symposium article

New strategies to identify molecular markers predicting chemotherapy activity and toxicity in breast cancer

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Despite significant improvements in the treatment and outcomes of early-stage breast cancer, the quest continues to find biological and molecular markers that would enable earlier diagnosis or better prediction of treatment efficacy and toxicity. Metabolomics—the latest and one of the most exciting of the 'omic' sciences—has shown early promise as a non-invasive diagnostic aid in ovarian cancer, and may allow the detection of subtle metabolic changes that could have diagnostic, prognostic or predictive value in breast cancer. Routine monitoring of circulating tumour cells (CTCs) has also been advocated as a novel means of detecting breast cancer progression earlier, and identifying alterations in tumour cells that might signal the need for therapy changes. Ongoing studies should help to answer important questions relating to the use of metabolomics and CTC evaluation as new strategies to monitor cancer progression and identify markers of chemotherapy activity and toxicity.

Key words: breast cancer, metabolomics, metabolic profiles, circulating tumour cells, molecular markers

introduction

Breast cancer is increasingly being diagnosed at an early stage, thanks to massive use of screening mammography programmes [1, 2]. Adjuvant chemotherapy and hormonal treatments have improved disease-free intervals and overall survival [3]; however, breast cancer treatment can still be viewed largely as a 'shot in the dark' and the tools available to help predict who will respond optimally to which treatment are still relatively crude. Thankfully, however, technological advances and the steady blurring of boundaries between oncology, biochemistry and physics have recently enabled the development of new strategies to identify molecular markers of breast cancer treatment activity and toxicity. Two of the most promising approaches for use in the clinical setting—metabolomics and circulating tumour cell analysis—may one day help us to tailor breast cancer treatment with ultimate precision.

a brief history of metabolomics

Metabolomics (or metabonomics) is the study of global metabolite profiles in a system (cell, tissue or organism) under a given set of conditions [4]. Metabolites result from the interaction of the system's genome with its environment; they are not merely the end product of gene expression, but form part of the regulatory system in an integrated manner. Metabolomics has its roots in early metabolic profiling studies, but is now a rapidly expanding field of scientific research,

and has justifiably taken its place alongside genomics, transcriptomics and proteomics as one of the latest and most exciting 'omic' sciences.

The potential of metabolomics in science and medicine is significant. As Mitchell et al. wrote in the *Biologist* in 2002 [5]: 'Occasionally, a new idea emerges that has the potential to revolutionize an entire field of scientific endeavour. It is now within our grasp to be able to detect subtle perturbations within the phenomenally complex biochemical matrix of living organisms. The discipline of metabolomics promises an all-encompassing approach to understanding total, yet fundamental, changes occurring in disease processes, drug toxicity and cell function.'.

Metabolite profiling first appeared in the scientific literature in the 1950s, but was relatively slow to take off as a research field in its own right. Early published work typically involved profiling the metabolites of specific pharmaceutical products (e.g. synthetic oestrogens) [6], and this work gradually expanded to include classes of compounds such as the catecholamines [7] and cyclo-oxygenases [8].

Metabolomic research interest began to burgeon in the 1990s, and by 2005, the number of metabolomic-related publications per year was more than double the total number in the preceeding two decades combined [9].

metabolomic principles and techniques

The human genome consists of ∼40 000 genes encoding up to 1 million proteins which, through the up- or down-regulation of metabolic enzymes, influence the synthesis or degradation of small molecules. The exact number of unique small

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molecules in the human metabolome is still under debate, although several thousand have so far been identified (Figure 1) [10]. The metabolic fingerprint for each individual varies considerably as a consequence of diet, lifestyle, environment and genetic effects. Disease states and drug treatments can also alter the metabolic phenotype of an individual, and this has been the driving force for the use of metabolomics in clinical medicine [11].

One of the major benefits of metabolomics in the study of disease and drug therapy is that metabolic profiling can usually be achieved using urine or plasma samples. A range of other fluids have been studied, including seminal fluid, amniotic fluid, cerebrospinal fluid, synovial fluid, lung aspirates and dialysis fluids. However, the accessibility of urine and plasma clearly makes these samples ideal for large-scale research [12].

Currently, three techniques are available for the metabolic analysis of biological samples: proton nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy and optical spectroscopy. Mass spectroscopy requires separation of the metabolic components using either gas chromatography after chemical derivatisation or liquid chromatography (LC), with the newer method of ultra-performance LC becoming increasingly popular. NMR spectroscopy is one of the most commonly used technologies in metabolomics research, providing detailed information on the molecular structure and concentrations of compounds and probing metabolite molecular dynamics and mobility [14].

NMR spectroscopy is based on the principle that the nuclei of all elements carry a charge. A nucleus with spin 1/2 will have two possible orientations. In the absence of an external magnetic field, these orientations are of equal energy. If a magnetic field is applied, the energy levels split. The overall spin of the charged nucleus generates a magnetic dipole along the spin axis, and the intrinsic magnitude of this dipole is a fundamental nuclear property called the nuclear magnetic moment.

Of particular interest to researchers are ^{1}H, ^{13}C, ^{19}F and ^{31}P, which represent key elements in the framework of most organic compounds. Each of these elements has its own magnetic momentum. When a biofluid, which often contains hundreds of organic compounds, is placed under the influence of an external magnetic field the atoms of each molecule will align either along (+1/2, lowest energy state) or against (−1/2, highest energy state) the external magnetic field, generating a difference of energy between the two spin states (Figure 2). Irradiation of a sample with a radiofrequency wave will then tip the nuclei away from a +1/2 state to the higher −1/2 spin state. When the radiofrequency emission is switched off, the nuclei will return to their original lowest energy state (+1/2). During this process they emit energy, which can be picked up by a radiofrequency receiver. An NMR spectrum is acquired by varying or sweeping the magnetic field over a small range while observing the radiofrequency signal from the sample [13].

All metabolomics studies result in complex multivariate datasets that require visualisation software and chemometric and bioinformatic methods for interpretation. The aim of these procedures is to produce biochemically-based fingerprints that are of diagnostic or classification value. A second stage—crucial in such studies—is to identify the substances causing the diagnosis or classification, and these become the combination of biomarkers that define the biological or clinical context. For a fuller description of the different approaches taken to analyzing NMR spectroscopy data, the reader is referred to the recent review by Claudino et al. (2007) [13].

applications of metabolomics in medicine

Metabolomics has become useful in many different areas of medicine as an aid to disease diagnosis or staging, and as a tool to predict or monitor treatment response or toxicity. Most recently, metabolomics has been used to identify inborn errors
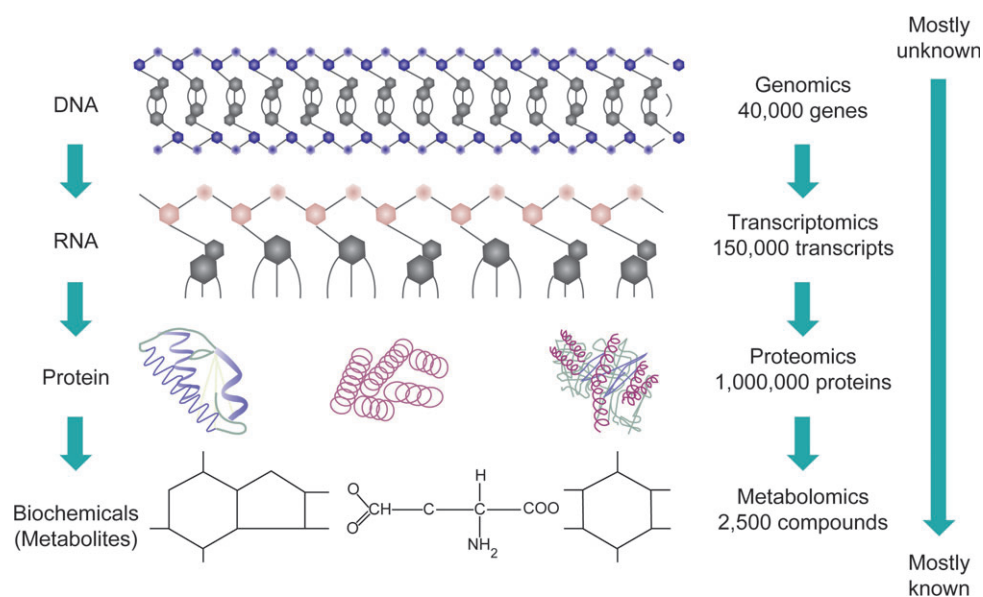
Figure 1. The central dogma of molecular biology. One of the great advantages of metabolomics is that the human metabolome is relatively small. To date, only ~2500 unique small molecules have been identified [10]. Reprinted with permission of Business Briefings Ltd, a subsidary of Tach Group PLC.
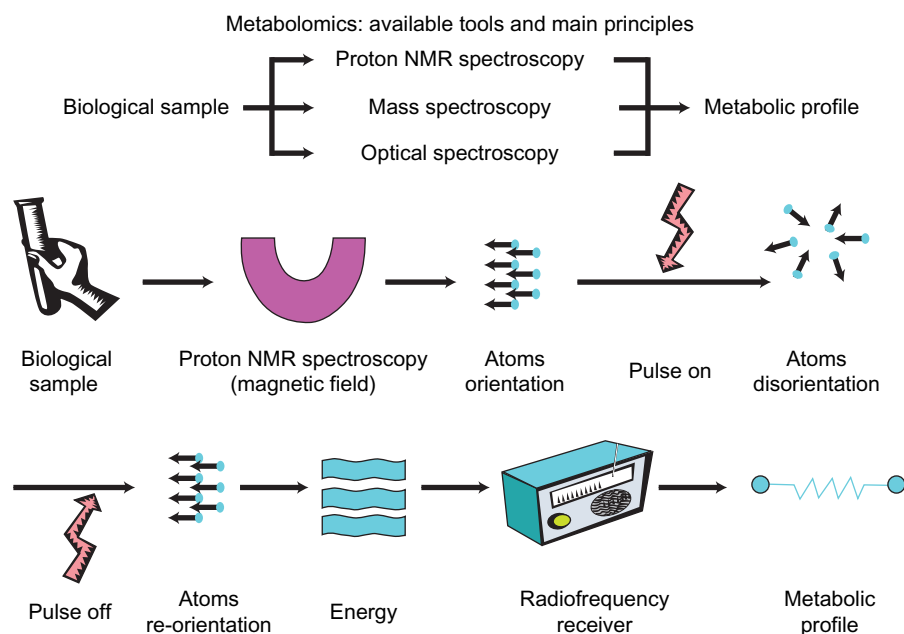
Metabolomics: available tools and main principles

Figure 2. Principle steps leading to the generation of a metabolic profile for a given biological sample.

of metabolism [14, 15], to predict the presence and severity of coronary heart disease [16], to rapidly diagnose meningitis and ventriculitis [17], and to predict the clinical outcome of subarachnoid haemorrhage [18].

The usefulness of NMR-based metabolomics for the evaluation of drug toxicity effects has also been comprehensively explored by the Consortium for Metabonomic Toxicity (COMET), which included five pharmaceutical companies and Imperial College in London [19]. The aim of the consortium was to develop methodologies for the acquisition of metabolomic data using ^{1}H-NMR spectroscopy of urine and blood from rats and mice for pre-clinical toxicological screening of candidate drugs, to build databases of spectra, and to develop an expert system for predicting organ toxicity and, at the last report, all of these goals had been achieved [19].

Another area of considerable interest in the field of metabolomics is that of personalised healthcare, whereby an individual's drug treatment is tailored so as to achieve maximal efficacy while avoiding adverse drug reactions. One of the approaches that has been used is to relate the genetic make-up of individuals to their varying ability to handle drug treatments (so-called pharmacogenomics). Another more recent approach has been to use metabolomics to predict the metabolism and toxicity of a dosed substance based solely on the analysis and modelling of a pre-dose metabolic profile [20].

applications of metabolomics in cancer research

Cancer research is one area of medical research that has witnessed great progress in the application of metabolomics. Although a significant role in cancer initiation and progression is attributed to changes in RNA and protein expression levels and regulation, a growing body of evidence supports an

important role for metabolic regulation in many cancers, including breast cancer [13]. Malignant cells undergo significant changes in metabolism including a redistribution of metabolic networks [21] and these changes can result in a metabolic landscape that differs markedly between cancer cells and normal cells.

early detection of cancer

The first report suggesting that proton NMR spectroscopy could be useful for cancer detection was published in 1986 by Fossel et al. [22]. The study was based on the measurements of ^{1}H-NMR spectra of plasma at either 360 or 400 MHz at 20–22°C from 331 subjects including controls and patients with various types of malignant and benign tumours, and suggested that it was possible to reliably distinguish between controls and individuals with malignancy. Subsequent studies were unable to reproduce such a clear distinction in similar mixed cancer populations, which led to a comprehensive evaluation of the reproducibility and accuracy of NMR spectroscopy as a test for cancer [23]. The test was ultimately declared to be reproducible, but was not considered sufficiently accurate to be a cancer screening tool in the general population [23]. In hindsight, this is unsurprising, given our current understanding of the varying biology, invasiveness, and metastatic potential of different tumours, and the susceptibility of metabolomic profiles to lifestyle and other factors.

More recently, Odunsi et al. [24] have used a metabolomic approach to try to discriminate between women with epithelial ovarian cancer (EOC) and healthy controls. In their study, ^{1}H-NMR spectroscopy was performed on serum samples from 38 pre-surgical patients with EOC (2 patients at stage I, 34 patients at stage IIIC, 2 patients at stage IV), 12 patients with benign ovarian cysts, and 53 healthy women (21 pre-menopausal and 32 post-menopausal). The ^{1}H-NMR spectra showed substantial differences between blood samples taken from the patients with EOC and healthy subjects (Figure 3).
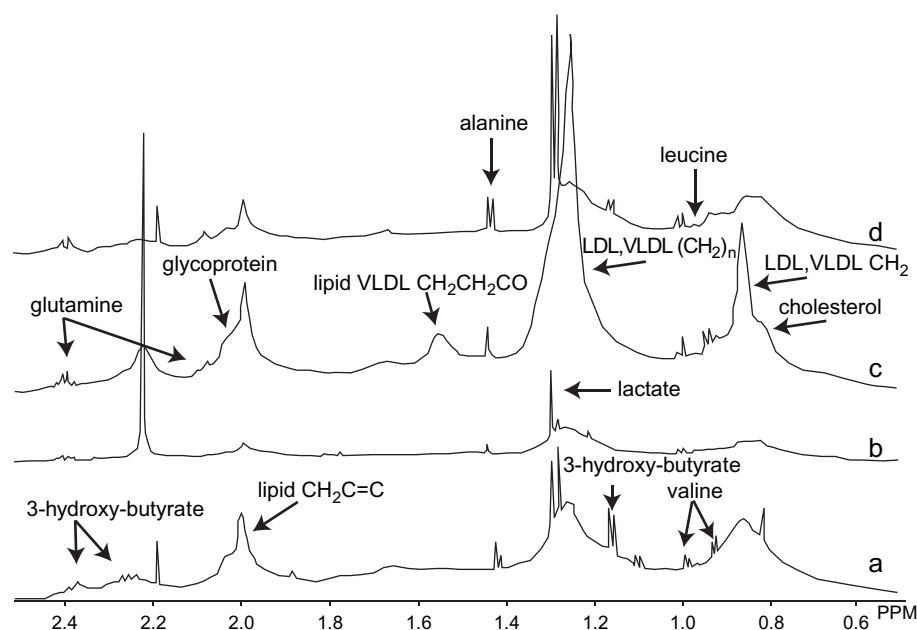
Figure 3. Comparison of patients with epithelial ovarian cancer (EOC) with healthy subjects [22]. The 600 MHz ^{1}H-NMR spectra of serum samples from (a) a post-menopausal stage I EOC patient, (b) a pre-menopausal health subject, (c) a post-menopausal healthy subject and (d) a patient with benign ovarian cyst (endometriosis). The chemical shifts of a selection of major metabolites are indicated (based on a comparison of published metabolites). Detection of epithelial ovarian cancer using ^{1}H-NMR-based metabolomics, Vol. 113, No. 5, 2005, 782–788. Copyright 2005 Odunsi K. Reprinted with permission of Wiley-Liss Inc, a subsidiary of John Wiley and Sons, Inc.

Principal Component Analysis (PCA) of the ^{1}H-NMR spectra (which groups metabolic signals with the strongest correlations with one another) allowed correct separation of all serum specimens from the 38 patients with EOC, all of the 21 pre-menopausal women and all the sera from patients with benign ovarian disease. Correct separation was also possible for 37 of 38 (97.4%) cancer specimens and from 31 of 32 (97%) post-menopausal control sera. The investigators concluded that metabolomic analysis of blood is a potential novel strategy for the early detection of epithelial ovarian cancer.

Studies in breast cancer epithelial cell lines suggest a sharp increase in metabolic activity of several pathways compared with normal cell lines, leading to, amongst other changes, an upregulation of fatty acid synthesis [25]. Katz-Brull et al (2002) have recently used ^{13}C-NMR-based spectroscopy to explore the biochemical pathways leading to the relatively high levels of water-soluble choline metabolites found in cancerous breast tumours, and reported differences in choline transport activity on the cell membranes of MCF7 human breast cancer cells compared with human mammary epithelial cells [26].

Cheng et al. (1998) performed a metabolomic analysis on 19 ductal carcinoma tissue biopsies excised from breast cancer patients. The spectroscopic analysis showed a dissimilar pattern of phosphocholine presence between the cancerous tissues and the surrounding normal breast tissue [27]. Mackinnon et al. (1997) have also explored the relationship between choline-containing metabolites and malignancy. These investigators evaluated 218 fine needle biopsy specimens taken from 191 patients undergoing breast surgery. Relying on the presence and intensity of choline peaks, the authors were able to discriminate between patients with invasive carcinoma, benign disease and carcinoma in situ [28]. All the above studies suggest that metabolic disturbances do

occur in breast cancer tumours; however, confirmation of these findings in larger studies is warranted.

Although still in its infancy, metabolomics may offer an attractive, non-invasive tool for the early identification of primary breast cancer and the detection of persisting disease after surgery. Sitter et al. (2006) analysed 85 tumour samples from breast cancer patients and 18 samples from adjacent non-tumour tissue using high-resolution magic-angle spinning MR spectroscopy [29]. The intensity of glycerophosphocholine, phosphocholine and choline signals were recorded and compared between the normal and tumour tissues. Principal component analysis enabled correct sample classification in the majority of cases (82% sensitivity, 100% specificity).

Mountford et al. (2001) undertook a similar NMR-based spectroscopic study using fine needle aspiration biopsies from 140 individuals with breast lumps (83 malignant and 57 benign) and were able to classify samples as malignant or benign with a sensitivity and specificity of 93 and 92%, respectively [30]. Additionally, pathological features such as nodal involvement and vascular invasion could be predicted with high accuracy using the NMR spectra [30].

These results are encouraging and should prompt a fuller integration of metabolomics into the context of cancer research. The technology has the potential to be used for early diagnosis through population screening and to predict prognosis after radical local treatment.

monitoring drug treatment response

Another intriguing potential application of metabolomics in cancer research is to monitor drug treatment response.

This potential has been highlighted through the work of Beloueche-Babari et al (2005) [29], who investigated four human carcinoma cell lines—three breast and one colorectal—to determine whether magnetic resonance spectroscopy could detect metabolic biomarkers associated with selective inhibition of mitogen-activated protein kinase (MAPK) by the MAPK inhibitor, U0126. These investigators reported time-dependent reductions in the levels of cellular phosphocholine as a result of the inhibition of MAPK signalling, suggesting that phosphocholine may be an important biomarker predicting objective responses to MAPK inhibitors.

early detection of drug toxicity

A third application of metabolomics in the field of cancer research is for the early detection of drug toxicity. We know, for example, that some chemotherapeutic agents—particularly anthracyclines—have a potential cardiotoxic effect, with toxicity being cumulative, dose-related, and irreversible. The most commonly recognised form of anthracycline-induced cardiotoxicity is left ventricular dysfunction progressing to congestive heart failure. The most likely pathophysiological mechanism underlying this dysfunction is oxidative stress, which induces changes in contractile protein turnover and mitochondrial function, leading to fibrillar disarray and apoptosis. Mechanisms to protect the cell from oxidative stress are highly conserved in the genome. One of these salvage pathways comprises a system that scavenges free-radicals, thus decreasing oxidative damage. The glutathione–glutathione peroxidase system promotes non-enzymatic detoxification of hydroxyl radicals, so enhancing the cell's capacity to tolerate oxidative damage. Thus, the balance between anthracycline-generated free radicals and salvage pathways is critical in determining myocyte damage [30]. Monitoring the early signs of cardiotoxicity offers the opportunity of early intervention, thereby allowing attenuation or abrogation of the full clinical expression of cardiac dysfunction. Metabolomics may offer the opportunity for screening and identifying sensitive and specific plasma or urine biomarkers that might prove to be specific markers of myocyte damage.

In summary, metabolomics has opened up new avenues in the field of cancer research, and offers the potential to identify novel diagnostic markers in breast cancer and other malignancies. We are particularly intrigued by the possibility of identifying new markers predicting chemotherapy activity and toxicity and hope to extend our understanding of the value of metabolic profiling in our own breast cancer studies. Metabolomics is undoubtedly an attractive tool offering a comprehensive approach to assessing the end products of many pathogenic pathways, and we look forward to more extensive use of this novel technology in oncology practice in the future.

circulating tumour cells

It has been known for some time that the presence of CTCs in peripheral blood is a clear indicator of a poor prognosis in metastatic carcinoma [31–33]. This is especially true for

metastatic breast cancer, although an association between the detection of CTCs and an unfavourable outcome has also been reported for metastatic prostate cancer [34], gastric cancer [35], colorectal cancer [36] and small cell lung cancer [37].

Some of the best and most rigorous studies demonstrating the prognostic potential of CTCs in metastatic breast cancer have been reported by Cristofanilli et al [38–40]. In their initial study, these investigators assessed CTC levels in 177 patients with measurable metastatic breast cancer before the initiation of a new line of therapy and reported that the number of CTCs before treatment was an independent predictor of progression-free survival and overall survival (Figure 4) [38]. Interestingly, CTC values obtained after one cycle of therapy predicted which patients were on ineffective treatment (Figure 4) [38].

Most recently, these investigators have reported that CTC levels have greater prognostic value than other conventional markers, and that detection of CTCs should now be considered for staging stratification of patients with metastatic breast cancer [40].

Several clinical studies have investigated the prognostic importance of CTCs in early-stage breast cancer [41–43]. Stathopoulou and colleagues (2002) [41] used a nested RT–PCR assay to detect cytokeratin-19-positive cells in the blood of 148 patients with operable breast cancer (stages I and II) and reported that cytokeratin-19 mRNA was detected in 44 patients (30%) before the start of adjuvant therapy and after removal of the tumour. These patients were followed up for a median of 28 months (range 7–62) and, during this period, 19 (13%) developed distant metastases and 8 (5%) died from breast cancer. The presence of cells positive for cytokeratin-19 mRNA in the peripheral blood was reported to have a significant independent influence on the disease-free interval and overall survival.

Xenidis et al (2006) [43] have recently analysed peripheral blood from 167 node-negative breast cancer patients before the initiation of systemic adjuvant chemotherapy and confirmed that detection of CTCs was an independent predictive and prognostic factor for a reduced disease-free interval and overall survival. Interestingly, the investigators also reported that the administration of CMF was an independent predictor of early relapse compared with the administration of the more intense T/EC regimen, leading them to suggest, albeit tentatively, that elimination of CTCs from a higher proportion of patients receiving the T/EC regimen might have resulted in the lower incidence of clinical recurrence reported.

Several other investigators have suggested that blood CTC monitoring in patients with primary breast cancer could help to identify high-risk patients and tailor treatment accordingly [44]. Meng and coworkers [44] hypothesized that CTCs may represent a 'real time' biopsy of metastatic tumours, allowing earlier detection of disease progression and individualised therapy. These investigators used a sensitive blood test to detect and characterise CTCs in terms of their HER-2 gene status in 31 patients with primary breast cancer and reported 97% concordance between the HER-2 status of the primary tumour and corresponding CTCs, with no false positives. In 10 patients with HER-2-positive tumours, the
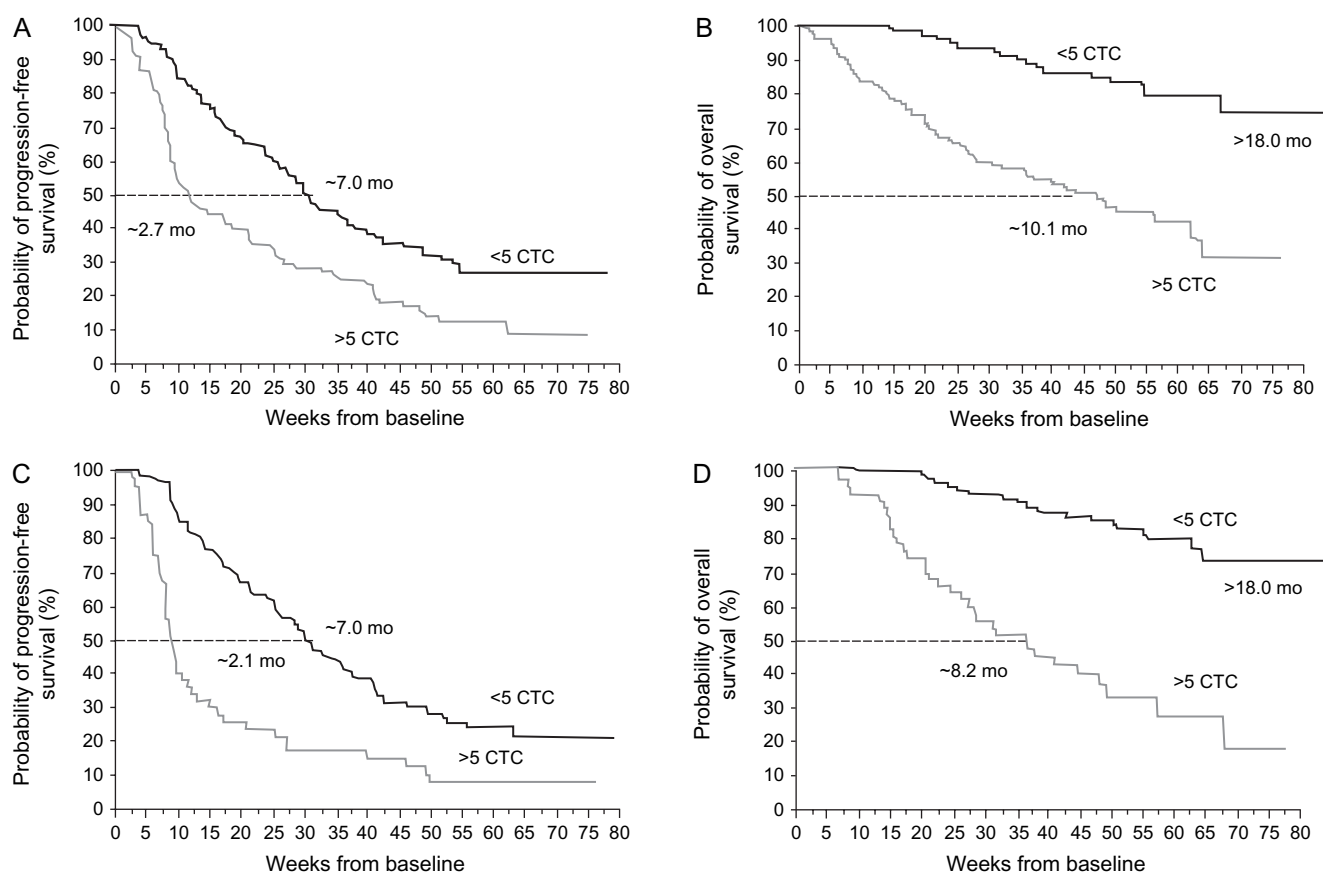
Figure 4. Kaplan–Meier curves demonstrating differences in progression-free survival (A and C) and overall survival (B and D) based upon high- and low-risk CTC classifications. Patients with elevated CTCs at baseline (A and B) and at first follow-up after one cycle of therapy (C and D) have significantly worse median progression-free survival and overall survival compared with the corresponding patients with low CTCs [38]. Reprinted with permission of the New England Journal of Medicine.

HER-2 chromosome enumerator probe 17 ratio in each tumour was twice that of the corresponding CTCs (mean 6.64 ± 2.72 versus 2.8 ± 0.6) demonstrating that the ratio of the CTCs was a reliable surrogate marker for the expected high ratio in the primary tumour. However, 9 (37.5%) of the 24 breast cancer patients whose primary tumour was HER-2 negative acquired HER-2 gene amplification in their CTCs during cancer progression. Four of the nine patients were subsequently treated with trastuzumab-containing therapy; one had a complete response and two had a partial response.

In our view, this study highlights beyond reasonable doubt the importance of monitoring CTCs in patients with breast cancer, regardless of their HER-2 status at the time of diagnosis. It seems clear that individuals whose tumours are HER-2 negative can acquire HER-2 gene amplification as breast cancer progresses, and that these patients may benefit significantly from additional target treatment.

The clinical studies reported so far suggest that CTC counting and biocharacterisation might in the future become an important tool to monitor treatment response and individualise anticancer therapies. Nevertheless, the available evidence is not strong enough to move this tool into daily practice. It still needs to be defined whether early detection of disease progression by CTC counting can lead to

a significant improvement in survival by early treatment activation. Moreover, only a well designed, adequately powered clinical trial can properly evaluate the efficacy of anti-HER-2 compounds in advanced breast cancer patients with a previous history of a HER-2 negative primary tumour and current evidence of HER-2-amplified CTCs. Ongoing trials will hopefully address these critical issues and define the clinical value of CTC counting and biocharacterisation in breast cancer patients.

conclusions

Metabolomics and the monitoring of CTCs are two of the most promising new strategies for molecular marker identification in breast cancer. Metabolomics holds the promise of a comprehensive, non-invasive analysis of metabolic biomarkers that could detect early-stage breast cancer, identify residual disease post-surgery, and help to monitor treatment response and detect early treatment toxicity. Routine screening for, and analysis of, CTCs could also help to identify patients at risk of breast cancer progression and allow targeted therapy for those patients whose tumour cells begin to express HER-2 and who could therefore benefit from additional treatment.

disclosures

The authors have no conflicts of interest to declare.

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