Breast Cancer Biomarkers: Development, Application and Management

Names of authors
Puleng R Matatiele* (PhD: Biochemistry and Molecular Biology)
Wilhelmina MJ van den Heever* (PhD: Haematology and Molecular Biology)
*School of Health Technology, Central University of Technology, Free State, Bloemfontein, South Africa

Authors' Institutional Affiliation and location
Private Bag X20539, School of Health Technology, Central University of Technology, Free State, Bloemfontein, 9300, South Africa

Correspondence to:
Dr PR Matatiele, Private Bag X20539, School of Health Technology, Central University of Technology, Free State, Bloemfontein, 9300, South Africa
Tel: +27 51-507 3853 · Fax: +27 51-507 3354 · Email: pmatatie@cut.ac.za

OR

Dr WMJ van den Heever, Private Bag X20539, School of Health Technology, Central University of Technology, Free State, Bloemfontein, 9300, South Africa
Tel: +27 51-507 3118 · Fax: +27 51-507 3355 · Email: evdheev@cut.ac.za

Abstract
At present, the most useful clinical biomarkers for breast cancer are the oestrogen/progesterone receptors and HER-2. The discovery of cancer biomarkers is currently a major focus of cancer research. Consequently, a large number of potential diagnostic and prognostic biomarkers for breast cancer are now known, although no single marker has so far proven useful as an independent method for breast cancer screening or diagnosis. This can be ascribed to the fact that many of these biomarkers have not yet been assessed in a reliable and reproducible way in order to determine their importance and usefulness in clinical patient management. Discovering biomarkers is a slow process, and numerous obstacles must be overcome in translating them from the research laboratory into clinical use. This paper gives an overview of the application of biomarkers in breast cancer management, the process of biomarker discovery and development and the future prospects regarding biomarker application in breast cancer management.

1. BIOMARKER DISCOVERY AND DEVELOPMENT

1.1 Introduction

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). The National Institute of Health (NIH) Working Group on Biomarkers and Surrogate Endpoints states, furthermore, that biomarkers can be anatomical, physiological, biochemical or molecular parameters, and that they are normally present in small amounts in biological samples such as blood serum, plasma, saliva, urine or other tissues. The expression of these substances has a connection to a person's physiological characteristics, so an alteration in their expression indicates that cancer may be present in the body.

Biomarkers are detectable and measurable by a variety of methods, including physical examination, laboratory assays and medical imaging. Recent advances in comprehensive molecular technology have led to the analysis of global gene expression or protein profiles in cancerous versus normal tissues, with the goal of identifying RNA or protein markers that are expressed differentially between benign and malignant tissues (Volinia *et al*., 2006)**.** Several nucleic acid-based studies have examined samples of normal versus cancerous tissues, established cell lines, and samples from before and after treatment with chemotherapeutic agents for differences in expression in a set of genes (Buchholz *et al*., 2002; Molich *et al*., 2004). Based on these studies, markers have been detected in human serum, breast nipple aspirate fluid and tissue samples (NCI report, 2004). Biomarker research has thus emerged as an exciting tool in disease prevention, particularly in cancer prevention and treatment. To date, many of the investigated markers have actually not yet been assessed in a reliable and reproducible way to determine their importance and usefulness in clinical patient management. As a result, most of the known markers have not been useful as independent screening or diagnostic tools for breast cancer - so much so that, despite such a vast corpus of information, breast cancer diagnosis and staging has not changed significantly over the last 20 years. Nevertheless, biomarkers cannot be totally discarded, as they remain useful as prognostic and predictive factors, directing the overall management of the disease. Therefore biomarker research has not been discontinued and we believe that, due to this continued effort, the integration of biomarkers into clinical care will eventually become a reality. The first part of this review summarises the available information on breast cancer biomarkers with regard to their application in the diagnosis, staging and prognosis of breast cancer, as well as the assessment of breast cancer risk. Reference is also made to the future prospects for biomarker application in breast cancer.

1.2 Types of Biomarkers

Biomarkers serve a variety of purposes in experimental research and clinical settings, namely screening for promising drugs, determining the dosage and scheduling of a drug, predicting the risk of developing disease, predicting a patient's chance of responding to a drug or having an adverse reaction to a drug, or serving as surrogate endpoints in a trial (Nelson, 2006). The growing enthusiasm for using biomarkers for early detection of breast cancer has led to extensive drug discovery and development. The various biomarkers used today reflect their disparate uses in preclinical development (Casey, 2005; Boguslavsky, 2006; Chitty, 2006). The different types of biomarkers are described below:

- *Antecedent biomarkers* are used to identify the risk of developing an illness, and enable identification of people who may be at risk of developing a disease but do not yet exhibit symptoms.
- *Early detection biomarkers* are used to identify disease states in the earliest stages of onset and progression.
- *Diagnostic biomarkers* enable identification of the presence or absence of a particular disease state. They may be used as synonymous with *disease biomarkers,* which are related to clinical outcomes and are measures of disease states.
- *Prognostic biomarkers* are used to determine the survival probabilities of patients, and their presence predicts what the clinical outcome would be if the disease were to be left untreated.
- *Predictive biomarkers* assist in predicting the efficacy of drug therapies in the treatment of disease by enabling prediction of the response of the disease to specific types of therapy. These biomarkers are needed to assess risk and derive safety margins for molecules being brought forward as candidate drugs.
- *Translation biomarkers* are biomarkers that have proved useful in a research setting, and have been translated into standard use in the clinical reference laboratory.
- *Efficacy biomarkers* (also called *surrogate endpoints*) reflect the beneficial effects of a given treatment. These are a special class of extensively evaluated biomarkers that generally correlate with desired clinical outcomes and can be used as a basis for corporate decisions, as well as for obtaining accelerated provisional regulatory approval of a drug. To date, no biomarkers have yet been validated as surrogate endpoints for cancer (Greenwald, 2002).
- *Surrogate biomarkers* are regarded as valid substitutes for measuring clinical outcomes. A surrogate marker may be associated with a specific phase of carcinogenesis, and/or it may be mechanistically linked to the mode of action of the agent being evaluated. In clinical trials, surrogate biomarkers are used to predict the effect of a given agent on the pri-

mary end point of disease incidence. They can also be used on a short-term basis to provide some preliminary insight into the effectiveness of the treatment strategy, thereby assisting in the selection of the most promising agents (and doses) for large-scale trials.

- *Toxicity biomarkers* report the toxicological effects of drugs on an *in vitro* or *in vivo* system. These biomarkers serve as surrogate end points of toxicity at the preclinical, clinical, and post-market stages.
- *Target biomarkers* report the interactions of drugs with their targets, i.e. the marker demonstrates whether the drug is effecting the appropriate target interaction.
- *Staging Biomarkers* distinguish between different stages of a chronic disorder.
- *Mechanism Biomarkers* report a downstream effect of a drug, and may consequently reveal the mechanism and extent of drug action.
- *Exploratory biomarkers* are based on general exploratory or research information such as broad gene expression screening or the collection of sera or tissue samples, and have not yet attained the status of a potentially valid biomarker.

Table 1.1 gives a list of cancer biomarker types in general, and examples with regard to breast cancer are also provided where possible in each category.

Table 1.1: Biomarkers of cancer risk, therapeutic benefit and toxicity (MacGregor, 2004) with examples in breast cancer.

Biomarker Function		Example(s) in breast cancer	References
Biomarkers of clinical therapeutic efficacy	Biochemical status of tumour	• Hormone and growth factor receptors - ER positivity predicts response to endocrine manipulation - EGF receptors are negatively correlated with ER and poorer prognosis • Oncogenes - Tumours that express C-erb-B2 oncogene likely to - be resistant to CMF chemotherapy - be resistant to hormonal therapy - respond to anthracycline - respond to taxols • Proteases - Urokinase and cathepsin D found in breast cancer - Presence confirms a poorer prognosis	Kaklamanos *et al*., 1999; Skasko *et al*., 2005; Harris *et al*., 1989; Toi *et al*., 1994; Treish *et al*., 2000; Surgical-tutor, 2006
	Genetic characteristics of tumour	• Germ cell mutations in tumour suppressor genes, e.g. *p53, BRCA-1, BRCA-2, PTEN* • Activation of oncogenes: e.g. *Ras*, HER-2/neu (erbB2),c-*myc*, HER-1, Skp2 and p27 • Sporadic mutations in the genome of normal somatic cells, e.g. E-Cadherin	Osbourne *et al*., 2004; Ethier, 2003; Traub *et al*., 2006
	Genetic characteristics of patient	Germline mutations in breast cancer genes BRCA-1 and BRCA-2, CHEK2 mutation carriers and inherited mutant TP53 allele	Fabian *et al*., 2001, Walsh *et al*., 2006
Biomarkers of toxicity	Cellular integrity	Telomere dysfunction and loss of heterozygosity (LOH)	Herbert *et al*., 2001
	Cellular function	• Decrease in high-density lipoprotein cholesterol, headache, hypercalcemia, and noncardiogenic pulmonary oedema. • Increase in serum triglycerides , lipoprotein cholesterol (HDL), total cholesterol (TC), lipoprotein and apolipoproteins	Lawrence *et al*., 2001, Atalay *et al*., 2004
	Molecular and cellular damage and damage response	CHK2 gene (a tumour suppressor), telomerase activity and 14-3-3σ (σ) down-regulation	Ingvarsson *et al*., 2002; Ferguson *et al*., 2000
Biomarkers of susceptibility	Biochemical status	Epidermal growth factor receptor (EGFR) status	Toi *et al*., 1994, Skasko *et al*., 2005
	Genetic susceptibility	Mutated BRCA1 and BRCA2 genes, and germline mutations in *CHEK2*, *TP53*, *RAD51* and *PTEN* genes	Ludwig and Weinstein, 2005
Biomarkers of tumour status and development	Tumour size, location and growth	Overexpression of $p16^{INK4A}$, p53, cyclin A, pRb, and $p21^{waf1}$	
	Tumour metabolic activity	Oestrogen-regulated gene sequence pLIV1, Cyclin D1	Kuijper, *et al*., 2005
	Proliferative changes	EGFR and c-erbB-2 oncoprotein, and DNA ploidy status	Skasko *et al*., 2005
	Genetic changes	Amplification of the erbB-2 receptor gene (HER-2)	MEDCEU, 2006
	Morphological changes	Presence of necrosis : Circulating DNA and tumour cells	Lee *et al*., 2006
	Biochemical changes	Cathepsin D over-expression	Garcia *et al*., 1996

2. Biomarker Application in Breast Cancer Management

The past decade has seen a proliferation of information regarding the understanding of normal breast biology, as well as parallel information regarding biomarker expression in breast cancer. Consequently, the knowledge of molecular mechanisms that influence normal and aberrant cell growth has advanced, and led to the identification of an increasing number of breast cancer biomarkers. A biomarker has now been identified for almost every pathway that eventually leads to breast cancer. However, despite the growing enthusiasm for biomarkers, their use for early detection of breast cancer, drug discovery and development is still in its early stages. In this section, the various types and applications of current and emerging breast cancer biomarkers are described. Issues that negatively affect the development and clinical use of biomarkers in breast cancer prevention and treatment are also highlighted.

2.1 Breast Cancer Predisposition and Screening

There has been confusion about the search for breast cancer biomarkers and inherited or germline mutations that affect the likelihood of developing breast cancer (Arrow *et al.,* 2005). Although the discovery of such mutations is important for assessing breast cancer risk and may ultimately lead to the identification of the causes of breast cancer, the mere presence of such mutations does not indicate or predict the presence of breast cancer in an individual (Arrow *et al*., 2005).

The germline mutations BRCA-1 and BRCA-2 are the most significant, accounting for 90-95% of familial breast cancer cases (Petrij-Bosch *et al*., 1997; Puget *et al*., 1999b). Both genes appear to be susceptible to carrying a wide mutational spectrum of high-penetrance, individually rare genomic rearrangements (Walsh *et al*., 2006). Other important mutations predisposing a person to breast cancer are found in the *CHEK2, TP53* and *PTEN* genes. Table 1.2 gives a list of other genes associated with breast cancer susceptibility (Ross *et al*., 2003).

Table 1.2: Genes associated with breast cancer susceptibility (Ross *et al*., 2003)

Gene	Notes	Hereditary breast cancer association	Sporadic breast cancer association	Other cancers
BRCA1	• DNA repair genes • Mutation (abnormality) in gene causes an increased risk of breast or ovarian cancer.	High (40%)	High	Ovary (colon, prostate)
BRCA2		High (40%)	High	No ovary, male breast (prostate)
p53	• A tumour suppressor gene (P53 protein present at minute level in any normal cells). • Functions to eliminate and inhibit the proliferation of abnormal cells • Germinal mutation: affected individuals are predisposed to developing sarcomas, osteosarcomas, leukemias and breast cancers at unusually early ages. • Somatic mutation: makes young adults susceptible to breast cancer, with more aggressive disease and lower chances of overall survival.	Low	High	Carcinomas, sarcomas, leukemias (Li-Fraumeni syndrome)
RAS (HRAS)	• Important in gene expression and controlling growth and development. • may be pathologically activated by overexpression of growth factor receptors	Low in young; higher in old	High in old	Carcinomas, sarcomas, leukemias
hMLH1 (lynch II)	• Mismatch repair genes • Mutate only occasionally in sporadic breast tumours	Low	Low	
hMSH2 PMS2, MSH6 (HNPCC)		Low	Low	Colon, skin, stomach
Androgen receptor	• A steroid hormone receptor that is expressed in breast tissue • Mediates the effects of steroid hormones • Expressed in the majority (50%) of ER-negative tumours • Expression predicts positive response to hormonal therapy and overall survival	Only males, low	Only males	Male breast
Ataxia telangectasia	• An autosomal recessive syndrome that occurs commonly in sporadic breast cancer	Low	Low	Lymphomas, leukemias
Neurofibromatosis 1	• Cancer of the nervous system • Caused by loss of heterozygosity in neurofibromin (NF1) gene, which controls cell division. • Affected individuals carry high risk of developing cancer in general	Very low	Low	Nerve, brain
PTEN	• Tumour suppressor gene • Germline mutations cause a multiple hamartoma syndrome (Cowden syndrome)			Thyroid cancer, endometrial cancer
STK11 (Peutz Jeghers)	• A tumour suppressor gene, which usually controls cell growth and cell death. • Mutation on both alleles causes Peutz-Jeghers syndrome			Stomach, oesophageal, colon, pancreatic and ovarian cancers

Attempts to develop new and improved biomarkers for screening cancer have focused on all levels of biochemistry, including the genes, RNA, proteins and metabolites; however, scientists have thus far been unable to screen unequivocally for any form of cancer (Shau *et al*., 2003). This failure can be ascribed chiefly to the fact that most of the cancer biomarkers are also produced by normal cells, which has made it difficult to precisely identify those enzymes whose activities actually change during the progression of the disease. Nevertheless, these substances are still considered markers because they are produced at the wrong time in a person's life, in excessive amounts or in an altered conformation. Markers have been identified using both old and new methods. Unfortunately, most of these markers have not been useful as independent diagnostic or screening tools; however, they are still useful during diagnosis and management of the disease.

2.2 Breast Cancer Diagnosis and Staging

The formal *TNM staging system of tumour classification* describes the anatomic extent of cancer by separately classifying the individual tumour (T), node (N), and metastasis (M) elements, and then grouping them into stages (Sobin, 2003). Since its inception in 1958, the TNM staging system has provided a standardised, anatomical basis for staging cancer by providing a basis for prediction of survival, choice of initial treatment, stratification of patients in clinical trials, accurate communication among healthcare providers, and uniform reporting of outcomes (Ludwig and Weinstein, 2005). Even though biomarkers have not yet been incorporated into the traditional TNM staging system, the value that the integration of biomarkers could add to the system is evident when considering the fact that some targeted therapeutic agents are effective in treatment selection only if their respective molecular markers are expressed. For instance, tumours that are oestrogen-receptor and progesterone-receptor positive usually signify a better prognosis, and are good candidates for hormonal therapy (e.g. tamoxifen) and aromatase inhibitors, while HER-2/*neu* positive tumours usually signify a poor prognosis and are candidates for Herceptin treatment (Ludwig and Weinstein, 2005). In both cases, the results are independent of the cancer TNM stage.

Risk assessment tools for pre-screening patients with family histories suggestive of germline mutations predisposing them to breast cancer are available, and the early detection of circulating breast cancer cells by morphologic methods is commonly used. These methods employ measurement of the serum tumour marker levels of carcinoembriogenic antigens CA15-3 and CA27-29 (Ross *et al*., 2003). These markers only reflect progression of the disease, since they are not sensitive enough for use in early breast cancer detection. Consequently, these older methods are currently being challenged by ultra-sensitive proteomic (Li *et al*., 2002) and PCR-based methods (Hu *et al*., 2000 and 2001; Cronin *et al*., 2004). Using these newer methods, two biomarkers - mammaglobin and maspin - have been identified as highly potential for use as markers of early breast cancer (Grunewald *et al*., 2000; Corridini *et al*., 2001; Mercatali *et al*., 2006).

Nevertheless, since the discovery of the relationship between breast cancer and mammaglobin and maspin expression about a decade ago, both biomarkers have not yet been incorporated into the clinical diagnosis system of breast cancer. Only recently, the first diagnostic test for breast cancer recurrence, Oncotype DX, has become clinically available (Nelson, 2006). This test measures the expression of 21 genes by RT-PCR in paraffin-embedded breast tumour samples, and may predict the likelihood of breast cancer recurrence in women diagnosed with node-negative, oestrogen-receptor positive breast cancer. The test also makes it possible to determine which women are likely to benefit from chemotherapy. Thus, only a few biomarkers have been accepted for use in the clinical management of breast cancer so far. However, the present use of high-throughput technology has led to the identification of a vast number of breast cancer biomarkers with both predictive and prognostic potential value. An enormous amount of data is presently becoming available from genomics and proteomics studies, adding to the already long list of breast cancer biomarkers. Table 1.3 provides a list of various types of breast cancer biomarkers, giving an indication of the diverse categories of breast cancer biomarkers identified so far.

2.3 Prognosis and Treatment

Due to the multiplicity of pathways leading to breast cancer development, scientists have already acknowledged that it may not be possible to find a single marker that will be perfectly suited to the detection and diagnosis of breast cancer (Beckmann *et al.,* 1997; Arciero *et al*., 2003). Therefore, the challenge is now to seek a panel of synergistic markers that can be used in concert as diagnostic, prognostic and/or predictive tools for different categories and stages of breast cancer. Major pharmaceutical companies are aggressively pursuing this approach, reflecting the general scientific opinion

Table 1.3: The different categories of breast cancer biomarkers identified so far, with examples.

Cell cycle associated markers	Growth factors and receptors	Oncogenes	Tumour suppressor genes	Cell adhesion molecules	Invasion-associated proteases and proteins	Oestrogen and Progesterone receptor proteins	Markers of Drug resistance
• Ki-67 antigen • Cyclin D1 (PRAD1 or bcl-1) • Cyclin E • p21 protein • (p21/WAF1/ Cip1) • p27 (kip1) and skp2	• Epidermal growth factor receptor (EGFR), also called c-*erb*-B-1 and HER-1 • HER-2/neu • Transforming growth factor (TGF)-α • TGF-β • Insulin and Insulin-like growth factors (IGF)-I and II • Platelet-derived growth factor (PDGF) • Fibroblast growth factors (FGF), including *Int*-2 and HST-1 genes • Vascular endothelial growth factor (VEGF) • Epidermal growth factor-Cripto-FRL1-Cryptic (EGF-CFC)	• *c-myc* proto-oncogene • H-*ras* and N-*ras* genes • Loss of heterozygosity (LOH) • *c-fos* and *c-jun* regulators, and *c-myb* • Jun activation domain-binding protein (JAB)-1	• p53 • MDM2 • Retinoblastoma (Rb) tumour suppressor gene • NM23 • p16INK1A tumour suppressor gene cyclin-dependent kinase inhibitor • PTEN tumour suppressor gene • Maspin = serine protease inhibitor • BRCA proteins	• Cadherin/ catenin complex • CD44 • Integrins and laminin • EpCAM	• Cathepsin D • Serine proteases =urokinase plasminogen activator (uPA) and plasminogen activator inhibitor (PAI)-1 • Matrix metalloproteases (MMPs) = MMP-2, -9 and -11	• Oestrogen receptor proteins • Progesterone receptor proteins	• Multidrug resistance gene (MDR1) • Glutathione S-transferase (GST)-π • pS2 • HSP 27 and HSP.70 • Apoptosis and apoptosis regulators • Transcription factors • Telomerase • DNA repair and microsatallite instability • DNA methylation

that the use of such biomarkers will give rise to improved diagnostic and therapeutic approaches in the cancer field (Wagner, 2004; Boguslavsky, 2006). Genomic and proteomic technologies are currently being employed to identify new diagnostic and prognostic biomarkers. These technologies have already been proven to successfully identify biomarker patterns of early-stage cancer, and to predict patient outcomes. For instance, Petricoin *et al*. (2002) have demonstrated that serum protein patterns can distinguish women with stage I ovarian cancer from unaffected women, and can facilitate early-stage diagnosis of this highly life-threatening cancer. The following biomarkers have been identified through several studies as novel prognostic markers of breast cancer:

Oncogene products: They play an important role in transmitting external signals to the inside of cells. If an abnormality occurs in any one of these proteins, cells become unable to perform normal functions such as proliferation and differentiation which in turn, leads to the development of cancer. Examples are Bcl-2, p53, HER-2/*neu*, cyclin D1 and Nm23.

Proteases: They promote the aggressive properties of metastatic breast tumours through support of their motile and invasive behaviour (Dickson *et al.,* 1994; Esteva and Hortobagyi, 2004). Examples include urokinase-type plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor (PAI-1), cathepsin D and tenascin C.

Markers of proliferation: Tumours positive for these markers have a high metastatic potential and warrant the possible use of early aggressive therapy. Examples are Ki-67, mitosin and LEA-135 (Jensen *et al*., 2001; Clark *et al*., 1997; Esteva and Hortobagyi, 2004; Liu *et al*., 2000).

At molecular level, breast cancers do not all look the same, hence the conclusion that breast cancer is not one disease (Beckmann *et al.,* 1997). There is no single pathway to breast cancer development; up to 62 genes and their protein products are potentially involved in breast cancer-related mechanisms (Arciero *et al*., 2003). This suggests that protein and gene expression profiles will be important new tools that will facilitate the stratification of patient populations with respect to the stage of tumour development and the selection of appropriate therapeutic regimens in the near future.

Whereas only one or two breast cancer treatment options (surgery and/or radiotherapy) were available in the past, there has been an explosion of life-saving treatment advances in recent years, bringing new hope and excitement. Today the main treatments for breast cancer are surgery, radiation, hormonal (anti-oestrogen) therapy and chemotherapy. As indicated previously, the choice of treatment is based mainly on the TNM staging of the tumour. With regard to biomarkers, three main categories of factors are recognised as critically important (Giancotti, 2005). These are the oestrogen and progesterone receptors, HER-2/*neu* and the kinases. These markers are used to guide treatment and the extent of surgical intervention. Current consensus recommends routine clinical use of only oestrogen (ER) and progesterone (PR) receptors as molecular biomarkers for patients with newly diagnosed breast cancer (Osborne *et al*., 2004). For monitoring the disease, CA15-3 and CA27-29 have become the most useful markers (Duffy, 2001). Expression of HER-2/*neu* has recently also been included in the management of breast cancer (Ross *et al*., 2003; Peoples *et al*., 2005). However, it has emerged in the past decade that presently available breast cancer treatments are effective for only a small fraction of people with a particular type of cancer, and that treatment is administered without knowing whether the tumour has already spread beyond its primary site. It is therefore generally accepted that there is a need for the development of more personalised medicine, so that the appropriate people can benefit from a drug (Nelson 2006). Biomarkers will play a key role in the future with regard to the personalisation of medicine, as they will be used to screen, detect and diagnose, to monitor treatment and to predict recurrence, as well as to assist in prognostic evaluation and clinical decision-making with regard to breast cancer.

2.4 Biomarker Identification and Development

The process of discovery for new biomarkers usually involves a comparison of physiological changes between normal and disease states. The physiological and biochemical conditions in disease states differ from those in normal states, resulting in differential gene and protein expression profiles and changes in metabolite profiles (Casey, 2005). After examining up-regulated and down-regulated genes, proteins and metabolites, researchers are able to identify potential biomarker compounds and genetic patterns associated with particular diseases that can serve as new biomarkers (Casey, 2005).

An enormous amount of data is presently becoming available from genomics and proteomics studies, adding to the already long list of breast cancer biomarkers (Perou *et al*., 1999; Ross *et al*., 2000; Shau *et al*., 2003; Seibert *et al*., 2005). With such a huge amount of information, the challenge is now to distinguish clinically useful biomarkers from non-useful ones with a view to developing biomarkers and drugs for cancer diagnosis and treatment. There are a number of critical factors that have an impact on the ability to move newly discovered biomarkers from a research setting into routine use in the clinical reference laboratory. The following three factors are generally recognised; namely biomarker discovery, validation and development.

2.5 Biomarker Discovery

The present biomarker challenge is to identify unique molecular signatures in complex biological mixtures that can be unambiguously correlated to biological events in order to validate novel biomarkers and predict prognosis. The key requirements to be considered when developing a new biomarker include the potential utility of the biomarker, the availability of a reliable and sensitive assay, and the selection of appropriate clinical or biological samples to validate its clinical utility (Biomarker validation, 2005). Upon technical and clinical confirmation, assays are moved systematically toward a standardised, reproducible, high-throughput format for clinical diagnostic implementation. The whole evidentiary process of linking a biomarker with biological and clinical endpoints is termed qualification (or clinical validation) of biomarkers (Wagner, 2004). The process is carried out with the sole purpose of validating the clinical utility of the marker for use in the detection and diagnosis of disease (see Figure 1.1).

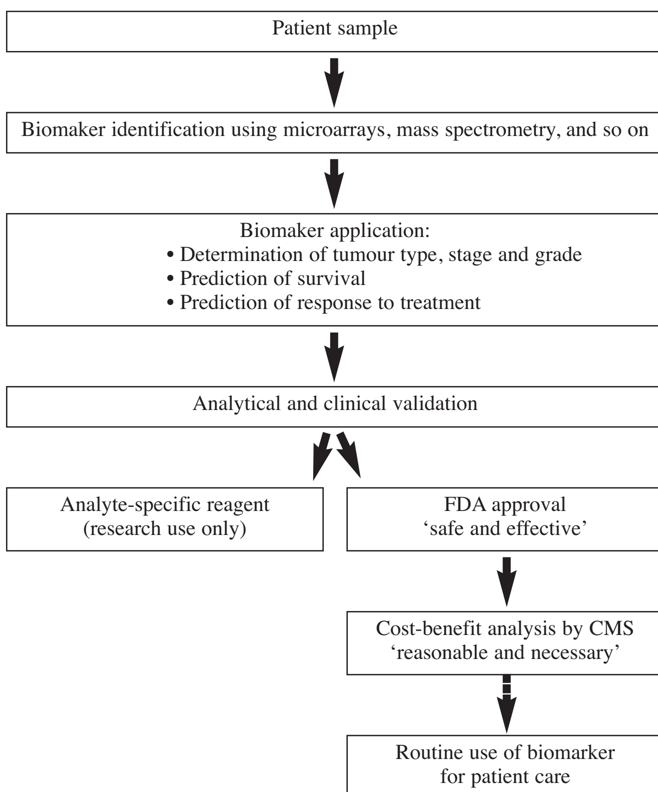

Figure 1.1: Chronology of biomarker development.
A biomarker is first identified, then evaluated for a particular clinical indication. Analytical and clinical validations must be performed before submission for US Food and Drug Administration (FDA) approval. Alternatively, the marker might bypass the FDA approval process if it is to be used for 'research purposes only'. Once a marker is FDA approved, the Center for Medicaid and Medicare Services (CMS) might determine that it is 'reasonable and necessary' for improved patient care and, therefore, reimbursable. Because CMS decisions indirectly influence coverage by private insurance carriers, a marker is not widely used in the clinic unless all of the steps in the process have been completed (Ludwig and Weinstein, 2005)

2.6 Biomarker Validation
After identifying potential biomarkers, the next step is to validate whether the putative biomarker compound or genetic pattern can really be useful as a biomarker; in other words, whether it is suitable for the contemplated purpose. The validation step is a systematic process of assessing the assay or measurement performance characteristics of the biomarker (Lesko and Atkinson, 2001; Wagner, 2004). The process involves modifying the putative biomarker and checking for phenotypic changes and alterations in the biochemical and physiological profiles thereof (Wagner, 2004; Casey, 2005). Depending on the nature of the putative biomarker (genetic or biochemical), the modification can be achieved through several methods such as gene knockout studies, RNA reference methods or the development of agonist and antagonist compounds, after which the physiological effects of such changes on the biomarker expression patterns are determined (Casey, 2005). This is a rigorous assessment of the procedures and criteria used to evaluate or validate biomarkers so that they can gain widespread acceptance, and requires increased effort.

2.7 Economics of Biomarker Development
The time and cost involved in evaluating and identifying additional breast cancer markers has always been a hurdle. The size and duration of the treatment effect are essential aspects of biomarker evaluation, as well as the sample size and study design. In some cases, the proposed clinical benefit (for instance, an effect on survival) might not be detectable in trials of reasonable duration or size, in which case the detection of such an effect may require enormously large studies of considerable duration (i.e. many years), thus becoming very costly. The cost of current efforts in biomarker discovery and validation and the development of associated molecular diagnostics is estimated at more than $1 billion annually (GeneOs, 2006). With such huge costs involved, it is absolutely crucial that the biomarker candidates selected for clinical trials have a good chance of making it through the trials and onto the market, a guarantee that is impossible for the research scientist to give. Consequently, funding for many research projects has dwindled, and it has become necessary to reduce the number of patients enrolled in a trial while maximising the chance of success with the drug or biomarker concerned (Next Generation Pharmaceutical, 2006). Thus, often due to lack of funding, adequate and well-controlled studies to evaluate biomarkers are not attempted, or are not feasible.

2.8 Biomarkers in Clinical Breast Cancer Management
2.8.1 Biomarker use in Breast Cancer
Breast cancer is the most common form of cancer and the leading cause of cancer deaths among women worldwide. The causes and natural history of breast cancer are presently unknown, but epidemiological research has revealed genetic, biological, environmental and lifestyle risk factors for this disease (Kols, 2002). Optimal management of a breast cancer patient requires a multidisciplinary approach, which includes an assay of certain biochemical markers. The rationale behind biomarkers is to be able to personalise medicine by providing the right drug, at the right dose, to the right patient, at the right time. This is achieved by using biomarkers to predict a patient's optimal route for treatment when presented with a particular disease. Clinically, the most useful biomarkers with regard to breast cancer at present are the oestrogen/progesterone receptors and HER-2, which are used to predict the response to hormone (e.g. tamoxifen) and antibody (Herceptin) therapy respectively. Biomarkers also play a key role in preclinical development, where they are used in screening for promising drugs, determining the dosage and scheduling of a drug, predicting the risk of developing a disease, predicting a patient's response and the risk of developing an adverse reaction to a drug, or serving as surrogate endpoints in a trial (Nelson, 2005). Consequently, the discovery of cancer biomarkers has become a major focus of cancer research in recent years. It is expected that, in future, cancer management will rely heavily on the use of biomarkers to guide physicians in every step of the disease management process.

Research in recent years has identified a large number of potential diagnostic and prognostic biomarkers for breast cancer. A lot of effort is currently being focused on understanding the clinical significance of these markers, finding relationships among them and discovering new ones. So far, single markers have unfortunately not proved of much clinical use as independent methods for the screening or diagnosis of cancer. Therefore, the ultimate goal of biomarker research is to establish a panel of biomarkers with sufficient and reliable predictive value for diagnosing any specific type of cancer (Shau *et al*., 2003). Unfortunately, biomarker discovery is a slow process and many obstacles have to be overcome in translating these biomarkers from the research laboratory into clinical care.

2.8.2 Methods of Biomarker Analysis in Breast Cancer Management
Routine medical management of breast cancer is currently based on histopathological features (e.g. tumour size, grade), anatomic staging (i.e. tumour-node-metastasis) and the expression of a few molecular markers, namely overexpression of oestrogen and progesterone receptors and HER-2/*neu* (Mariani, 2003). Currently, most biomarkers, particularly the hormonal and epidermal growth factor receptors are being utilised for breast cancer prognosis. However, as already mentioned, no adequate breast cancer biomarker has yet been identified; none of the biomarkers in use have sufficient diagnostic, prognostic and/or predictive power across all categories and stages of breast cancer (Arciero *et al*., 2003). This is true despite the fact that knowledge of the molecular mechanisms that influence normal and aberrant cell growth has advanced, leading to the identification of an increasing number of breast cancer biomarkers.

An overview of laboratory methods currently being used to characterise the above-mentioned clinical biomarker candidates using the types of samples most commonly available, is given below; the limitations associated with each method are also highlighted.

- **Immunohistochemistry**

Immunohistochemistry (immunocytochemistry) is a method of analysing and identifying cell types based on the binding of antibodies to specific components of the cell. Immunohistochemistry (IHC) is used in breast cancer to predict outcome for a particular grade of the disease by staining a tissue specimen from a biopsy sample obtained from the patient at the time of diagnosis. The expression of receptors for oestrogen and progesterone, as well as HER-2/*neu* amplification, are studied by IHC with a view to predicting the tumour's likely response to antihormonal therapies. This method can be carried out on fresh (frozen) and archival (paraffin-embedded) tissues using standard laboratory equipment, and is therefore the method of choice for most clinical laboratories. However, the immunohistochemical detection of oestrogen receptor (ER) and HER-2/*neu* status does not necessarily reflect its functional competence. IHC analysis relies on the surgical collection of biopsy samples, which is an invasive technique. Moreover, it involves subjective interpretation of the staining intensity and extent of tumour cells within a section in order to assign an expression score. IHC uses different antibodies with different binding affinities and epitope specificities for the determination of HER-2 overexpression, thereby creating differences in HER-2 overexpression rates. When IHC staining techniques that are too sensitive are employed, it becomes problematic to differentiate between normal HER-2 protein levels versus the high levels that are associated with gene amplification (Ross *et al*., 2003; MEDCEU, 2006).

- **Fluorescent *in-situ* hybridisation**

Fluorescent *in-situ* hybridisation (FISH) involves the hybridisation of specific DNA probes that are visible under a fluorescent microscope, and can be performed on routinely processed formalin-fixed or paraffin-embedded tissues. Although IHC is the most frequently used method to assess the overexpression of HER-2 protein, FISH is recognized as the "gold standard" for the determination of HER-2/*neu* status in breast cancer (see Figure 2.1). It is compatible with all kinds of tissue, even after storage for many years. In addition, FISH only requires small tissue samples, and the extreme sensitivity of the procedure makes it possible to detect amplification from a histologic section. However, FISH is not widely available in hospital laboratories due to its higher cost and the fact that it requires a fluorescent microscope. (Tavassoli *et al*., 2001; Singer, 2006).

- **Chromogenic *in-situ* hybridisation**

Chromogenic *in-situ* hybridisation (CISH) is a modification of FISH that uses *in situ* hybridisation technology, but also takes advantage of the chromogenic signal detection of IHC. In CISH, a DNA probe is detected using a simple IHC-like peroxidase reaction, and positive signals can be detected with an ordinary light microscope. The technique is easily integrated into routine laboratory testing, particularly with a view to confirming the immunohistochemical staining results (Tanner *et al*., 2000). CISH is used to detect HER-2/*neu* gene amplification and it can minimise, if not eliminate, the false positive fraction obtained with the IHC procedure (Madrid and Lo, 2004). The advantage of CISH over FISH is that the assay is practical and cost-effective, and can be used when fluorescent microscopy is not available. The method appears to be a valid alternative to FISH in testing for gene alteration, especially in centres working primarily with IHC. However, CISH, like IHC relies on subjective interpretation of data, with the result that CISH results are sometimes called in question (Nistor *et al*., 2006).

- **Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assay (ELISA) is a sensitive analytical technique in which an enzyme is complexed to an antigen or antibody (i.e. it is an immunochemical test). A substrate is then added, which generates a colour proportional to the extent of binding. In contrast to IHC and FISH, which measure HER-2 receptor protein (mostly in an intracellular sense), ELISA can detect HER-2/*neu* as a serum marker, since it specifically measures levels of the extracellular HER-2 receptor proteins released into the plasma from HER-2 overexpressing tumours (MEDCEU, 2006). The use of body fluids that can be collected non-invasively bypasses the need for a tissue specimen, and may make earlier HER-2/*neu* detection possible. Compared with the other methods of HER-2/*neu* detection ELISA does have considerable advantages, namely simplicity, high-throughput processing, specificity, and reliability of data (Nugent *et al*., 1992; Singer, 2006). This technique has therefore been widely accepted as a clinical tool.

- **Real-time PCR**

More recently, a real-time polymerase chain reaction (PCR) assay using the LightCycler® was developed for quantifying HER-2/*neu* gene amplification

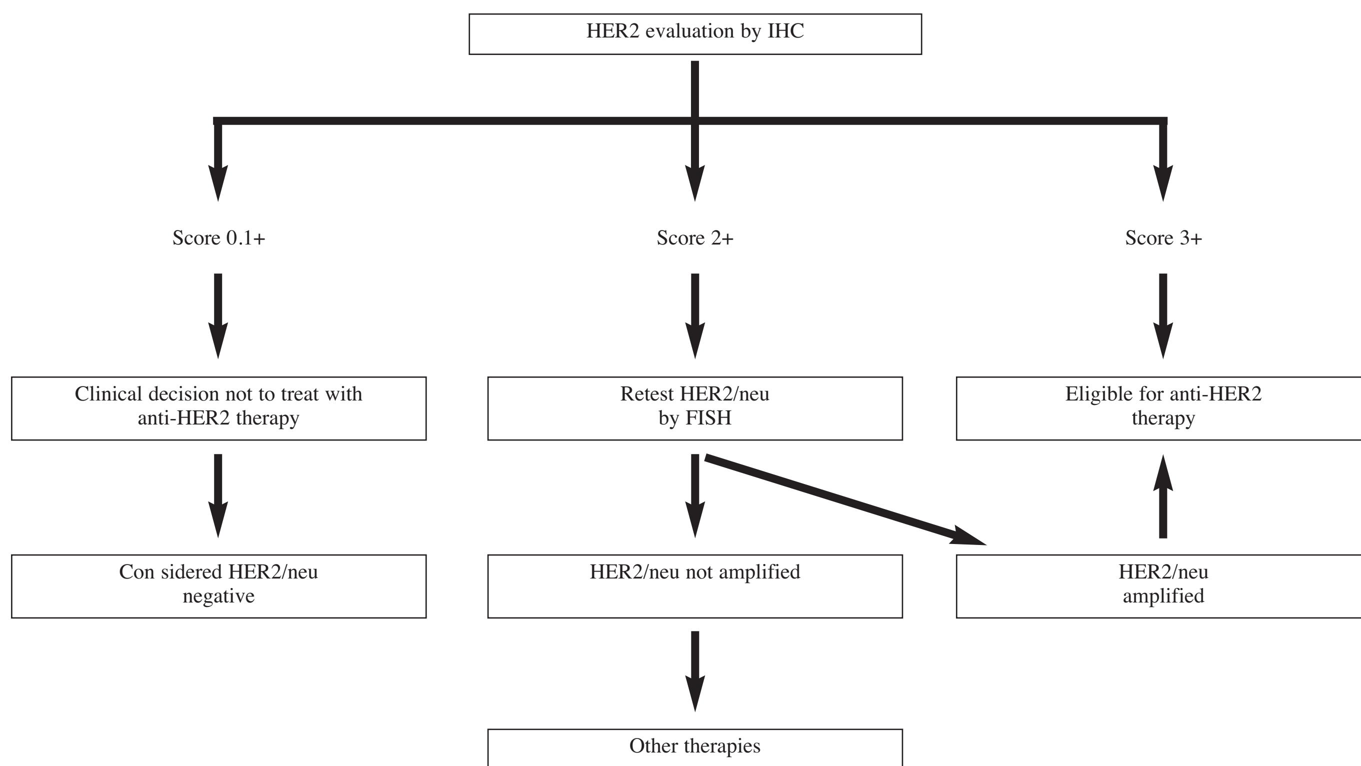

Figure 2.1: Proposed diagnostic algorithm for HER2 testing.
The specimen should be tested with use of an approved immunohistochemistry (IHC) kit and standardized scoring. If the score is 0.1+, or 3+, testing by fluorescence in situ hybridization (FISH) is done on a case-by-case bases. If the IHC HER2 score is 2+, reflex HER-2/*neu* testing by FISH is done. (Perez *et al*., 2002; Ross *et al*., 2003).

(Nistor *et al*., 2006). The results obtained, show that a combined IHC and real-time PCR approach for determining HER-2/*neu* amplification in breast cancer patients might be an effective and efficient strategy. Unfortunately, in real-time PCR methods, template/primer combinations may be very sensitive to subtle temperature differences that could exist in real-time PCR instruments, which can lead to unpredictable variation in the results. This makes such assays unsuitable for the precise and reliable quantitative analysis of gene copy number (Königshoff *et al*., 2003). Moreover, results from real-time PCR quantitative assays can be affected by contamination of the tumour sample by nucleic acids from non-tumour surrounding tissues (Nistor *et al*., 2006). Nevertheless, the technique still offers some advantages in that it is simple, convenient, non-radioactive and rapid, making it highly suitable for a routine clinical laboratory. No expertise beyond accurate and aseptic pipetting technique is required.

2.9 Future Prospects for Breast Cancer Biomarkers

Despite the fact that measurement of HER2 overexpression has been central to the development and current clinical breast cancer management, only 30% of patients identified by this biomarker have responded positively to treatment, while the remaining 70% are subjected to chemotherapy risks with no benefit (Nelson, 2005; Lesko and Atkinson, 2001). It is therefore necessary to incorporate additional biomarkers into clinical breast cancer management in order to increase this low predictive ability.

In addition, IHC and FISH are routine methods used for assessing HER2 overexpression, and no quality control programmes are in place to assess whether individual laboratories can perform these tests accurately and reproducibly, even though it is a known fact that interpretation of their results is operator-dependent. Problems of this nature will also be overcome as several biomarkers, which would increase specificity and sensitivity, can be incorporated into clinical practice. One more hurdle in biomarker research is that the small quantity of tumour material available from breast carcinomas does not allow simultaneous analysis of several biomarkers from one sample. In this case, the use of molecular biology in the early detection of breast cancer has become very important. Characterisation by molecular techniques (quantitative real-time PCR and RT-PCR) has the advantage of being able to identify even very small numbers of tumour cells in a heterogeneous population of cells (Ludwig and Weinstein, 2005). In addition, analysis of biomarkers by these molecular methods can be carried out with greater precision, even from frozen and preserved sections of tissue (Nistor *et al*., 2006; Konigshoff *et al*., 2003).

A very attractive approach, proposed as a readily accessible biological parameter that may be useful for monitoring disease progression, is to measure gene expression at the mRNA level of the genes normally non-expressed in tissues, by RT-PCR. In fact, quantitative real-time RT-PCR is generally considered the 'gold standard' against which other methods are validated (Ludwig and Weinstein, 2005). The analysis of many potential DNA markers has also been achieved using the sensitive methylation-specific PCR technique (Tooke and Patterson, 2004). This technique enables the detection of silenced genes - a very common phenomenon in carcinogenesis, arising from CpG methylation (Herman *et al.,* 1999). Recent completion of the sequencing of the human genome and advances in technology have also made proteomic profiling of cancer biomarkers possible (Shau *et al*., 2003; Seibert *et al*., 2005). Cancer gene profiling with microarrays is being pursued vigorously, and the potential of being able to not only diagnose cancer, but even categorise it into different subtypes using this technique, seems quite high (Cooper, 2001; Ramaswamy and Perou, 2003; Chang *et al*., 2005; Ahmed and Brenton, 2005). This development has strongly advanced the prospects of discovering biomarkers that are characteristic of early cancer growth, and may soon make it possible to discover cancer at an earlier stage than is presently the case.

The new high-throughput "omic" technologies have yielded many potential biomarkers and biomarker patterns, and the general feeling is that some of these markers will soon prove to be clinically useful in the diagnosis, staging and grading of cancers (MacGregor, 2003; Ludwig and Weinstein, 2005). Presently, the need is to translate recent discoveries in oncology research into clinical practice, and this requires objective, robust, and cost-effective molecular techniques for clinical trials, and ultimately for routine use. It is expected that there will be a surge in the number of molecularly targeted breast cancer drugs in the near future. However, realising this potential will require co-operation among academics and pharmaceutical and diagnostics companies working in the field of oncology with regard to developing predictive diagnostic tests for individual therapies, reaching the consensus necessary to bring biomarkers into practice and authorising the use of drugs based on test results.

The medical economics of biomarkers is a final obstacle to their widespread integration. Clinical trials are expensive and the cost involved in obtaining approval for new therapeutics is very high; this results in a lack of investors to back biomarker research. Unfortunately, proven approaches and best practices that will help save time, resources and money throughout the

process of biomarker discovery and drug development, are not yet available. However, the industry would do well to realise that, as more potential biomarkers are discovered, their limitations in clinical use during the discovery phase are reduced, and are reduced even more during the validation step – hence making rapid application in clinical practice possible.

3. Conclusions

Although much progress has been made to identify potential breast cancer biomarkers, the challenge remains to validate, in a robust manner, their accuracy, reproducibility, specificity, and sensitivity, and to assess the feasibility and cost-effectiveness of applying biomarkers in large population-based studies. The identification, validation and use of biomarkers in breast cancer depends fundamentally on an increased understanding of the mechanism of action and the role of molecular and biochemical pathways with regard to this disease. Proteomic profiling of breast cancer biomarkers by numerous laboratories worldwide is currently under investigation, and many new biomarkers are undergoing preclinical and clinical development in pharmaceutical pipelines around the world (Wagna, 2004; Boguslavsky, 2006). These advances are likely to result in the development of biomarkers that will play important roles as entry, stratification or exclusion criteria for clinical trials, and will subsequently guide the optimisation of drug prescription for individual patients. We therefore expect that, in the near future, there will be a surge in the number of clinically useful biomarkers discovered and breast cancer targeting drugs that are developed.

Moreover, strengthening the medical approach will require an increase in training and multidisciplinary collaboration such as prevention clinical trials, which are based on molecular biological leads and include biomarkers or imaging methods to define eligibility or to provide intermediate endpoints. The cancer research community must also be prepared to work with those in other fields of research to pursue strategies for the broadest public benefit. For instance, without losing its focus on clinical trials, the medical approach towards breast cancer prevention could be strengthened by adopting complementary lifestyle approaches, such as moderate alcohol consumption and proper weight management.

In future, the translation of newly discovered markers into clinical use will be faster and more cost-effective as uniform standards are applied across all laboratories. Although numerous and difficult practical problems will be experienced, the flood of potential biomarkers is paving the way to a more personalised practice of breast cancer management. Biomarkers carry the promise of detecting breast cancer early, staging it accurately, and assessing treatment benefit. Practising physicians, patients and medical insurance companies should therefore be better prepared to accept and adopt the inevitable integration of biomarkers into the routine clinical management of breast cancer in the near future.

REFERENCES

1. Ahmed A.A., Brenton J.D. *Microarrays and breast cancer clinical studies: forgetting what we have not yet learnt. Breast Cancer Res,* 2005; **7:** 96-99.
2. Arciero C., Somiari S.B., Shriver C.D. *et al. Functional relationship and gene ontology classification of breast cancer biomarkers. Int. J. Biol. Markers, 2003;* **18(4):** 241-72.
3. Arrow K.J. *Saving women's lives; strategies for improving breast cancer detection and diagnosis. National Academies Press,* 2005; p157, http://www.nap.edu/openbook/0309092132/html/157.html (accessed on 23-05-2006).
4. Atalay G., Dirix L., Biganzoli L. *et al. The effect of exemestane on serum lipid profile in postmenopausal women with metastatic breast cancer: a companion study to EORTC Trial 10951, 'Randomized phase II study in first line hormonal treatment for metastatic breast cancer with exemestane or tamoxifen in postmenopausal patients'. Ann. Oncol,* 2004; **15(2):**211-7.
5. Beckmann M.W., Niederacher D., Schnurch H.G. *et al. Multistep carcinogenesis of breast cancer and tumour heterogeneity. J. Mol. Med,* 1997; **75(6):**429-39.
6. Biomarker validation: http://www.healthtech.com/2005/bmv/day1.asp. Accessed on 14-03-2006.
7. Biomarkers Definition Working Group. *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin.Pharm. Ther,* 2001; **69:**89-95.
8. Boguslavsky J. *Biomarkers as Checkpoints: Pharmaceutical companies rely on biomarker data for more informed candidate selection, early toxicology and pharmacodynamic evaluation, patient stratification and clinical trial diagnosis. Drug discovery and Development,* 2006; http://www.dddmag.com. Accessed on May 10, 2006.
9. Buchholz T.A., Stivers D.N., Stec J. *et al. Global gene expression changes during neoadjuvant chemotherapy for human breast cancer. Cancer J,* 2002; **8(6):**461-8.
10. Casey R.M. *Bioinformatics in Biomarker Discovery*, 2005; http://www.b-eye-network.com/view/1574?jsessionid=8417aae931842 (Accessed on May 10, 2006).
11. Chang J.C., Hilsenbeck S.G., Fuqua S.A.W. *The promise of microarrays in the management and treatment of breast cancer. Breast Cancer Res*, 2005; **7:**100-104.
12. Chen C.C., Hou M.F., Wang J.Y. *et al. Simultaneous detection of multiple mRNA markers CK19, CEA, c-Met, HER-2/neu and hMAM with membrane array, an innovative technique with a great potential for breast cancer diagnosis. Cancer Lett,* 2005; Nov 11.
13. Chitty M. *Pharmaceutical Biomarkers glossary,* 2006; http://www.genomicglossaries.com/content/printpage.asp?REF=/content/Biomarkers.asp.(Last revised March 15, 2006)
14. Clark G.M., Allred D.C., Hilsenbeck S.G. *et al. Mitosin (a new proliferation marker) correlates with clinical outcome in node-negative breast cancer. Cancer Res,* 1997; **57(24):** 5505-5508.
15. Cooper C.S. *Applications of microarray technology in Breast Cancer Research. Breast Cancer Res,* 2001; **3:**158-175.
16. Corradini P., Voena C., Astolfi M. *et al. Maspin and mammaglobin genes are specific markers for RT-PCR detection of minimal residual disease in patients with breast cancer. Ann. Oncol,* 2001; **12(12):**1693-8.
17. Cronin M., Pho M., Dutta D. *et al. Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. Am. J. Pathol*, 2004; **164:**35-42.
18. Dickson R.B., Shi Y.E., Johnson M.D. *Matrix-degrading proteases in hormone-dependent breast cancer. Breast Cancer Res. Treat*, 1994; **31(2-3):**167-73.
19. Duffy M.J. *Biochemical markers in breast cancer: which ones are clinically useful? Clin. Biochem*, 2001; **34(5):**347-352.
20. Esteva F.J., Hortobagyi G.N. *Prognostic molecular markers in early breast cancer. Breast Cancer Res,* 2004; **6:**109-118.
21. Ethier S.P. *Identifying and validating causal genetic alterations in human breast cancer. Breast Cancer Res. Treat*, 2003; **78(3):**285-7.
22. Fabian C.J. (2001) *Breast cancer chemoprevention: beyond tamoxifen. Breast Cancer Res*, 2001; **3(2):** 99–103.
23. Ferguson A.T., Evron E., Umbricht C.B. *et al. High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc. Natl. Acad. Sci. U.S.A*, 2000; **97(11):** 6049-54.
24. Garcia M., Platet N., Liaudet E. *et al. Biological and Clinical Significance of Cathepsin D in Breast Cancer Metastasis. Stem Cells,* 1996; **14(6):** 642-650.
25. GeneOS: http://www.geneos.fi/nxl/39443234/biomarker_page.en.html. Accessed on 15-05- 2006.
26. Giancotti V. *Breast cancer markers. Cancer Lett*., 2006 [Epub ahead of print].
27. Greenwald P. *Science, medicine, and the future: Cancer chemoprevention. Biomed. J*, 2002; **324(7339):** 714–718.
28. Grunewald K., Haun M., Urbanek M. *et al. Mammaglobin gene expression: a superior marker of breast cancer cells in peripheral blood in comparison to epidermal growth factor receptor and cytokeratin-19. Lab. Invest*, 2000; **80(7):** 1071-7.
29. Harris A.L., Nicholson S., Sainsbury J.R. *et al. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. J. Ster. Biochem,* 1989; **34(1-6):** 123-31.
30. Herbert B.S., Wright W.E., Shay J.W. *Telomerase and breast cancer. Breast Cancer Res,* 2001; **3(3):** 146–149.
31. Herman J.G. *Hypermethylation of tumor suppressor genes in cancer. Semin. Cancer Biol,* 1999; **9(5):** 359-67.
32. Hu X.C., Chow L.W. *Detection of circulating breast cancer cells by reverse transcriptase polymerase chain reaction (RT-PCR). Eur. J. Surg. Oncol,* 2000; **26(6):** 530-5.
33. Hu X.C., Chow L.W. *Detection of circulating breast cancer cells with multiple-marker RT-PCR assay. Anticancer Res,* 2001; **21(1A):** 421-4.
34. Hu Y., Zhang S., Yu J., Liu J., Zheng S. *SELDI-TOF-MS: the proteomics and bioinformatics approaches in the diagnosis of breast cancer. Breast*, 2005; **14(4):** 250-5.
35. Ingvarsson S., Sigbjornsdottir B.I., Huiping C. *et al. Mutation analysis of the CHK2 gene in breast carcinoma and other cancers. Breast Cancer Res,* 2002; **4(3):** R4.
36. Jensen E.V., Cheng G., Palmieri C. *et al. Estrogen receptors and proliferation markers in primary and recurrent breast cancer. Proc. Natl Acad. Sci. U S A,* 2001; **98(26):** 15197-202.
37. Kaklamanos I.G., Bathe O.F., Franceschi D. *et al. Expression of receptors for estrogen and progesterone in malignant colonic mucosa as a prognostic factor for patient survival. J. Surg. Oncol,* 1999; **72(4):** 225-9.
38. Kols A. *Breast cancer: Increasing incidence, limited options. Outlook*, Jun 2002; **19(4 revised):** 1-8. Available at http://www.path.org/files/eol19_4-rev.pdf (Accessed on 29-06-06).
39. Konigshoff M., Wilhelm J., Bohle R.M., Pingoud A., Hahn M. *HER-2/neu Gene Copy Number Quantified by Real-Time PCR: Comparison of Gene Amplification, Heterozygosity, and Immunohistochemical Status in Breast Cancer Tissue. Clin Chem*., 2003; **49:** 219-229.

40. Kuijper A., de Vos R.A., Lagendijk J.H. *et al*. *Progressive Deregulation of the Cell Cycle with Higher Tumour Grade in the Stroma of Breast Phyllodes Tumours. Am. J. Pathol,* 2005; **123:** 690-698.
41. Lawrence J.A., Adamson P.C., Caruso R. *et al*. *Phase I clinical trial of alitretinoin and tamoxifen in breast cancer patients: toxicity, pharmacokinetic, and biomarker evaluations. J. Clin. Oncol,* 2001; **19(10):** 2754-63.
42. Lee A.H., Gillett C.E., Ryder K. *et al*. *Different patterns of inflammation and prognosis in invasive carcinoma of the breast. Histopath,* 2006; **48(6):** 692-701.
43. Lesko L.J., Atkinson A.J. *Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies· Annu Rev Pharmacol Toxicol*., 2001; **41:** 347-366.
44. Li J., Zhang Z., Rosenzweig J., Wang Y.Y., Chan D.W. *Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem*., 2002; **48(8):** 1296-304.
45. Liu D., Naritoku W.Y., Tsao-Wei D. *et al*. *LEA.135 expression: an independent and favourable prognostic biomarker for patients with primary invasive breast cancer. Int. J. Cancer*, 2000; **89(3):** 224-9.
46. Ludwig J.A., Weinstein J.N. *Biomarkers in cancer staging, prognosis and treatment selection. Nat. Rev*, 2005; **5(11):** 845-856.
47. MacGregor J.T. *Biomarkers of Cancer Risk and Therapeutic Benefit: New Technologies, New Opportunities, and Some Challenges. Toxicol. Path*, 2004; **32(1):** 99–105.
48. Madrid M.A., Lo R.W. *Chromogenic In Situ Hybridization (CISH): a Novel Alternative in Screening Archival Breast Cancer Tissue Samples for HER-2/neu Status*, 2004.
(Available at http://www.medscape.com/viewarticle/487451).
49. Mariani S.M. *Conference Report - Breast Cancer Markers: What next? Med. Gen. Med,* 2003; **5(4)** (Available at www.medscape.com).
50. MEDCEU. *Breast Cancer and HER-2*, 2006;
http://www.medceu.com/course-no-test.cfm?CID=307). Accesssed on 15-05-2006.
51. Mercatali L., Valenti V., Calistri D. *et al*. *RT-PCR determination of maspin and mammaglobin B in peripheral blood of healthy donors and breast cancer patients. Ann. Oncol,* 2006; **17(3):** 424-8.
National Cancer Institute (NCI). *Women's Health Report, Fiscal Years 2003-2004* (http://women.cancer.gov/planning/whr0304/whr0304.pdf). Accessed on 15-05-2006.
52. Nelson N.J. *Experts wrestle with problems developing biomarkers, search for new tests, Oxford University Press,* 2006; **DOI: 10.1093/jnci/djj200.**
53. Next Generation Pharmaceutical. *Biomarkers: yesterday's tomorrow today*, 2006;
http://www.ngpharma.com/pastissue/article.asp?art=25533&issue=143. Accessed on 07-02-2006.
54. Nistor A., Watson P.H., Pettigrew N., Tabiti K., Dawson A., Myal Y. *Real-time PCR complements immunohistochemistry in the determination of HER-2/neu status in breast cancer. BMC Clin Pathol.,* 2006; 6(1):2.
55. Nugent A., McDermott E., Duffy K., O'Higgins N., Fennelly JJ., Duffy MJ. *Enzyme-linked immunosorbent assay of c-erbB-2 oncoprotein in breast cancer. Clin Chem.,* 1992; **38:** 1471-1474.
56. Osborne C., Wilson P., Tripathy D. *Oncogenes and tumour suppressor genes in breast cancer: potential diagnostic and therapeutic applications. Oncol,* 2004; **9(4):** 361-77.
57. Peoples G.E., Gurney J.M., Hueman M.T. *et al*. *Clinical trial results of a HER-2/neu (E75) vaccine to prevent recurrence in high-risk breast cancer patients. J. Clin. Oncol,* 2005; **23(30):** 7536-45.
58. Perez E.A., Roche P.C., Jenkins R.B., Reynolds C.A., Halling K.C., Ingle J.N., Wold L.E. *HER2 Testing in Patients With Breast Cancer: Poor Correlation Between Weak Positivity by Immunohistochemistry and Gene Amplification by Fluorescence In Situ Hybridization. Mayo Clin Proc*., 2002; 77: 148-154.
59. Perou C.M., Jeffrey S.S., van de Rijn M. *et al*. *Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc. Natl. Acad. Sci. U.S.A,* 1999; **96(16):** 9212-7.
60. Petricoin E.F., Ardekani A.M., Hitt B.A. *et al*. *Use of proteomic patterns in serum to identify ovarian cancer. Lancet*, 2002; **359(9306):** 572-7.
61. Petrij-Bosch A., Peelen T., van Vliet M. *et al*. *BRCA1 genomic deletions are major founder mutations in Dutch breast cancer patients. Nat. Gen,* 1997; **17:** 341–345.
62. Puget N., Stoppa-Lyonnet D., Sinilnikova O.M. *et al*. *Screening for genomic rearrangements and regulatory mutations in BRCA1 led to the identification of four new deletions. Cancer Res,* 1999b; **59:** 455–461
63. Ramaswamy S., Perou C.M. *DNA microarrays in breast cancer: the promise of personalised medicine. Lancet*, 2003; **361(9369):** 1576-1577.
64. Ross D.T., Scherf U., Eisen M.B. *et al*. *Systematic variation in gene expression patterns in human cancer cell lines. Nat Genet.,* 2000; **24(3):** 227-35.
65. *Ross J.S., Fletcher J.A., Linette G.P.* et al. *The HER-2/neu Gene and Protein in Breast Cancer: Biomarker and Target of Therapy. Oncol*, 2003; **8(4):** 307–325.
66. Seibert V., Ebert M.P., Buschmann T. *Advances in clinical cancer proteomics: SELDI-ToF-mass spectrometry and biomarker discovery. Brief Funct Genomic Proteomic*., 2005; **4(1):** 16-26.
67. Shau H., Chandler G.S., Whitelegge J.P. *et al*. *Proteomic profiling of cancer biomarkers. Brief. Funct. Genomic Proteomic*, 2003; **2(2):** 147-58.
68. Singer M.S. *Breast Cancer and HER-2/neu: Diagnostic Tools for Targeted Therapy,* 2006. Available at
http://www.medcompare.com/spotlight.asp?spotlightid=194).
Accessed on 15-05-2006.
69. Skasko E., Paszko Z., Mazur S. *A new look at the prognostic value of the estrogen, progesterone and epidermal growth factor receptors in breast cancer tissue. Neoplasma,* 2005; **52(1):** 10-7.
70. Sobin L.H. *TNM: evolution and relation to other prognostic factors. Sem. Surg. Oncol,* 2003; **21(1):** 3-7.
71. Surgical-tutor. *Breast Cancer,* 2006;
http://www.surgical-tutor.org.uk/default-home.htm?specialities/general/breast_cancer.htm~right. Accessed on 15-05-2006.
72. Tanner M., Gancberg D., Di Leo A. *et al*. *Chromogenic in Situ Hybridization: A Practical Alternative for Fluorescence in Situ Hybridization to Detect HER-2/neu Oncogene Amplification in Archival Breast Cancer Samples. Am J Pathol*., 2000; **157:** 1467-1472.
73. Tavassoli F., Costa J., Flynn S.D., Martel M., Ocal I.T. *Breast pathology*. Available at http://www.yalepath.org/DEPT/diagunits/breast.htm. 2001. Accesssed on 15-05-2006
74. Toi M., Tominaga T., Osaki A. *et al*. *Role of epidermal growth factor receptor expression in primary breast cancer: results of a biochemical study and an immunocytochemical study. Breast Cancer Res. Treat,* 1994; **29(1):** 51-8.
75. N., Pettersson M. *CpG methylation in clinical studies: utility, methods, and quality assurance, Medical Device Link,* 2004;
http://www.devicelink.com/ivdt/archive/04/11/002.html.
Accessed on 20-03-2006.
76. Traub F., Mengel M., Luck H.J. *et al*. *Prognostic impact of Skp2 and p27 in human breast cancer. Breast Cancer Res. Treat*, 2006; Apr 25.
77. Treish I., Schwartz R., Lindley C. *Pharmacology and therapeutic use of trastuzumab in breast cancer. Am. J. Health Syst. Pharm*, 2000; **57(22):** 2063-76.
78. Volinia S., Calin G.A., Liu C.G. *et al*. *A microRNA expression signature of human solid tumors defines cancer gene targets. Proc. Natl. Acad. Sci. U.S.A*, 2006; **103(7):** 2257-61.
79. Wagner J. *Biomarkers in Rational Drug Development*, 2004; (http://bigdaddy.scripps.edu/darlene/Asilomar/pages/abstracts/jwagner.htm) Accessed on 20-03-2006.
80. Walsh T., Casadei S., Coats K.H. *et al*. *Spectrum of Mutations in BRCA1, BRCA2, CHEK2, and TP53 in Families at High Risk of Breast Cancer. JAMA*, 2006; **295(12):** 1379–1388.
81. Yaziji H., Gown A.M. *Controversies and guidelines in tissue-based HER-2/neu testing in breast cancer. MLO Med Lab Obs*. 2002; **34(6):** 12-6, 20-1.