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SELDI-TOF MS Proteomics in Breast Cancer

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Abstract
Background Proteomic profiling is a rapidly developing technology that may enable early disease screening and diagnosis. Surface-enhanced laser desorption ionization–time of flight mass spectrometry (SELDI-TOF MS) has demonstrated promising results in screening and early detection of many diseases. In particular, it has emerged as a high-throughput tool for detection and differentiation of several cancer types. This review aims to appraise published data on the impact of SELDI-TOF MS in breast cancer.
Methods A systematic literature search between 1965 and 2009 was conducted using the PubMed, EMBASE, and Cochrane Library databases. Studies covering different aspects of breast cancer proteomic profiling using SELDI-TOF MS technology were critically reviewed by researchers and specialists in the field.
Results Fourteen key studies involving breast cancer biomarker discovery using SELDI-TOF MS proteomic profiling were identified. The studies differed in their inclusion and exclusion criteria, biologic samples, preparation protocols, arrays used, and analytical settings. Taken together, the numerous studies suggest that SELDI-TOF MS methodology may be used as a fast and robust approach to study the breast cancer proteome and enable the analysis of the correlations between proteomic expression patterns and breast cancer.
Conclusion SELDI-TOF MS is a promising high-throughput technology with potential applications in breast cancer screening, detection, and prognostication. Further studies are needed to resolve current limitations and facilitate clinical utility.

Keywords Breast cancer · SELDI · Proteomics · Biomarkers

Abbreviations

ADH	Atypical ductal hyperplasia
BI-RADS	Breast Imaging Reporting and Data System
BRCA	Breast cancer gene
Da	Dalton
DCIS	Ductal carcinoma in situ
EGF	Epidermal growth factor
ER	Estrogen receptor
FLC	Ferritin light chain
HNP	Human neutrophil peptides
IBC	Invasive breast carcinoma
IHC	Immunohistochemistry
IMAC	Immobilized metal affinity capture
kDa	Kilodaltons

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LMW	Low molecular weight
m/z	Mass to charge
MALDI	Matrix-assisted laser desorption ionization MS
NAF	Nipple aspiration fluid
PND	Pathological nipple discharge
PR	Progesterone receptor
SAX	Strong anion exchange (Q10)
SELDI	Surface-enhanced laser desorption ionization
SLN	Sentinel lymph node
WCX	Weak cation exchange (CM10)

Introduction

Surface-enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS) blends together the principles of retention chromatography and mass spectrometry (Figs. 1 and 2), providing a rapid, high-throughput, and relatively sensitive screening method capable of analyzing complex protein samples. This technology is also capable of rapid separation, detection, and analysis of proteins at the femtomole level, directly from biological samples. It is a tool, per se, that enables multi-analyte discovery and facilitates analyses of large numbers of different samples with the simultaneous study of multiple biological variables.

The limitations of current diagnostic techniques for asymptomatic breast disease have been well documented [1–3]. While breast screening by mammography can reduce mortality from breast cancer [4–6], the sensitivity of mammography is limited and reported at 63–87% [7] only. In addition, mammographic sensitivity is lower in young women due to increased breast density [1–3], and so younger women and patients with a genetic predisposition for early onset breast cancer (for example BRCA1 or BRCA2 mutations), frequently miss the diagnostic screening window [8]. These factors have prompted the search for alternative approaches to improve the sensitivity of breast cancer diagnosis and screening.

Alterations in individual or groups of proteins, their abundance, structure, or function, can act as useful indicators of pathological abnormalities prior to development of clinical symptoms. Similar to other malignancies, the multifactorial nature of breast cancer lends itself towards the use of multiple biomarkers for early detection, prognostication, and the monitoring of response to therapy. Consequently, analysis of panels of biomarkers may prove more reliable for the detection and subtyping of breast cancer than using single markers. Furthermore, markers detecting early stage disease would be valuable since they could identify the disease at an early curable phase. For example, while ductal carcinoma in situ (DCIS) is potentially curable if detected early [9, 10], more than one third of all breast cancers are disseminated at diagnosis [9]. The emerging field of proteomics allows the discovery of early stage biomarkers in cellular or biological fluids. SELDI-TOF MS combines an "on-array" separation of complex protein mixtures via retention coupled directly with mass spectrometry detection. This technique enables profiling of proteins from different biological samples such as cancer cell lines, serum, nipple aspirate, plasma, urine, and tissue extracts, and has the advantage of high sample throughput, which allows generation of sufficient data to adequately power statistical tests. The principles and clinical applications of SELDI have been reviewed and have been successfully used in biomarker discovery on various tissues and samples [11, 12]. Only a few microliters of the sample of interest are deposited on a selective chromatographic surface (array), which retains proteins from biological mixtures according to their physico-chemical properties. Consequently, the proteins of interest are captured on the chromatographic surface depending on the arrays' properties (Table 1). Special biochemically active surfaces can also be used to exploit specific molecular recognition mechanisms such as antibody–antigen, enzyme–substrate, receptor–ligand, and protein–DNA interactions. This is a unique advantage of SELDI over other forms of MS where crude samples cannot be reliably used for protein analysis. Mass testing and comparison between protein peaks can pinpoint significant differences in protein abundance between samples. SELDI-TOF MS, therefore, allows potent protein analysis from a variety of sample types, with minimal sample consumption and processing.

If the analysis reveals candidate biomarkers or a combination (panel) of biomarkers, classification rules can then be used to discriminate between the various groups. Validation with a larger cohort is then required ideally using independent blinded samples from different centers to reduce sources of bias and avoid extraneous variation. Once the peptide peak of a potential biomarker is established, purification and identification of the biomarker is clearly an additional complementary step.

The use of SELDI-TOF MS and other mass spectrometry platforms, such as matrix-assisted laser desorption ionization (MALDI MS) for proteomic profiling of body fluids has many potential uses and advantages as a clinical assay. These techniques use readily accessible clinical samples and have proven to be reliable and reproducible [11, 13, 14]. SELDI is a modification of MALDI but has the advantage of immobilizing proteins from complex biological samples on selective chromatographic surfaces. It is thus designed to detect subtle changes in protein properties by the different conditions of each surface. Moreover, fractionation of samples prior to analysis with SELDI increases the possibility of low abundance protein discovery. Different spectra

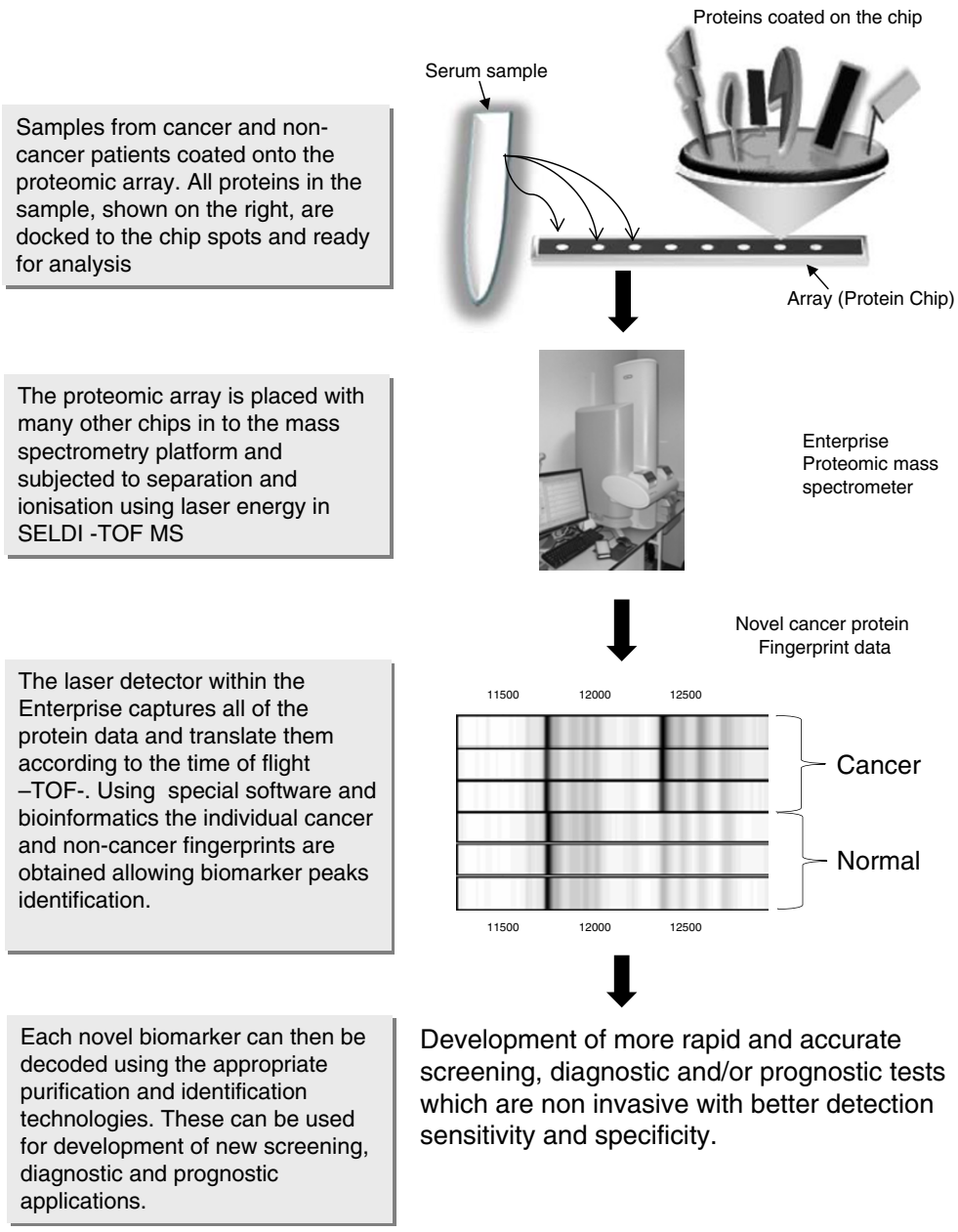

Fig. 1 An illustration of the SELDI-TOF MS experimental steps. This starts with sample preparation followed by its application onto a chromatographic array. The samples will then be loaded into the SELDI-TOF MS and targeted with laser. This will ionize the proteins within the sample and will allow them to fly to an oppositely charge electrode in a vacuum chamber. A laser detector will then measure the time of flight (TOF) of each protein which reflects its molecular weight. Using special software the masses will be presented in waves corresponding to *m*/*z*. This can then be analyzed using different bioinformatic tools to detect any differentially expressed peaks between different groups

may be generated from the same sample using different surfaces and sample fractions, increasing the venues for deep protein mining. In this article, we present a critical review of the role of SELDI-TOF MS in breast cancer profiling. We highlight the significant findings to date and discuss weaknesses and limitations of existing studies with suggestions for improvement and optimization of the use of SELDI-TOF MS in this field.

Serum Proteomic Profiling in Breast Cancer

Serum has been widely used for study by SELDI proteomics as it is relatively minimally invasive, reproducible, and cheap to obtain. Based on the hypothesis that cancer-related protein/peptide signatures in sera can be used in the early detection of cancer, serum proteomic expression profiles have been studied as potential tools for early diagnosis. During tumorogenesis, differentially expressed serum proteins can originate from different sources. Direct or indirect communication of cells with the blood may lead to the release of tissue specific proteins upon cell damage or death. Serum profiles could also reflect specific cancer excreted proteins, host response, acute phase reactants, or elevated normal body protein levels in response to tumors. Since these profiles could represent altered phenotypic events during neoplastic transformation and progression, it is reasonable to expect characteristic proteomic profiles in the presence of cancer.

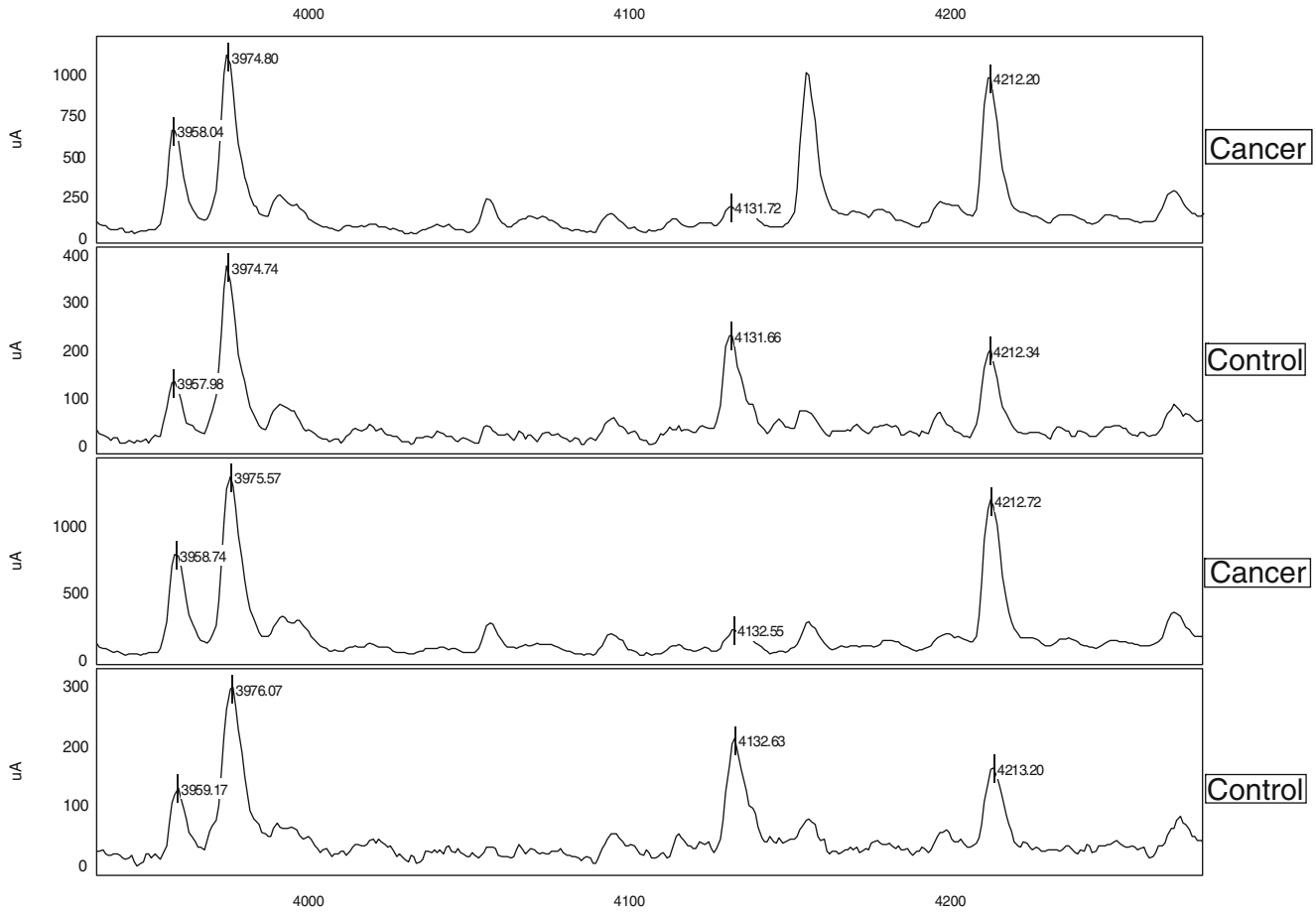

Fig. 2 An Illustration of peak clusters generated by SELDI-TOF MS proteomic profiling. *Marked values* represent significant mass/charge (*m*/*z*) protein peaks. The differential expression of proteins between cancer and control spectra is illustrated in this figure. Few of the differentially expressed peaks between the two groups are detected in a very narrow region of the spectra between 3.6 and 4.2 kDa. *Horizontal and vertical axes* represent *m*/*z* in Daltons and peak intensity in microampere, respectively

Serum Proteomic Profiling in Breast Cancer Prediction

One of the early pilot studies of SELDI proteomic profiling in breast cancer was a prospective analysis of 134 pre-treatment serum samples by Vlahou et al. [15] (Table 2), to determine if protein profiling could distinguish between patients and controls. Multivariate analysis revealed three discriminatory peaks differentiating cancer from normal

Table 1 Summary of the description and applications of most commonly used ProteinChips in SELDI-TOF MS proteomic profiling

Array	Description	Applications and requirements
IMAC30	Immobilized metal affinity capture (IMAC), with nitriloacetic acid surface	Transition metal activation is needed prior to use. Phosphorylated proteins and polyhistidine-tagged proteins capture. Protein profiling and identification
CM10 (WCX)	Weak cation exchanger with negatively charged carboxylated surface	Protein profiling and identification
Q10 (SAX)	Strong anion exchanger with positively charged quaternary amine surface	Protein profiling and identification
H50	Hydrophobic interaction surface Reverse phase	Protein profiling and identification
NP20	Normal phase surface	Quality control assay and protein molecular weight validation
PS10, PS20, RS100	Pre-activated surfaces	Specific protein–protein binding (e.g., protein–ligand or antibody–antigen binding)

Information adapted from Bio-Rad Inc

Table 2 Summary of the candidate biomarkers detected in different studies

	Study			
	Vlahou et al. [15]	Li et al. [18]	Li et al. [17]	Mathelin et al. [19]
Number	134 45 patients with breast cancer, 42 benign disease, 47 healthy controls	169 99 stage I–III 4 DCIS 41 healthy controls 25 benign disease	176 61 patients locally invasive breast cancers (IBC), 32 DCIS, 37 benign breast lesions (19 atypical ductal hyperplasia (ADH)), 46 age-matched healthy subject	89 49 patients with breast cancer, 13 patients with benign breast disease, 27 from healthy controls
Sample	Serum	Serum	Serum	Serum
Array used	IMAC30, Q10	IMAC-Ni	IMAC-Ni	IMAC-Ni
Differential peaks	**Cancer vs. control** **2.95 kDa** **3.68 kDa** **4.27 kDa** **Cancer vs. benign** **6.43 kDa** **7.48 kDa** **8.61 kDa**	**BC1: 4.3 kDa↓[a]** **BC2: 8.1 kDa ↑[a]** **BC3: 8.9 kDa ↑[a]**	**BC2: 8.1 kDa ↑[a]** **BC3: 8.9 kDa ↑[a]**	**BC1: 4.3 kDa ↓[a]** **BC1a: 4.286 kDa ↓[a]** **BC1b: 4.302 kDa ↓[a]** **BC3: 8.9 kDa ↑[a]** **BC3a: 8.919 kDa ↑[a]** **BC3b: 8.961 kDa ↑[a]**
Comments	Using combination of peaks from two different chips improved sensitivity and specificity of detection to 90 and 93% respectively	BC1, BC2, and BC3 were suggested as potential breast cancer biomarkers. They achieved sensitivity and specificity of 93% and 91%, respectively	The study failed to validate BC1: 4.3 kDa BC2 and BC3 were identified as complement components BC2 and BC3 did not correlate to size, grade, nodal, or ER/PR status	Failed to recover BC2: 8.1 kDa from Li et al. [16] No correlation was found between the markers and age, nodal status, metastasis, vascular invasion, ER/PR status, or Ca 15.3

groups (Table 2). In addition, three different peaks segregated cancer serum sample proteomic profiles from the benign group. Cross validation analysis of cancer versus normal samples revealed a sensitivity and specificity of 80% and 79%, respectively, and for cancer versus benign disease of 79% and 83% respectively. The use of multiple chromatographic binding surfaces (immobilized metal affinity capture (IMAC) and strong anion exchange (SAX)) further improved sensitivity and specificity in this study.

Laronga et al. [16] utilized the optimized assay conditions described by Vlahou et al. [15]. The main peaks to discriminate between breast cancer patients and controls on cross validation were selected. Using this approach, they were able to differentiate between patients with cancer and controls with a sensitivity of 87% and a specificity of 93% on cross-validation. This is consistent with the findings of Vlahou on which many of the parameters of this study were based [17].

In a further pilot study, Li et al. [18] performed screening for potential biomarkers on the serum of 169 patients with breast cancer and healthy controls. Serum from participants was tested by chromatography using SELDI-TOF MS following standardized sample preparation [18]. A panel of three markers (BC1 4.3 kDa, BC2 8.1 kDa, BC3 8.9 kDa) [18] were selected based on their ability to distinguish between invasive disease and controls, or in situ disease. When applied to the same cohort, they achieved 93% sensitivity and 91% specificity in distinguishing between these groups. Nevertheless, as there were only four patients with DCIS, and this study could not test the ability of these markers in identifying the disease at an early in situ stage. In a separate study, the authors tested the same markers in a different patient population [17]. Serum samples obtained from 176 women prior to medical intervention were used. Similar to the original study [18], two peaks—BC2 (8.1 kDa) and BC3 (8.9 kDa)—were differentially overexpressed in breast cancer patients compared to healthy subjects, demonstrating the reproducibility of SELDI-TOF MS across different patient populations. Furthermore, the authors were able to identify these peaks as complement components C3adesArg and a C-terminal-truncated form of this component. This illustrates one of the concerns in such studies, where non-specific candidates qualify as discriminatory biomarkers. This study was unable to validate the third candidate peak previously

Table 2 (continued)

	Study		
	Van Winden et al. [20]	Laronga et al. [16]	Becker et al. [23]
Number	96 48 primary invasive breast cancer vs. 48 healthy	16 cancer pre- and post-surgery samples 15 healthy	62 15 BRCA1 cancer 15 BRCA1 carrier 16 SBC 16 Norml
Sample	Serum	Serum	Serum
Array used	IMAC-Ni	IMAC-Cu	IMAC-Cu
Differential peaks	**Cancer vs. control** **BC1: 4.3 kDa ↓**[a] **BC3: 8.9 kDa ↓**[a]	Pre- vs. post-surgery 6.194 kDa↑ 2.276 kDa ↑ 3.892 kDa ↑ BRCA1 cancer vs. carrier **5.9 kDa ↓**[a] SLN + ve vs. SLN −ve SAX: **5.9065** ↓[a] 4.0277, 7.4144 kDa IMAC-Cu: 1.437, 1.003, 1.349 kDa	BRCA1 cancer vs. carrier **8.138 kDa ↑**[a] **5.909 kDa ↓**[a] BRCA1 cancer vs. SBC **8.1 kDa ↑**[a]
Comments	The study detected a possible BC2 peak at 8.1 kDa which was not differentially expressed between the two groups	All three differentially expressed peaks are overexpressed post-surgery	BRCA1 carriers and healthy had similar profiles
	No correlation between any candidate biomarker and tumor characteristics	5 peaks were retained by cancer patients post-treatment, whether this correlates to residual disease, poorer response/outcome, or DFS is yet to be studied	BRCA1 cancer had 8.1 kDa compared to BRCA1 carrier/SBC, but not from healthy patients
	Addition to discrepancy in validation studies		Limited study—small sample size, needs validation

detected, BC1 (4.3 kDa), which was identified as the truncated form of interalpha-trypsin inhibitor heavy chain H4 [17]. This peak was underexpressed in the breast cancer group within the first set [18] and overexpressed in the validation breast cancer population [17]. Clearly, reproducibility and robustness of the SELDI-TOF MS technique necessitates consistent collection, storage, handling, and sample preparation techniques, with similar protein array surfaces and analytical and bioinformatics settings.

In a second validation of this study, Mathelin and coworkers [19] also found that the combination of the two peaks BC1 (4.3 kDa) and BC3 (8.9 kDa) differentiated between breast cancer patients and healthy subjects. The BC2 marker recorded by Li et al. [18] was not recovered. This later found four peaks which could correspond to the BC1 and BC3 peaks identified in the previous work. The first two corresponding peaks, termed BC1a (4,286 Da) and BC1b (4,302 Da) could correspond to BC1 (4.3 kDa) from the previous work of Li et al., and were similarly underexpressed in the breast cancer patients. Likewise, the peaks named BC3a (8,919 Da) and BC3b (8,961 Da) appeared to correspond to the BC3 (8.9 kDa) peak from Li et al. and were overexpressed in the cancer group. Division of the four peaks into stringent (no error) and flexible (<10% error) enabled identification of 33% and 45% of patients with breast cancer, from patients with benign disease and controls, respectively. Interestingly, the proteomic expression patterns in patients with benign disease were similar to controls. In a trial to combine use of these peaks with an existing clinical tumor marker, Ca 15.3, the predictive power of the combination of these markers improved the ability for cancer detection. While this partially validated the earlier study of Li et al., the efficiency of these markers in indentifying patients with breast cancer was moderate, and in particular, the ability of the technique to detect early in situ disease remained unproven.

A recent study by Van Winden and colleagues aimed to further validate the BC1, BC2, and BC3 peaks [20]. In this study, the SELDI spectra of 48 breast cancer patients were compared to 48 controls. Storage duration and/or batch effects but not participants' age seemed to significantly affect the overall peak intensity but not the discriminatory pattern between the groups. Only one peak (BC1 4.3 kDa) was validated in this study and was underexpressed in patients with breast cancer, as previously predicted. Two ionic peaks possibly corresponding to the BC2 and the BC3

Study		
Belluco et al. [21]	Sauter et al. [26]	Li et al. [13]
310	114	95
155 patients with stage I invasive ductal carcinoma (IDC) of the breast 155 healthy controls	81 benign diagnosis, 6 ADH, 5 DCIS, and 22 invasive breast cancer (IBC)	
Serum	NAF	NAF and ductal lavage fluid
IMAC-Cu	H4, NP and Q10	IMAC30
5.99255 kDa ↓[a]	Cancer vs. healthy NAF: 5.2, 11.9, and 13.9 kDa	3.38 kDa
5.83850 kDa ↓	DCIS vs. benign disease differentially expressed peaks: 5.2 and 33.4 kDa	
2.95470 kDa ↓	ADH vs. benign disease: 5.2 kDa	3.45 kDa
5.88770 kDa ↓	Breast cancer vs. benign lesion: 13.9 kDa	3.49 kDa
9.01770 kDa ↑		
8.65720 kDa ↑		
8.95650 kDa ↑[a]		
These 7 peaks were validated and tested to differentiate stage I breast cancer from healthy samples, and had strong sensitivity and specificity	Strongest peak was the 15.94 kDa identified as the β chain of hemoglobin	Identified as human neutrophils peptides (HNP) 1–3
	Interestingly, the same duplicate samples' peaks were detected on the 3 chips in 90% of the runs	
	Peaks differentiated DCIS from benign disease, ADH and IBC	

peaks were noted; however, their expression was inconsistent with the original study. This study demonstrates the difficulties in validating the results of SELDI, especially with the absence of subsequent protein sequencing platforms.

A further well-designed and internally validated study involving 310 subjects explored the ability of mass spectrometry for distinguishing between patients with early breast cancer (stage I) and healthy subjects [21]. Mammography was used to exclude cancer in the control group, and patients with a family history of breast cancer were excluded from the control group. Belluco and colleagues [21] used 109 controls and 109 patients with invasive ductal carcinoma (IDC), a blinded testing set of 46 controls and 46 patients with IDC, and finally an independent validation set of 46 patients (15 healthy controls and 31 with IDC) whose samples were collected and analyzed 14 months later. This study focused on the enrichment and selection of carrier proteins to which most of the lower molecular weight (LMW) biomarkers are linked. Specifically, an IMAC chip surface which has a high affinity for albumin was used to enable analysis of bound LMW proteins. In the training set, seven ion peaks were identified that discriminated between the groups with a sensitivity and specificity of 95% and 85%, respectively (Table 2). In the validation study, all the 17 T1a tumors were identified. This work demonstrates that SELDI profiling of serum can generate a robust classification of stage I breast cancer. When the SELDI-TOF results were used to further classify patients with equivocal mammography (34 patients), 94% of the patients with Breast Imaging Reporting and Data System score of 4 were correctly diagnosed. This potential clinical utility of mass spectrometry in improving the positive predictive value of mammography was clear in this work. This has led to the development of an ongoing prospective multicenter project between Italy and the USA involving 4,000 subjects to further consolidate these findings [21].

Serum Proteomic Biomarkers in BRCA1 Mutation Patients

BRCA1 mutations account for 7–10% of breast cancers and are associated with early development of breast cancer and poor outcome [22]. Up to 80% of patients with BRCA1 mutations ultimately develop breast cancer, and identification of cases that are less likely to develop cancer would enable more accurate targeting of risk reducing and therapeutic strategies. As such, BRCA1 mutation carrier testing is another promising application of SELDI-TOF

Table 2 (continued)

	Study		
	Noble et al. [24]	Ricolleau et al. [33]	Streckfus et al. [43]
Number	65 21 cancer 44 healthy	60	6
Sample	NAF	Cytosol	Saliva
Array used	IMAC30	IMAC-Cu, Q10	CM10
Differential peaks	Breast cancer vs. healthy: 6.5, 8.0, 15.9, 28.1, and 31.8 kDa Ipsilateral affected breast vs. healthy breast CM10: **3.471** , 3.511, **4.151**, 4.586, **4.646**, 4.698 kDa IMAC30: **3.501**, **3.627**, **4.147** kDa Contralateral breast vs. healthy breast CM10: **3.471**, 3.869, **4.151**, **4.646**, 14.720 kDa IMAC30: **3.501**, **3.627**, **4.417**, 4.376, 5.890 kDa	8.5 kDa IMAC30 (ubiquitin) 19.8 kDa SAX (Ferritin light chain) FLC	18.113 kDa 170 kDa 228 kDa 287 kDa
Comments	No differentially expressed peaks were detected comparing the affected and contralateral breast NAF in the breast cancer group, which might suggest systemic changes in women with breast cancer	Combining St Gallen score to the 2 peaks led to strong prediction of none elapsing group ($p<$ 0.0001) which could mean avoiding chemotherapy over treatment in 40% of breast cancer patients post-surgery	This small pilot study managed for the first time to detect significant proteomic peaks that differentiated DCIS from healthy subjects in human saliva

Bold peaks are either validated or detected in different studies or binding surfaces

[a] Repeatedly detected/validated peaks which present a potential biomarker

MS. Studies by Becker [23] and Laronga et al. [16] have examined the role of SELDI proteomic profiling in patients with BRCA mutations.

Differentiation Between BRCA1 Cancer and BRCA1 Carrier Groups

In a promising proteomic study, serum samples from 30 BRCA1 carriers, 16 patients with sporadic breast cancer (SBC) and 16 healthy controls were used (Table 2) [16, 23]. Of the 30 patients with BRCA1 mutations, 15 patients developed breast cancer within 3 years of follow-up, whereas the other 15 carriers were cancer-free after 7 years of follow-up. The mean age of those who developed breast cancer was 44.2 years and the mean age of those who did not was 44.6. It is conceivable, given the young age of the two groups, that many of those who had not developed breast cancer at the time of the study would ultimately develop breast cancer despite being placed in the "cancer-free" group. Nevertheless, the study demonstrated two peaks that best distinguished between the BRCA1 cancer and carrier groups. One peak occurred at 8.1 kDa and was overexpressed in patients who developed breast cancer, and the other peak was noted at 5.9 kDa and was underexpressed in the same group (sensitivity and specificity 100% in the testing set; 87% on cross validation). The latter peak was the primary determinant between the two groups. SELDI-TOF MS accurately differentiated 13 out of the 15 women with BRCA1 mutations who developed cancer from the 15 BRCA1 mutation carriers who did not (87% sensitivity and specificity). Whether the distinction in protein profiles found between the two BRCA1 mutation groups represents an early detection of a pre-cancerous state or an occult malignancy remains unclear.

Becker et al. [23] also examined proteomic profiles in patients with SBC and healthy controls. Differentiation between BRCA1 cancer and SBC groups was possible with a sensitivity of 94% and a specificity of 100% on cross validation. Additionally, between BRCA1 cancer and the control group, peak separation was achieved with 87% sensitivity and 94% specificity. As expected, no single discriminatory peak was detected and separation between groups was based on a panel of peaks. Finally, in differentiating between BRCA1 carriers compared to control groups, the peaks revealed interchangeable profiles which were not discriminatory. The peaks were, however, different from both the SBC and BRCA1 cancer groups. This, therefore, confirmed the distinction between healthy controls and the SBC group. It is notable that differentiation between BRCA1 cancer group from the BRCA1 carrier group and the SBC group involved the BC2 (8.1 kDa) peak. This was overexpressed in the BRCA1 cancer group and not the other two. Interestingly, a similar size peak was also shown to be significantly overexpressed in breast cancer serum samples studied by Li et al. [17, 18] and Mathelin et al. [19]. This certainly requires verification and identification of each protein and could lead to new era in the molecular understanding of breast cancer. Despite the small number of samples, the reproducibility of SELDI proteomic profiling published by multiple investigators is intriguing and necessitates further large-scale validation.

Comparison of Proteomic Profiles Pre- and Post-surgery

Laronga and coworkers performed proteomic profiling on paired serum samples from 16 breast cancer patients pre- and post-surgery and 15 age-matched healthy samples [16] (Table 2). Profiling yielded three significant differentially expressed ion peaks between the pre- and post-surgery groups, and it was possible to differentiate 14 out of 16 of the post-treatment group from the pre-treatment samples, with a sensitivity and specificity of 75% and 87%, respectively.

Work was also performed to investigate whether samples from breast cancer patients post-surgery could be differentiated from healthy controls. This is of interest as if removing the tumor leads to a restoration of the normal healthy profiles it would be reasonable to link the characteristic peaks in the profile to the proteins shed or inhibited by the tumor. In contrast, however, the SELDI analysis clearly differentiated protein profiles between the controls and breast cancer patients post-surgery, with a final separation sensitivity of 93% and specificity of 73%. The post-treatment group partially retained protein profiles after surgery, which were absent in healthy individuals [14].

The Relationship Between Serum Proteomic Biomarker Profiles and Clinicopathological Variables

Studying the associations between potential proteomic biomarkers and clinicopathological factors may lend insight into the biological significance of proteomic profiles. For example, profiles strongly associated with estrogen receptor (ER) status might be expected to be due to proteins involved in hormonal signaling. It is also possible, however, that the absence of such a correlation with known clinicopathological variables might be due to the identification of new independent factors. However, in the study by Li et al. [17], BC2 and BC3 peaks did not correlate with tumor size, nodal involvement, tumor grade, or ER/progesterone receptor (PR) status. The authors hypothesized that these complement peaks are overexpressed in early breast cancer stages but underexpressed in advanced and metastatic disease. In addition, no correlation was found between the four peaks identified by Mathelin and colleagues and any clinical or histological parameters including age, nodal status, metastasis, vascular invasion, ER/PR, and Ca15.3, suggesting that the four biomarkers are independent to these factors [19]. Other groups have further confirmed these findings, demonstrating a lack of association between these proteomic markers and tumor characteristics; however, a reduction in the 8.1-kDa peak intensity noted in the post-menopausal subgroup warrants further evaluation [20].

Laronga et al. attempted to use SELDI-TOF to distinguish sentinel lymph node (SLN)-positive from SLN-negative patients in 98 women [14]. Out of the node-positive group, 22/27 samples were correctly classified compared to 55/71 correctly classified samples in the node-negative group. This produced a classification tree with a sensitivity of 81% and a specificity of 77%. The main differentiating factors were three low mass peaks on the IMAC-Cu and three high mass peaks on the SAX surfaces. If axillary node status could be accurately determined using proteomic profiling approaches, node-negative patients could be spared unnecessary axillary surgery with its attendant complications.

Nipple Aspirate Fluid Proteomic Profiling

Intraductal sampling and diagnostic techniques include nipple aspiration, ductal lavage, and duct endoscopy. These are minimally invasive and allow direct access to the ductal system where most breast malignancies arise. This could be particularly useful in young women where mammographic sensitivities are reduced. Breast duct fluid is a rich source of protein from the immediate vicinity of tumors and their local microenvironment and may represent a superior source of cancer biomarkers due to this close proximity. Similarly, in early stage in situ (DCIS)

disease where the basement membrane remains intact, ductal fluid may contain tumor markers that are excluded from the systemic circulation. The main disadvantage of nipple aspirate fluid (NAF), however, lies in the low cellular yield making cytological analysis difficult. Recent technological advances have enabled rapid protein biomarker identification from small volume NAF using SELDI-TOF MS.

Nipple Aspirate Fluid Proteomics of the Ipsilateral Affected Breast in Patients with Breast Cancer Compared to Controls

Noble et al. [24] compared NAF proteomes from women with unilateral breast cancer and healthy female controls. NAF was collected from both breasts of 21 patients newly diagnosed with unilateral breast cancer and a further 44 samples from healthy subjects with a family history of breast cancer.

Comparing proteomic profiles of NAF from the ipsilateral affected breast and NAF from healthy controls revealed six peaks on the CM10 surface and three on the IMAC that were significantly different between the two groups (Table 2). Five breast cancer biomarker peaks were identified that were more frequently expressed in women with breast cancer compared to women without [25]. The most promising peak that best distinguished between the two groups was a 15.9-kDa protein. This protein was identified on an H4 array with the aid of the virtual tryptic digest database as the β chain of hemoglobin. Two other peaks at 8 and 31.8 kDa were identified as the doubly charged and diameric forms of the same protein, respectively. Clearly, validating this biomarker was critical in this instance.

To validate this pilot study and to establish a breast cancer predictive model using NAF protein profiling, Sauter et al. [26] analyzed NAF from 114 women (Table 2). None of the previously identified protein peaks were found to be associated with breast cancer in this study. Seven new candidates were detected, of which three were associated with breast cancer (Table 2). These remained significant discriminators even after excluding women with pathological nipple discharge and duct papillomas. Furthermore, two peaks at 5.2 and 33.4 kDa differentiated between DCIS and benign disease, and the peak at 33.4 kDa differentiated DCIS from IBC. Importantly, these peaks were consistent in differentiating DCIS from the benign group, which may provide an early prediction of the development of neoplastic disease. Overexpression of the peaks at 5.2, 13.88, and 33.4 kDa was also shown to be associated with both DCIS and IBC but not with atypical ductal hyperplasia (ADH) or benign disease. The authors also attempted to build a predictive module using the newly discovered peaks in association with other clinical variables. A model including age, parity, and the peak at 11,880 Da using the H4 chip was found best with a sensitivity and specificity of 40% and 94%, respectively.

In a multicenter study involving 95 patients, Li and colleagues [13] attempted to profile breast fluid proteomes obtained from both ductal lavage and NAF. Using an optimized immobilized metal affinity surface protocol, Li et al. [13] identified three potential biomarkers which differentiated cancer fluids from controls. Three differentially expressed peaks (3.375, 3.447, and 3.490 kDa) were identified as human neutrophil peptides (HNP)1−3 (Table 2). This was also verified using SELDI-TOF MS immunocapture assay on breast cancer samples which originally revealed higher HNP1–3 peak intensities. Moreover, Li et al. [13] used enzyme-linked immunosorbent assay to quantify the three biomarkers providing further validation of their findings using SELDI. HNP was shown not to be the result of blood contamination and had been previously detected in tissue samples from lung, oral, colorectal, and renal cancers [27–30]. Their high level in cancers might be explained by tumor cell invasion and release of neutrophils and eosinophils, or by direct secretion of these peptides.

NAF Proteomics of the Contralateral Unaffected Breast in Patients with Breast Cancer Compared to Ipsilateral Affected Breast Cancer

While previous studies applying different profiling techniques have shown significant proteomic differences between NAF from tumor-bearing and normal breasts in breast cancer patients [31, 32], Noble et al. [24] found no significant differences between these paired profiles using SELDI. Comparative profiling between NAF from the unaffected breast in patients with breast cancer and NAF from controls was also performed [24], and interestingly, no differences were noted. While further validation studies are required, the absence of discriminatory peaks between the NAF from the ipsilateral affected breast and the contralateral unaffected breast is striking. These results led the researchers to hypothesize that in breast cancer a field change occurs across both breasts, and that NAF proteomics may have more value in breast cancer risk assessment, rather than in diagnosis or screening. While comparison between the two sides from the same individual is conceivably an attractive approach providing an internal control for hormonal and environmental effects, it would not be as informative if this hypothesized field change takes place. Yet again, this is a suitable setting for paired SELDI and other proteomic profiling studies using one cohort to address these conflicting results.

Cytosolic Protein Profiling in Breast Cancer

Low abundance biomarker proteins related to tumor cells or their microenvironment might be diluted, altered, or fragmented once shed in the bloodstream. This may have an adverse effect on the results obtained by MS profiling. Cytosolic extracts represent an alternative source of tissue, possibly with higher concentrations of the relevant proteins. Moreover, profiling the cytoplasmic proteome has the advantage of helping detect novel intracellular pathways and interactions involved in tumorogenesis. Alterations in mitochondrial proteins have also been linked to cancer development and progression and could be detected by this method. Overall, studying the protein from the native tumor may lead to important insights into tumor differentiation, invasiveness, and responsiveness to adjuvant therapies. Conversely, however, this type of study is less likely to help with early detection of carcinogenesis, since by definition, samples are taken from the tumor.

Node status remains the strongest prognostic marker in breast cancer and helps determine the need for adjuvant therapies. Nevertheless, a significant proportion of node-negative patients develop disease recurrence and earlier identification and treatment of this high risk group may be advantageous.

Ricolleau et al. [33] performed cytosolic protein profiling on 60 samples from patients with node-negative sporadic breast cancer. Thirty of these patients had developed a distant relapse and 30 remained disease-free. Samples were analyzed using IMAC-Cu and SAX chips. Seventy-three peaks were generated from both arms, and two of these peaks at 8.5 and 19.8 kDa had prognostic potential. This was further confirmed by repeated random sampling, and the 8.5-kDa peak was found to be expressed at lower levels in patients with metastatic disease while the 19.8-kDa peak was found at higher levels in these patients. These peaks were further identified as ubiquitin and ferritin light chain (FLC), respectively, and the results validated by western blotting and immunohistochemistry. Combining these new molecular biomarkers of disease relapse with a pre-existing model for prognostication (The St. Gallen risk profile age <35, size >20 mm, negative hormone receptors, grade 3) led to improved discrimination between the groups of patients who suffered a systemic relapse and those who did not. This promising line of work, therefore, supports the notion that proteomic profiling may one day help improve our ability to classify patients based on molecular parameters and helps determine treatment options.

A further study was conducted by Brozkova and coworkers [14]. Focusing on linking proteomic patterns to tumor clinicopathological characteristics, this group examined non-metastatic breast cancer tissue lysates from 105 patients on IMAC30 ProteinChip Arrays. One hundred thirty peaks were detected, which could cluster the tumors into five distinct groups differing on tumor type, nuclear grade, presence of hormonal receptors, mucin 1 and cytokeratin 5/6, or cytokeratin 14 expression. This study also showed clustering of biomarker patterns into five further smaller subgroups associated with tumor type, hormonal receptor status, and nuclear grade. Hormone receptor expressing luminal group, HER2/neu positive and the basal high proliferation gene expression subtypes were generated by hierarchical clustering. Strikingly, this molecular tumor classification was identical to those generated by complementary DNA genomic expression profiling [34]. In addition, this study successfully identified heat shock protein (HSP)27 and Annexin V within the tumor subgroup biomarker classifiers. Both proteins were mainly overexpressed in the luminal subcohort. Overall, this study illustrates the potential utility of SELDI-TOF MS proteomic approaches in identifying clinically relevant tumor subgroups and expanding our understanding of breast cancer tumor behavior, which is a key step towards individualization of patient therapy [14, 35].

Salivary Proteomic Profiling in Breast Cancer

The level of many salivary constituents is altered in disease states compared to healthy controls. For example, there are differences in the levels of kallikreins, CA125, epidermal growth factor, and cErb2 in the saliva of healthy compared to diseased subjects [36–39]. Additionally, the collection of samples is safe, simple, inexpensive, easily repeatable, and most importantly non-invasive. Researchers have previously studied the possibility that saliva may be used to diagnose systemic diseases [40–42], and Streckfus et al. [43] used SELDI to detect putative breast cancer markers in saliva. Pooled reference samples and individual saliva from three healthy controls and three patients with ductal carcinoma in situ were analyzed and four differentially expressed protein peaks detected (Table 2). While this is a small exploratory study, it demonstrates the sensitivity and threshold of SELDI-TOF MS in detecting significant differentially expressed peaks in saliva between different patient groups. In particular, this feasibility study proved that SELDI can be used to detect alterations of salivary proteins in cancer. While the results of this pilot study are encouraging, larger and blinded validation studies will be required to confirm the value of the differentially expressed protein peaks detected. In a more recent study using LC–MS/MS, Streckfus et al. have provided further support for the notion that salivary protein profiles can provide useful information to classify patient populations [44].

Shortfalls in SELDI-TOF MS Profiling

With the aid of recent advances in proteomic technologies and mining techniques, the limited number of proteins detected using serum samples can be augmented. SELDI as a high-throughput solid phase extraction technology is a useful means of sample protein extraction, fractionation, and detection. Accordingly, SELDI was perceived as a suitable platform to enhance the power of profiling studies through increasing the analysis of both samples and proteomic signals. Nevertheless, a low molecular mass detection preference, added to its selective surfaces and its semi-quantitative abilities, are all factors that could limit the detection of biomarkers. Other technical aspects including its low resolution, the slow progression to validation, identification, and clinical utility, have all contributed to the negative views of SELDI-based approaches.

Variability and the lack of sequencing abilities currently limit the use of SELDI. In addition, discrepant reports failing to validate earlier SELDI results have cast doubts on SELDI as a technique [45]. It is known that biased non-biological variability can result from any minor non-standardized step(s) in any proteomic profiling work, starting from sample collection and ending by data processing and analysis [46–48]. Studies testing the effects of different variables including storage tubes, clotting time, incubation temperature, storage temperature, and handling proved the importance of uniform handling to exclude systemic pre-analytical inconsistency and false discovery [49, 50]. Non-standardized protocols in different validation studies have generated conflicting results including clear variations in the discriminatory power and direction of several putative biomarkers. These factors have all raised concerns on the performance of SELDI [51]. In a real world setting, however, unifying patient cohorts, collection protocols, storage conditions, handling, and analysis settings is a challenging task. Nevertheless, adequately powered upstream studies analyzing large cohorts would attenuate this variability effect and select for potential clinically valid biomarker patterns. Moving from detection to identification of biomarkers is a well-known limitation of this MS-based tool. Nonetheless, potent cancer biomarker signatures can be transiently used in multicenter validation providing a proof supporting or dismissing their relivance. Identification of these markers can then be established using other conventional downstream methods.

The identification of acute phase proteins or proteins resulting from exoprotease activities as candidate biomarkers have also raised concerns about the reliability of this type of profiling [52]. We believe these findings do not imply underperformance per se, given that any signal including proteolytic products and acute response players can reflect a milestone in tumorogenesis. As long as candidate biomarkers are validated and do not fall within the false-positive bracket, their role in cancer evolution and diagnosis remains viable.

Transparent and standardized biomarker discovery programs are crucial for any unbiassed studies. Candidate biomarker validation, verification, and authentication should be the basis for any future biomarker discovery agendas. Adopting this strategy with strict standardized protocols would reduce false positives and recover some of the missing confidence in this exciting field.

Discussion

Expression profiling and biomarker discovery aims to provide means for tumor diagnosis, classification, and prediction of response to therapy and prognosis. This could potentially lead to the building of robust early detection modules and personalized effective breast cancer therapies which would improve outcomes. Genomic expression profiling can reliably classify and predict outcome in breast cancer [34, 53]. While huge strides have been made by the application of such genetic techniques, many crucial changes at the protein level including post-translational modifications may clearly be important in carcinogenesis. Yet, these will not be detected by genomic profiling. It is, therefore, important to correlate genomic studies with protein expression patterns and relate these to tumor classification and clinical outcomes. Proteomic profiling using SELDI-TOF MS possesses huge potential but requires considerable further study and development. For example, Brozkova et al. [14] succeeded in classifying breast cancer patients into subclasses identical to the ones established by gene expression profiling [34]. This along with identification of two potential biomarkers HSP27 and Annexin V provides evidence to the importance, validity, and complementary role of SELDI-based profiling in drawing a complete picture of the molecular basis of breast cancer.

It has been argued that the LMW serum proteins discovered by SELDI-based profiling are non-specific rather than cancer-related proteins. Additionally, recent studies have also showed that even though some of these biomarkers may be acute phase reactants belonging to common coagulation, complement pathways, or epiphenomena of tumor presence [45], they do appear to indicate tumor cell presence and/or activity [54]. Further work is clearly required to assess the specificity of these markers for different diseases and tumor subtypes. To date, discriminatory proteomic biomarkers identified through SELDI have been detected in other cancers. Koopman et al. [55] compared the serum of 60 patients with pancreatic adenocarcinoma to 60 with benign pancreatic disease and a

further 60 healthy controls by SELDI. They were able to discriminate cancers from healthy controls with a sensitivity and specificity of 78% and 97%, respectively. Ornstein found that proteomic ion patterns achieved 100% sensitivity and 67% specificity in distinguishing patients with and without prostate cancer [56], and Petricoin and colleagues reported a proteomic pattern with 100% sensitivity and 95% specificity in ovarian cancer detection [57]. Breast cancer serum profiling, however, remains the focus of many of the studies applying this MS technology [13, 18, 21, 26, 33, 43, 58]. While caution should be exerted in the interpretation of these figures due to methodological limitations of some of the studies concerned, and shortcomings inherent to the use of sensitivity and specificity figures, these reports illustrate the applications of SELDI outside of breast cancer, and some of the potential promise shown by SELDI in early work.

SELDI-TOF MS with its high-throughput capabilities and its abilities to process crude biological samples on different chromatographic surfaces represents a promising opportunity for large-scale breast cancer proteomic profiling. The current variability of detected biomarkers in different studies could well be a reflection of biological variation. As such, this is anticipated to lead to a wide range of proteomic mapping covering different aspects of diseases. In this review, the initial results from different groups applying this technology indicated a broad spectrum of potential biomarkers. Each study examined slightly different aspects of breast cancer with diverse cohort characteristics, sample collection and storage techniques, and varied analysis protocols. The detection of unmatched ion peaks and identified biomarkers in different studies is, therefore, not a surprising phenomenon and could also be related to the natural population differences, stage of the disease when samples were collected, collection and storage duration, the use of different up-front sample preparation and/or fractionation techniques, differences in chips, instrument settings, and other pre-analytical factors. Evidently, it is difficult at present to compare results of different studies, as there are huge variations in pre-analytical conditions used which would lead to discovery of different subsets of biomarkers. In addition, the bioinformatics involved in data analysis represents a further source of variability in this evolving technology which if unified, could potentially consolidate proteomic analysis using SELDI.

Avenues for improvements in the application of SELDI technology in cancer detection would involve comparative analysis of different types of samples, biostatistical interpretations, as well as involving larger multicenter studies and standardizing protocols (Zeidan et al., submitted for publication). One advantage of the SELDI technique is the ability to use selective surfaces to analyze different characteristics from the same cohort of samples but the downside of this is the variability of detected peaks found in different studies. Planned multicenter studies applying multiple surface analysis and adjunct tools could eventually lead to robust biomarker discovery. Since SELDI proteomic profiling is a relatively newborn technology, it will be undoubtedly difficult to unify pre-analytical preparation and analysis. However, where extensive projects are being carried out, collaboration between groups may facilitate an organized, supervised, and eventually unified progression of proteomic mapping of breast and other cancers. Major clinical trials should also provide a favorable focus for proteomic analysis within predefined patient groups.

This review describes the potential for early breast cancer detection and outcome prediction through identification of protein/peptides biomarker profiles using SELDI-TOF MS. This supports the rationale for development of SELDI-TOF and more advanced MS technologies, and the need for larger-scale standardized validation studies of candidate markers and their identification.

Key to any biomarker discovery is the translation to valid bed side application. SELDI profiling as such is not a valid clinical diagnostic tool. Moreover, a clinical version of SELDI mined breast cancer biomarkers remains absent. This could be partly due to a challenging biomarker identification stage and a time-consuming quantitative assay optimization and validation. Overall, a lag usually heralds the transition between biomarker discovery and clinical application. Here, an immune-based clinical test would be most optimal. It is anticipated that future work would reveal new biomarkers or profiles which may ultimately find practical clinical application in cancer screening, diagnosis and outcome prediction, and patient-tailored treatment strategies.

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