the information explosion in molecular understanding of breast cancer, approximately 40% of patients with breast cancer die from their disease; a situation that has not changed much in the last 50 years. One million new cases of breast cancer were diagnosed per year for the period 1990-2000 which represented an increase of 24% when compared to the previous decade (Duffy, 1999). Breast cancer is a complex systemic disease which poses many perplexing problems. Despite the ability to diagnose the disease at an earlier stage of its evolution and refinements in treatment (both of which have collectively resulted in improvements in 5 year survivals), breast cancer mortality rates have not generally improved. Despite the tremendous knowledge we are now able to generate at a comparatively rapid and almost logarithmic pace,. in science today we know more than we have been able to comprehend at a comparable speed.

It is becoming increasing apparent that several changes to mammary cells produce the transformed phenotype. The need for proper validation of the influence of time on new markers must be investigated. Traditional morphological indices remain unsurpassed as indicators but are inadequate to predict the behavior and response to treatment of a significant number of breast cancer tumours.

7.2 p53 auto-antibodies and breast cancer

Serological analysis of p53 antibodies in patients with breast cancer can be considered a

complementary procedure in assessing the functional state of the p53 gene and is a useful adjunct, along with other approaches (molecular and biochemical methods), for studying p53 gene alteration. Detection of anti-p53, using recombinant wild-type p53 protein, used in the ELISA system in this study, recognized different alterations in the p53 gene and represent the true prevalence of serum auto-antibodies in this investigation. Results confirm previous reports (Schlichtholz *et al.*, 1992; Angelopoulou *et al.*, 1993; Umekita *et al.*, 1994; Peyrat *et al.*, 1995) that p53 immune response may be associated with an aggressive biological phenotype of cancer.

This study demonstrated elevated levels of p53 auto-antibodies in the post-operative serum of 12% of breast cancer patients, but cannot propose that this marker is a clinically useful diagnostic or prognostic factor. Rather it suggests that the anti-p53 ELISA is a convenient and reasonably specific test which reflects a significant host anti-tumour response to alterations in p53 gene expression and could be of value in the characterization of patients with breast cancer. Western blot assays are suitable for large scale diagnostic testing, whereas ELISA techniques are easy to perform. The ELISA system used in this study used recombinant wild-type p53 protein to detect anti-p53. This enabled recognition of different alterations in the p53 gene and should represent the true prevalence of serum auto-antibodies in this population. From the limited number of positive sera identified in this study, antibodies were detected in sera from patients with a variety of breast cancer types. As with all biochemical markers in current use, positive p53 or elevated c-erb B-2 concentrations are not organ-specific. These measurements alone are therefore of little use in identifying primary breast cancers.

- **The hypothesis that certain p53 mutations may produce a more immunogeneic protein requires further investigation.**

Evidence from several studies has shown that patients' sera recognize both wild-type and p53 mutants in a similar manner (Labrecque *et al.*, 1993; Schlichtholtz, *et al.*, 1992). There is no evidence that anti-p53 antibodies preferentially recognize mutant p53 proteins. A strong correlation between levels of p53 in cells and the presence of p53 antibodies was cited by Winter *et al.*, (1992), suggesting that p53 stabilization (?hsp70) and its resulting overexpression are essential prerequisites for the presence of p53 antibodies. The location of the mutation in the p53 protein does not appear

to be a major determinant in the immune response of these patients (Schlichtholz *et al.*, 1994).

Why only some women with breast cancer elicit an immune response against mutant p53 is nebulous. The reasons why factually and theoretically some women with breast cancer may not have detectable antibodies at a given point over the course of their clinical history are: [1] p53-Abs are thought to and in some diseases have been shown to associate with mutations in the p53 gene, which occur in only 22% of breast cancer patients; [2] p53 mutations are not immunogenic in all individuals; [3] positive patients may be sampled at points when their titres have ebbed below detection sensitivity levels or [4] and possibly at times when they are suffering the immunosuppressive effects of therapy, however currently the reason why the incidence is not higher is not completely understood or fully known. [Refer to page 6.9 for references supporting these above observations].

7.3 c-erb B-2

- **It is recommended that serum determination be performed before surgery as inappropriate timing of sampling could lead to false-negative predictions.**

The impact of surgery and medication on the oncoprotein level needs to be defined to determine it's clinical utility. The data indicates that post-operative serum c-erb B-2 does not correlate with clinicopathological indices and therefore does not presently justify its use in the clinical setting. A relation between serum levels and clinical status was indistinct in the literature (Kath *et al.*, 1992; Volas *et al.*, 1992). Literature regarding c-erb B-2 and prognosis are inconclusive, particularly for node-negative patients. Results from this study reiterated evidence that post-operative serum c-erb B-2 is not diagnostically helpful but may reflect tumour burden accurately. Surprisingly, positive c-erb B-2 concentrations at the time of detection of metastases were not associated with c-erb B-2 positivity at the time of primary surgery in the study by Jager *et al.*, (1996). Although there were significant pathologic differences between patients, there was no prognostic selection bias for sub-groups. Further prospective studies should be undertaken on pre-operative serum in order to accurately assess if c-erb B-2 ELISA could be used as an additional clinical parameter in breast cancer.

- **Whether ELISAs are detecting all forms of c-erb B-2 with similar efficiencies remains unclear.**

A consideration in interpreting results is that experimental problems cannot be excluded. Since p120 is a glycoprotein, there is a possibility that p120, derived from tumour and normal cells, differ in the composition of their carbohydrate moieties and hence lectin reactivity. A sensitive assay specific for serum p120, needs to be devised because the current commercial assay for c-erb B-2 protein does not differentiate between p120 and p185 molecules. The presence of different lectin reactivities between tumour cell and normal cell derived p120, should assist in developing a specific p120 assay which reflects tumour activity.

7.4 *Quo Vadis ?*

Results indicate the necessity of further research if we are to see a decrease in breast cancer mortality. Since the demonstration of axillary lymph node micrometastases has a limited value in predicting survival, the study of new biologic factors in primary tumours must be developed. Immunodiagnosis of breast cancer does not presently constitute a reliably accurate method of choice for detection of carcinogenesis. It appears rare, if possible, to attribute the metamorphosis of a normal cell to a cancer cell solely on the activation of a single gene since a range of genetic changes appears to dictate the behavior of breast cancer. BRCA 1;2;3 has now provided the opportunity to predict which women are at most risk for breast cancer. Maybe, with time, researchers in gene therapy may discover how to replace the missing functions of the p53 gene in the 22% of women shown to have a p53-related tumour.. This may prove easier than correcting the aberrant behavior of an oncogene such as c-erb B–2. Breast cancer posses a dual threat to women; it attacks their femininity as well as their lives. As the incidence continues to rise among women, the need for a paradigm shift in our understanding of breast cancer becomes more compelling.

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ADDENDUMS

I General definition of the TNM pathological classification

II Histological types for Breast Cancer

III Breast Patient Questionnaire - English

IV Breast Patient Questionnaire - Zulu

V Treatment Protocol - Oncology Department

- 1 Staging
- 2 Radiotherapy technique
- 3 Chemotherapy regimes
- 4 Hormone therapy

VI Researchers Case Study Form

VII Raw data

Addendum I

Histological types of breast cancer : General definitions of the pathological classification (pTNM)

The following general definitions are used.

pT : Primary tumour

pT_x	Primary tumour cannot be assessed histologically
pT_0	No histological evidence of primary tumour
pT_{is}	Carcinoma *in situ*
pT_1, pT_2, pT_3, pT_4	Increasing extent of the primary tumour histologically

pN : Regional lymph nodes

pN_x	Regional lymph nodes cannot be assessed histologically
pN_0	No regional lymph node metastasis histologically
pN_1, pN_2, pN_3	Increasing involvement of regional lymph nodes histologically

Direct extension of the primary tumour into lymph nodes is classified as lymph node metastasis. When size is a criterion for pN classification, as in breast carcinoma, measurement is made of the metastasis not of the entire lymph node.

pM : Distant metastasis

pM_x	Presence of distant metastasis cannot be assessed microscopically
pM_x	No distant metastasis microscopically
pM_x	Distant metastasis microscopically

The category pM_1 may be further specified in the same way as M_1 (See above)

The category M_1 may be further specified according to the following notation

Pulmonary	PUL
Osseous	OSS
Hepatic	HEP
Brain	BRA
Lymph nodes	LYM
Bone marrow	MAR
Pleura	PLE
Peritoneum	PER
Adrenals	ADR
Skin	SKI
Others	OTH

Subdivision of TNM

Subdivisions of some main categories are available when greater specificity is needed, for example, T_{1a}, T_{1b}, or N_{2a}, N_{2b}.

For most tumour sites and entities the definitions of the pT, pN and pM categories correspond to the T, N and M categories. Different clinical and pathological definitions exist for the N classification of breast carcinoma, T classification of malignant melanoma of conjuctiva and retinoblastoma, and T and N classification of nephroblastoma, neuroblastoma, and childhood soft tissue carcinomas.

Procedure in doubtful cases

If there is doubt about the correct T, N or M category to which a particular case should be allotted, then the lower (that is, less advanced) category should be selected.

BREAST CANCER HISTOLOGY

1. Duct cell carcinoma
2. Medullary carcinoma
3. Lobular carcinoma
4. Mucinous carcinoma
5. Tubular carcinoma
6. Adenoid cystic carcinoma
7. Papillary carcinoma
8. Apocrine carcinoma
9. Squamous cell carcinoma
10. Paget's disease (oestrogen receptor, CEA, EMA, Cytokeratin S.100 protein)
11. Intraductal carcinoma
12. Cystosarcoma phyllodes (bi-phasic tumour)

BENIGN CONDITIONS

1. Breast cysts:
   a. simple and apocrine cysts
   b. papillary
2. Inflammatory lesions:
   a. Acute mastitis
   b. Abscess formation
   c. Plasma cell mastitis
3. Lipomas (* interchangeable)
4. Fat necrosis and subareolar abscess
5. Fibrocystic disease:
   a. Apocrine metaplasia
   b. Granuloma of the breast
   c. Duct ectasia

Benign tumours

6. Juvenile papillomatosis
7. Fibroadenoma
8. Fibro-adenosis and adenosis tumour
9. Adenomas
10. Lactating adenomas
11. Granular cell tumour
12. Granulomas
13. Localized amyloid tumour
14. Papilloma (stage 0)
15. Gynaecomastia

(Comprehensive cytopathology edited by Marluce Bibbo M.D., 1991)

BREAST CANCER QUESTIONNAIRE

PARTICIPANTS WILL REMAIN ANONYMOUS

SECTION A: QUESTIONNAIRE

1a	Pregnancies		1st child	year	1	9				
			2nd child	year	1	9				
			3rd child	year	1	9				
			4th child	year	1	9				
			5th child	year	1	9				
			6th child	year	1	9				
b	Did you breast feed?		YES				NO			
	If YES, for how long?	First baby	Months				Weeks			
		Second baby	Months				Weeks			
		Third baby	Months				Weeks			
2a	Have you had an abortion		YES				NO			
	If YES, state in which year	First		year	1	9				
		Second		year	1	9				
b	Have you had a miscarriage?		YES				NO			
	If yes, state in which year	First		year	1	9				
		Second		year	1	9				
3	Does your family has a history of breast cancer		YES				NO			
4	If YES, who?		Mother				Sister			
			Aunt				Grand-mother			
			Other							
5	How old are you?		20-29				30-39			
			40-49				50-59			
			60-69				70-79			
6a	What is your weight in kilograms?									
b	Do you eat sensibly?	SOMETIMES	YES				NO			
c	Do you eat fresh fruit and vegetables?	SOMETIMES	YES				NO			
7	Do you smoke?	SOMETIMES	YES				NO			
8	Do you drink alcohol?	SOMETIMES	YES				NO			
9	Are you aware of self examination		YES				NO			
10	Have you ever had a Mammogram?		YES				NO			
11	If YES, how many?									

12 Have you ever had a medical problem with your breasts?
For example:

Mastitis	YES		NO	
Abscess	YES		NO	
Lumpy breasts	YES		NO	
Pain	YES		NO	
Bleeding nipples	YES		NO	
Milk from nipples (Not when lactating)	YES		NO	
Any other problem	YES		NO	

Please give details on the above, if necessary

13 Are you menopausal? YES NO

14 If NO, give date of last period DD MM YY

Was this period NORMAL HEAVY LIGHT

15 If YES, are you taking hormonal replacement therapy (HRT) YES NO

16 If YES, for how many years? - CROSS applicable block

1-2	2-3	3-4
5-7	7-8	9-10
Longer		

17 Cancer has long been regarded as a genetically based disorder. Progress to full malignancy appears to depend on the mutation or deletion of the p53 gene. This study affords the opportunity to expound on whether differences exist, which affect prognosis, for the different population groups of South Africa. For this reason, please state your race.

Asian	Black	Coloured	White

SECTION B: COMMENTS

THANK YOU VERY MUCH FOR PARTICIPATING IN THIS STUDY.

Addendum IV

IMIBUZO NGOBUCAYI BOMDLAVUZA WEBELE ABAPHENDULI BALEMIBUZO ABAYIKUVEZWWA

SECTION A: IMIBUZO

1.	a	(i)	Ukhulelwe kangaki?	.....			
		(ii)	Yisho iminyaka owakhulelwa ngayo	.....			
	b	(i)	Wawubancelisa abantwana na?	YEBO /	QHA		
		(ii)	Uma impendulo yangenhla ingu yebo 1	Inyanga	.....	Amaviki	.....
			Wancelisa isikhathi esingakanani 2	Inyanga	.....	Amaviki	.....
			Emunye 3	Inyanga	.....	Amaviki	.....
2	a		Usuke waskhipha isisu?	YEBO /	QHA		
	b	(i)	Wake waphuphunyelwa isisu?	YEBO /	QHA		
		(ii)	Uma impendula ka b(i) ingu Yebo ngoyaka yisho unyaka	19.....			
3			Kwabomndeni wakho kukhona owake waphathwa umdlavuza?	YEBO /	QHA		
4			Uma impendula ka 3 ingu yebo khombisa	UMAMA		UGOGO	
			kulezikhala ukuthi kwakungobani	UANTI		ABANYE	
				UDADEWETHU			
5			Uneminyaka emingaki?	20-29		30-39	
				40-49		50-59	
				60-69		70-79	
6	a		Isisindo sakho singakanani ngokwamakilogramu?	.....			
	b		Udla ngokwanele osukwini?	YEBO	QHA	Kokunye	
	c		Uyayidla imifino nezithelo?	YEBO	QHA	Angivamile	
7			Uyawubhema ugwayi?	YEBO	QHA	Kokunye	
8			Uyabuphuza utshwala na?	YEBO	QHA	Kuqabukela	
9	a		Ungakwazi kanjani ukuzihlola amabele akho?	YEBO /	QHA		
	b.		Uma uvuma, ungakwenza lokhu?	YEBO /	QHA		
10			Wake wathathwa isithombe sebele esibhedlela?	YEBO /	QHA		

11			Uma impendulo ka 9 ingu yebo, wathathwa kangaki?	YEBO /	QHA		
12	a		Wake waba nenkinga yokuguliswa yibele?	YEBO /	QHA		
	b		Cacisa ukuthi yinhloboni yokugula, uma impendulo	Ithumba		Iguludla	
				Ukuvuvuka		ubuhlungu	
				Kwebele		Bebeleni	
	c		ube ungancelisi izingono zakho ziphuma	Ubisi		igazi	
	d		Uma kunesidingo chaza kabanzi ngesigulo sakho sebele	.....			
13	a		Usunazo izinkombazokuphela kokuya esikhathini?	YEBO /	QHA		
	b		Uma impendulo kungu Qha. Ugcine nini esikhathini?. Isho inyanga nonyaka.	.....			
	c		Isikhathi sakho siza kangakanani?	Kancan		Ngamandla	
				Ngokujwayelekile			
14			Uma impendula ka 13 ingu yebo, ikhona yini ihomoni oyithola ukusiza isimo okusona?	YEBO /	QHA		
15			Uma impendulo ka 14 ingu yebo, iminyaka emingaki uyithatha lehomoni?	.....			
16			Umdlavuza uthathwa njengesifo sofuzo ukugulisa kwawo okunzioma kudalwa ukishintsha okanye ukulahleka kwejini uP53. Lenhlolo luvo inika ithuba lokucwaninga, noma ukuhlasela kwawo kuya gobuhlanga. Yini kungasho ukuthi lomgudu wokuqubuka kwawo usuka ngezinhlanga ezikhona eNingizimu Africa ngenxayalokho yisho ubuzwe bakho:				
				Ndiva		Mnyana	
				Khaladi		Mhlophe	

SECTION B: OKUPHAWUKEKAYO OKUNYE

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UKUBAMBA KWAKHO IQHAZA KULENHLOLO LUVO KUYABONGEKA KAKHULU

BREAST CANCER TREATMENT PROTOCOL : ONCOLOGY DEPARTMENT

King Edward VIII Hospital, Durban, South Africa
Addington Hospital, Durban, South Africa

STAGING INVESTIGATIONS

1. Full medical, surgical, gynaecological and family history

2. Examination
   - general
   - local
     - mass
       - size - 2 diameters by 2 observers
       - site/consistency/
       - fixity to skin/pectoralis/chest wall
     - skin
       - erythema/oedema/tethering/nodules
     - nipple
       - retraction/discoloration/thickening/
       - reddening/erosion/discharge/destruction
   - nodes
     - axillary
       - number/mobility/size/significance
     - supraclavicular/infraclavicular
     - cervical

3. Laboratory
   - FBC/U&E/Creatinine/Calcium/LFT/CA 549

4. Radiology
   - Chest x-ray/Bilateral mammography
   - Appropriate skeletal x-rays
   - Bone scan (in all patients)
   - Cardiac ejection fraction (MUGA) if treatment plan involves epiadriamycin or mitoxantrone in patients with cardiac risk factors (DM/HT/IHD/CCF)
   - Ultrasound or CT liver (if liver enzymes raised)

5. Pathology
   - Cytology/Needle biopsy (not recommended)/Excision biopsy
   - Cytology positive - proceed to wide local excision
   - Cytology negative x 2 and mammogram positive with high clinical suspicion - Proceed to wide local excision.

The following information is required of the pathology report:

Type of specimen
- Trucut/Incisional/Excisional biopsy/Mastectomy.

Gross Description
- Tumour
  - size of two maximum diameters.
  - depth - distance from skin or chest wall.
  - gross margins of involvement.
- Nodes
  - total number identified.
  - number with metastatic tumour
  - presence or absence nodal capsular violation.

6. Histology
- Classification (based on revised WHO classification dominant pattern.)
- Histologic grade - scored out of three (HG I-III).
- Microscopic margins.
- Involvement of adjacent structures (skin/nipple/muscle/chest wall/ dermal lymphatics).
- If previous biopsy performed, comment on whether there was residue of tumour, and whether or not this is now clear.
- Oestrogen and progesterone receptor levels (separate fresh specimen required).

MANAGEMENT (See appendix 1 for the currently used TNM staging system)

STAGES I & II

SURGERY

1. Primary site — Wide local excision (WLE) with a minimum gross margin in 1 cm is considered the surgical treatment of choice in patients whose breast will allow such surgery without prejudice to the cosmetic outcome. Simple mastectomy (SM) is the alternative. Attention should be paid to optimal placement of incisions and drains for easy placement of radiation fields.

   A pathology report indicating close or involved surgical margins is an indication for re-excision with an adequate margin. This may entail another WLE or mastectomy.

2. Axilla — The required procedure is a Level II clearance, leaving the perivascular fascia around the axillary vein intact. When WLE is performed the axilla should be approached through a separate incision. The nodes should be dissected free of the axillary fat by the surgeon in theatre and counted, prior to submission to the laboratory. Axillary 'sampling' is considered inadequate.

RADIOTHERAPY

1. Post WLE — Whole breast megavoltage radiotherapy. In node positive disease attempts to include as much axilla in the 2 field technique is made.

2. Post SM — Node negative disease requires no radiotherapy. Node positive disease required orthovoltage chest wall radiation. Supraclavicular and axillary fields are added for N2 disease.

See Appendix 2 for details of radiotherapy technique.

CHEMOTHERAPY

1. N+ disease — All patients should be offered adjuvant chemotherapy unless specific contraindications exist. Standard is 12 cycles of polychemotherapy (PCT), with review after 6.

2. NO disease — Patients with 2 or more of the following poor prognostic features should be offered adjuvant chemotherapy. Standard is 6 cycles of PCT:

    1. Steroid receptor negativity
    2. Histological grade III (poorly differentiated).
    3. Vascular or lymphatic invasion.
    4. Young age (< 40) /Premenopausal.

See appendix 3 for details of chemotherapy regimes.

Contraindications to the use of adjuvant chemotherapy include:

1. Age > 70 years.
2. Performance status > 2 (ECOG scale).
3. Specific cardiac or other medical conditions.

HORMONE THERAPY

All patients who are oestrogen or progesterone receptor positive or unknown or who are receptor negative but more than 5 years post menopause should receive hormone therapy - see appendix 4 for details. Most early stage patients will receive hormone therapy as adjuvant although it may be used as primary treatment in elderly women who are not immediately suitable for surgery.

STAGE III

The risk of systemic disease is high in node positive and locally advanced breast cancer and systemic treatment (i.e. chemotherapy and hormone therapy) is central to the management of these patients. Local control of disease is, however, very important.

SURGERY

Complete removal of the primary tumour is the objective and may be achieved in any manner which will result in the most acceptable cosmetic result. WLE or SM are both options for treatment within the confines of the above statement. Neoadjuvant chemothrapy may precede surgery if an initially inoperable tumour is rendered operable by chemotherapy (usually 3 cycles of PCT). If neoadjuvant chemotherapy is used completion of adequate local surgery should not be overlooked, even in the face of a complete response to the chemotherapy. Reconstructive surgery using myocutaneous flaps may be required to close a large defect left following excision of a large tumour.

RADIOTHERAPY

This modality is used to achieve or consolidate local control and is recognised not to influence the survival of patients with Stage III disease which is considered to be systemic from the outset.

1. Post WLE — Whole breast megavoltage radiotherapy using a 2 field, tangential technique, to include as much axilla as possible.

2. Post SM — Orthovoltage chest wall radiotherapy (2 field in N0/N1 disease or 4/5 field in N2/N3 disease).

3. Palliative — In patients with symptomatic, locally advanced disease.

See appendix 2 for details of radiotherapy technique

CHEMOTHERAPY

1. Neoadjuvant chemotherapy - 3 or more cycles of PCT given for tumour shrinkage and systemic control in patients with marginally operable or inoperable breast cancer.

2. All patients with Stage III disease should receive postoperative chemotherapy, 12 cycles of PCT, unless there are specific contraindications. Review after 6 cycles is mandatory to exclude progression or intolerance.

HORMONE THERAPY

All patients who are oestrogen or progesterone receptor positive or unknown or who are recptor negative but more than 5 years post meopausae should receive hormone therapy - see appendix 4 for details.

STAGE IV

Metastatic disease of the breast is not considered curable. Long term survival and good palliation can be achieved with the judicious use of chemotherapy, surgery and radiotherapy. Symptom control is the principle objective of treatment and careful, critical assessment of treatment related toxicity should be undertaken. Prolonged use of chemotherapy without objective evidence of response is to be avoided.

SURGERY

The role of this modality is to provide histology and steroid receptor status and to secure local control. Systemic treatment should not be delayed. Surgical treatment of the primary usually involves the use of a 'toilet' procedure to gain local control when the area escapes control by chemotherapy. Surgical procedures, such as fixation of the pathological fracture or potential fracture may be necessary.

RADIOTHERAPY

Being a locally active modality, the same principles apply. Symptom control either at the primary site (ulceration/pain/haemorrhage) or at metastatic sites is the aim. Again systemic treatment should not be delayed.

CHEMOTHERAPY

This is the prime modality of management and PCT is the standard therapy with EMV or MMM used as second line or alternative therapy. Twelve cycles is the aim of treatment with some patients proceeding beyond this depending upon tolerance and response. Cumulative maximum doses of anthracyclines should be respected.

HORMONE THERAPY

All patients who are oestrogen or progesterone receptor positive or unknown or who are receptor negative but more than 5 years post menopause should receive hormone therapy - see appendix 4 for details.

FOLLOW-UP

While on treatment with chemotherapy and for 6 months thereafter, patients are seen with a blood count every 5 weeks, or as the chemotherapy regimen dictates. Review intervals are then extended to 3 months x 4 blood counts, 6 months x 4 blood counts and then yearly.

Tumour marker levels (CA 549/CA 153 Cathepsin-D) will be used, when available, at the discretion of the attending doctor. Chest x-ray, FBC, U&E, LFT should be obtained yearly and mammography (in appropriate circumstances), isotope bone and liver scans are performed according to clinical indication.

SPECIAL CASES

1. Elderly patients - arbitrarily defined as > 70 years - may be maintained on hormonal therapy as either primary or adjuvant therapy. Lack of response of a primary lesion indicates the need for local therapy.

   Chemotherapy adjuvant therapy using IV or oral monochemotherapy (see appendix 3) may be used in patients with adequate performance status scores.

   Metastatic disease may be treated by hormonal therapy with or without chemotherapy depending on the condition of the patient. The emphasis on symptom relief and quality of life is important. Radiotherapy can make a significant contribution to the management of these patients.

2. Pregnancy - Careful discussion with the patient is important. Decisions rest upon the stage of both the pregnancy and the malignancy. Appropriate treatment given as early as possible results in the best prognosis, but survival rates are significantly impaired by the simultaneous occurrence of breast cancer and pregnancy. Adjuvant therapy should be considered in all cases because of the poorer prognosis.

   In general patients presenting in the 1st trimester should receive adequate local surgical therapy (which is feasible with minimal risk to the foetus). If the patient requires adjuvant therapy she should be advised to terminate the pregnancy to facilitate this. The wishes of the patient are, however, of paramount importance and in the face of literature indicating no advantage for termination, excessive pressure to terminate is not indicated.

   Patients in the last trimester can reasonably be treated after induction of delivery.

   Patients in the middle trimester should be treated on merit considering all disease and patient related factors.

   Stages of disease which are associated with systemic spread require aggressive therapy. Radiation and chemotherapy should not, as a rule, be given to a patient carrying a viable foetus and if treatment of the disease is identified as a priority in a particular patient, and the foetus is not viable then termination should be undertaken.

   When there is a need for adjuvant therapy in the immediate post partum period breastfeeing should be stopped and lactation suppressed.

3. Males - treated as for females, stage for stage. The hormone receptor positivity rate is higher in males and hormonal adjuvant therapy (used in manner similar to that for female patients) plays a more important role.

4. Inflammatory carcinoma - has a poor prognosis because of a tendency for early systemic spread. Primary chemotherapy with delayed radiation and surgery is the treatment to choice.

5. Ductal carcinoma-*in-situ* (DCIS) - recognised standard therapy is mastectomy. To date the curability of DCIS by conservative surgery and radiotherapy has not matched mastectomy. Mortality from breast cancer, in patients with DCIS treated conservatively, can be kept below 3% if there is careful attention paid to surgical margins with re-excision if necessary, full dose radiotherapy and meticulous follow-up with regular mammography. Axillary dissection is not necessary when conservative therapy is used.

6. Lobular carcinoma-*in-situ* - this appears to occur in predominantly young women, is rarely associated with a lump or microcalcification, is often multifocal and is associated with a 50% risk of malignant progression in either breast annually. The multifocality and systemic nature of LCIS dictate adequate, but not necessarily ablative, surgery with radiation and an emphasis on hormonal adjuvant therapy. Decisions regarding chemotherapy are standard.

7. Paget's disease - is associated with an underlying mass in 60% of cases. All are associated with in-situ or invasive carcinoma in the underlying breast. Treatment is dictated by the presentation:

   No mass - WLE of the involved skin and nipple ducts, axillary dissection and postoperative radiotherapy with adjuvant hormone and chemotherapy given according to the normal guidelines.

   Superficial mass - WLE, axillary dissection, and postoperative radiotherapy with adjuvant hormone and chemotherapy given according to the normal guidelines.

   Deep mass - SM with postoperative adjuvant radiotherapy, hormone and chemotherapy given according to the normal guidelines.

1 : STAGING

Stage		Description
Tx		Primary tumour cannot be assessed
T0		No evidence of primary tumour
T1S		Carcinoma-*in-situ* (including DCIS, LCIS & Paget's with no mass)
T1		Tumour < 2.0 cm in greatest dimension
	a	< 0.5 cm
	b	0.5 - 1.0 cm
	c	1.0 - 2.0 cm
T2		Tumour 2.0 - 5.0 cm in greatest dimension
T3		Tumour >5.0 cm in greatest dimension
T4		Tumour of any size with direct extension to chest wall or skin
	a	Extension to chest wall
	b	Oedema (*including peau d'orange*) OR skin ulceration OR satellite nodules confined to the same breast.
	c	Simultaneous chest wall and skin extension
	d	Inflammatory carcinoma
Nx		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		*Metastasis to movable ipsilateral axillary nodes*
N2		Metastasis to ipsilateral axillary nodes fixed to one another or to other structures
N3		Metastasis to ipsilateral internal mammary nodes
Mx		Haematogenous metastases cannot be assessed
M0		No haematogenous metastases
M1		Haematogenous metastases

2 - RADIOTHERAPY TECHNIQUE

1. **Whole breast megavoltage** - ^{60}Cobalt or a linear accelerator beam.

Medial and lateral oblique, tangent fields are used with appropriate combinations of wedged and open fields to treat the whole breast to the chest wall, including nor more than 2 cm of lung in the field at maximum diameter. Does variance across the tumour volume should ideally not exceed 10%.

Dose: 2,0 Gy daily (5/7) to the tumour volume, x 23 to 46,0 Gy total.
Bolus to be applied to the scar and drain sites in the field.

Electron boost to the scar with a margin. Depth to be calculated from CT or ultrasound scan measurements.

Dose: 2,0 Gy daily (5/7) to the 80% isodose, x 5 or 6 to total of 10/12 Gy.

Total tumour dose will reach 58 Gy and maximum point dose will be 65,6 Gy.

2. **Chest wall orthovoltage** - Dxr beam

Medial and lateral tangent fields applied with 'breast box'. Maximum IFD is 20 cm and field is typically shielded from a 10 x 20 cm applicator.

Dose: 31,0 Gy midline dose in 15 or 17 fractions daily (5/7)
250 kV beam with a Thoraeus II filter
Bolus bags are used to fill in around the treated breast

Anterior and posterior supraclavicular fields with appropriate shielding of the larynx and humeral head. Typical field size is 20 x 10 cm.

Dose: 31,0 Gy midline dose in 15 or 17 fractions daily (5/7)
250 kV beam with a Thoraeus II filter

Parasternal field applied directly to the sternal region with a 2 cm margin around each sternal edge. Typical field size 6 x 8 or 6 x 12 cm.

Dose: 33.5 Gy given dose in 15 or 17 fractions daily (5/7)
250 kV beam with a Thoraeus II filter

3 - CHEMOTHERAPY REGIMES

1. Polychemotherapy (PCT)

EPIADRIAMYCIN	50 mg/m^2 IV q5wk (max. Cumulative dose = 900 mg/m^2)
VINDESINE	3 mg/m^2 IV q5wk
5 FLUOROURACIL	600 mg/m^2 IV q5wk
CYCLOPHOSPHAMIDE	250 mg/m^2 PO weekly
METHOTREXATE	2,5 mg/q6h PO weekly

Either 6 (N0) or 12 (N+/M1) courses of treatment will be delivered with dose adjustment according to toxicity. Upon completion and restaging the oral cyclophosphamide component is continued for a further year.

2. EMV

ETOPOSIDE	400 mg/m^2 IV q4wk
MITOXANTRONE	10 mg/m^2 IV q4wk
VINCRISTINE	1 mg/m^2 IV q4wk

3. MMM

MITOMYCIN	8 mg/m^2 IV q8wk
MITOXANTRONE	10 mg/m^2 IV q8wk
METHOTREXATE	35 mg/m^2 IV q8wk

EMV and MMM are reserved for salvage or alternative treatment when PCT is contraindicated. The number of cycles to be delivered will be determined by the response and toxicity with critical reassessment occurring at least every 3 cycles.

4. Mitoxantrone

MITOXANTRONE	10 mg/m^2 IV q4wk

5. Oral Etoposide

ETOPOSIDE	100 mg daily PO Monday - Thursday weekly x 3 weeks, repeated on a monthly cycle.

This monotherapy is utilised for patients whose age or general condition precludes aggressive therapy. Critical response and toxicity assessment is necessary.

4 - HORMONE THERAPY

The following drugs have been utilized and in metastatic disease in the following sequence:

1.	Tamoxifen	20 mg PO daily
2.	Aminoglutethimide	250 mg PO q12h
3.	Medroxyprogesterone	100 mg PO q8h (Provera)
		500 mg PO q12h/daily (Farlutal)
4.	Decadurabolin	50 mg IM q3wk
5.	Tamoxifen	20 mg PO daily
6.	Prednisone	5 mg PO q8h

Adjuvant hormone therapy utilised Tamoxifen and is continued for at least 2 years. The length of treatment is unsettled at present and it is current policy to continue indefinitely.

RESEARCHERS CASE STUDY FORM **ADDENDUM VI**

PRIVATE AND CONFIDENTIAL

SURNAME					
FIRST NAME					
AGE (in years)					
DATE OF BLOOD COLLECTION					
PLACE OF BLOOD COLLECTION					
HOSPITAL NUMBER					
ONCOLOGY NUMBER					
DIAGNOSIS:	RIGHT BREAST				
	LEFT BREAST				
DATE OF FIRST CONSULTATION					
CHEMOTHERAGY (CT) COMMENCED		DATE		TYPE	
BIOPSY		DATE		RESULTS	
ER STATUS					
MASTECTOMY		YES		NO	
		IF YES, DATE:			
MAMMOGRAM		YES		NO	
		IF YES, DATE/S:			
HISTOLOGY NUMBER					
NODAL STATUS					
GRADE					
STAGE					
MENTRUAL STATUS		HRT	MENOPAUSAL	LMP	
OTHER RESULTS		Bone Scan			
		Ca125	Ca15.3		
		CEA	Ca549		
COMMENTS					
ANTI-p53 RESULTS		Date	Date	Date	
c-erb B-2 RESULTS		Date	Date	Date	
WEIGHT (±)		Slim	Normal	Obese	

ELISA p53 Auto-antibody
Well position, patient and controls

	Patient No	Index	OD: Well 1/OD: Well 2	Mean	Valuation
1	1	-.065	.011/		NEG
2	2	+.706	.233/.192	.213	POS
3	3	-.046	.016/.016	.016	NEG
4	4	+.034	.038/.035	.037	POS
5	5	-.038	.017/.018	.018	NEG
6	7	-.080	.009/.004	.007	NEG
7	8	-.050	.012/.017	.015	NEG
8	9	-.038	.016/.019	.018	NEG
9	10	-.095	.003/.002	.003	NEG
10	12	-.095	.001/.005	.003	NEG
11	13	-.046	.018/.014	.016	NEG
12	21	-.073	.008/.010	.009	NEG
13	22		.026/.030	.028	R
14	25	-.057	.013/.013	.013	NEG
15	29	-.076	.005/.010	.008	NEG
16	30	-.065	.005/.017	.011	NEG
17	31	-.069	.010/.009	.010	NEG
18	32	-.092	.003/.005	.004	NEG
19	33	-.073	.006/.011	.009	NEG
20	36	-.065	.013/.008	.011	NEG
21	40	-.080	.008/.006	.007	NEG
22	42	+.847	.256/.243	.250	POS
23	43	-.076	.007/.008	.008	NEG
24	44	-.095	.001/.005	.003	NEG
25	46	-.069	.011/.009	.010	NEG
26	47	-.099	.005/.008	.002	NEG
27	48	-.050	.013/.016	.015	NEG
28	49	-.046	.016/.016	.016	NEG
29	52	-.092	.005/.003	.004	NEG
30	60	-.065	.013/.008	.011	NEG
31	61	-.034	.013/.024	.019	NEG
32	64	+.115	.057/.059	.058	POS

33	70	-.099	.004/.000	.002	NEG
34	73	-.057	.013/.013	.013	NEG
35	74	-.057	.010/.015	.013	NEG
36	75	+.328	.112/.115	.114	POS
37	78	-.061	.014/.010	.012	NEG
38	79	-.087	.004/.014	.005	NEG
39	81	-.057	.013/.012	.013	NEG
40	83	-.084	.004/.008	.006	NEG
41	86	-.095	.004/.002	.003	NEG
42	88		.017/.046		R
43	89		.029/.016		R
44	90	-.053	.014/.014	.014	NEG
45	91	-.080	.009/.005	.007	NEG
46	92				NEG
					NEG

ELISA p53 Auto-antibody
Breast Cancer Control Group

	Control No	Well 1 : No.	Well 2 : No.	Well 1	Well2	
1	1	H15	G14	.032	.012	NEG
2	2	F13	E12	.039	.042	NEG
3	6	D11	C10	.039	.044	NEG
4	9	B9	A8	.008	.011	NEG
5	17	H23	G22	.039	.032	NEG
6	21	F21	E20	.040	.042	NEG
7	22	D19	C18	.017	.021	NEG
8	24	B17	A16	.004	.002	NEG
9	28	H31	G30	.029	.024	NEG
10	30	F29	E28	.005	.011	NEG
11	34	D27	C26	.017	.020	NEG
12	35	B25	A24	.055	.054	NEG
13	36	A39	G38	.011	.013	NEG
14	39	F37	E36	.046	.039	NEG
15	40	D35	C34	.017	.020	NEG
16	41	B33	A32	.043	.048	NEG
17	42	H47	G46	.024	.019	NEG
18	54	F45	E44	.009	.018	NEG
19	60	D43	C42	.024	.031	NEG
20	50	B41	F140	.025	.008	NEG
21	71	H55	G54	.056	.037	NEG
22	59	F53	E52	.029	.039	NEG
23	69	D51	C50	.034	.36	NEG
24	87	B49	A48	.027	.023	NEG
25	88	H63	G62	.031	.032	NEG
26	89	F61	E60	.019	.025	NEG
27	91	D59	C58	.028	.031	NEG
28	92	B57	B56	.055	.052	NEG

29	95	H71	G70	.059	.031	NEG
30	96	F69	E68	.036	.038	NEG
31	98	D67	C66	.032	.036	NEG
32	100	B65	A64	.032	.028	NEG
33	103	H79	G78	.035	.036	NEG
34	130	F77	E76	.038	.043	NEG
35	131	D75	C74	.013	.012	NEG
36	132	B73	A72	.028	.019	NEG
37	133	H87	G86	.021	.023	NEG
38	134	F85	E84	.070	.072	NEG
39	135	D83	C82	.027	.034	NEG
40	76	B81	A80	.026	.025	NEG
41	77	A1		.037		NEG
42	82	H95	G94	.069	.063	NEG
43	84	E93	E92	.050	.055	NEG
44	85	D91	C90	.064	.056	NEG
45	87	B89	A88	.032	.029	NEG

MANUAL FAST MODE READINGS

ANTHOS 2001 Fastmode Printout 9-DEC-1997 11:58:34

Meas. Filt: 492 nm

	1	2	3	4	5	6	7	8	9	10	11	12	
A	.411	2.824	OVERFLOW	2.461	2.446	2.824	2.654	2.578	2.500	2.824	2.500	2.703	A
B	.402	2.703	OVERFLOW	2.484	2.469	2.824	2.572	2.553	2.418	2.745	2.509	2.028	B
C	.259	1.112	OVERFLOW	OVERFLOW	OVERFLOW	2.567	2.449	2.873	2.650	2.407	2.854	1.343	C
D	.258	1.090	OVERFLOW	OVERFLOW	OVERFLOW	2.301	2.409	2.785	2.572	2.572	2.745	1.175	D
E	.213	.878	2.286	2.495	OVERFLOW	2.585	2.266	2.495	2.638	2.770	2.538	2.662	E
F	.204	.864	2.272	2.523	OVERFLOW	2.578	2.341	2.498	2.631	2.745	2.569	2.608	F
G	.193	.679	2.585	2.526	2.854	2.638	2.770	2.699	2.616	2.854	2.754	2.983	G
H	.048	.710	2.602	2.373	2.824	2.658	2.790	2.759	2.582	2.824	2.745	2.943	H
	1	2	3	4	5	6	7	8	9	10	11	12	