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Review

Blood-based protein biomarkers in breast cancer

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ABSTRACT

Breast cancer (BCa) is a significant healthcare problem on women worldwide. Thus, early detection is very important to reduce mortality. Furthermore, better BCa prognosis could improve selection of patients eligible for adjuvant therapy. New markers for early diagnosis, accurate prognosis and prediction of response to treatment are necessary to improve BCa care. The present review summarizes important aspects of the potential usefulness of modern technologies, strategies, and scientific findings in proteomic research for discovery of breast cancer-associated blood-based protein biomarkers in the clinic.

Abbreviations list: AAL, aleuria aurantia lectin; ACM, antibody colocalization microarray; ACN, acetonitrile; AD, area-based breast density; AFP, α-fetoprotein; AHSG, alpha 2HS-glycoprotein; ANX A3, annexin A3; APOA1, apolipoprotein A-I; APOA2, apolipoprotein A-II,; APOC1, apolipoprotein C-I; APOC2, apolipoprotein C-II; APOC3, apolipoprotein C-III; APOE, apolipoprotein E; APOH, apolipoprotein H; ATII, angiotensin II; AUC, area under the curve; BBC, BRCA1 mutant breast cancer; BBD, benign breast disease; BCa, breast cancer; BH, BRCA1 mutant healthy; BM, bone metastasis; BTD, biotinidase; CA15-3, carbohydrate antigen 15-3; CA19-9, carbohydrate antigen 19-9; CA27.29, carbohydrate antigen 27.29; CA125, carbohydrate antigen 125; C3a desArg, C3a des-arginine anaphylatoxin; C4BPB, complement component 4 binding protein β; CDH5, cadherin-5; CEA, carcinoembryonic antigen; CFHR3, complement factor H-related 3; CGB, champedak galactose binding; COL10A1, collagen 10a1; COL11A1, collagen11a1; COMP, collagen oligomeric matrix protein; 1CTP, pyridinoline crosslinked carboxyterminal telopeptide of type I collagen; DCIS, ductal carcinoma in situ; DE, differentially expressed; DEP, differentially expressed proteins; DL, detection limit; DM, distant metastases; dPC, digital ProteomesChip; DPV, differential pulse voltammetry; DR, dynamic range; 2D-DIGE, 2-dimensional difference gel electrophoresis; 2D-nanoLC-MS/MS, two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry; ECL, electrochemiluminescent; ELISA, enzyme-linked immunosorbent assay; ELLA, enzyme-linked lectin assay; ER, estrogen receptor; ESI, electrospray ionization; EVs, extracellular vesicles; FGA, fibrinogen alpha; GO, graphene oxides; HC, healthy controls; HER2, human epidermal growth factor receptor-2; HIC, hydrophobic interaction chromatography; HILIC, hydrophilic interaction chromatography; HP, human plasma; HPA, *helix pomatia* agglutinin; HS, human serum; HSPs, heat shock proteins; HSP90A, heat shock protein 90A; IAP, inhibitor of apoptosis; IDC, invasive ductal carcinoma; IEF, isoelectric focusing; IHC, immunohistochemistry; IGFBP3, insulin-like growth factor-binding protein 3; IgG Fc, immunoglobulin G crystallizable fragment; IMAC, immobilized metal affinity chromatography; ITIH4, inter-alpha trypsin inhibitor heavy chain family member H4; iTRAQ, isobaric tags for relative and absolute quantification; LAC, lectin affinity chromatography; LC, label-free quantification; LC-MS/MS, liquid chromatography tandem-mass spectrometry; LNM, lymph node metastasis; LR, local recurrence; LTA, *lotus tetragonolobus* agglutinin; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MBs, magnetic beads; MIF, migration inhibitory factor; M-LAC, multi-lectin affinity chromatography; MLR, multiple logistic regression; MRM, multireaction monitoring; MWCNTs, multiwalled carbon nanotubes; *m/z*, mass/charge ratio.; NCIs, non-cancerous individuals; NFX1, nuclear transcription factor, X box-binding protein 1; NSR, no sign of recurrence; NTNBC, non-triple-negative breast cancer; NTX, *N*-terminal crosslinking telopeptides of type I collagen; OPN, osteopontin; ORM-1, α-glycoprotein orosomucoid 1; OS, overall survival; PAI-1, plasminogen activator inhibitor-1; PAPPA, pappalysin-1; PCA, perchloric acid; PDD, primary disseminated disease; PDGF, platelet-derived growth factor; PEP, protein elution plate; pIgR, polymeric immunoglobulin receptor; PKG1, cGMP-dependent protein kinase1; PR, progesterone receptor; PRM, parallel reaction monitoring; PTMs, post-translational modifications; PTHrP, parathyroid hormone-related protein; PZP, pregnancy zone protein; RALGAPA2, Ral GTPase-activating protein subunit alpha-2; RANTES/CCL5, regulated on activation normal T cell expressed and secreted/chemokine (C-C motif) ligand 5; REC, recurrent breast cancer; ROC, receiver operating characteristic; RP, reverse phase; RPC, reversed phase chromatography; SAP, serum amyloid protein; SBA, antibody suspensión bead array; SBC, sporadic breast cancer; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; SEREX, serological analysis of recombinant cDNA expression libraries; SISCAPA, stable isotope standards and capture by anti-peptide antibodies; SLPI, secretory leukocyte protease inhibitor; SPB, serum protein biomarker; sTfR, soluble form of transferrin receptor; TAA, tumor-associated antigens; TAAb, tumor associaced autoantibodies; TJP2, tight junction protein 2; TNBC, triple-negative breast cancer; TPA, tissue polypeptide antigen; TPS, tissue polypeptide specific antigen; UPLC, ultraperformance liquid chromatography; WCX, weak cation exchange; WT, wild type

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1. Introduction

Breast cancer (BCa) is one of the most common cancers in women and accounts for about 14% of cancer-related deaths in females around the world [1]. Nonetheless, 5-year survival rate for BCa can approach 90% in developed countries if detected in the early phase [1]. Thus, early detection of BCa is important for improvement of prognosis and survival rate.

Until now, mammography has been one of the most important early diagnostic methods for BCa, but it is less effective for young women (under 40 years old), with a sensitivity of 25–59%. Diagnosing breast cancer in younger women is more difficult because their breast tissue is generally more dense than the breast tissue in older women [2]. Furthermore, mammography shows other limitations such as high rates of false-negative (between 4 and 34%), and there is a high rate of false-positives that lead to unnecessary biopsy procedures [3].

The prognostic tests in current clinical use require tumour tissue to be obtained by biopsy or other surgical approaches. It is desirable to find novel noninvasive biomarkers for the early detection of BCa in asymptomatic individuals, precise prognosis and prediction of response to treatment, and clinical detection of BCa metastasis Please write [4-6].

In this way, plasma/serum biomarkers have great potential in cancer screening and their role could extend further from general population risk assessment to treatment response estimation and recurrence monitoring. The rich content of different molecular and cellular elements in blood, which give information about the health status of an individual, make it a perfect compartment to develop noninvasive diagnostics for cancer [7]. Nevertheless, although several reports related to biomarkers for common cancers, blood based diagnostic tests that inform about the presence of cancer at an early stage and predict treatment response have been difficult to develop [8,9,10,11].

Proteomics has become an attractive approach to search for novel biomarkers in biological fluids of cancer patients using protein and peptide profiling [12]. In this way, mass spectrometry (MS) has been used to compare proteomic patterns in cancer patients and healthy controls (HC) [13]. The detection of early-stage cancer is based on the paradigm that the disease develops by increasing deviations from the normal status. Thus, potential biomarkers could be found among the specific proteins or peptides that are up or down-regulated in serum proteomic profiling in cancer patients compared with controls [14]. Furthermore, proteomics analysis could also complement gene analyses in its use in the prognosis and evaluation of disease [15].

Although no validated protein biomarker currently exists for use in routine clinical practice for breast cancer early detection, prognosis and the prediction of treatment response [16], this review summarizes recent progress linked to the application of proteomics in this field.

2. Protein biomarkers for breast cancer screening and diagnosis

Besides the prevention, detection at an prompt stage continues to be the way to decrease BCa associated mortality. Even though important advances in breast imaging, the capacity to precisely detect BCa remains a challenge. With the discovery of protein signatures and strategic biomarkers for BCa, proteomic technologies are prepared to work as an ideal diagnostic adjunct to imaging (see Table 1).

Some blood-borne tumour markers have showed capability to detect malignancy before clinical diagnosis and are presently being evaluated in screening trials for certain cancers; for example, carbohydrate antigen 125 (CA-125) in ovarian cancer [17]. Though candidate markers such as carcinoembryonic antigen (CEA) [18], the oncogenic protein RS/DJ-1 [19], the human epidermal growth factor receptor-2 (HER2) [20] and circulating cytokeratin fragments (tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS) and CYFRA 21-1) have been recommented as diagnostic markers, they were defficient in sensitivity and specificity for early BCa detection [21]. However, carbohydrate antigen 15-3 (CA15-3), was the most widely used serum marker for routine BCa screening, monitoring and follow-up of patients with BCa [22,23].

In order to develop novel strategies for the ultrasensitive detection of CA15-3, an electrochemical nanostructured immunosensor was fabricated using non-covalent functionalized graphene oxides (GO/Py-COOH) and multiwalled carbon nanotube (MWCNTs)-supported numerous ferritin as labels [24]. CA15-3 was selectively detected as low as 0.009 ± 0.0006 U/mL in human serum (HS) samples. As advantages, this system showed an excellent selectivity and it can be regenerated for multiple uses, having a great potential for future development of the point-of-care cancer diagnostics. On this way, perchloric acid (PCA) was used to improve the detection of serum O-glycosylated proteins (such as carbohydrate antigen 27.29 (CA27.29) and CA15-3) using an earlier developed sandwich enzyme-linked lectin assay (ELLA) [25]. By subjecting pre-coated champedak galactose binding (CGB) lectin-captured glycoprotein fractions of serum PCA isolates of the stage 0 ($n = 31$) and stage I ($n = 48$) BCa patients and those of controls ($n = 105$) to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), substantial inverse altered abundance of plasma protease C1 inhibitor and proteoglycan 4 were detected in both the early stages of BCa patients related to the controls. Although further validation in clinically representative populations is needed, ratio of serum proteoglycan 4 to protease C1 inhibitor may play crucial role in early BCa screening.

Furthermore, the potential of different biomarker panels (containing the CA15-3) were explored for the diagnosis of early BCa. It was found that the set of ten potential BCa serum biomarkers and cancer antigens (haptoglobin, osteopontin (OPN), CA15-3, CA125, carbohydrate antigen 19–9 (CA19–9), CEA, prolactin, α-fetoprotein (AFP), leptin and migration inhibitory factor (MIF)) cannot be used to predict early stage BCa [26]. Similarly, none of these 9 candidate markers (CA15-3, RANTES/CCL5 (regulated on activation, normal T cell expressed and secreted/chemokine (C-C motif) ligand 5), OPN, PAI-1 (plasminogen activator inhibitor-1), SLPI (secretory leukocyte protease inhibitor), HSP90A (heat shock protein 90A), IGFBP3 (insulin-like growth factor-binding protein 3), APOC1 (apolipoprotein C-I) and PAPPA (pappalysin-1) or combinations was useful for screening BCa, and only links with clinico-pathological elements correlated to prognosis were found for the candidates CA15-3, HSP90A and PAI-1 [27].

However, a panel of complex antigens consisting of B11 (LGALS3), B18 (PHB2), B119 (MUC1) and B130 (GK2) along with CA15-3 significantly increased the sensitivity (87%), specificity (76%), and overall survival (OS) (82.7%) in the diagnosis of BCa at as early as stage $T_1N_0M_0$, compared with CA15-3 alone [28]. Even though this panel of complex antigens required to be validated using more BCa samples, it may be a promise instrument to detect early-stage BCa. Furthermore, CA15-3 was also included in the diagnostic panel constituted of 4 protein peaks [m/z 3972, 6850, 8115 (Bc2) and 8949 (Bc3)] used to distinguish 62 BCa patients with invasive ductal carcinoma (IDC) from 16 HC and 31 patients with benign breast diseases (BBDs) [29]. Importantly, the resultant 4 peaks panel together with CA15-3 was demonstrated to have good sensitivity and specificity for the diagnosis of BCa. However, further investigation using a larger sample size should be performed to verify these results.

The potential of CA15-3 was also explored for the early diagnosis of metastatic BCa [30]. In this fashion, the sensitivity of CA15-3, CEA and HER2 was investigated, and it was found that the combination of two tumour markers enhanced the sensitivity for detection of metastatic BCa, and the determination of all three tumour markers only improved the sensitivity vaguely. These authors suggested the combination of CEA and HER2 in tissue HER2 positive tumours and the combination of CA15-3 and CEA in tissue HER2 negative tumours. Nevertheless, sizeable prospective clinical randomised trials are required to explore the clinical benefits of early detection and treatment of metastatic disease.

The efficacy of other serum biomarkers on early detection of BCa

Table 1
Summary of proteomic studies in plasma/serum to identify proteins related to breast cancer diagnosis.

Type of samples	Enrichment strategy	Techniques (determination)	Candidate biomarkers	Results	Reference
HS samples with concentrations of CA 15-3 ranging from 0.02 to 100 U/mL	(GO/Py-COOH) as sensor probe MWCNTs-supported numerous ferritin as labels	DPV	CA 15-3	DR: 0.05 and 100 U/mL DL: 0.009 ± 0.0006 U/mL	[24]
HS PCA isolates of HC (n = 105) and BC patients with stage 0 (n = 31) and stage I (n = 48)	Enrichment of serum glycoproteins using PCA	ELLA	Ratio of serum proteoglycan 4 to protease C1 inhibitor	Significant inverse altered abundance of proteoglycan 4 and plasma protease C1 inhibitor in BC patients compared to HC	[25]
HS from 68 women diagnosed with BC up to three years after enrollment and 68 matched HC	–	Quantitative bead-based multiplexed assay	Biomarker panel: OPN, haptoglobin, CA15-3, CEA, CA-125, prolactin, CA19-9, AFP, leptin, MIF	This panel cannot be used for diagnosis of early breast cancer	[26]
HS from 239 women who subsequently developed BC and 239 matched HC	–	ELISA	Biomarker panel: CA15-3, RANTES, IGFBP3, OPN, PAI-1, SLPI, HSP90A, PAPPA and APOC1	Potential as early prognostic markers: CA15-3, HSP90A and PAI-1	[27]
HS from 100 BC patients and 50 HC	–	SEREX in combination with phage display technology	Biomarker panel: B11 (LGALS3), B18 (PHB2), B119 (MUC1), B130 (GK2), and CA15-3	The complex autoantigens identified along with CA15-3 significantly increased the sensitivity (87%), specificity (76%), and OS in the diagnosis of BC at as early as stage $T_1N_0M_0$, compared with CA15-3 alone	[28]
HS samples from 62 patients with IDC and 47 NCIs (16 HC and 31 patients with BBD)	IMAC	SELDI-TOF MS	4 protein peak set: m/z 3972, 6850 and 8115 (BC2) and 8949 (BC3)	The identified 4 peaks combined with CA15-3 expression may be used as a protein profiling test to diagnose BC	[29]
HS from 107 patients with recurrence after BC: 15 with PDD, 9 developed LR during the follow-up period and 83 developed DM	–	ADVIA Centaur automated assay (two-site sandwich immunoassay using direct chemiluminescent Technology)	CA 15-3, CEA and HER2	For the detection of metastatic breast cancer: combination of CEA and HER2 (in tissue HER2+ tumours) and CA 15-3 and CEA (in tissue HER2− tumours)	[30]
HS samples from 27 BC patients, 24 women with BBD and 37 HC	IMAC30 protein chips loaded with Cu^{2+} metal	SELDI-TOF MS	Bc1, Bc2, Bc3	Bc2 possesses the highest individual diagnostic power	[31]
HS from 50 BC patients and 26 HC	Protein immobilization	Multiplex immunoassays on micro-structured protein microarray	Seven proteins belonging to the HSPs family (HSPB1, HSPD1, HSP70, HSP90, HSP110, HSPA5, HSP90B1) and one oncoprotein (p53)	Discrimination of BC patients (50) from HC (26) with a sensitivity of 86% and a specificity of 100%	[39]
HS from 36 newly diagnosed patients with stage II BC and those from 36 HC	Proteoprep® 20 Plasma Immunodepletion kit	2D electrophoresis, Western blot and MALDI-TOF MS	AHSG	Detection of autoantibodies against AHSG in BC patients with a sensitivity of 91.7%	[40]
HS samples from 18 HC, 92 participants diagnosed with BBD and 100 participants diagnosed with BC	–	ECL-based ELISA	22 SPB and 24 TAAb	The benefit of the integration of SPB and TAAb data in a combinatorial proteomic approaches for detecting BC	[41]
HS samples from 15 BC with estrogen receptor (ER+) histological staining harboring one or more lymph node metastases and 11 HC	–	ACM	ENG, LEP, OPN, IL-1B, TNF-α, and uPAR	The ability of the ACM to distinguish between healthy and breast disease using protein levels in patient sera	[42]
40 HP samples from women with BC (diagnosed with a stage II or III or earlier breast cancer) and 40 HP samples from HC	–	Identification by LC/MS/MS and quantification using the LC/MS-based label-free protein quantification software licensed from Eli Lilly and Company	90 alternative splicing isoforms in 38 genes were found, which showed statistically significant ($q < 0.05$) differences between BC and HC samples	The signature identified 92.5% BC samples and 72.5% of normal samples	[47]
HS samples for BC patients and HC were pooled with equal volumen (100 µL each)	Albuvoid™ beas	2-D gel separation and subsequent PEP	metabolic enzymes (hexokinases) and proteases	Qualitative and quantitative differences between BC patients and HC	[48]

NA, not available.

were also considered. For example, after the evaluation of the efficacy of Bc1 (peak at 4.3 kDa), Bc2 (peak at 8.1 kDa), and Bc3 (peak at 8.9 kDa) serum biomarkers on early detection of BCa, only Bc2 (peak at 8.1 kDa) was statistically significant in comparison between the malignant disease group, control group and benign disease group [31].

On other hand, it is well known that BCa is a heterogeneous disease in which cancer cells can express a variety of aberrant proteins (tumor-associated antigens: TAA) that are capable of eliciting an immune response (antibody production). Interestingly, this immune response appears months or years before the clinical diagnosis of the malignancy [32,33]. TAA and their specific antibodies may offer *in vivo* amplification of an early carcinogenic signal, thus possibly allowing earlier detection of cancer than methods used currently.

In particular, serum possesses several circulating antigens and antibodies related with cancer progression and development [34,35]. The presence of autoantibodies in serum against several tumor antigens, such as p53, antineural/antinuclear antigens, and embryonic neural proteins, has been also assessed in BCa [36].

Cancer antigens have demonstrated incredible importance in the clinic for screening and as prognostic indicators [37,38]. Particularly, heat shock proteins (HSPs), over-expressed in a extensive range of human cancers, caused the stimulation of the immune system and accordingly in elevated concentration of anti-HSP autoantibodies, that are associated with tumor metastasis in BCa patients. Consequently, screening these autoantibodies could be of prognostic and diagnostic values. In this way, L. Shi et al. [39] immobilized seven proteins belonging to the heat shock protein family (HSPB1, HSPD1, HSP70, HSP90, HSPA5, HSP90B1) and one oncoprotein, P53, in six different surface chemistries. Two surface chemistries (COOH and chitosan) were employed to detect antitumor antigen autoantibodies in 26 HC and 50 BCa sera. The detection of a single autoantibody did not allow significantly discriminating BCa sera from healthy sera, whereas combining seven autoantibodies (autoantibodies against HSPB1, HSPD1, HSP70, HSP90, HSPA5, HSP90B1, and P53) increased the specificity and sensitivity of the test (with a specificity of 100% and a sensitivity of 86%). In this study, they have demonstrated that customized protein microarrays could be effective tools for the rapid screening of thousands of biomarkers in a parallel and high-throughput fashion. The performance of protein microarray is influenced by many parameters such as spotting buffer, surface chemistry, and protein concentration. However, larger cohorts of BCa patients and HC are needed to validate its performance.

An immune proteomic approach also suggested that the presence of serum autoantibodies against alpha 2HS-glycoprotein (AHSG) protein could be helpful as serum biomarkers for early-stage BCa minimally invasive diagnosis and screening [40]. However, the AHSG will need to be tested and validated by multiple independent studies utilizing an adequately sized test and a training set of sera samples from very-early-stage BCa. Moreover, further verification with samples from patients with ductal carcinomain situ and BCa in stages III and IV would aid in confirming the specificity of AHSG autoantibodies in this subset of patients with BCa. This research provided additional preliminary, but important, data on the potential advantage for clinical serological screening of autoantibody measurement to detect small tumors in early stages, because autoantibody biomarkers have also been identified in BCa, the majority of these have only been reported in the late-stage, but not in the early-stage BCa.

Breast tumors were found to be related with systemic changes in levels of both serum protein biomarkers (SPB) and tumor associated autoantibodies (TAAb). Meredith C. Henderson et al. [41] evaluated for the first time the independent and combinatorial contribution of SPB and TAAb expression data for identifying BCa using a retrospective cohort of prebiopsy serum samples from 18 participants with no evidence of breast disease (ND), 92 participants diagnosed with Benign Breast Disease (BBD) and 100 participants diagnosed with BCa, including ductal carcinoma in situ (DCIS). It is important to mention that when modeling integrated data from both SPB and TAAb, the clinical sensitivity and specificity for detection of BCa improved to 81.0% and 78.8%, respectively. These data showed the advantage of the combination of SPB and TAAb data and toughly sustained the development of other similar combinatorial proteomic approaches for detecting BCa in the future.

A novel concept for multiplexing without mixing named antibody colocalization microarray (ACM) was introduced by M. Pla-Roca et al. [42]. This technique was validated by profiling 32 proteins in the serum of (i) 11 controls from age-matched patients undergoing reduction mammoplasties, (ii) 15 patients with primary BCa overexpressing the estrogen receptor (ER) in the primary tumor (ER positive subtype). It was found that six proteins (ENG, LEP, OPN, IL-1B, TNF-α, and uPAR) were associated with the cancer grade of the patient. The candidate biomarkers that were identified agree with the findings of previous studies which described increased concentrations of uPAR [43], TNF-RII [44], IL-1B [45], and ENG [46]. However, as disadvantages, all of them need to be veried in follow-up studies with more patients and controls.

Besides, recognizing and characterizing different forms of a protein (isoforms) are critial to the study of molecular mechanisms and early detection of complex diseases such as BCa. In this way, F. Zhang et al. [47] showed that isoform-specific peptides could differenciate normal breast from BCa, identifying 92.5% cancer samples and 72.5% of normal samples in an independent set of 40 normal samples and 40 BCa samples. It showed that alternative splicing isoform makers could act as independent markers of BCa.

In a study developed by D. L. Wang et al. [48], a functional proteomics technology was used to monitor protease activities and metabolic enzymes (hexokinases) from resolved serum proteins produced by a modified 2-D gel separation and subsequent Protein Elution Plate (PEP). For the first time, substantial differences were found between BCa patient serum and normal serum in both families of enzymes implicated in the cancer development and metastasis, giving excellent biomarker candidates for BCa diagnosis and drug development.

2.1. Protein profiling for diagnosis of breast cancer

Protein and peptide profiling was used to find novel biomarkers in biological fluids (as serum and plasma) of cancer patients [12] (see Table 2). Among the specific proteins or peptides that are up or down-regulated in serum proteomic profiling in cancer patients compared with controls, potential biomarkers could be found [14]. Particularly, several studies focused on protein profiling using the two laser desorption/ionisation (LDI) platforms (as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [49] and its variant surface enhanced laser desorption/ionisation time-of-flight (SELDI-TOF) MS were developed to search novel BCa biomarkers [50].

On the other hand, recently novel sample preparation techniques based on nanomaterials have developed, and applied to the separation and enrichment of peptides and proteins in biological samples [51]. Particularly, magnetic microspheres with the properties of the easiness to surface modification, high dispersibility and magnetic responsivity, were considered as a promising material for the convenient and efficient enrichment of peptides or proteins [52,53].

Commercial *n*-alkyl magnetic polymeric beads (1–10 μm diameter) have widely been used in the enrichment of low-abundance peptides and proteins in biological samples [54,55]. However, the commercial magnetic beads (MBs) have usually showed poor magnetic response.

C8-functionalized magnetic nanoparticles (about 50 nm diameter) with high dispersibility, large surface area and excellent magnetic responsibility, were successfully applied for convenient, fast and efficient enrichment of low-abundance peptides from tryptic protein digest and HS, followed by a direct MALDI-TOF MS analysis [56]. Furthermore, weak cation exchange magnetic beads (WCX-MBs) were used for the effective enrichment of peptides and proteins in biological samples.

Table 2
Summary of plasma/serum protein profiling studies for the diagnosis of breast cancer.

Type of sample	Enrichment strategy	Techniques (determination)	Proteins identified	Validation/verification	Candidate biomarkers	Ref.
HS samples from 46 BC patients and 46 HC	WCX-MBs	MALDI-TOF MS	14 peaks expressed differentially ($p < .05$) between the BC and HC cohorts. Sensitivity of 88.3% and specificity of 85.8%	NA	NA	[58]
HS samples from 45 BC patients and 46 HC	HIC-C8-MBs, HIC-C18-MBs and WCX-MBs	SELDI-TOF and MALDI-TOF-TOF MS	14 peptides expressed differentially between the BC and HC cohorts. Sensitivity of 89% and specificity of 67%	NA	NA	[59]
HS samples from 105 BC patients and 202 HC	WCX-MBs and RP-C18-MBs	MALDI-TOF MS	6 significant peaks for discriminating BC from HC for WCX and 16 for RCP18	NA	NA	[66]
HS samples from 96 BC patients and 64 HC	WCX-MBs	LC-ESI-MS/MS	3 serum peptide biomarkers were identified: FGA605-629, ITIH4 347-356, and APOA2 43-52	NA	NA	[67]
111 HP samples from 3 independent BC patient cohorts with and without clinical evidence of BM	WCX protein chip array (CM10)	SELDI-TOF MS	PTHrP(12-48) significantly increased in plasma of BM patients compared with patients without BM ($p < .0001$)	NA	NA	[68]
HS samples from 60 BC patients (22, TNBC; 38 NTNBC) and 20 HC	WCX protein chip array	SELDI-TOF MS, SDS-PAGE electrophoresis and MALDI-TOF/TOF	*m/z* 6447.9 identified as apolipoprotein C-I (ApoC-I)	NA	NA	[69]
HS samples from 37 BC patients 36 HC	IMAC-Cu-MBs	MALDI-TOF MS	24 significantly differentiated peptides (3 peaks at *m/z* 698, 720 and 1866 were used to construct the peptidome patterns with 91.78% accuracy)	Electrochemiluminescence immunoassay	CA15–3	[70]
HS samples from 68 BC patients and 68 HC	IMAC30 Protein Chip arrays activated with nickel	SELDI-TOF MS, iTRAQ and 2D-nanoLC-MS/MS	High levels in pre-diagnostic BC serum of: SELDI-TOF MS: APOC1, C3a desArg 2D-nanoLC-MS/MS: afamin, APO E and ITIH4	NA	NA	[71]
HS samples from 73 incident BC patients cases and 73 HC	IMAC30 array, WCX (CM10) array and the strong anion exchange (Q10) array	SELDI-TOF MS	8 significantly differentiated peptides corresponding to: APOC2, oxidized APOC2, APOC3, fragment of coagulation factor XIIIa, heterodimer of APOA1 and APOA2, hemoglobin B-chain and post-translational modified hemoglobin	NA	NA	[72]
HS samples from 32 BC patientes with early stages I-II IDC and 30 HC	WCX-MBs and IMAC-Cu-MBs	MALDI-TOF MS	Biomarkers upregulated in patients with IDC by MB-WCX: (*m/z*: 4209 and 4264) Biomarkers overexpressed in patients with IDC by MB-IMAC-Cu (*m/z*: 4263 and 4208)	NA	NA	[72]
HS samples from 10 stage IV BC patients and 10 HC	CaptureSelectTM Transferrin Affinity Matrix (affinity resin purified and isolated Transferrin and associated boud proteins)	LC-MS/MS analysis	21 significant proteins associated with the complement and coagulation systems	ELISA	Fibrinogen, Fibronectin and CA15-3	[74]
HS samples from 92 BC patients diagnosed at stages I and II and 104 HC	Amicon Ultra-4 membrane (50 kDa cut-off)	MALDI-TOF MS	A biomarker built from three components (*m/z* values of 2303, 2866 and 3579) showed 88% sensitivity and 78% specificity	NA	NA	[75]
HS samples from 219 invasive BC patients, 192 BBD and 630 HC	–	SELDI and MALDI-TOF MS	ANX A3	MS biomarker validation, ANX A3 ELISA and immunohistochemistry validation	ANX A3 was significantly upregulated in the benign disease group sera compared with other groups ($p < .0005$)	[76]
Post-translational modifications						
HS samples from 5 stage 2 BC patients and 5 HC	High abundance protein depletion, M-LAC and IEF separation by a dPC	LC-MS analysis	Thrombospondin-1 and 5, alpha-1B-glycoprotein, serum amyloid P-component and tenascin-X	NA	NA	[87]

(*continued on next page*)

Table 2 (*continued*)

Type of sample	Enrichment strategy	Techniques (determination)	Proteins identified	Validation/verification	Candidate biomarkers	Ref.
Pool of HS from 5 breast cancer serum and pool from 5 HC	High abundance protein depletion, two lectin columns specific to fucose (AAL and LTA, fractionation of the captured glycoproteins by RPC	LC-MS analysis	35 DEP from the combined LTA and AAL captured proteins and a narrower panel of 8 DEP that were commonly differentially expressed in both LTA and AAL captured proteins	NA	NA	[88]
HS samples from 25 metastatic BC patients with REC and 23 BC patients with NSR 5 years after diagnosis of their primary tumour	LAC (HPA-affinity chromatography) and 2D-DIGE	LC-MS/MS	PZP, CDH5 and pIgR	PZP, CDH5 and pIgR ELISA assays	CDH5 as a novel biomarker of metastatic breast cancer	[89]
HS samples from 62 newly diagnosed BC patients and 107 HC	HILIC	UPLC	Statistically significant alterations were observed in BC patients compared with HC: an increase in sialylation, branching, and outer-arm fucosylation and a decrease in highmannosylated and biantennary core-fucosylated glycans	NA	NA	[91]
HS samples from 90 BC and 54 HC	N-glycans from IgGs were extracted and purified using NuTip 10 (Carbon; Glygen, Columbia, MD, USA) and were labeled with 3AQ/CA	MALDI-MS	IgG Fc region *N*-glycans	Internal statistical validations	An IgG Fc region N-glycan-based multiple logistic regression model could distinguish BC patients from NC controls (AUC = 0.874)	[92]
HP samples from 18 BC patients and 6 HC	Microvesicles and exosomes were isolated from human plasma samples through high-speed and ultra-high-speed centrifugations	Label-free LC-MS/MS analysis	144 phosphoproteins in plasma EVs that are significantly higher in BC patients compared with HC	PRM and MRM	RALGAPA2, PKG1, TJP2 and NFX1	[94]
HS samples from 4 BC patients	Immunoprecipitation (using modified Protein A agarose beads) and IMAC enrichment (with a nickel-agarose magnetic beads)	LC-MS/MS without depletion of abundant proteins	Label-free quantitative analysis of lysine acetylation (AcK) and arginine mono-methylation (Rme)	NA	NA	[96]
Breast cancer subtypes						
HP samples from 29 BC patients (6 luminal A, 5 luminal B, 8 HER2+ and 10 TNBC) and 18 HC	Immunodepletion using a GenWay Seppro® IgY-12 spin columns	nanoLC-MS/MS	Differential: Luminal B: 8 upregulated and 12 downregulated compared to HC TNBC: 2 upregulated and 20 downregulated compared to HC	NA	NA	[103]
HP samples from 68 BC patients (24 luminal A, 15 luminal B, 10 HER2+, 19 TNBC) and 80 HC	–	LC-MS/MS	Clustering analysis: luminal A and luminal B, and basal-like and HER2+, were clustered together	NA	NA	[104]
HS samples from 76 BC patients (19 luminal A, 19 luminal B, 19 HER2+, 19 TNBC) and 20 HC	IgG and Albumin depletion kit (GE Healthcare)	2D-DIGE, iTRAQ and SWATH-MS	Differential proteins: luminal A: 24, luminal B: 38, HER2+: 17, TNBC: 10	Immunoblotting and mass spectrometry based SRM	Immunobloting: ZA2G, VTN, A1AT, FETUA, KNG1, AMBP. SRM assays: APOL1, AATM, TTHY, THRB, AACT, APOD, CPN2, CD5L	[105]
HP samples from 420 prediagnostic ER+ BC and 420 HC	Immunodepletion using a Hu-6 column (4.6 × 205 mm, Agilent)	nanoLC-MS/MS	Differential proteins: 57	ELISA	EGFR	[106]
HP samples from 420 prediagnostic ER+ BC and 420 controls	Immunodepletion using a Hu-6 column (4.6 × 205 mm, Agilent)	nanoLC-MS/MS	Differential proteins: 467	NA	NA	[107]
HP samples from 20 ER+/HER2- *vs* 18 HC; 24 ER+ HER2+ *vs* 21 HC; 18 ER-/HER2+ BC *vs* 18 HC; 24 ER-/HER2- *vs* 21 HC	–	Antibody microarray	Differential proteins: ER+/HER2-: 4, ER+/HER2+: 2, ER-/HER2+: 3, ER-/HER2-: 2	NA	NA	[108]

(*continued on next page*)

Table 2 (*continued*)

Type of sample	Enrichment strategy	Techniques (determination)	Proteins identified	Validation/verification	Candidate biomarkers	Ref.
HP samples from 14 TNBC patients and 14 HC	ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit (Sigma Chemical, St. Louis, MO)	Antibody microarray	Differential proteins: 93	Validation: antibody microarray	DUSP9, EED, EFNA5, ITGB1, PPMT1	[109]
HP samples from 10 patients with small size HER2-positive BC (5 patients with LNM+ *vs* 5 patients without LNM-)	ProteoMiner Protein Enrichment Kits to deplete high abundance proteins	LC-MS/MS analysis (TripleTOF 5600+ mass spectrometer coupled with the Eksigent Nano LC system)	Differential proteins: 33 (24 proteins were over-expressed in LNM+ group)	Western blotting	RARB was greatly increased in LNM+ group and FBLN5 was slightly elevated	[110]

NA, not available.

Both enrichment methods were applied for the detection of BCa [57,58].

Using magnetic bead-hydrophobic interaction chromatography C8 and C18 (HIC-C8-MBs and HIC-C18-MBs), and WCX beads for the enrichment of proteins presented in HS samples, 14 biomarkers were found, whose combination detects BCa patients from non-cancer controls with a sensitivity of 89% and specificity of 67% [59]. Of them, five biomarkers were comparable with previously identified proteins from published data using similar approaches (peaks at 4283 and 3972 Da [60,61], 3972 Da [62], 6630 and 6629 Da [63,64] and 6429 Da [65]). In addition, this biomarker panel were able to discriminate low-risk (tumor grade G1 or tumor grade G2 with a low level of uPA and PAI-1) and high-risk BCa patients (tumor grade G3 or tumor grade G2 with a high level of uPA and PAI-1) with a high sensitivity (75%) and specificity (100%). However, further validation of biomarkers could potentially facilitate the early diagnosis of BCa as an aid to imaging diagnostics.

In a similar way, combining the data resulting from two complementary workup procedures (WCX-MBs and reversed-phase (RP) C18 MBs) improved the classification of BCa, and sensitivity and specificity increased up to 84 and 95%, respectively [66]. Although MALDI-TOF peptide and protein profiles can be used for classification of BCa, larger patient sets must be analyzed for validation and MS/MS be used to identify the discriminating proteins and peptides for its use in BCa screening programs. More recently, WCX-MBs fractionation provided predictive model for BCa *versus* HC with 79.04% sensitivity and 82.18% specificity. Furthermore, fibrinogen alpha (FGA) encompassing residues 605–629, isoform 1 of inter-alpha trypsin inhibitor heavy chain family member H4 (ITIH4) 347–356 and apolipoprotein A-II (APOA2) 43–52 were found as potential peptide biomarkers [67].

Using WCX fractionation and MS protein profiling, C. L. Washam et al. [68] found that 12–48aa peptide fragment of parathyroid hormone-related protein PTHrP(12–48) was significantly increased in the plasma of bone metastasis (BM) patients compared with patients without BM ($p < .0001$). Importantly, the clinical measurement of PTHrP(12–48) in plasma in combination with NTX in serum improved the detection of breast cancer BM (diagnostic specificity and accuracy (AUC = 0.99). This result could provide novel opportunities for the improved diagnosis of bone metastasis, however, some limitations of this study are that is retrospective, the sample size was somewhat small; and it may also suffer from selection bias. Using the same methodology, Y. Sun et al. [69] found that the candidate biomarker positioned at *m/z* 6447.9 identified as APOC1) was significantly decreased in BCa patients, and its expression intensity was weaker in the triple negative breast cancer (TNBC) and pre-surgery group compared with the non-triple negative breast cancer (NTNBC) and post-surgery group. These results suggest that ApoC-I peptides may be a potential diagnostic biomarker and therapeutic approach for BCa. However, further replicated experiments with more samples are necessary to verify this possible protein biomarker.

Many reviews reported the advantages of immobilized metal ion affinity chromatography (IMAC) on the purification of peptides and proteins [51]. Using this approach for the serum proteomic analysis of 36 HC and 37 BCa patients, three peaks at *m/z* 698, 720 and 1866 were idetintified and used to construct the peptidome patterns with 91.78% accuracy [70]. Using an independent group for the validation, it was found that the peptidome patterns could differentiate the validation group achieving a sensitivity of 91.89% (34/37) and a specitivity of 91.67% (33/36) (> CA15-3, $p < .05$).

Using the IMAC enrichment approach, several serum proteins that differed in concentration between women with asymptomatic BCa and matched HC were also detected [71]. Particularly, two SELDI-TOF MS peaks with *m/z* 3323 (doubly charged APOC1) and *m/z* 8939 (C3a desarginine anaphylatoxin ($C3a_{desArg}$)), and with 2D-nano liquid chromatography tandem-mass spectrometry (LC-MS/MS), afamin, apolipoprotein E (APOE) and isoform 1 of ITIH4 were higher in pre-diagnostic BCa

serum. Particularly, $C3a_{desArg}$ and ITIH4 have previously been related to the presence of symptomatic and/or mammographically detectable BCa. However, the currently identified proteins were high abundant and they were unlikely to be BCa specific. In order to find low abundant and probably more specific tumor markers, other techniques would be employed to give insight into 'the deeper/low abundant proteome'.

Similarly, performing serum fractionation by IMAC30 array, WCX (CM10) array and strong anion exchange chromatography preceding protein profiling with SELDI-TOF MS, eight peaks showed statistical significance difference in the intensity between incident BCa cases and controls ($p < .05$) [72]. Seven of these peaks were tentatively identified as heterodimer of apolipoprotein A-I (APOA1) and apolipoprotein AII (APOA2) (m/z 45,435), apolipoprotein C-II (APOC2) (m/z 8909), oxidized apolipoprotein C-II (m/z 8925), apolipoprotein C-III (APOC3) (m/z 8746), fragment of coagulation factor XIIIa (m/z 3959), hemoglobin B-chain (m/z 15,915), and post-translational modified hemoglobin (m/z 15,346). However, similar to the previous study [71], discriminating proteins were still high abundant.

In an attempt to combine both purification methods mentioned above, J. Yang et al. [73] used magnetic bead-based MB-WCX chromatography and MB-IMAC-Cu to preanalyze 32 patients with early stage (stages I-II) of IDC and 30 HC serum samples. It was found that the serum samples purified in the MB-WCX group provided a better proteomic pattern than MB-IMAC-Cu. However, both accurately distinguished patients with early stage IDC from HC. It was found that two candidate biomarkers (m/z 4209 and 4264) were upregulated in patients with IDC by MB-WCX purification, while similar potential biomarkers (m/z: 4263 and 4208) identified by MB-IMAC-Cu purification were also overexpressed in IDC breast cancer patients. Thus, these two candidate biomarkers will be further identified by expanding samples from patients with IDC.

In a different approach, 10 normal control and 10 stage IV BCa patient serum samples were analysed by label-free MS using a CaptureSelect™ Transferrin Affinity Matrix [74], identifying 21 potential candidate biomarkers. After selecting fibronectin and fibrinogen for further analysis in a larger cohort of patient samples along with CA15-3, it was found that these molecules were significantly altered when comparing the controls groups to stage IV BCa, highlighting the usefulness of analysing the high-abundant fraction associated proteins.

Other studies to identify serum proteome patterns specific for early stage BCa [75] and invasive BCa patients [76] using MALDI-TOF MS were also developed. In the latter case, a novel mammary biomarker, regulator and therapeutic target, Annexin A3 (ANX A3), was identified, however large scale prospective studies together with long term follow-up and detailed molecular analysis are required to elucidate the role and mechanism(s) by which ANX A3 might impact breast pathology, diagnostics, and tumourigenesis.

2.1.1. Protein profiling for diagnosis of breast cancer: post-translational modifications

To date, the majority of serum/plasma proteomic work has been conducted to analyze total protein level abundance, with only a few studies to analyze post-translational modifications (PTMs) [77], usually glycosylation [78,79]. As one of the most important mechanisms for regulating protein function, PTMs, including phosphorylation, acetylation, ubiquitination, and methylation, have been identified and validated as critical for signaling transduction, protein degradation, and transcriptional regulation [80]. The known importance of PTMs in cellular signaling provided the impetus for a large-scale survey of PTMs other than glycosylation by immunoaffinity enrichment of PTM-containing peptides.

2.1.1.1. Glycosylation. Breast carcinomas develop from mammary epithelial cells through genetic alterations, and interactions with the surrounding stromal tissue are essential for malignant transformation and for progression of the disease [81]. Glycosylation of cell surface proteins and lipids is a common post-translational event that regulates the interaction between epithelial cells and the microenvironment by altering adhesion properties, cell-cell interaction, and the immune system and by affecting the cells' migration properties [82].

Several studies, on both breast and other cancer types, have shown that cancer-related alterations in glycosylation are reflected in serum [83]. Thus, the characterization of glycan structures is expected to broaden the scope of discovery studies beyond the protein level, and thus improves the clinical values of existing biomarkers. For this reason, the development and application of techniques and methodologies for enriching or fractionating the glycoproteome has become an emerging field [51].

Experimentally, a main advantage of targeting glycosylation is that glycopeptides or glycoproteins can be effectively enriched over non-glycosylated molecules. In proteomics, enrichment targeted to *N*-glycosylation has typically been performed using lectin based enrichment [84,85] or hydrazide chemistry [86].

In the particular case of BCa, multi-lectin affinity chromatography (M-LAC) was used to isolate the serum glycoproteome. Subsequently, proteins with altered abundance and glycosylation that could act as biomarkers were identified: thrombospondin-1 and 5, alpha-1B-glycoprotein, serum amyloid P-component and tenascin-X. They will be further investigated in future studies to confirm these glycoprotein biomarker candidates in a significant group of patients for the improved early detection for BCa [87].

S. Selvaraju et al. [88] developed a fully integrated platform for capturing/fractionating human fucome from disease-free and BCa sera using two lectin columns specific to fucose, namely Aleuria aurantia lectin (AAL) and *Lotus tetragonolobus* agglutinin (LTA). After the comparison of the fucosylated proteins in both groups (disease-free and BCa patients), a broad panel of 35 differentially expressed proteins (DEP) from the combined LTA and AAL captured proteins and a narrower panel of 8 DEP that were commonly differentially expressed in both LTA and AAL captured proteins, were obtained. As advantages, the platform allowed the "cascading" of the serum sample from column-to-column in the liquid phase with no sample manipulation between the various steps. This guaranteed no sample loss and no propagation and dilution or experimental biases between the various columns when comparing the diseased serum fucome to the disease-free fucome by LC-MS/MS.

Lectin affinity chromatography (LAC) in conjunction with 2-dimensional difference gel electrophoresis (2D-DIGE) and LC-MS/MS were also used to identify serum markers of metastatic BCa [89]. In this case, *Helix pomatia* agglutinin (HPA) was used to isolate glycoproteins from pooled BCa serum samples due to their properties of binding aberrant glycans associated with metastatic BCa. Following proteomic identification of HPA binding glycoproteins, cadherin-5 (CDH5), pregnancy zone protein (PZP) and the polymeric immunoglobulin receptor (pIgR) emerged as potential markers of metastasis. It was also observed that CDH5 discriminated patients with no sign of recurrence (NSR) from those with recurrent BCa with 90% specificity. CDH5 showed to be a potential marker of metastatic BCa with both protein levels and HPA binding contributing to a test that is comparable to CA15.3 in terms of specificity. As evidenced by the CDH5 data, the glycoproteomic and validatory approach employed here presented the advantage to identify novel markers of BCa metastasis.

Hydrophilic interaction chromatography (HILIC) separates both neutral and charged glycans in a single separation, which facilitates total glycan characterization with single-column chemistry. Separation is based on the hydrophilic potential of the glycan, which is affected by its size, charge, composition, structure, linkage, and oligosaccharide branching [90]. HILIC glycan analysis is continuously advancing, and a method based on ultraperformance liquid chromatography (UPLC) has recently been developed for *N*-glycan separation of various samples with high efficiency [90]. Improving on HPLC, UPLC allows for a decrease in the run times and greatly increased resolution, partially due to

sub-2 μm stationary phase technology.

In previous studies using HPLC-HILIC, K. Marino et al. [83] found significant changes in glycosylation including sialylation, fucosylation, and branching in BCa, especially indicating the presence of metastasis, spread to the lymph nodes and correlation with tumor circulating cells. More recently, R. Saldova et al. [91] described the greatly improved separation of HS *N*-glycans on UPLC, as compared with HPLC, where > 140 *N*-glycans were assigned using this technique after profiling serum samples from HC and newly diagnosed BCa patients. Particularly, they also found decreases in high-mannosylated and biantennary corefucosylated glycans in BCa patients compared with controls. They found that bisected biantennary nonfucosylated glycans were decreased in patients with progesterone receptor (PR) positive tumors, and core-fucosylated biantennary bisected monogalactosylated glycans were decreased in patients with tumor TP53 mutation. In conclusion, this UPLC-based glycan analysis technique revealed highly significant differences between HC and BCa patients. Furthermore, significant associations with breast carcinoma and systemic features were also described.

Recently, BCa patients were distinguished from cancer-free (NC) controls by serum immunoglobulin G (IgG) crystallizable fragment (Fc) region *N*-glycosylation profiling using MALDI-TOF MS [92]. These results suggested that an unknown humoral factor or soluble mediator affects IgGs from the earliest stage of BCa, and also suggested that IgG Fc region *N*-glycosylation could play a role in tumor biology. Although the sample size in the present study was small, the IgG Fc region *N*-glycan multiple logistic regression (MLR) model developed presented the advantage to be used as a diagnostic biomarker and may provide the insights into tumor immunology.

2.1.1.2. Phosphorylation. Although the status of phosphorylation events could provide evidences regarding disease status, few phosphoproteins have been developed as disease markers. Furthermore, with several highly abundant proteins representing > 95% of the mass in blood, few phosphorylated proteins in plasma/serum can be identified with detectable and stable concentrations [93].

Recently, using label-free quantitative phosphoproteomics, 144 phosphoproteins significantly higher in patients diagnosed with BCa compared with HC were indentified in plasma extracellular vesicles (EVs) [94]. Four biomarkers were initially validated in individual patients using paralleled reaction monitoring (PRM) for targeted quantitation: cGMP-dependent protein kinase1 (PKG1), ral GTPase-activating protein subunit alpha-2 (RALGAPA2), tight junction protein 2 (TJP2), and nuclear transcription factor, X box-binding protein 1 (NFX1). These four phosphoproteins showed significant phosphorylation up-regulation in patients with cancer, and have been associated in several BCa studies [95]. Although this study demonstrated that the development of phosphoproteins in plasma EVs as disease biomarkers could transform cancer screening, as disadvantage it relied on the isolation of a good quantity of EVs with high reproducibility and the development of phosphoproteins as biomarkers was also strictly limited by the availability of phosphospecific antibodies.

H. Gu et al. [96] combined immunoaffinity purification and LC-MS/MS without depletion of abundant proteins for the enrichment and quantitative analysis of PTMs in serum samples of patients with BCa. It was found that lysine acetylation (AcK) and arginine mono-methylation (Rme) were more prevalent than other PTMs, finding several AcK and Rme sites with distinct abundance distribution patterns. Therefore, this approach could be very useful for patient profiling and biomarker discovery research.

2.1.2. Protein profiling studies related to breast cacer subtypes

Studies on BCa showed substantial tumor heterogeneity consisting of different molecular subtypes, each with distinct clinical and biological and individualities [97]: luminal, HER2-enriched, basal-like and normal breast-like subtype. The criteria to classify subtypes were recently refined, in that moderate of a strong expression of PR and Ki-67 level defined the subtypes as: luminal A-ER positive, HER2 negative, Ki-67 low, and PR high; luminal B (HER2 negative)-ER positive, HER2 negative, and either Ki-67 high or PR low; luminal B-like (HER2 positive)-ER positive, HER2 overexpressed or amplified, any Ki-67, and any PR; HER2 positive-HER2 over-expressed or amplified, ER and PR absent; and triple negative-ER and PR absent and HER2 negative [98,99].

Due to its lower costs and easy implementation into standard pathology workflow, immunohistochemistry (IHC) is the method used to define surrogate protein biomarkers for the classification of BCa [100]. More recently, the molecular classification by microarray analysis agreed well to IHC classification of different breast carcinomas [101,102]. Subsequently, IHC and molecular classifications are concurrently used to define the BCa subtypes.

In this way, proteomics could also detect additional proteins or protein profiles to improve current BCa classifications. Besides, proteomics might show biological insights and recognize protein biomarkers outlining differences in therapy resistance, prognosis and metastatic spread within a specific subtype. In this section, recent proteomic studies developed in relation with the molecular classification of BCa will be discussed.

In a proteomic study developed by H. Nakshatri et al. [103] was found that the plasma proteome of luminal A and HER2 positive BCa patients did not differ significantly from HC, however, in the luminal B subtype, eight proteins involved in immune response (as α-glycoprotein orosomucoid 1 (ORM-1) and serum amyloid protein (SAP)) were significantly increased, whereas 12 proteins involved in free radical scavenging were significantly decreased. Two complement factors (complement factor H-related 3 (CFHR3) and complement component 4 binding protein β (C4BPB)) were identified being elevated in TNBC compared with HC, and a significant number of plasma proteins (20) were downregulated compared with healthy individuals.

An integrative clustering analysis of BCa subtypes from plasma proteome samples showed that luminal A and luminal B subtypes were clustered together, as well as the basal-like and HER2 positive. Furthermore, luminal A and luminal B were more close to each other than basal-like and HER2 positive to each other. The results also showed that proteomic pathway-assisted clustering of BCa subtypes could offer relationships and biological insight into the intrinsic mechanisms between the diverse BCa subtypes [104].

More rencently, a multipronged quantitative proteomic approaches identified 307 differentially regulated subtype specific proteins: luminal A subtype consisted of 24, luminal B subtype 38, HER2 enriched subtype 17 and triple negative breast cancer subtype 10 [105]. These specific proteins were further subjected to bioinformatic tools which revealed the involvement in platelet degranulation, fibrinolysis, lipid metabolism, immune response, complement activation, blood coagulation, glycolysis and cancer signaling pathways in the subtypes of the BCa.

SRM assays in a different cohort of samples verified and confirmed that Biotinidase (BTD) is down-regulated in LA when compared with other subtypes. Similarly, APOL1, AATM, TTHY, THRB, AACT, and APOD found to be up-regulated in LB. SRM assays also confirmed that CPN2 showed increased expression in TN whereas CD5L showed elevated expression in LB and HE subtypes.

In a work to find protein biomarkers for early detection of ER positive BCa, it was found that the pre-diagnostic level of plasma EGFR was significant elevated in women who developed BCa. However, this biomarker showed moderate specificity and sensitivity and for early diagnosis [106]. In another study from the same group [107], an increased level of glycolysis-related proteins in plasma of BCa patients compared with that in controls was detected. Nevertheless, as disadvantage, the cause of these glycolysis proteins as well as their function in ER positive BCa was not elucidated until the moment.

Using an antibody microarray, a panel of 23 proteins was screened

in human plasma (HP) from patients with an actual diagnosis of BCa of different subtypes (defined by ER and HER2 status) or benign breast disease [108]. In comparison with women with benign breast disease, four proteins (amphiregulin, RANTES, heparin-binding EGF, TGFa) were increased in plasma of ER positive/HER2 negative BCa patients, three proteins (heparin-binding EGF, EGF, RANTES) in ER negative/HER2 positive BCa patients, two proteins (RANTES and platelet-derived growth factor (PDGF)) in ER positive/HER2 positive BCa patients, and two proteins (RANTES and VEGF) in plasma of ER negative/HER2 negative of BCa patients.

In other antibody-based assay, C. I. Li et al. [109] analyzed plasma samples from HC and TNBC patients identifying 93 differential proteins. 29 proteins were confirmed by the validation in an independent cohort. Using a strict criteria, five proteins (DUSP9, EED, EFNA5, ITGB1 and PTPMT1) were found and colud be exploited as markers for early detection of TNBC.

Lymph node status is a crucial predictor for the OS of invasive BCa. However, lymph node involvement is only detected in about half of HER2 positive patients. Since patients with lymph node involvement has less favourable prognosis and higher risk of recurrence, it is important to develop plasma protein biomarkers for distinguishing lymph node metastasis. In this way, L. Chen et al. [110] applied label-free quantitative proteomic strategy to construct plasma proteomes of ten patients with small size HER2 positive BCa (five patients with lymph node metastasis (LNM+) *versus* five patients without lymph node metastasis (LNM-)). A total of 388 proteins were identified, of which 33 proteins were differentially expressed (DE), and 24 proteins were overexpressed in LNM+ group. Western blotting analysis showed that RARB was greatly increased in LNM+ group and FBLN5 was slightly elevated, indicating the results of label-free quantification is highly consistent with western blotting. As advantage, this aproach is low-cost and high-efficiency in initial screening of plasma biomarkers, however the present dataset only provided a list of DE plasma proteins which could be used for further screening of genuine biomarkers.

3. Protein biomarkers for breast cancer prognosis

Compared to diagnostic studies, proteomics studies to discover novel markers to improve BCa prognostication are rather limited (see Table 3).

It was found that adjuvant use of bisphosphonate diminished recurrence in BCa patients through suppression of bone resorption. To determine the prognostic impact of bone resorption markers, M. Imamura et al. [111] investigated serum levels of the pyridinoline crosslinked carboxyterminal telopeptide of type I collagen (1CTP) and *N*-terminal crosslinking telopeptides of type I collagen (NTX). Relapse-free survival of 130 patients whose 1CTP changed from low at baseline to high at 6 months postoperatively showed RFS almost as poor as that for patients with high 1CTP throughout. As advantage, 1CTP could be suitable not only for categorizing patients with adverse prognosis, but also for choosing patients who colud benefit from administration of bone modifying agents in an adjuvant scenary.

Recently, it was developed an study to identify density-associated proteins to improve the understanding of mammographic breast density as a risk factor for BCa [112]. Particularly, it was found that ABCC11, TNFRSF10D, F11R and ERRF were positively associated with area-based breast density (AD), and SHC1, CFLAR, ACOX2, ITGB6, RASSF1, FANCD2 and IRX5 were negatively associated with AD. These data provided insights into the aetiology of breast density as a prominent risk factor for BCa. Furthermore, this study showed that stroma-specific and epithelial-specific proteins could be found in blood as a consequence of tissue leakage, which would make them key candidates for future individual risk stratification. However, this study showed the disadvantages that further validation and follow-up studies of the shortlisted protein candidates in independent cohorts were needed to infer their role in breast density and also its progression in premenopausal and postmenopausal women.

Among the major risk factors of BCa, other important role is played by familial history of BCa. Germ-line mutations in BRCA1/2 genes account for most of the hereditary ovarian and/or breast cancers. Gene expression profiling studies revealed particular molecular signatures for BRCA1/2-related breast tumors as compared to sporadic cases, which could facilitate diagnosis and clinical follow-up. Even though, a clear hallmark of BRCA1/2-positive BCa is still needing.

Tumor-specific changes in the plasma proteome of BCa patients and healthy family members sharing the same BRCA1 gene founder mutation (5083del19) were investigated by L. Tammè et al. [113]. The proteomic analysis revealed that gelsolin was down-expressed in plasma samples of patients with hereditary BCa, and that its levels were associated with the BRCA1 mutation status, showung that this relevant tumor suppressor gene could stimulate BCa cell proliferation, migration and invasion, also thorough the down-regulation of gelsolin.

Women with inherited BRCA1 mutations are more likely to develop BCa; however, not every carrier will progress to BCa. The aim of the study developed by J. Fan et al. [114] was to identify and characterize circulating peptides that correlate with BCa patients carrying BRCA1 mutations. After the enrichment of circulating peptides using a nanoporous silica thin films (NanoTraps), peptides $KNG1_{K438\text{-}R457}$ and $C3f_{S1304\text{-}R1320}$ were identified as putative peptide candidates to differentiate BRCA1 mutant BCa from sporadic BCa and cancer-free BRCA1 mutant carriers. Therefore, the expression level of both peptides were associated with cancer status in BRCA1 carriers. This approach could offer an answer to the dilema of who is going to get cancer. The long-term longitudinal information would also be greatly beneficial, particularly for cancer-free BRCA1 mutation carriers who maintain their high-risk status. We intend for this strategy to improve the early examination of cancer in the BRCA1 carriers based on the suggestions from the blood-based test. However, in our opinion, future prospective studies are required to validate these findings.

It was described that the inhibitor of apoptosis (IAP) protein Survivin and its variants (Survivin-ΔEx3 and Survivin-2B) are differentially expressed in BCa tissues. Furhermore, Survivin is released from tumor cells via small membrane-bound vesicles called exosomes. Thus, S. Khan et al. [115] developed the analysis of exosomal Survivin, Survivin-ΔEx3 and Survivin-2B in BCa patient sera in parallel with paired breast tumor tissue. After exsosomal investigation, Survivin and Survivin-ΔEx3 were detected in all of the samples examined, however, Survivin-2B was differentially expressed depending on the disease aggressiveness: expressed mostly in primary tumors in early stage disease, low or no expression was found in high-grade tumors and absent in most distant metastasis (DM). Therefore, in our opinion, exosomal Survivin-2B could be further investigated as an early diagnostic or prognostic marker in BCa.

M. Giussani et al. [116] tested plasma samples from HC and from patients with malignant or benign breast disease by enzyme-linked immunosorbent assay (ELISA) for the presence of collagen11a1 (COL11A1), collagen oligomeric matrix protein (COMP), and collagen 10a1 (COL10A1). Importantly, the combination of COL11A1, COMP, and COL10A1 was identified as potentially informative in discriminating BCa patients from those with benign disease. The three molecules resulted expressed in the stroma of BCa tissue samples, thus circulating COL11A1, COMP, and COL10A1 could be very advantageous in diagnostic assessment of suspicious breast nodules.

4. Biomarkers for response prediction and treatment monitoring of breast cancer

Though accurate prediction of chemosensitivity in cancer therapy would allow personalized therapy, thus avoiding toxic side effects and the use of ineffective agents, protein profiling studies searching for markers for response prediction and treatment monitoring of BCa are scarce (see Table 3).

Table 3
Summary of proteomic studies in plasma/serum to identify proteins related to breast cancer prognosis or predictive of response.

Type of sample	Enrichment strategy	Techniques (determination)	Proteins determined	Validation/verification	Candidate biomarkers	Ref.
HS samples from 469 BC patients operated	–	Dual-antibody radioimmunoassay method and the competitive ELISA	Pyridinoline crosslinked carboxyterminal 1CTP and N-terminal crosslinking telopeptides of NTX	NA	NA	[111]
HP samples from 1329 HC (woman without prior story of breast cancer)	–	SBA assays: 382 antobodies included in the first suspensión bead array (SBA1) and 393 antobodies included in the second bead array (SBA2)	Proteins positively associated with AD: ABCC11, TNFRSF10D, F11R and ERRF.Proteins negatively associated with AD: SHC1, CFLAR, ACOX2, ITGB6, RASSF1, FANCD2 and IRX5.	NA	NA	[112]
HP samples from 4 patients with inherited BC (bearing a founder mutation on the BRCA1 gene), 4 healthy family members sharing the same mutation and 4 healthy relatives, free of BRCA1-gene defects	Depletion of high abundant proteins with a Multiple Affinity Human-7 (Hu-7) Removal System (Agilent Technologies)	Nanoscale LC-MS/MS analysis	Gelsolin was down-expressed in HP samples of patients with hereditary BC	Western Blotting on plasma samples to verify the expression of gelsolin in cancer and healthy carrier patients compared to HC	Gelsolin	[113]
132 serum samples from 38 WT, 39 SBC, 27 BH, 28 BBC	Circulating peptides were enriched using our well-designed nanoporous silica thin films (NanoTraps)	MALDI-TOF MS detection and peptide identification by LC-MS/MS	KNG1K438-R457 and C 3fS1304-R1320 peptides were identified as candidates to differentiate BBC from sporadic BC and cancer-free BRCA1 mutant carriers	–		[114]
HS samples from 40 BC patients and 10 HC who were disease free for 5 years after treatment	Exosomes were isolated from this serum using the commercially available ExoQuick kit	Survivin protein levels in serum were analyzed using ELISA and the exosomal survivin by Western blotting	Survivin, Survivin-ΔEx3 and Survivin-2B. Exosomal Survivin-2B (proapoptotic) was expressed mostly in primary tumors in early stage disease	NA	NA	[115]
HP samples from 52 patients with invasive BC, 42 patients with benign breast disease, and 50 HC	–	ELISA	The combination of COL11A1, COMP, and COL10A1 as potentially informative in discriminating BC patients from those with benign disease	NA	NA	[116]
331 patients who underwent an operation for infiltrating BC	Dynabeads® RPC 18 (Life Technologies Dynal, Irvine, CA) with a C18 alkyl-modified surface	MALDI-TOF MS	*m/z* 1046.49 was associated with BC-specific mortality	ELISA	ATII (m/z 1046.46)	[120]
HS samples from 99 BC patients and 51 HC	Four adsorptive chip surfaces: Q10 (strong anion-exchange), Cu^{2+}-IMAC30 (immobilized metal affinity capture), CM10 (weak cation-exchange), and H50 (hydrophobic)	SELDI-TOF MS	Five protein peaks (*m/z* 3808, *m/z* 6624, *m/z* 8916, m/z 13,870, and *m/z* 28,268) that together classified BC and HC	Immunoprecipitation (Protein G Dynabeads) and western blotting	APOH (*m/z* 3808), APOC1 (*m/z* 6624), complement C3a (*m/z* 8916), transthyretin (*m/z* 13,870), and APOA1 (*m/z* 28,268) significantly predicted disease-free survival (P = .005)	[121]
Sixty pairs of HS samples from BC patients pre- and post-chemotherapy and serum from 60 HC	Peptide immunoaffinity enrichment: SISCAPA	LC-MS/MS	Differential levels of sTfR in breast cancer patients pre- (1.77 ± 0.53 μg/ml) and post- (0.98 ± 0.26 μg/ml) chemotherapy were obtained: sTfR may be a potential indicator of transfusion requirement	NA	NA	[122]
HS samples from 45 BC patients and 78 HC	Solid-phase extraction on silica C2 columns	LC-MS/MS	Significantly decreased serum levels after surgery for seven of the $ITIH_4$-derived peptides ($p < .02$): potential for the follow-up of breast cancer after surgery	NA	NA	[125]

NA, not available.

In recent years, several studies have reported the diagnostic utility of the low molecular weight fraction of the HS peptidome in BCa [58,117]; however, to the best of our knowledge, only two studies investigated the putative use of peptide signals as biomarkers to predict tumour outcome following surgery [118,119]. M. C. Gast et al. [118] demonstrated a strong association between serum haptoglobin phenotype and recurrence-free survival in a group of 63 high-risk early BCa patients. A. Gonçalves et al. [119] investigated post-operative sera of 83 high-risk BCa patients, identifying a 40-protein signature that correctly predicted the outcome in 83% of the cases. Major components of this signature include haptoglobin alpha-1, C3a, transferrin and APOA1 and APOC1. However, as disadvantage, both groups were unable to subsequently validate their results in an independent patient set.

In this way, recently F. Boccardo et al. [120] employed MALDI-TOF MS to recognise serum peptidome profiles predictive of mortality in 331 patients who underwent an operation for infiltrating BCa. At a median follow-up time of 25.5 years (range 1.3 to 26.9 years), 68 of the 102 patients were deceased, and 45 of these deaths were attributed to BCa-related causes. It was found that four signals were increased in deceased patients compared with living patients and only one having mass/charge ratio (m/z) 1046.49 was associated with BCa-specific mortality. This peak was identified as Angiotensin II (ATII) whose levels were increased in women who exhibited worse mortality outcomes, reinforcing the evidence that this peptide potentially significantly affects the natural history of early BCa.

After clustering analysis of protein spectra to identify protein patterns related to BCa and HC groups [121], five protein peaks (m/z 3808, m/z 6624, m/z 8916, m/z 13,870, and m/z 28,268) were indentified that together classified BCa and HC with a receiver operating characteristic (ROC) area-under-the-curve value of 0.961. These proteins were identified as a fragment of apolipoprotein H (APOH, m/z 3808), APOC1 (m/z 6624), complement C3a (m/z 8916), transthyretin (m/z 13,870), and APOA1 (m/z 28,268). Importantly, this panel significantly predicted disease-free survival ($P = .005$), with and efficacy greater in women with ER negative tumors ($n = 50$, $P = .003$) that in ER positive ($n = 131$, $P = .161$). Furthermore, in women with ER negative tumors, the combined biomarker could be used as an adjunct to other pathological variables in predicting patient outcome. However, in our opinion, this method needs to be confirmed in larger patient cohorts, and also to be developed in a new format (for example, a multiplexed ELISA kit) that could facilitate its further application.

Although the soluble form of transferrin receptor (sTfR) is frequently used to identify iron deficiency anaemia [122], a few studies have reported its elevation in a variety of cancers, including BCa [123]. In this way, Q. Xu et al. [124] developed and validated an advanced LC-MS/MS-based targeted proteomics assay coupled with peptide immunoaffinity enrichment (SISCAPA) for the quantification of low-level sTfR (100 ng/ml) in BCa patients after the onset of chemotherapy. Using this assay, 60 pairs of serum samples pre- and post-chemotherapy and the corresponding control samples from 60 HC were determined and compared. The results confirmed sTfR suppression during chemotherapy and suggested that sTfR may be a potential indicator of transfusion requirement. However, further studies using large sample sizes in preclinical and clinical trials will be required to confirm the value of this assay.

Using a fully validated liquid chromatography-tandem mass spectrometric method, I. van den Broek et al. [125] have compared absolute serum concentrations of eight peptides derived from ITIH4 ($ITIH_{4\,[658–687]}$ to $_{[667–687]}$ ($ITIH_4$–30 to −21)) before and after surgical substraction of the tumor. Intra-individual comparisons of serum obtained before and after surgery showed significantly decreased serum levels after surgery for seven of the $ITIH_4$-derived peptides ($p < .02$). The obtained results particularly suggest potential for these $ITIH_4$-derived peptides in the follow-up of BCa after surgery.

5. Summary

Many of the proteins found in HS and HP could be potential biomarkers for cancer early detection and drug efficacy evaluation. The development of clinical biomarkers using proteomic strategies usually undergoes three stages, including initial discovery, large-scale verification and clinical validation steps.

In this way, different proteomic approaches have been used for biomarker discovery: 2D-DIGE (lack of real high-throughput capability), protein microarrays (allows simultaneous analysis of multiple proteins using capture molecules spotted on a surface) and MS (enabled large-scale proteomic analyses).

Verification of proteomic findings with a different assay offers further information about data validity, while validation of proteins in a large patient cohort supports potential clinical application as a biomarker. Commonly used methods appropiate for these functions use an antibody-based approach and include ELISA, Western blot and IHC. Importantly, targeted proteomic method based on selected reaction monitoring (SRM) are believed to replace traditional western blot as the new golden standard for protein expression verification.

In this review, we have provided a comprehensive overview on the discovery and development of promising blood-based protein biomarkers for the early detection of BCa, precise prognosis and prediction of response to a treatment. It was found that the majority of the protein profiling studies performed in BCa have searched for novel diagnostic markers, while the search for new prognostic and predictive biomarkers is restrained to only few studies. Importanly, many of the identified candidate BCa markers indicated a general lack of tumour-specificity, because they have been found to bear diagnostic potential for other cancer types as well (e.g. apolipoprotein A-I in ovarian cancer, $C3a_{desArg}$ in colorectal cancer).

It is important to mention that various preanalytical parameters, such as clotting temperature, sampling device, and storage time, can thus all exercise a distinct effect on the serum proteome. Furthermore, the majority of markers has been reported by single breast cancer studies, in which only limited numbers of samples were investigated, thereby compromising the generalisibility of results. Hence, validation of biomarker candidates by quantitative methods and/or in new study populations is crucial. However, such validation studies have been performed for only few of the candidate biomarkers detected in proteomic studies and none of the candidate biomarkers discussed in this overview has been validated sufficiently to be used for clinical patient care. Thus, future research to evaluate these biomarkers in large-scale clinical validation studies is needed in order to expedite the use of biomarkers in clinical practice.

Conflict of interest

The authors declare no conflict of interest.

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