SOLUBLE c-erbB-2 FRAGMENT IN SERUM CORRELATES WITH DISEASE STAGE AND PREDICTS FOR SHORTENED SURVIVAL IN PATIENTS WITH EARLY STAGE AND ADVANCED BREAST CANCER.

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PREFACE

Breast cancer is a major health problem, afflicting up to 1 in 9 women in developed countries with Western diet and life style. While screening programs have led to earlier diagnosis, including diagnosis at a pre-invasive stage in a number of women, the majority of patients with breast cancer still present with clinically detectable, invasive breast cancer, which even if clinically localised still carries the risk of systemic micrometastases. Such patients have been shown to benefit both in terms of disease free as well as of overall survival from the addition of adjuvant systemic treatment.

The identification of progostic factors which can be used to tailor specific forms of adjuvant treatment to the patient's disease has been an important goal of breast cancer research during the last 20 years. A particularly important goal is the early identification of poor risk patients, who may benefit from aggressive intervention with intensive chemotherapy.

While many prognostic markers, including nodal status, hormone-receptor-status, ploidy and growth fraction and the expression of various oncogenes and proto-oncogenes by the tumor cells have been proposed as prognostic factors, the results, to date, have been equivocal for a number of these. Recently there has been much interest in the prognostic importance of c-erbB-2 protein in breast cancer. Most of these studies have concentrated on immunohistochemically staining the c-erbB-2 in tumor tissue. This dissertation focusses on the prognostic impact of the soluble c-erbB-2 protein in the serum of breast cancer patients treated

at the Breast Clinic of the Johannesburg Hospital and University of the Witwatersrand. The results of this investigation have been reported under the title "Soluble c-erbB-2 fragment in Serum Correlates with Disease Stage and Predicts for Shortened Survival in Patients with Early Stage and Advanced Breast Cancer" by H. Kandl, L. Seymour & W.R. Bezwoda. Published in British Journal of Cancer, Vol 70 p739-742, 1994.

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1. AIMS AND SCOPE OF THIS DISSERTATION

Historically prognostic factors have been used to classify patients into subsets with different outcome in order to help make treatment decisions for individual patients. Studies of prognostic factors in breast cancer have, moreover, helped to identify biologic factors which may be of fundamental importance in the pathogenesis and progression of malignant disease. A topic which has received considerable attention, both as a prognostic factor as well as a factor modulating the growth and behaviour of breast cancer is the c-erbB-2 oncogene and its protein product, which has been identified as a putative growth factor receptor.

The aim of this dissertation is to review the biology and prognostic import of c-erbB-2 in the context of the hormone-growth factor/receptor-oncogene nexus in breast cancer. The general review is followed by the presentation of the results of a study of the significance of soluble c-erbB-2 in the serum of patients with breast cancer.

2. PROGNOSTIC FACTORS IN BREAST CANCER

Prognostic factors in breast cancer may be used to:

1) identify very good risk patients, requiring no further therapy.
2) identify very poor risk patients and more specifically to identify those patients whose prognosis is so poor that it is not modified by conventional treatment approaches and where other, more investigative, therapies may be warranted.
3) identify response to specific therapies - these factors might also be termed predictive factors.

2.1 Classification of Prognostic Factors:

There is general agreement that lymph node involvement is the most important prognostic variable in breast cancer.[1-3] While lymph node involvement may be considered to provide the most definitive index of the metastatic potential of the patient's tumour, there are wide variations in prognosis within both the node negative and node positive patient subsets.

Apart from lymph node status a number of other prognostic factors have been found to be of importance in various series. The relative order of importance and the classification of these additional prognostic factors is, however, less clear. The scheme that has been adopted here is to group prognostic factors by type and then to

consider those factors which may indicate a very good prognosis, those which identify very poor risk patients and those which predict for resistance to treatment.

Table 1 shows more than 30 factors which have, usually in more than a single study, been shown to have prognostic importance in breast cancer.

It should, however, be pointed out that a number of these categories overlap since factors initially identified as oncogenes (e.g. c-erbB-2) may function as growth factor receptors, while transforming growth factors such as TGFα could just as readily be classified as oncogene products.

The extensive and ever growing list of factors identified as being prognostic in breast cancer has created the situation where various laboratory based groups compete for samples of decreasing size as patients are diagnosed with smaller and smaller tumors. The proliferation of prognostic factors also appears to have exceeded our ability to apply this mass of data, particularly when good and bad prognostic factors co-exist in the same patient making some rationalisation necessary. What follows is an attempt to give an overview of the more important prognostic factors in breast cancer.

2.1.1 Identification of Good Risk Patients; At the most basic level the TNM classification[3] combines information from tumour size, lymph node status and the presence or

absence of metastases into the simplest prognostic index. However, when the various T and N Categories are combined e.g. into Stage 1 (T1a and T1b, T2, No,Mo) the clinical outcomes are found to be quite heterogenous. A recent NCI consensus conference[4] concluded that patients with node negative tumors < 1 cm in diameter comprise a group within Stage 1 with particularly good prognosis and recommended that these patients not receive adjuvant chemotherapy. While this conclusion is supported by data from the Surveillance, Epidemiology and End Result (SEER) program of the National Cancer Institute (USA)[1] and from Rosen and co-workers[5] it is not clear that all tumors < 1 cm which were only detected by mammographic screening are biologically similar to tumors of the same size that are detected clinically. Indeed studies have shown that the benefit from mammographic screening programs may be confined to patients of specific age groups[6-13] suggesting that tumor behaviour cannot be predicted by a single parameter such as size, even at the phase of the earliest detectable disease.

One of the most successful approaches to the diagnosis of tumors with a particularly good prognosis appears to be the Nottingham Prognostic Index[14,15] which combines tumor stage, lymph node status and histopathologic grade. In a series of 1 629 cases a group (comprising 29 % of the total patient population studied) with small tumors, negative lymphnodes and good histopathologic grades (Grades I and II) were found to have a 15 year survival of 80 %. The 15 year survival of an age matched female

population was 83 %. These results suggest that there would be little place for systemic adjuvant therapy in this subset of patients.

2.1.2 Identification of Poor Risk Patients: Some breast cancer patients recur rapidly after diagnosis despite the addition of conventional adjuvant therapy to the primary treatment modality.[15] Patients with 10 or more lymph nodes involved at the time of diagnosis are at particularly high risk of dying of breast cancer despite the use of conventional dose adjuvant chemo- or chemo-hormone therapy. Such patients might well be candidates for experimental treatment approaches, such as high dose chemotherapy with hematologic supportive procedures. While this group is fairly readily identifiable, it includes only a minority (approximately 9 %) of patients with clinically localised breast cancer.[17]

Using multivariate models such as the Cox proportional hazards model[18] a number of groups have defined additional patient subsets, many with fewer than 10 positive nodes, that had varying grades of poor prognosis or even a prognosis as poor as that of patients with 10 or more nodes positive. The factors other than lymph node involvement which appear to most potently predict a particularly poor prognosis include tumor size, S-phase fraction and estrogen (ER) and progesterone (PR) receptor levels and patient age.[19-25] In other studies factors such as EGF-R[26] and c-erbB-2[27-29] overexpression have been found to be equally, if not more important

prognostically; or to interact with or negate some of the other apparently good prognostic factors (see Pg. 22 below).

Table 1: Prognostic Factors in Clinically Localised Breast Cancers

1) Patient Factors:
   Age
   Race?

2) Pathologic Indices:
   Nodal involvement (N category of TNM classification)
   Tumour size (T catefory in TNM classification)
   Histologic subtype
   Histopathologic grade
   Angio-invasion
   Ploidy

3) Markers of Proliferation:
   Thymidine labelling
   S-phase fraction
   Ki-67
   PCNA/cyclin
   Topoisomerase II
   Histone H3
   Thymidilate synthetase

4) Oncogenes/Tumour Supressor Genes:
   c-erbB-2/HER-2/neu
   int-2
   c-myc
   ras
   p53
   RB (retinoblastoma gene)

5) Hormone receptors, Growth Factors/Receptors:
   ER (estrogen receptor)
   PR (progesterone receptor)
   EGF-R (epidermal growth factor receptor)
   ILGF/R (insulin like growth factors/receptor)
   TGF's (transforming growth factors)

Table 1: Prognostic Factors in Clinically Localised Breast Cancers (Continued)

6) Invasion Related Factors:
Cathepsin D
UPA/PA-I (plasminogen activator)
Stromelysin-3
Angiogenesis factor

7) Miscellaneous:
p52
NM23
Heat shock protein

8) Markers of Drug Resistance:
c-erbB-2 co-expression
P-glycoprotein ?

2.2 Markers of Drug Resistance:

The best known factor predicting for response to therapy in breast cancer is the estrogen receptor (ER) status of the tumor.[30-32] It should be noted, however, that the prediction is mostly a negative one, in that negative receptors reliably define a group of patients (at least in the setting of advanced disease) who have a minimal chance of response to hormonal treatment, while patients with ER+ tumors generally have only an approximately 50 % chance of response to hormone therapy, although response rate is dependant on level of ER.[31,32]

Identification of patients who will not respond to conventional dose chemotherapy is also of considerable clinical importance.

Recently there has been considerable interest in the membrane associated multidrug resistance gene (mdR-1) product, p-glycoprotein, as a marker of resistance to chemotherapy in breast cancer. Studies in breast cancer have, however, shown conflicting results so far[33-35] and P-glycoprotein expression cannot be considered to be an established prognostic or even predictive factor in breast cancer.

2.3 The Integration of Various Prognostic Factors in Breast Cancer:

One of the challenges for the future will be the integration of the various factors listed in Table 1 into a usable prognostic scheme. While one would expect the prognosis

of patients in whom a particular prognostic factor has not been determined to be distributed randomly between that of patients for whom the factor has been determined to be present and that of patients in whom it was not detected, this is in fact not always so. For example, Clark and co-workers[16] (Table 2) have shown that when considering the combination of c-erbB-2 and ploidy as a maker of prognosis there appears to be some sort of interaction which results in a non-random effect on survival.

While there are a number of reasons why a particular prognostic factor investigation may not be able to be done in a specific instance, one of these must surely be the practical limitation of only having a finite amount of tissue available or acessable for investigation. These considerations illustrate the potential importance of using ancillary investigations such as blood tests in attempting to derive as much prognostic information as possible. This aspect will be dealt with later (chapter 4) in relation to the potential utility of serum soluble c-erbB-2 estimation in patients with breast cancer.

It should also be pointed out that a rational understanding of the importance of prognostic factors depends on undertanding the mechanisms by which these factors influence disease biology. A brief sketch of the action of hormones and growth factors (Chapter 3) and more specifically of the mechanism of action of the protein

encoded by the c-erbB-2 gene (see Pg. 17-23) is presented in the following sections.

Table 2: Prognostic Factors in Breast Cancer: Interaction of c-erbB-2 Expression and Tumour Ploidy on Disease Free Survival

c-erbB-2 expression	Tumor Ploidy	5 year Disease Free Survival
Absent	Diploid	89 %
Increased	Aneuploid	50 %
Unknown	Unknown	70 %
Absent	Unknown	70 %
Unknown	Aneuploid	70 %
Unknown	Diploid	70 %

After Clark GM. Breast Cancer Res. Treat. 25: 31-34, 1993

If disease free survival of patients in whom prognostic factor information was missing were randomly distributed, the expectation would be that the unknown groups form a continuum between the best (c-erbB-2 negative, diploid tumours) prognostic group (5 year DFS 89 %) and the worst (c-erbB-2 positive aneuploid) group (5 year DFS 50 %). While this appears to be so when both factors are unknown, determination of one factor (either c-erbB-2 or ploidy, alone) does not serve any discriminatory function.

3. HORMONAL - GROWTH FACTOR INTERACTIONS IN BREAST CANCER

Based on studies with the MCF 7 breast cancer cell line, a cell line which displays hormone receptors and which is responsive both to growth stimulation by oestrogens and to growth inhibition by tamoxifen, Lippman and co-workers first showed a relationship between hormones and growth factors in breast cancer.[36,37]

Growth factors are small peptides of between 10 - 20,000 daltons produced by a range of cells and tissues which serve to regulate growth and differentiation.[38-51] A number of these factors (Table 3) have been isolated and purified and more recently genes coding for these factors have been cloned. Aberrant expression of growth factors or insertion of genes for growth factors into cells in an unregulated fashion was, from the inception of this field of research, considered to be a possible mechanism for stimulation of tumour growth.

Growth factors were also shown to have specific cellular receptors.[43-46] The interaction of growth factor and receptor serving as a signal for cellular responses, most prominent among them being cell growth. Aberrant receptor expression or receptor like function may also be a stimulous to tumour growth.

A number of growth factors or their receptors have been identified as products of

oncogenes - those genes thought initially to be specifically involved in the genesis of cancer, but now increasingly being recognised as important cellular control genes in normal cells.

Table 3: Growth Factors, Source and Target Cell Population

Name	Source	Target Cell
Platelet Derived Growth Factor (PDGF)	Human Platelets	Fibroblasts Glial ceils Smooth muscle cells
Epidermal Growth Factor (EGF)	Mouse submaxillary gland	Fibroblasts Epithelial cells Epidermal cells
Nerve Growth Factor (NGF)	Mouse submaxillary gland	Sympathetic nerve Endothelial cells Chondrocytes
Transforming Growth Factors TGFα TGFß	Transformed cells Solid neoplasms Human placenta Human platelets	Transformed cells Fibroblasts Epithelial cells

Not just stimulation of growth of pre-existing tumours but indeed tumour like behaviour in otherwise normal cells can be demonstrated as a result of the action of certain growth factors.[44-48,50,51] This property lead to the definition of the so-called transforming growth factors.

Growth factors (including transforming growth factors) may also exert negative effects and inhibit growth of specific types of cell. The functional expression of growth factor effects is influenced both by the type of receptor and the cell or tissue under consideration.[48-50]

In breast cancer, the initial model proposed by Lippman and co-workers was that hormonal effects on growth of MCF 7 cells were indirect, by stimulating the elaboration and release of other, either growth stimulatory or growth inhibitory factors. In the MCF 7 cell line, TGFα (induced by estrogens) acts as a positive growth signal while TGFß (which is upregulated by tamoxifen) appears to act as a negative growth factor (refs) (Fig. 1). This model is an example of the wider field of autoregulatory phenomena and autocrine growth stimulation.[49-52]

The definition of c-erbB-2 as a membrane bound growth factor receptor (see section 3.1) appears to be an additional example of autocrine growth regulatory loops which may be important in the control of breast cancer growth. The c-erbB-2

receptor/ligand mechanism appears to be an additional link modulating the interaction of estrogenic hormones and growth factors on breast epithelial cell growth.

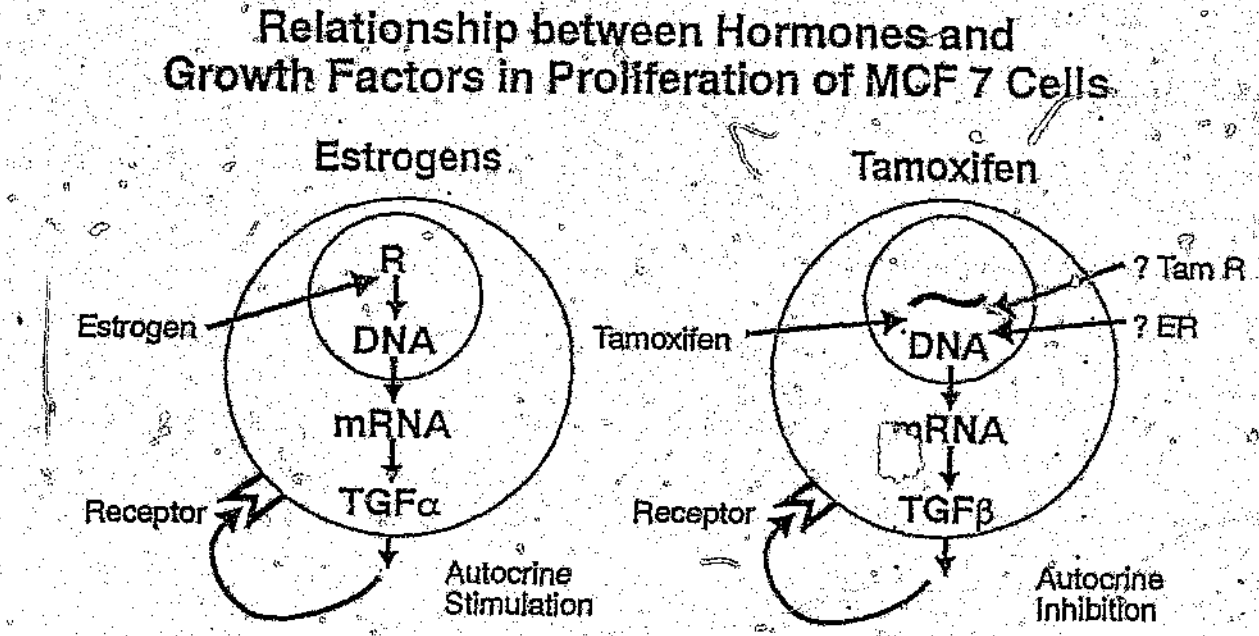

Figure 1: Hormone - Growth factor interactions in breast cancer; based on MCF7 cell line model. Estrogenic stimulation results in production of a positive growth factor TGFα, while tamoxifen administration results in elaboration of an inhibitory growth factor, TGFß.

3.1 c-erbB-2 Protein its Biology and Role as a Growth Factor Receptor:

The c-erbB-2 gene was originally identified in ethylnitrosurea induced rat neuroblastoma cell lines as an activated oncogene called neu.[53,54] In the rat cell lines the oncogenic neu gene product differs from that of the cellular neu gene by a single base change (glutamic acid to valine) in the putative transmembrane region.[53] The human homolog of the rat neu oncogene is the c-erbB-2/HER-2 neu proto-oncogene which is located on band 21q of chromosome 17[54].

The product encoded by the c-erbB-2 (HER-2/neu) gene is a 185,000 Kd protein with an extracellular domain, a transmembrane domain and an intracellular, tyrosine kinase, domain. This protein is termed $p185^{neu}$ or $p185^{erbB-2}$. The structural features, of p185, which are very similar to those of the epidermal growth factor receptor, suggested that c-erbB-2 was likely to be a cellular receptor for an as yet not fully characterised ligand.[55-58]

Further support for a growth regulatory role of c-erbB-2 came from the observation that a monoclonal antibody reactive with the extracellular domain of c-erbB-2 protein exerted a growth suppressive effect on neu transformed NIF/3T3 cells.[59] Monoclonal antibodies directed against the extracellular domain of c-erbB-2, also specifically inhibited the growth of breast-cancer derived cell lines overexpressing the HER/c-

erbB-2 gene product and prevented HER/c-erbB-2 transformed NIH/3T3 cells from forming colonies on soft agar.[60]

3.1.1 c-erbB-2 and the Pathogenesis of Breast-Cancer: By immunohistochemical analysis, staining for c-erbB-2 protein has been observed at low levels in the epithelial cells of most organs in normal human adults, and at slightly higher levels in fetal tissue. The c-erbB-2 gene however is not amplified in these normal tissues, nor specifically in normal breast tissue.[61,62]

Wang and co-workers[63] used retrovirus-vector mediated gene transfer to introduce neu genes into mammary epithelial cells in rats and thereby to induce mammary tumors. In these experiments the neu oncogene was activated by a point mutation, in contrast to to the findings in human breast-cancer where there appears to be overexpression of the normal c-erbB-2 gene. While such gene transfer was able to induce mammary tumors, this effect was abolished by prior oophorectomy suggesting that the mutated neu oncogene product is by itself not sufficient to predispose the transfected cell to undergo full malignant transformation. Other endogenous factors, including estrogens, are required to facilitate this transformation.

Di Fiore and co-workers found that c-erbB-2 needed to be overexpressed some 5 - 10 fold in NIH/3T3 cells in order to be transforming.[64] However, the fact that c-erbB-2 could be transforming merely by being overexpressed, without having the point mutation observed in the neu oncogenes of rat cell lines, suggests that overexpression of the c-erbB-2 gene in breast-cancer could have significance for the pathogenesis of

the disease.

Barnes and co-workers demonstrated positive immunohistochemical staining for the c-erbB-2 protein in the in-situ and in the infiltrating components of mammary carcinomas, strongly suggesting a role for c-erbB-2 gene amplification in the early steps of the pathogenesis of breast-cancer.[65] Important further evidence supporting the role of the c-erbB-2 in the pathogenesis of breast-cancer was the finding that out of 149 benign breast lesions examined none demonstrated c-erbB-2 overexpression.[61]

3.1.2 Proposed Ligands of the p185 neu Extracellular Domain: Huang and co-workers partially purified a neu protein specific activating factor (NAF) that was able to stimulate the tyrosine specific kinase activity of the neu protein ($p185^{neu}$).[66] Exposure of cells expressing of $p185^{neu}$ to NAF resulted in dimerization and internalization, of the neu protein as well as increased cell growth. These effects of NAF were mediated by an interaction with the extracellular domain of $p185^{neu}$. NAF appeared to be $p185^{neu}$ specific and does not cross react with the epidermal-growth-factor-receptor.[67]

Lupu and co-workers purified two potential ligands for $p185^{erbB-2}$ from the hormone responsive MCF-7 breast cancer cell line.[68,69] A 30 Kd peptide, gp30, stimulated phosphorylation of $p185^{erb-2}$ in cells overexpressing c-erbB-2 and also bound to the

epidermal-growth-factor-receptor[69]. Another ligand, p75, a 75 Kd protein also bound to the $p185^{erb-2}$ and increased tyrosine phosphorylation by 10 - 20 fold. p75 did not, however, induce tyrosine phosphorylation of the epidermal-growth-factor-receptor.[70] The effect of p75 on $p185^{erbB-2}$ tyrosine phosphorylation was inhibited by the presence, in the growth medium, of soluble fragments of the extracellular domain of the c-erbB-2 molecule. This inhibitory effect could, however, be reversed by the addition of maximally growth stimulating doses of p75.

The evidence suggests that cells overexpressing the c-erbB-2 oncoprotein also secrete one or other of its ligands, which are required for proliferation, and in addition that such cells may release soluble c-erbB-2 fragments, implying the existence of an autocrine loop, with a complex pathway relulating $p185^{c\text{-}erbB\text{-}2}$ function.[67]

3.1.3 The Influence of Estrogens on c-erbB-2 Signalling Pathways: Using the c-erbB-2 expressing, hormonally dependant BT474 cell line as a model to study the interactions of estrogens and c-erbB-2 and its ligand Lupu and Lippman[71] found that both estrogen (10^{-9} M) exposure and exposure to high concentrations of gp30 (2 mg/ml) resulted in down regulation of ER concentration. While estrogen exposure increased PR (progesterone) receptor concentration by some 2.5 fold, gp30 had no effect on PR. When estradiol and gp30 were added simultaneously, gp30 at low concentrations blocked the down-regulation of ER by estradiol.

Incubation of BT474 cells with estradiol also resulted in a decrease of c-erbB-2 expression by approximately 45 %, whereas treatment with gp30 (2 mg/ml) did not significantly change c-erbB-2 levels. However, this concentration of gp30 completely abrogated the down regulating effect on c-erbB-2 induced by 17 ß-estradiol.

Since estrogens are growth stimulatory through reaction with ER in this cell line, but at the same time estrogens down regulate ER, the implication appears to be that the interaction of c-erbB-2 ligand (gp30) with its receptor (membrane bound c-erbB2) may be a mechanism whereby estrogenic effects are enhanced or perpetuated instead of being abrogated or autoregulated by simultaneous receptor down regulation.

3.1.4 Correlation of c-erbB-2 with Histological Subtypes of Breast-Cancer:

Immunohistochemically stainable c-erbB-2 protein was initially observed in 44/79 (59 %) cases of ductal-carcinomas-in-situ but in none of 48 lobular carcinomas in-situ.[63] Van de Vijiver and co-workers[72] described a significantly higher incidence of c-erbB-2 overexpression in the large cell-comedo type ductal carcinoma-in-situ as compared to invasive ductal carcinoma, suggesting either that the invasive ductal carcinomas that are c-erbB-2 positive are derived from a specific type (large-cell-comedo type) of ductal carcinoma-in-situ, or alternatively that c-erbB-2 expression may be lost in a proportion of cases during tumor invasion and progression. Evidence supporting the first theory was provided by Maguire and co-workers[73] who found that tumors

with c-erbB-2 negative in-situ components had immunonegative invasive components, while tumors with immunopositive in-situ components had immunopositive invasive ductal carcinomas. It should be pointed out, however, that the immunostaining was frequently more intense in the in-situ components, than in the invasive carcinomas and the question of the association c-erbB-2 expression and histologic type of cancer has not been fully resolved. A problem is the apparent contradiction of finding a high frequency and lower intensity of intense staining for c-erbB-2 in the least aggressive form of breast cancer (i.e. carcinoma in-situ) on the one hand, while in invasive carcinomas there appears to be a lower frequency of c-erbB-2 staining, but when it is present it appears to indicate a poor prognosis.

3.1.5 c-erbB-2 Protein Expression and the Treatment of Breast-Cancer: Overexpression of c-erbB-2 protein may be a useful marker to identify patients who are most likely to benefit from high dose adjuvant chemotherapy. Tumors with overexpression of the c-erbB-2 oncogene were found to be less responsive to CMF (cyclophosphamide, methotrexate, 5-fluoro-uracil) adjuvant chemotherapy regimens than those with a normal amount of gene product[74]. Muss and co-workers[75] demonstrated that patients with node positive breast-cancer with tumors that overexpressed c-erbB-2 had a significantly longer disease free interval and overall survival when treated with a high dose anthracycline containing adjuvant chemotherapy regimen (cyclophosphamide, doxorubicin, fluoro-uracil) as compared to those treated with conventional doses of the same chemotherapy. There was no such dose response effect in patients with minimal or no c-erbB-2 expression. An explanation for this finding might be that breast-cancer cells overexpressing c-erbB-2, frequently also overexpressed topoisomerase IIα, a target enzyme for doxorubicin.[76]

Patients with tumors that are positive for estrogen receptors derive the greatest benefit from endocrine therapy.[77] c-erbB-2 overexpression may be a marker for breast-cancers that are not responsive to endocrine manipulation, despite being positive for estrogen receptor. In a study by Klijn and co-workers[78], responsiveness of breast-cancer to endocrine therapy was associated with low or absent c-erbB-2 expression. These findings were confirmed by Wright and co-

workers[79], who found that patients with c-erbB-2 positive breast-cancers had a significantly poorer response to endocrine therapy than did those whose tumors were c-erbB-2 negative. Co-expression of c-erbB-2 reduced the response rate of estrogen-receptor (ER) positive patients from 48 % to 20 %, and of ER-negative cases from 27 % to 0 %.

Co-expression of epidermal-growth-factor-receptor and c-erbB-2 also has a negative effect on response to endocrine therapy.[79,80] None of the epidermal growth factor positive and c-erbB-2 positive tumors responded to hormone treatment.

A possible therapeutic approach to treating breast-cancer was suggested by experimental models using monoclonal antibody against the extracellular epitope of the c-erbB-2 protein. In in-vitro investigations the use of such monoclonal antibodies enhanced the cytotoxicity of cisplatin against human breast- and ovarian cancer cell lines overexpressing c-erbB-2 protein. Athymic mice bearing subcutaneous xenografts of human tumor cells expressing high levels of $p185^{c\text{-}erbB\text{-}2}$ showed a greatly enhanced inhibition of tumor growth when treated with cisplatin together with the monoclonal antibody compared to treatment with either the antibody or cisplatin alone. This effect was specific in as much as the antibody did not enhance the growth inhibitory effect of cisplatin on tumor xenografts not expressing $p185^{c\text{-}erbB\text{-}2}$.[81]

3.2 The Soluble Extracellular Domain Fragment of c-erbB-2 and its Biology:

Soluble forms of a number of membrane receptors have been demonstrated in cell free supernatants obtained from a variety of cell lines and also in human serum. These include soluble forms of the insulin receptor,[82] of the epidermal growth factor receptor[83] or and of the receptor for interleukin-2[84]. The quantitation of soluble IL-2 receptors by enzyme-linked immunosorbent assay (ELISA) in human serum has been shown to reliably correlated with disease activity in a number of autoimmune and inflammatory disorders, in transplantation rejection, and also in a number of infectious diseases. Soluble IL2 receptor levels also appear to reflect tumor burden and response to therapy in human T-lymphotropic-retrovirus-type I associated adult-T-cell-lymphomas and in hairy cell leukemia.[84] The c-erbB-2 fragment found in serum and malignant effusion of patients with breast and other cancers joins the growing list of soluble receptor molecules or fragments the biology of which is only beginning to be investigated.

Mori and co-workers[85] developed an enzyme-linked immunosorbent assay to measure soluble c-erbB-2 protein in culture supernatants of various human cell lines and in sera of patients suffering from recurrent breast-carcinomas. These workers found a good correlation between soluble c-erbB-2 protein as measured by ELISA and immunohistochemical staining of the corresponsing tumors for c-erbB-2. The study sample was, however, small (3 samples seropositive/2 samples

immunohistochemically positive for the c-erbB-2 protein). A soluble, immunoreactive form of the c-erbB-2 receptor has also been detected in the sera of nude mice bearing SK-OV-3 ovarian cancer xenografts. Antigen levels correlated with both overexpression of the tissue c-erbB-2 protein and with tumor volume.[86]

Narita and co-workers[87] detected markedly elevated soluble c-erbB-2 levels in serum of patients with breast carcinoma with distant metastasis, suggesting that serum c erbB-2 levels may reflect tumor burden. This study also showed a correlation between soluble c-erbB-2 in serum and the presence of immunohistochemically stainable c-erbB-2 in tumor cells, but again only small numbers of patients and only patients with stage 3 and stage 4 breast-cancers were investigated.

All these studies taken together, however, suggest that measurement of serum soluble c-erbB-2 may be useful in the assessment of patients with breast cancer. Before dealing with the measurement of serum soluble c-erbB-2 in more detail, some consideration must be given to the questionof the source of the soluble c-erbB-2 fragment in serum and the possible physiologic role of such soluble recpetor molecules.

3.2.1 Proposed Mechanisms of Release of the Soluble c-erbB-2 Protein Fragments: Soluble c-erbB-2 found in serum has been shown to be a fragment of the complete c-erbB-2

protein molecule consisting of the truncated extracellular domain of c-erbB-2. As previously noted this soluble fragment retains ligand binding properties which may have important patholphysiologic consequences. Such soluble fragments may be produced in one of a number of ways.

Soluble c-erbB-2 could be produced by post-translational processing of the full length c-erbB-2 gene by cell surface proteolysis.[88] Such a mechanism of production has been suggested for the soluble form of the IL-2 receptor.

Alternatively a truncated m-RNA could code for the soluble extracellular domain of c-erbB-2 that is found in serum. Transfection experiments have shown that truncated mRNAs can be translated into secreted proteins. Expression of a truncated c-erbB-2 mRNA transcript in COS-1 cells results in the production of both secreted and of intracellular forms of the extracellular domain of c-erbB-2. Such, transcriptionally generated truncated c-erbB-2 fragments were concentrated within the perinuclear cytoplasm of the cells without membrane expression of c-erbB-2.[89]

While these studies show that truncated or alternatively spliced mRNAs could be responsible for the generation of soluble c-erbB-2 fragments, it has not yet been determined whether human breast cancers express or co-express such differing or alternatively spliced mRNAs and the mechanisms involved in the production of the

soluble c-erbB-2 fragment found in the serum of patients with breast cancer has not been elucidated.

3.2.2 Possible Physiologic Functions of the Soluble Fragment of c-erbB-2: The soluble form of human c-erbB-2 could function as either;

a) a potential antagonist of normal cell-surface-receptor ligand interaction.

or

b) as a carrier for the transportation, shielding or destruction of ligand.

Transfection studies indicate that excess production of c-erbB-2 extracellular domain in human tumor cells overexpressing full length c-erbB-2 receptor can result in resistance to the growth inhibitory effects of anti-c-erbB-2 monoclonal antibodies.[89] These findings may imply that soluble c-erbB-2 could modulate the interaction of membrane bound c-erbB-2 and its ligand.

While such putative functions have been ascribed to the soluble extracellular domain, c-erbB-2 fragment it could also be a non-functional byproduct of proteolytic action which appears to be important in the normal process of receptor-down-regulation.[88] However, even if the soluble c-erbB-2 fragment was the result of proteolytic degradation, c-erbB-2 overexpression and release of soluble c-erbB-2

fragments may still be important both physiologically and prognostically. Proteolytic degradation would normally result in receptor down regulation with release of only small amounts of soluble fragments. When c-erbB-2 is over amplified and overexpressed, proteolytic degradation might be insufficient to abrogate receptor function but yet result in increased release of soluble fragments with elevated serum levels.

Whether the soluble c-erbB-2 fragment found in the serum of patients with breast cancer has any in-vivo pathophysiologic function or is merely an indication of disease bulk and/or membrane turnover has not yet been established.

4. SOLUBLE C-ERBB-2 FRAGMENT IN SERUM CORRELATES WITH DISEASE STAGE AND PREDICTS FOR SHORTENED SURVIVAL IN PATIENTS WITH EARLY STAGE AND ADVANCED BREAST CANCER.

4.1 Aims:

The aim of the present study was to evaluate the prognostic significance of serum soluble c-erbB-2 in patients with breast cancer.

4.2 Material and methods:

Blood samples were obtained from 79 patients attending the Breast Clinic of the Johannesburg Hospital between 1986 and 1993. Serum was stored at -20°C until assay. Sampling was performed at the time of diagnosis of recurrent or metastatic disease. The 110 Kd, serum, soluble c-erbB-2 fragment was measured using a serum c-erbB-2, enzyme-linked-immunoassay kit (Triton Diagnostics, Alameda, California). Briefly, monoclonal anti-c-erbB-2 antibody conjugates were added to aliquots of serum, incubated for 2 hours, followed by addition of linking solution and then chromogen-substrate. Absorbance was read in a spectrophotometer at 450 nm. Control and calibrator samples were run with each assay. Controls included samples from 24 healthy women falling into the same age range as the patients with breast cancer. The amount of c-erbB-2 protein was calculated from a standard curve.

Results are expressed as u/ml of serum. Serum levels ≥ 10 u/ml were deemed positive. This level was chosen as being 2 standard deviations above mean for healthy women and was also the upper limit for the negative controls supplied with the kit. The antibody has no significant cross reactivity with epidermal growth factor (EGF), and reacts only with the external domain of the c-erbB-2 molecule. Western blotting of samples with elevated levels confirmed the presence of a 100 kd protein in serum which showed reactivity with this antibody.

Estrogen receptor status (ER) and tissue c-erbB-2 were also determined, where suitable specimens were available. ER was measured using the ERICA kit (Abbott Laboratories) kit method according to the manufacturers instructions. Tissue c-erbB-2 determination was by means of immunohistochemistry using a monoclonal antibody to the external domain of c-erbB-2 (Triton Diagnostics) and a standard avidin-biotin procedure. Briefly, endogenous peroxidase was blocked using methanolic peroxide, and then blocking antibody, primary and control antibodies, secondary antibody, ABC (Vectastain) and DAB were layered on sequentially. Specimens were deemed positive if clear membrane immuno-staining was observed. Suitable positive and negative controls were incorporated into each assay procedure.

4.2.1 Statistical analysis: Disease free survival, overall survival, and survival from disease progression were analyzed using SAS statistical software. Additional variables

analysed included age, sex, site of disease, initial stage of disease, menopausal status and ER status (where available). Survival curves were generated using the method of Kaplan and Meier, and were compared using the log-rank statistic.

4.2.2 Patient Characteristics: Forty-four out of 79 (56 %) patients were postmenopausal at time of diagnosis. The mean age at presentation was 51.4 ± 13.1 (range 24 -85) years. Further patient characteristics are shown in Tables 1 and 2.

4.2.3 Ethical considerations: All patients gave informed consent prior to entry into the study. The study was approved by the Committee on Ethics of Human Research of the University of the Witwatersrand and was carried out in accordance with the principles of the Declaration of Helsinki.

4.3 Results:

4.3.1 Serum soluble c-erbB-2 levels: Serum levels of the soluble fraction of c-erbB-2 ranged from 2-278 with a mean of 35 ± 57.6 u/ml. Intra and interassay variation was < 2 %. Intra-patient variation of serum c-erbB-2 levels, when levels were tested in blood samples from 17 patients who had clinically stable disease and who had 2 or > seperate blood samples taken at intervals of 14 to ≤ 42 days was also < 2 % .

39 patients (49 %) had serum soluble c-erbB-2 levels of ≥ 10 u/ml. There was no correlation between the presence of elevated serum soluble c-erbB-2 level and menstrual status (p=0.66) [Table 5].

There was a significant correlation between serum level and the type of treatment chosen for patients with stage 4 disease [27 of 42 (64 %) patients receiving chemotherapy were seropositive compared to 11 of 32 (34 %) receiving hormonal therapy] (Table 6). There was no correlation between the presence of raised serum soluble c-erbB-2 level and any specific site of relapse.

4.3.2 Soluble c-erbB-2 and tissue c-erbB-2 immunostaining: 24 patients had contemporaneous tumour tissue and serum samples available for c-erbB-2 determination. Tissue immunostaining did not correlate with the presence of soluble c-erbB-2 in serum (Table 7). The presence of positive tissue immunostaining had no impact on overall survival, time to relapse, or on survival from progression (p = 0.26).

4.3.3 Soluble c-erbB-2 estrogen receptor levels: There was no significant correlation between estrogen receptor expression among 37 patients of known receptor status, and serum soluble c-erbB-2 levels (p=0.6) [Table 8].

4.3.4 Soluble c-erbB-2 and survival and time to relapse: The median overall survival of this cohort of patients from time of initial diagnosis was 44 ± 7.4 months (range 1 - 254 months). Among the 74 patients who either presented with or who had progressed to stage 4 disease, the median survival time from progression was 19 ± 24.7 months (range 1 - 158). The presence of soluble c-erbB-2 fragment in serum at the time progression was diagnosed had a significant impact on overall survival of these patients. Seropositive patients had a median survival of 21 months vs 64 months for seronegative patients ($p = 0.03$) Fig. 2. The prognostic impact of soluble c-erbB-2 on survival was lost when the analysis was confined to ER positive patients, possibly due to the low number of such patients.

4.3.5 Soluble c-erbB-2 and response to therapy: Serum soluble c-erbB-2 had no influence on response to either initial ($p = 0.64$) [Table 9] among 74 patients assessable for treatment response. Nor was there any relation between c-erbB-2 and response to salvage treatment for stage IV disease ($p = 0.78$).

4.4 Discussion

The c-erbB-2 protein is a 185kD transmembrane protein with tyrosine kinase activity. It comprises both an extracellular and an intracellular domain. While the extracellular domain has ligand binding activity, the ligands have yet to be clearly defined but is thought to act as a growth factor receptor.[52-59,66-70] Antibodies to c-erbB-2 have been shown to inhibit both anchorage dependent and anchorage independent growth in-vivo.[91]

C-erbB-2 has been found to be amplified in 20-30% of primary breast cancers, and gene amplification correlates with oncoprotein overexpression. C-erbB-2's impact on prognosis is, however, somewhat controversial. A number of investigators have reported a correlation between c-erbB-2 amplification and survival in node positive primary breast cancer.[92,93] However, both Zhou and co-workers[94] and Toikkanen and co-workers[95] failed to demonstrate any impact of c-erbB-2 expression on survival in node positive patients. Conflicting results have also been reported in node negative patients[96,97]. Allred and co-workers[98] found a highly significant correlation between disease free survival and c-erbB-2 expression, but only in specific subsets of patients (small tumour size, ER positive and no significant in-situ component), so-called 'low-risk patients'. Furthermore, while Gusterson and co-workers[99] found c-erbB-2 immunostaining to have an overall prognostic impact only in patients with node

positive disease, c-erbB-2 positive, node negative patients, receiving adjuvant chemotherapy fared less well, in their study, than those who were c-erbB-2 negative. These findings tended to suggest that whatever influence the presence of c-erbB-2 expression has on the biology of breast cancer, this effect is confined to the earlier clinical phases of the illness.

In addition it has been suggested that c-erbB-2 amplification and protein expression correlate both with poor histological grade and lack of oestrogen receptor (ER) expression.[100,101] Poller and co-workers[102] demonstrated that overexpression of c-erbB-2 is significantly correlated with S-phase and proliferative index in ductal carcinoma in-situ ($p = 0.001$), as well as in early invasive duct carcinoma ($p = 0.04$).

There is also evidence to suggest that c-erbB-2 overexpression may be preferentially associated with certain histological subtypes of breast cancer. Van de Vijver and co-workers[72] described a high incidence of c-erbB-2 overexpression in large cell, comedo-type ductal carcinoma-in-situ as compared to invasive ductal carcinoma, suggesting either that the invasive ductal carcinomas that are c-erbB-2 positive are derived from a specific type (large cell comedo) of ductal carcinoma-in-situ, or that c-erbB-2 expression may be lost during tumour invasion and progression. Evidence supporting the first theory is provided by Maguire and co-workers[73] who found that

while tumours with c-erbB-2 negative in-situ components had immunonegative invasive components, tumours with immunopositive comedo type in-situ components had immunopositive invasive ductal carcinoma. It should be pointed out, however, that the immunostaining was frequently more intense in the in situ components, than in the invasive carcinomas.

Soluble forms of the c-erbB-2 protein have been reported in the serum of patients with breast cancer,[87,103] and in addition have been shown to correlate with disease bulk as well as with tissue overexpression in an animal model.[86] No reports have, however, been published to date on the prognostic significance of soluble c-erbB-2 in patients with breast cancer.

The present study examined a group of 79 women with advanced breast cancer. The method used in this study measured a 100 Kd c-erbB-2 antigen fragment, which is not detected in the serum of normal controls or in patients with benign breast disease[104]. A surprisingly high frequency of elevated serum soluble c-erbB-2 levels was found, possibly due to the fact that this study included mainly patients with aggressive disease, with 25 patients presenting with advanced breast cancer and all but 5 of the remainder having progressed to stage 4 disease. In addition there was a statistically significantly higher incidence of seropositivity in patients given chemotherapy as first line therapy for progressive disease - indicative of the perception that these patients

were suffering from aggressive disease. In addition, the presence of serum soluble c-erbB-2 fragment concentrations of > 10 u/ml had a significant impact on overall survival from diagnosis of metastatic disease.

The lack of correlation between seropositivity and tissue expression of c-erbB-2 raises some interesting possibilities. While the lack of correlation may be due to low sample number, definite tissue staining was demonstrated in 10 of the 24 patients with tumour samples available for examination. This frequency was, again, a relatively high rate of c-erbB-2 expression. While it may be argued that lack of tissue immunostaining is related to the sensitivity of the method, both tissue positive and serum negative as well as tissue negative and serum positive cases were found. Among the serum soluble c-erbB-2 negative patients with negative tissue expression there were 3 patients with extremely high serum levels (range 120 - 160 u/ml) and with extensive disease, while all 4 patients with negative serum soluble c-erbB-2 tissue and positive tissue staining demonstrated strong positive staining and also had extensive disease. Since both the serum and tissue assays were performed using a monoclonal antibody specific for the external domain of c-erbB-2, these findings suggest the possibility that loss of tissue expression may result either from proteolytic cleavage, with release of the external domain and transfer into the blood, rendering the tissue negative to reaction with the antibody used or that the serum component represents an alternatively spliced variant lacking the membrane domain. This question will be

addressed in future studies by using antibodies to both the internal domain as well as to the external domain of the c-erbB-2 molecule.

Whatever the pathophysiologic explanation, assay for soluble c-erbB-2 in serum is a relatively simple test, requiring only a blood sample rather than the availability of tissue. Soluble c-erbB-2 may to offer prognostic information with seropositivity being a predictor of shorter survival in patients with breast cancer.

Table 4: **Serum soluble c-erbB-2: Patient Characteristics and Seropositivity Rate.**

Patient Characteristics			Soluble c-erbB-2				
			Positive		Negative		
	Number	(%)	Number	(%)	Number	(%)	p-value
Menopausal status							
pre	35	(44)	17	(49)	18	(51)	NS
post	44	(56)	22	(50)	22	(50)	
Stage at presentation							
I	4	(5)	1	(25)	3	(75)	
II	31	(39)	11	(35)	20	(65)	p<0.05
III	19	(24)	10	(53)	9	(47)	
IV	25	(32)	17	(68)	8	(32	
ER Status							
positive	15	(20)	7	(47)	8	(53)	
negative	25	(33)	12	(48)	13	(52)	NS
unknown	39	(47)	20	(51)	19	(49)	

Table 5: Serum Soluble c-erbB-2 and Menstrual Status

	c-erbB-2 ≤ 10 u/ml	c-erbB-2 > 10 u/ml	Total
Premenopausal	21	19	40
Post menopausal	21	18	39
Total	42	37	79

Table 6: Serum soluble c-erbB-2: Therapy and Response to Treatment for stage IV Dise

	Soluble c-erbB-2						
	Positive				Negative		
	Number	(%)	Number	(%)	Number	(%)	p-value
Treatment for stage IV disease							
hormonal	32	(42)	11	(34)	21	(66)	$p<0.05$
chemotherapy	42	(48)	27	(66)	15	(34)	

Table 7: **Serum Soluble c-erbB-2 and the Presence of Breast Tumour Tissue Expression of c-erbB-2 Protein**

	Serum soluble c-erbB-2 positive	Serum soluble c-erbB-2negative
Tissue Immunostaining c-erbB2		
Positive	6	4
Tissue Immunostaining c-erbB2		
Negative	6	8

Table 8: **Soluble c-erbB-2 and Estrogen Receptor Status**

	c-erbB-2 ≤ 10 u/ml	c-erbB-2 > 10 u/ml	Total
ER+	12	7	19
ER-	10	8	18
TOTAL	22	15	37

Table 9: **Soluble c-erbB-2 and Response to Systemic Treatmeat for Recurrent/Metastatic Breast Cancer**

	Response to Treatment		
	CR	**PR**	**NR**
c-erbB-2 ≤ 10 u/ml	7	13	16
c-erbB-2 > 10 u/ml	9	11	18
TOTALS	16	24	34

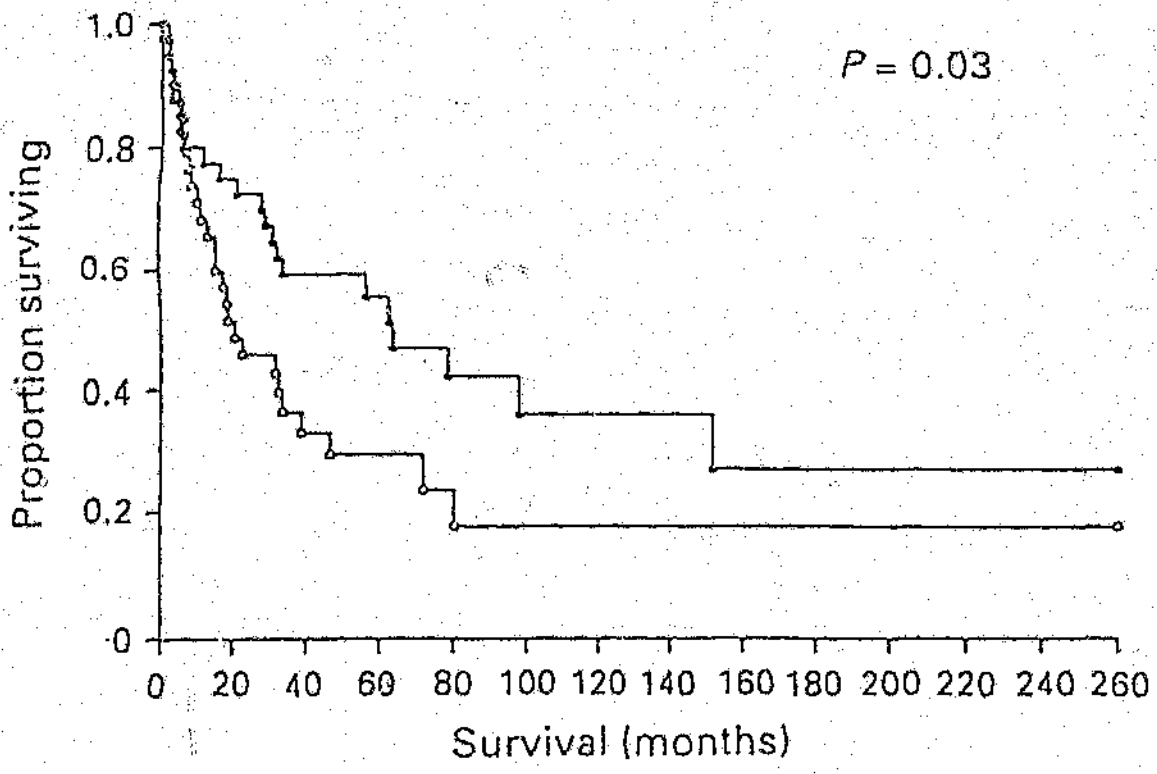

Figure 2: Influence of the presence of soluble c-erbB-2 fragment in the serum on prognosis of patients with breast cancer. Overall survival. ● patients with serum soluble c-erbB-2 < 10 μ/ml; o patients with serum soluble c-erbB-2 ≥ 10 μ/ml.

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