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The surface proteomic profile of serum extracellular vesicles as a diagnostic and prognostic tool in breast cancer

Giada Corti[1], Rene Buchet[2], Andrea Magrini[3], Pietro Ciancaglini[4], Saida Mebarek[2] and Massimo Bottini[1,5]

The diagnosis of breast cancer in the early stage is essential for a favorable prognosis. Extracellular vesicles isolated from body fluids have a central role in breast cancer development due to their biochemical components. Among the biochemical components, surface proteins mediate vesicle interactions with elements of the extracellular milieu, the extracellular matrix, and neighboring cells. The identification of specific surface proteomic profile has been regarded as an easy and reproducible means to define cancer parameters, identify markers for a diagnosis, and determine targets for therapeutical treatments. In this review, we will focus on annexins, tetraspanins, integrins, immune checkpoint proteins, and growth factor receptors that have been identified on the surface of extracellular vesicles isolated from the serum of patients with breast cancer and that have been found to be relevant diagnostic and prognostic biomarkers.

Addresses
[1] Department of Experimental Medicine, University of Rome Tor Vergata, 00133 Rome, Italy
[2] Université Lyon 1, ICBMS UMR CNRS 5246, UFR Biosciences, 69 622 Villeurbanne Cedex, France
[3] Department of Biomedicine and Prevention, University of Rome Tor Vergata, 00133 Rome, Italy
[4] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto -Universidade de São Paulo, 14040-900 Ribeirão Preto, Brazil
[5] Sanford Burnham Prebys, La Jolla, CA 92037, USA

Corresponding author: Bottini, Massimo (massimo.bottini@uniroma2.it, mbottini@sbpdiscovery.org)

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Introduction

Breast cancer is the most diagnosed cancer in females across the globe [1]. Even though breast cancer mortality has been steadily reducing in recent years, there is still a wide disparity between the prognosis of women diagnosed with early-stage breast cancer compared with those diagnosed with metastatic breast cancer [1].

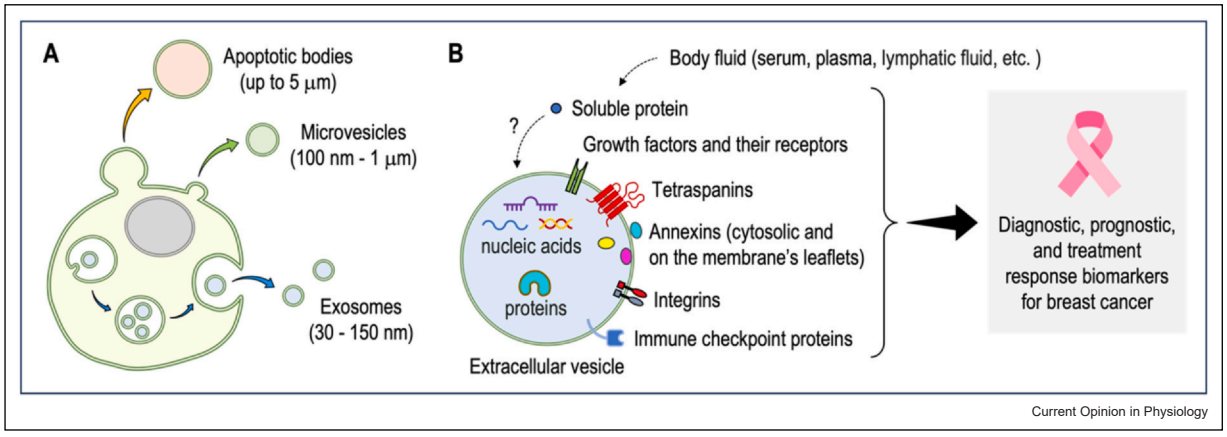
Extracellular vesicles are lipid bilayer-delimited particles released by all cell types to carry out diverse functions, including remove unnecessary molecules from the cells' cytosol and plasma membrane, deliver information cargoes into target cells, and initiate biomineralization (Figure 1) [2,4,5]. Different classes of extracellular vesicles (e.g. exosomes, microvesicles, and apoptotic bodies) can be detected in cancer patients' tissues and body fluids (e.g. blood, urine, saliva, and lymphatic drainage fluid) where some of them are a source of biomarkers clinically valuable in providing insight into patients' diagnosis, prognosis, and treatment response [6]. The secretion of extracellular vesicles from breast cancer cells can trigger metastasis, and/or stimulate the tumoral activity by the transfer of nucleic acids and proteins from the extracellular vesicles' lumen to healthy cells [5] (Figure 1). The interactions between extracellular vesicles and their target cells are triggered by their external membrane components [5]. The extracellular vesicles' membrane proteins can be potential biomarkers, aiming to achieve benefits in breast cancer diagnosis, prognosis, monitoring, and treatment [7]. In this review, we will focus on annexins, tetraspanins, integrins, immune checkpoint proteins, and growth factor receptors that have been recently identified on the surface of extracellular vesicles isolated from the serum of breast cancer patients and that have been found to be relevant diagnostic and prognostic biomarkers.

Surface proteins of serum extracellular vesicles as breast cancer biomarkers

Tetraspanins

Tetraspanins are type-III transmembrane proteins expressed on both the plasma and intracellular membranes where they form microdomains involved in membrane trafficking and fusion, as well as cell motility and signaling [8,9]. Tetraspanins are also distributed on the

Figure 1



The extracellular vesicles' surface proteins as diagnostic and prognostic biomarkers in breast cancer. **(a)** Extracellular vesicles have been divided into three major classes (e.g. exosomes, microvesicles, and apoptotic bodies) based on their biogenesis process and size [2]. **(b)** Extracellular vesicles harbor the complete set of biochemical components needed to carry out their functions [2]. Surface proteins enable extracellular vesicles to interact with elements of the extracellular matrix, the extracellular milieu, and neighboring cells [3]. By doing so, surface proteins mediate physiologic and pathologic processes, and have been regarded as clinically valuable biomarkers [4].

extracellular vesicles' membrane where they initiate vesicle docking and uptake [10]. Proteomics indicates that cluster of differentiation 82 (CD82) [11] and Raph blood group (CD151) [12] were significantly enriched on the surface of extracellular vesicles derived from the serum of breast cancer patients than that of healthy individuals, suggesting possible diagnostic biomarkers (Table 1). Serum level of extracellular vesicle CD82 increased with breast cancer malignancy [11]. The treatment of MDA-MB-231 (highly invasive) and MDA-MB-468 cells (scarcely invasive) with CD151-devoid extracellular vesicles decreased cell motility, suggesting that CD151-expressing extracellular vesicles promoted the migration of breast cancer cells. regardless of their invasiveness, and might be used as a therapeutic target (Table 1) [12].

Annexins

Annexins are Ca^{2+}/phospholipid-binding proteins that are involved in exocytosis, membrane repair, and apoptosis [13]. In cells, annexins are mostly cytosolic, but they were also found inside organelles as well as on the plasma membrane's leaflets [14,15]. Similarly, annexins were found simultaneously in different locations in extracellular vesicles [16].

Extracellular vesicles from breast cancer cell lines contain annexin A2 (AnxA2) [17]. The AnxA2 amount in extracellular vesicles correlated positively with cell invasiveness *in vitro*, and AnxA2-positive extracellular vesicles induced angiogenesis and promoted metastasis *in vivo* [17]. A study based on race-derived patient cohorts found that the serum level of extracellular vesicle AnxA2 was significantly higher in breast cancer patients than in healthy individuals and was associated with tumor grade and poor overall survival (Table 1) [18]. The serum level of extracellular vesicle AnxA2 was also higher in triple-negative breast cancer (TNBC) than in other cancer subtypes (human epidermal growth factor receptor 2 [$HER2^+$] and estrogen receptor [ER^+]), and it was higher in African-American than in Caucasian–American patients with TNBC and correlated with tumor grade [18]. Thus, extracellular vesicle AnxA2 could be exploited as a diagnostic and prognostic marker of TNBC in African-descent women. Studies to assess the relationship of extracellular vesicle AnxA2 with other ethnicities and breast cancer subtypes are warranted.

Extracellular vesicle annexin A6 (AnxA6) mediated the prometastatic capacity of neoadjuvant chemotherapy [19]. The effects of neoadjuvant chemotherapy on the plasma level of extracellular vesicle AnxA6 were assessed in a small cohort (n = 6 including IA, IIB, and IIIA stages) of breast cancer patients undergoing neoadjuvant therapy (Table 1). The plasma level of extracellular vesicle AnxA6 increased at mid-treatment compared with pretreatment levels [19]. Notably, at the end of the therapy, and before curative operation, the level of extracellular vesicle AnxA6 decreased in the patients (n = 5) who achieved a complete or partial response, while it increased in one patient with no response. Thus, extracellular vesicle AnxA6 could be exploited as a predictive marker of metastasis and an outcome marker after neoadjuvant chemotherapy.

Integrins

Integrins are type-I transmembrane proteins mediating several key processes, including cell–cell and cell–extracellular

Table 1

Surface protein markers found in extracellular vesicles extracted from fluids of human patients or from cancer cells.

Isolation method	Fluid	Patients	Breast cancer cell lines	Markers	Ref.
- ExoQuick kit - UC (1500*g*, 30 min) - UC (1500*g*, 5 min)	Serum	Breast cancer patients (80) Patients with benign breast diseases (80) Healthy individuals (80)		CD82 diagnostic	[11]
- UF (0,22 μm) - UC [150,000*g*, 3 h (×2)]	Serum	TNBC patients (30) Healthy individuals (37)		CD151 diagnostic, potentially	[12]
- UC (1000*g*, 10 min) - UC (10,000*g*, 30 min) - UF (0,22 μm) - UC (100,000*g*, 2 h)			MDA-MB-468 MDA-MD-231	therapeutic	
- UC (300*g*, 10 min) - UC (16,500*g*, 20 min) - UF (0,2 μm) - UC (120,000*g*, 70 min)			MDA-MD-231	AnxA2 diagnostic, prognostic	[17]
- UC (2000*g*, 30 min) - Incubation with total exosome isolation reagent - UC (10,000*g*, 10 min)	Serum	Breast cancer patients [TNBC (58), ER^+ (50), $HER2^+$ (59)] Healthy individuals (68)			[18]
- UC 134,000*g*, 70 min (×2)	Plasma	Breast cancer patients (6)		AnxA6 prognostic	[19]
- UC (1600*g*, 15 min) - UC (100,000*g*, 70 min)	Blood	Breast cancer patients (128)		Integrins α_V, β_1 prognostic	[25]
- UC (355*g*, 10 min) - UC (1422*g*, 15 min) - UC (7199*g*, 15 min) - UF (0,45 μm) - UC (100,00*g*, 90 min) in sucrose - UC (110,00*g*, 12 h) in sucrose - SEC			MDA-MB-231 MCF10CA1a 4T1 4T07		
- UC (500*g*, 10 min) - UC (12,000*g*, 20 min) - UC (100,000*g*, 70 min)		Breast cancer patients (22) Healthy individuals (6)	MDA-MB-231 organotropic lines	Integrins α_1, α_2, α_3, α_6, β_1, β_3, β_4 prognostic	[26]
- UC (300*g*) - UC (3000*g*) - UF (0,45 μm) - UC (110,000*g*, 80 min)	Plasma	Breast cancer patients (18 +48)		Integrins β_3, β_4, $\alpha_V\beta_5$ prognostic	[27]
- UF (0,2 μm) - UC (110,000*g*, 75 min)	Serum	Breast cancer patients (30)		PD-L1 diagnostic, treatment response	[34]
- UC (300*g*,10 min) - UC (2000*g*, 10 min) - UC (10,000*g*, 30 min) - UC [100,000*g*, 70 min (×2)] - UF (0,22 μm)			T cells MDA-MB-231	PD-1 potentially prognostic	[35]
- UC (1500*g*, 20 min) - UC (16,000*g*, 45 min) - UC (100,000*g*, 2 h) - UF (0,22 μm)	Blood	Breast cancer patients (46) Healthy individuals (20)		TGFβR2 diagnostic (early), prognostic	[39]
- UC (300*g*, 5 min) - UC (2000*g*, 15 min) - UC (16,000*g*, 45 min) - UC (100,000*g*, 2 h) - UF (0,22 μm)			MDA-MB-231 MCF7 4T07 4T1		
- UC (3000*g*, 10 min) - UC (15,000*g*, 35 min) - UF (100 kDa) (can be omitted) - UC [100,000*g* (×2)] *Alternatively* - UC (3000*g*, 10 min) - ExoQuick kit			MDA-MB-231 MCF7	ADAM10 GLUT1 GPC1 potentially prognostic	[42]

Abbreviations: SEC = size-exclusion chromatography; UC = ultrafiltration; UF = ultrafiltration.

matrix interactions and signal transduction [20]. They are heterodimers composed of an α- and a β-subunit [20]. Integrins on both the plasma membrane and extracellular vesicles' membrane actively contribute to several steps of cancer development [21–23].

A large-scale proteomic study showed that extracellular vesicles released from breast cancer cells expressed integrins α_1, α_2, α_3, α_5, α_6, α_V, β_1, β_4, and β_5, and the level of integrins α_1, α_6, α_V, and β_1 in extracellular vesicles correlated with cancer stage [24]. The level of integrins α_2, α_3, α_V, and β_1 in extracellular vesicles correlated with the cell metastatic potential [25]. Additionally, the expression of integrin α_V in plasma CD63-expressing extracellular vesicles correlated with cancer stage in both breast cancer patients and xenografted mice bearing metastatic tumors (Table 1) [25]. These findings suggested that the extracellular vesicles' integrin profile may be exploited as a prognostic biomarker.

A landmark study showed that the extracellular vesicles' integrin profile can be used as an organotropic biomarker to predict the metastasis site [26]. Organotropic cancer cells release extracellular vesicles with a unique integrin profile such that, when the extracellular vesicles were injected in nude mice, they exploited their integrin profile as an 'address code' to accumulate in the metastatic organ as their cells of origin and prepare pre-metastatic niches by targeting specific resident cells [26]. Extracellular vesicles expressing integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ targeted fibroblasts and epithelial cells in the lungs, while extracellular vesicles expressing integrin $\alpha_V\beta_5$ targeted Kupffer cells in the liver *in vivo* [26]. The expression of integrin β_4 was higher in extracellular vesicles from a small cohort (n = 2) of breast cancer patients that developed lung metastasis [27]. The association of the extracellular-vesicle expression of integrin β_4 with lung metastasis was also confirmed in two cohorts of breast cancer patients in a recent study [27]. While several pieces of evidence indicate that breast cancer cells secrete extracellular vesicles with one or several integrins, the mechanisms to secrete exosomes with a specific integrin and how their relative proportions could direct exosomes toward a specific tissue or cells remain unclear.

Immune checkpoint proteins

Immune checkpoint proteins are signaling pathway molecules expressed by immune cells to modulate immune responses while preserving self-tolerance [28]. Immune checkpoint proteins are either inhibitory or stimulatory [29]. Inhibitory immune checkpoint proteins have been detected in tumor cell-derived exosomes and it has been suggested that they are exploited by tumor cells to promote tumor progression and metastasis by inactivating cytotoxic T cells [30–32].

To date, only the level of the inhibitory immune checkpoint programmed death ligand 1 (PD-L1) from extracellular vesicles has been evaluated as a diagnostic tool in breast cancer. A positive correlation of serum level of extracellular vesicle PD-L1 with the stage of breast cancer was found by an electrochemical sensor [33]. HER2 is overexpressed in ~25% of breast cancers and is associated with poorest prognosis [34]. HER2-targeted drug resistance in HER2-positive breast cancer cells correlates with increased levels of the immunosuppressive molecules transforming growth factor β1 (TGFβ1) and PD-L1 [34]. However, the serum level of extracellular vesicle PD-L1 did not significantly associate with the response to HER2-targeted neoadjuvant therapy (trastuzumab with or without lapatinib) in a small cohort (n = 30) of $HER2^+$ breast cancer patients [34]. Conversely, extracellular vesicle TGFβ1 — a molecule that regulates the expression of immune checkpoint proteins and co-expressed with PD-L1 on extracellular vesicles [32] — was significantly higher in patients who did not respond to the therapy compared with those who exhibited partial or complete response [34]. This would suggest that extracellular vesicle TGFβ1 might be a better outcome marker than extracellular vesicle PD-L1.

Activated cytotoxic T cells release extracellular vesicles expressing programmed cell death protein-1 (PD-1) (Table 1) that can restore immune surveillance in a breast cancer mouse model by PD-L1 internalization *via* clathrin-mediated endocytosis, and thereby prevent subsequent cellular PD-L1:PD-1 interaction [35]. Thus, the serum level of extracellular vesicle PD-1 secreted by T cells could be exploited as a tumor prognostic marker. It is worth noting that, unlike the continuous release of extracellular vesicles from cancer cells, the release of extracellular vesicles from T cells is triggered by rapid immune synapse during activation, which may make T-cell-derived extracellular vesicles difficult to detect and unreliable as prognostic tools [36–38]. A possible solution of this problem would be to detect extracellular vesicle PD-1 along with other immune checkpoint receptors.

Growth factor receptors

A seminal report has shown that metastatic breast cancer cells release transforming growth factor-β receptor-2 (TGFβR2)-positive extracellular vesicles that promote tumor metastatic outgrowth and cytotoxic T-cell exhaustion *in vivo* [39]. The diagnostic and prognostic usefulness was also assessed on breast cancer patients (n = 46). Patients with TNBC exhibited higher serum level of extracellular vesicle TGFβR2 than both $HER2^+$ and luminal patients [39]. Additionally, the level of extracellular vesicle TGFβR2 can be used as a biomarker for the early detection of metastasis with high sensitivity and specificity and predicts the overall and metastasis-free survival.

Other surface proteins of serum extracellular vesicles

An *in vitro* study showed that the transmembrane proteins glucose transporter 1 (GLUT1), glypican 1 (GPC1), and a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) were expressed in the extracellular vesicles released by MDA-MB-231, a metastatic breast cancer cell line, but not MCF-10A, a noncancerous epithelial breast cell line, suggesting their potential diagnostic use as extracellular-vesicle biomarkers [40].

Concluding remarks

There is solid scientific evidence to suggest that the surface proteomic profile of the extracellular vesicles isolated from the breast cancer patients' serum can be exploited to achieve information about, for instance, the cancer stage and malignant parameters as well as the outcome after neoadjuvant chemotherapy. The surface proteomic profile might be also exploited to achieve information about the parent cells and design novel cell-specific therapies, also with the aid of nanomedicine approaches [41,42]. However, many pieces of the puzzle are still missing to fully understand how to exploit the extracellular vesicles' surface proteomic profile as diagnostic, prognostic, and therapeutic biomarkers in breast cancer. One key point would be to understand how the body fluid from which the vesicles are isolated affects their surface proteomic profile. Currently, extracellular vesicles are described as released by parent cells equipped with the complete set of surface proteins (*native* surface proteins). However, numerous pieces of evidence have led to formulate the model that soluble proteins are added to the extracellular vesicles' surface from the extracellular milieu (*acquired* surface proteins or protein corona) (Figure 1) [3,43–47]. This would suggest that the body fluid, along with parent cells, can modulate the vesicles' surface proteomic landscape and, in turn, its use as a biomarker. However, the validation of this paradigm is currently hampered by the difficulty to preserve the protein corona during the isolation of extracellular vesicles [3]. An additional limitation derives from the fact that several distinct heterogeneous populations of extracellular vesicles are isolated by the different isolation methods [3]. The comparison of the biochemical properties of the extracellular vesicles obtained from different body fluids by using different isolation methods shall contribute to achieve the total retrieval of acquired surface proteins and support their use as biomarker tools in breast cancer.

Data Availability

No data were used for the research described in this article.

Declaration of Competing Interest

The authors of this article declare to have no disclosures or conflicting interests.

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