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REVIEW ARTICLE

Non-invasive proteomics—thinking about personalized breast cancer screening and treatment

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Abstract The early diagnosis of breast cancer in potentially curable stages improves prognosis and consecutively reduces mortality of breast cancer patients. Established screening programs have an unfavorable connotation due to significant rates of false negative as well as false positive results leading to overdiagnosis and overtherapy. The combination of a non-invasive *breast-cancer-suspectability-biomarker* with established clinical diagnostics could help to increase the acceptance of population based breast cancer screening programs by creating an individual risk profile, which is irrespective of mammography quality and interpretation. Recently, non-invasive proteomic biomarkers obtained from blood, saliva or nipple aspiration fluid have been extensively investigated and might play a future role in the personalized management of breast cancer screening. A simple, robust and inexpensive, non-invasive test for screening and diagnosis could easily be performed in every medical practice leading to an affordable, high-throughput instrument. This review describes recently investigated proteomic screening biomarkers that could improve the early diagnosis of breast cancer in the following years.

Keywords Breast cancer · Proteomics · Personalized screening · Personalized treatment

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Introduction

Breast cancer is the most commonly diagnosed type of cancer in women and is responsible for 15% of cancer related deaths in the United States [1]. Up to now clinical breast examination, imaging by mammography as well as tumor biopsy are the only recommended methods for breast cancer screening in the non-high risk population [2]. A non-invasive test for the early diagnosis of breast cancer would be an efficient step into personalized breast cancer screening and could significantly improve breast cancer survival by bringing the time of diagnosis at an earlier and therefore still curable stage [3, 4]. As an early diagnosis is the key for the successful treatment of breast cancer, much effort has been made to develop non-invasive biomarkers for the detection of early-stage breast cancer. But so far there is no reliable non-invasive test available for the clinical routine [2, 5].

In this review we would like to give an overview of investigated proteomic screening biomarkers in breast cancer research that could gain success in non-invasive breast cancer screening in the following years.

Non-invasive proteomics

Protein profiling-methods

As we know, cancer arises from genetic changes, by which numerous cellular processes such as growth regulation, proliferation and apoptosis are altered [6, 7]. Consequently, first approaches towards cancer-specific biomarkers were based on Genomics and Transcriptomics to get further insights into the genetic basis of cancer development [8–11]. In the process of developing an individual risk profile

for the development of breast cancer two breast cancer susceptibility genes BRCA1 and BRCA2 were identified in families with strong patterns of breast cancer [8, 11]. These two genes are considered to be tumor suppressors and can be found in all women as well as men. Women carrying a mutation in either BRCA1 or BRCA2 are in significantly higher risk of developing breast cancer or ovarian cancer [12]. Thus genetic testing for alterations in BRCA1 and BRCA2 can be used for predicting the individual risk of breast cancer development in patients with familial anamnesis of hereditary breast cancer. Nevertheless, there are major concerns regarding the use of BRCA1 and BRCA2 for genetic testing due to the variability of the eventual onset of breast cancer as well as the final outcome. In addition, women with familial risk without any mutations in BRCA1 or BRCA2 are still at risk of developing breast cancer after a negative testing for one of these genes. Hence, both—negative as well as positive results—may lead to mental distress and concerns regarding further management [13–15]. Alternative splicing of mRNA as well as proteins in combination with the multiplicity of post-translational alterations such as phosphorylation, ubiquitination or nitration explain why one single gene code for different protein species [16, 17]. Thus, Genetics by itself shows just an incomplete pattern of cancer development, whereas Proteomics reflects the genetic program of each cell as well as its final influence on the cellular physiology in a more dynamic way [18, 19].

The quest for screening biomarkers in cancer was initially based on two-dimensional gel electrophoresis (Fig. 1) [20]. Thereby specific protein fingerprints in malignant conditions are detected by their apparent molecular mass and isoelectric point. For protein detection the electrophoretically separated polypeptides are visualized by sensitive staining methods such as silver staining. In a further step the patterns of patients' samples and healthy controls are screened for differences in protein patterns. The spots of interest are excised from the gel, subjected to protease digestion by what peptide fragments can be examined in further analyses. Nevertheless, it is a time consuming process that is difficult to automate and to reproduce. Furthermore, it cannot adequately resolve the large number of protein modifications within a tumor sample.

Later on, advances in analytical technologies regarding mass spectroscopy (MS) facilitated large-scale proteomic analyses (Fig. 2) [21]. MS measures the mass to charge ratio (m/z) of ionized proteins as they travel through an electric or a magnetic field. Proteins are analyzed and identified based on unique spectrometric signatures, which reveals even structural features such as phosphorylation or methylation.

Blood-based protein profiling

Reflecting the physiological and pathological status of the human body in combination with its easy collection and availability blood represents the most extensively studied substance in the search of cancer biomarkers [22]. Specific tumor-secreted proteins in conjunction with cancer related patterns of digested tissue and plasma proteins make blood the ideal source for proteomic analyses [18, 23].

Numerous serum- and plasma-based SELDI-TOF MS and MALDI-TOF MS analyses were performed during the last years to differentiate between breast cancer patients, benign disease and healthy controls [24–27]. Each study described specific patterns of protein peaks, whereas all of them kept structurally unidentified and their validation in an independent sample set is still outstanding. Contrariwise, Li et al. screened the serum of 103 stage I–III breast cancer patients, 25 benign controls as well as 41 healthy women and verified two increased peaks (8.1 kDa, 8.9 kDa) as well as one decreased peak (4.3 kDa) in the serum of breast cancer patients which have been structurally identified as the C-terminal truncated form of complement component C3a (CtC3a, 8.1 kDa), complement component C3a itself

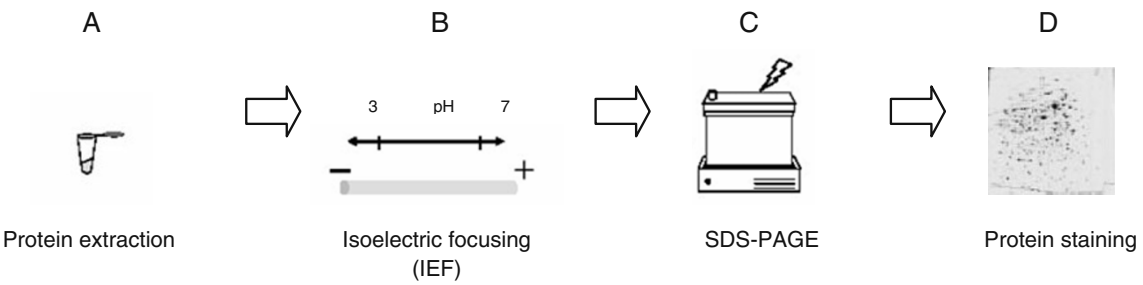

Fig. 1 Protein identification by two-dimensional gel electrophoresis (2D-PAGE). **A**: Protein extraction of patients' samples and healthy controls. **B**: By isoelectric focusing (IEF) proteins are separated on their isoelectric point (pI) in a strong electric field using stripes with an immobilized pH gradient (IPG-Stripe). **C**: After IEF an electric potential is applied to the IPG-Stripe at a 90 degree angle from the first field and proteins are separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel acts as a molecular sieve by separating the proteins on the basis of their relative molecular mass. **D**: Proteins are visualized by sensitive staining methods such as silver staining

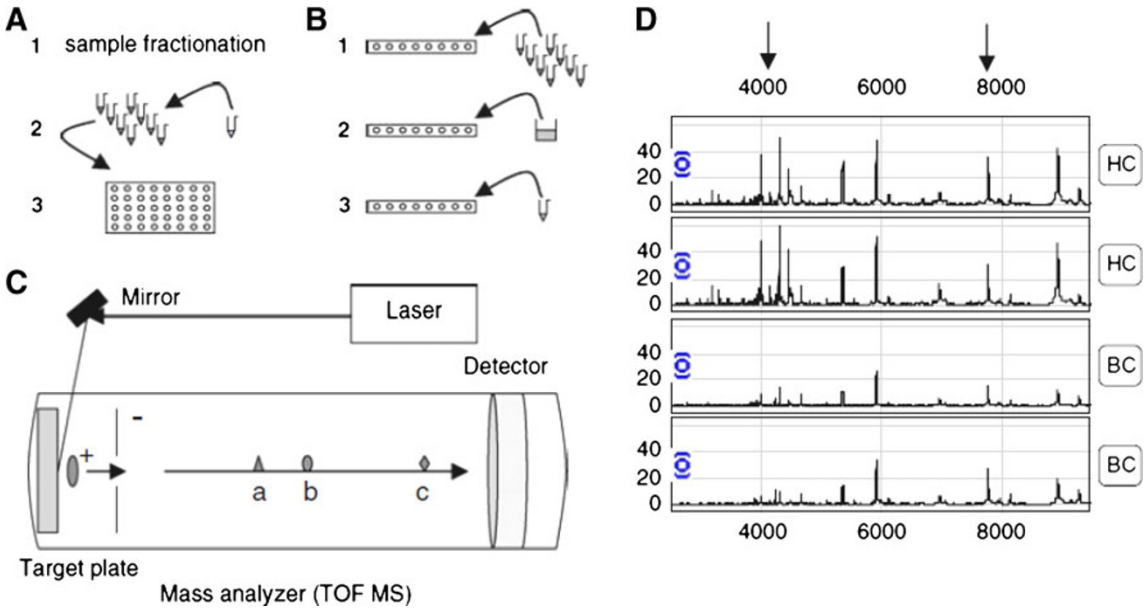

Fig. 2 Schematic demonstration of MALDI- and SELDI-TOF MS (adapted from [20]). **A**1–**A**3: Performing matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) the samples are fractionated off-line using chromatographic beads. An energy absorbing matrix e.g. cinnamic acid is added to the fractionated samples and this mix is applicated to an inert plate for laser desorption/ionization in a time of flight (TOF) mass analyzer (C). **B**1–**B**3: For surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) the sample is loaded onto a protein-selective array with a hydrophilic, hydrophobic, cationic, anionic or immobilized-metal affinity capture moiety in an appropriate binding buffer. The sample is purified by one or more washing steps and the bound proteins are treated with an energy-absorbing matrix such as cinnamic acid for desorption/ionization by laser in a TOF mass analyzer (C). **C**: The MALDI target plate or SELDI array is inserted into the analyzer, where bound proteins are treated with laser irradiation for desorption and ionization. Owing to the electric field, they are accelerated through a TOF-analyzer and separated by a mass per charge ratio (m/z): small proteins (c) migrate faster than multiply charged (b) or large ones (a). **D**: In a resulting mass spectrum the m/z-ratio is displayed on the x-axis, whereas the y-axis reflects the protein abundance. Here, SELDI-TOF MS shows up-regulations in the serum of breast cancer patients (BC) compared to healthy controls (HC) are visible at m/z 3980, m/z 4292 and m/z 8939

(8.9 kDa) and a fragment of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4, 4.3 kDa) [28, 29]. In a further independent sample set only the C-terminal truncated form of complement component C3a (8.1 kDa) and complement component C3a (8.9 kDa) could be verified, whereas the 8.1 kDa-fragment also lost its significance in further validation [29–31]. In comparable studies the decreased ITIH4-fragment at 4.3 kDa was found to be increased in the serum of breast cancer patients or even lost its ability to distinguish between cancer patients and healthy controls [32–34]. Due to the inhomogeneous results regarding ITIH4 and the C3-complement components described above, which have recently been reconfirmed to be up-regulated in breast cancer patients, the diagnostic strength of these markers can not be finally evaluated yet [35]. Fan et al. described also a down-regulated candidate protein (6.6 kDa), which was identified as apolipoprotein C-1 (APO-C1) that might play a certain role in breast cancer carcinogenesis [35]. Further candidate biomarkers of the lipoprotein-group (APO A-IV) had also been described by Villanueva et al. before, whereas these findings as well as the diagnostic power of further candidate markers such as fibrinogen alpha, bradykinin, factor XIII or transthyretin have to be validated in larger studies before their future clinical implementation [34]. In a pathology-specific blood proteome analysis by Braun et al. expression patterns of circulating leucokytes were investigated in breast cancer patients, patients with benign tumors and healthy controls [36]. Performing protein mapping this group demonstrated significant alterations in the repertoire of microfilamental network-associated proteins in circulating leukocytes of breast cancer patients compared to healthy controls: Calgranulin A (S100), LyGDI (RhoGDIβ), RhoA and profilin 1. Investigating cellular subfractions by two-dimensional gel electrophoresis (2D-PAGE), nuclear matrix proteins (NMP) have been shown to be cell and cancer specific [37–40]. Hence, circulating NMP are ideal candidates for non-invasive cancer diagnosis in several cancer entities [41–43]. Unlike established protocols of separating NMP by two-dimensional gel electrophoresis, NMP66 (28.3 kDa) was found to be specific for breast cancer by a serum proteomic screening strategy using SELDI-TOF MS [44]. But up to now NMP66 did not make its way to clinical routine. Based on our expertise with CCSA-2 in the development of a serum-based immunoassay in colon cancer we are investigating the use of NMP in breast

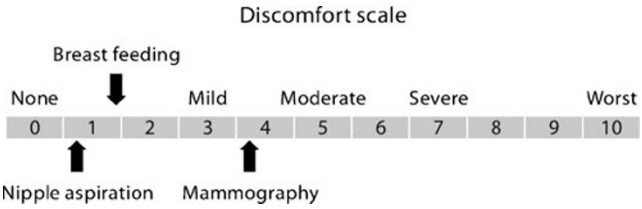

Fig. 3 Discomfort of nipple aspiration in comparison with mammography and breast feeding in a study on female healthy volunteers [48]

cancer by two-dimensional gel electrophoresis in an ongoing study [43].

Diagnostic proteomics in nipple aspiration

Nipple fluid, that contains breast epithelial cells, is produced in the breast ducts and can be collected by vacuum aspiration or ductal lavage. Based on the idea that the concentrated source of proteins from breast cancer ducts might be better able to identify tumor-specific protein patterns, attention has been paid to the proteomic analysis of nipple aspiration fluid (NAF) and ductal lavage fluid (DLF). Performing SELDI-TOF MS of 114 NAF-samples obtained from 27 breast cancer patients and 87 healthy controls Sauter et al. defined a 11.8 kDa protein that appeared to be specific for breast cancer patients [45]. Pawlik et al. employed the use of SELDI-TOF MS to NAF in 23 women with stage I and II breast cancer to compare them to 5 healthy volunteers [46]. In this study 17 peaks were overexpressed in cancer-bearing breasts compared to breasts of healthy controls ($p<0.0005$). When spectra from the nontumor-bearing breasts of breast cancer patients were compared with spectra from breasts of healthy controls, two distinct peaks were noted to be overexpressed in breast cancer patients and one peak was underexpressed ($p<0.0027$). These results get support from Noble et al. who found nine protein peaks to be significantly different between the cancer-bearing breast compared with healthy women as well as 10 peaks of the contralateral healthy breast and healthy women ($p<0.05$) [47]. Performing two-dimensional gel electrophoretic separation and MALDI-TOF analysis in 20 NAF-samples of 10 breast cancer patients and 10 healthy controls Alexander et al. distinguished between three peaks that were up-regulated in three or more breast cancer patients [48]. These peaks were identified as gross cystic disease fluid protein-15 (GCDFB-15), apolipoprotein-D (apo-D) and alpha-1-acid glycoprotein (AAG). For further validation 105 NAF-samples (52 cancerous and 53 controls) were analyzed by ELISA, whereas GCDFB-15 levels were showed to be lower ($p<0.001$) and AAG levels to be higher ($p<0.001$) in breast cancer patients. Apo-D levels were not associated with breast cancer in this control.

Based on recent findings of Suijkerbuijk et al., who described the advantages of Oxytocin-supported nipple aspiration affiliated with reduced discomfort (Fig. 3), NAF might be a promising instrument for breast cancer diagnosis and should be validated in further studies [49].

Salivary protein profiling

Saliva is the secretion of the salivary glands representing a source of easily accessible body fluids. In 1999 the use of saliva for diagnostic purposes has been demonstrated by the detection of elevated levels of CA15.3 and c-erB-2 in breast cancer patients [50]. Inspired by the absolute non-invasive character as well as the ease of sample handling Steckfus et al. investigated the feasibility of salivary protein profiling for diagnostic purposes in breast cancer [51]. Using SELDI-TOF MS five high molecular weight peaks were found to be overexpressed in breast cancer patients compared to healthy controls. Although these peaks were never structurally identified this group was able to demonstrate the potential use of saliva for breast cancer diagnosis. Recently, the initial findings were supported by salivary protein profiles that are unique to fibroadenoma and ductual carcinoma in-situ of the breast [52].

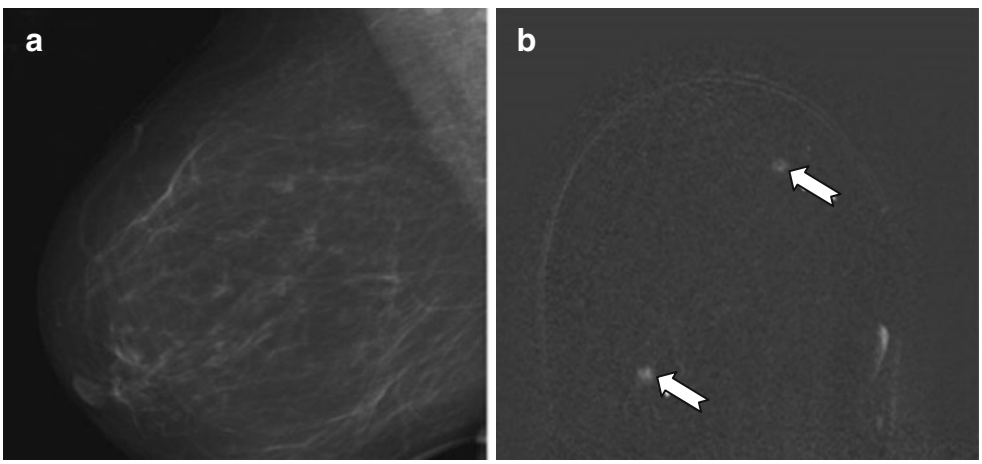

Fig. 4 False-negative mediolateral oblique mammogram of a 50 years old female patient in routine breast cancer screening (**a**). The additional Breast-MRI (**b**) identifies two small enhancing foci (*arrows*). MR-guided biopsy revealed multifocal ductal invasive breast cancer

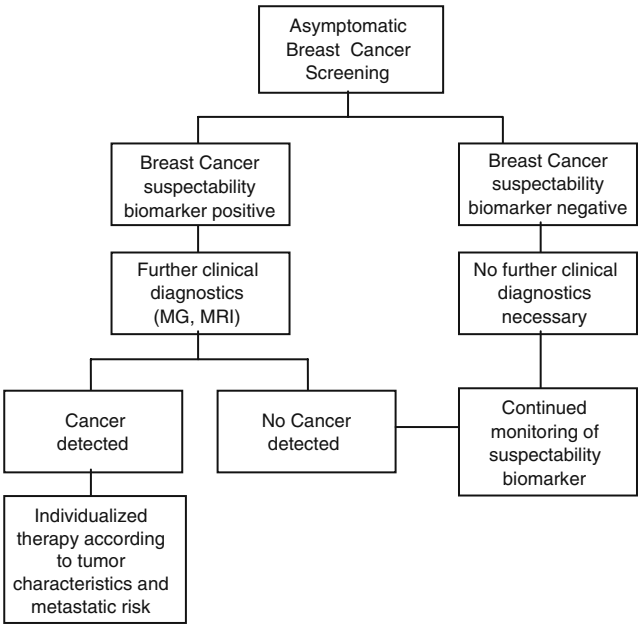

Fig. 5 Flow-chart: Personalized Screening for breast cancer using a screening biomarker as gatekeeper before further clinical diagnostics

Potential behind non-invasive diagnostic technologies

The early diagnosis of breast cancer in a potentially curable stage improves the prognosis and consecutively reduces mortality of breast cancer patients [3, 4]. The established screening by breast examination and mammography is able to detect breast cancer in early stages and has been shown to reduce mortality [53, 54]. Nevertheless, screening by mammography is controversially discussed due to significant rates of false negative results (Fig. 4) as well as false positive results leading to overdiagnosis and overtherapy [55]. Especially young women (<50 years) with high breast density show low sensitivities in mammography, wherefore established mammography screening programs are mainly addressed to older patients (50–69 years) [56, 57]. Keeping in mind that 1 of 5 breast cancers occurs in this non-screened subgroup and these patients suffer from more aggressive and fast growing forms of breast cancer the proposed non-invasive screening biomarker would be of outstanding value [58, 59].

Thus it is necessary to implement a reliable non-invasive test as a gatekeeper for further diagnostics among asymptomatic patients (Fig. 5). In a second step only patients with a positive *breast-cancer-suspectability-biomarker* would receive further imaging or biopsy. Asymptomatic patients showing a negative *breast-cancer-suspectability-biomarker* are not in need of additional clinical diagnostics and are spared uncertainties and anxiety due to ambiguous imaging results. Table 1 summarizes candidate non-invasive proteomic biomarkers that could improve the early diagnosis of breast cancer in the following years.

Table 1 Non-invasive Proteomic biomarkers for breast cancer discussed in this review

Medium	Biomarker	Profiling method
Serum	C3a	SELDI-TOF MS
Serum	CtC3a	SELDI-TOF MS
Serum	ITIH4	SELDI-TOF MS
Serum	APO-C1	SELDI-TOF MS
Serum	APO A-IV	MALDI-TOF MS
Serum	Fibrinogen alpha	MALDI-TOF MS
Serum	Bradykinin	MALDI-TOF MS
Serum	Factor XIII	MALDI-TOF MS
Serum	Transthyretin	MALDI-TOF MS
Blood (Leukocytes)	Calgranulin A	2D-PAGE, MALDI-TOF MS
Blood (Leukocytes)	LyGDI (RhoGDIβ)	2D-PAGE, MALDI-TOF MS
Blood (Leukocytes)	RhoA	2D-PAGE, MALDI-TOF MS
Blood (Leukocytes)	Profilin 1	2D-PAGE, MALDI-TOF MS
Serum	NMP66	SELDI-TOF MS
Nipple fluid	11.8 kDa peak (structurally unidentified)	SELDI-TOF MS
Nipple fluid	GCDFB-15	2D-PAGE, MALDI-TOF MS
Nipple fluid	APO-D	2D-PAGE, MALDI-TOF MS
Nipple fluid	AAG	2D-PAGE, MALDI-TOF MS
Saliva	18 kDa peak (structurally unidentified)	SELDI-TOF MS
Saliva	113 kDa peak (structurally unidentified)	SELDI-TOF MS
Saliva	170 kDa peak (structurally unidentified)	SELDI-TOF MS
Saliva	228 kDa peak (structurally unidentified)	SELDI-TOF MS
Saliva	287 kDa peak (structurally unidentified)	SELDI-TOF MS

Clinical impact of predictive medicine in breast cancer treatment

In a further step towards personalized medicine recent biomarker developments regarding innate tumor characteristics could contribute to a more individualized breast cancer management. It has been shown that each tumor presents unique characteristics with different outcomes, relapse and responsiveness to therapy [60–62]. Traditionally, breast cancer prognosis was determined by clinical as well as histopathological features such as age, tumor size, lymph node status, grading, hormone receptor status and Her2/neu status [63–65]. Recently, these traditional factors have been completed by multi gene expression tools like Mammaprint, Oncotype DX or Rotterdam Signature that can be used to predict the risk of recurrence or distant metastases in node-negative disease [9, 10, 66–68]. In patients with newly diagnosed node-negative breast cancer urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1) might be used for prognostic evaluation as well [69]. It has been shown that elevated levels of both factors, uPA and PAI-1, in tumor tissue are associated with a higher risk of recurrence and death, wherefore especially these patients may benefit from adjuvant treatment [70–72]. Another target of individualized breast cancer management should be residing on predictive biomarkers determining the response to certain adjuvant regimes in a particular patient. It is known that Anthracycline-based treatment alone as well as in combination with Taxanes shows significant benefits in patients with lymph node-positive breast cancer and Her2/neu-overexpression [73–75]. Further factors like Topoisomerase II-overexpression, which suggests greater benefit from Anthracycline-based chemotherapy, or low expression of Microtubule-associated protein tau, which is associated with increased chemosensitivity to Taxanes, are under ongoing investigation [76–78]. Contrariwise, high levels of Tau correlate with estrogen receptor (ER)-positive breast cancer and may predict endocrine sensitivity in this subgroup of patients [79]. Consequentially, an increased expression of Microtubule-associated protein tau may define ER-positive breast cancers with increased sensitivity to endocrine therapy, whereas lower Tau levels may indicate better chemosensitivity to Taxanes ER-positive breast cancer patients [80].

Outlook

Biomarkers that detect cancer, predict cancer outcome and influence treatment choices will play a major role in the future management of breast cancer. The proposed combination of an objective *breast-cancer-suspectability-biomarker* with established clinical diagnostics could help to increase the acceptance of population based breast cancer screening programs by creating an individual risk profile, which is irrespective of mammography quality and interpretation. A simple, robust and inexpensive, non-invasive test for screening and diagnosis could easily be performed in every medical practice leading to an affordable, high-throughput instrument. Recent technological improvements to identify and characterize proteins by two-dimensional gel-electrophoresis and mass spectroscopy in combination with improved bioinformatical databases and analysis software make Proteomics a powerful tool in the quest for new tumor markers. Thus, outmost effort should be put into the development, validation and clinical implementation of reliable screening biomarkers.

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