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REVIEW

Breast cancer circulating biomarkers: advantages, drawbacks, and new insights

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Abstract As of today, the level of individualization of cancer therapies has reached a level that 20 years ago would be considered visionary. However, most of the diagnostic, prognostic, and therapy-predictive procedures which aim to improve the overall level of personalization are based on the evaluation of tumor tissue samples, therefore requiring surgical operations with consequent low compliance for patients and high costs for the hospital. Hence, the research of a panel of circulating indicators which may serve as source of information about tumor characteristics and which may be obtainable by a simple withdrawal of peripheral blood today represents a growing field of interest. This review aims to objectively summarize the characteristics of the currently available breast cancer circulating biomarkers, also providing an overview about the multitude of novel potential soluble predictors which are still under evaluation. Specifically, the usefulness of a so-called "liquid biopsy" will be discussed in terms of improvements of diagnosis, prognosis, and therapy-prediction, but an overview will be given also on the potentiality of the molecular characterization arising from the isolation of circulating biomarkers and cells. Although this review will focus on the specific case of the breast, in the future liquid biopsies will hopefully be available for virtually any type of neoplasms.

Keywords Breast cancer circulating biomarkers · Breast cancer liquid biopsy · Circulating DNA · Circulating microRNA · Circulating tumor cells · Microvesicles and exosomes

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Introduction

Breast cancer (BC), in the year 2015, is estimated to cause the death of 90,800 women among European Union (EU) and of 40,290 women in USA, a number which is still too high even though the last decades have been a golden age in terms of diagnostic and therapeutic progress [1, 2]. For developing countries, a reduced incidence is overall documented, although late diagnoses dramatically contribute to increase the mortality [3].

The technical advances achieved in screening procedures and molecular characterization of the different BC subtypes can offer nowadays an overall good expectation of survival for an early diagnosed patient, also considering the growing spectrum of individualized therapeutic options currently available. Overall, diagnostic, prognostic and therapy-monitoring procedures already offer elevated throughput and detailed results; however, they usually require a proper biopsy. Since biopsies typically mean very low compliance and relatively high expense, a panel of indicators which may be obtained by the simple withdrawal of peripheral blood, a so-called liquid biopsy, would represent a big advance so far (Table 1). Currently

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Table 1 Advantages and drawbacks of tumor liquid biopsies compared to standard tissue-based biopsies

Circulating biomarkers	Tumor biopsy
Advantages	Advantages
• Diagnosis	• Diagnosis
• Prognosis	• Prognosis
• Real-time therapy follow-up	• Material obtained from the primary lesion (high specificity)
• Low cost	
• Minimally invasive—higher compliance	
Drawbacks	Drawbacks
• Lack of well-defined biopanels	• Highly invasive—lower compliance
• Relatively overall lower specificity/sensitivity, especially if considered singularly	• Relatively expensive
	• Outcome strictly dependent from the correctness of the procedure

some soluble cancer biomarkers are already available (CEA, CA15-3), but if taken singularly, they do not offer complete reliability due to their intrinsic lack of both sensitivity and specificity. Other panels, instead, possess high predictive values only in the setting of particular conditions, such as circulating tumor cells (CTCs) for metastatic carcinomas. Recently, the attention has been redirected to the diagnostic, prognostic, and predictive role of circulating and exosomal nucleic acids, such as circulating cell-free DNA (ccf-DNA) and microRNAs (miRs), formerly thought not to possess any biological relevance (Fig. 1). It has become increasingly evident that these molecules may be a powerful source of information about the status of a malignancy, since their quantification and qualitative evaluation would represent an easily-accessible monitor coming directly from the primary lesion [4].

This review proposes to objectively summarize the most relevant BC circulating biomarkers, fully aware of the unavoidable limitations (Table 2). The variety of soluble indicators which are becoming systematically available may offer the possibility of setting-up a panel which could take advantage of the combination of the various biomarkers and exploit their union to counterbalance the drawbacks of the elements taken singularly.

A reliable panel of circulating cancer biomarkers would be helpful in relation to:

-screening and diagnostic procedures;

-prediction of prognosis;

-selection of therapeutic options, including experimental ones;

-follow-up, detection of eventual inefficacy of an ongoing therapy and prediction of side-effects;

-detection of eventual recurrence.

Classic markers

Cancer antigen 15-3 (CA 15-3) is the soluble form of MUC-1, a transmembrane protein which is normally expressed on the external layer of epithelial cells, especially in ducts and lumens. Beyond its physiological function of protection and lubrication, MUC-1 is typically seen to be overexpressed on breast tumor cells, identified by aberrant glycosylated patterns. Since MUC-1 has been defined as a cancer antigen, its shed form CA15-3 is therefore considered as a soluble cancer biomarker. The diagnostic value of CA15-3 is relatively low, being characterized by an intrinsic lack of both sensitivity and specificity; increased serum values can indeed be detected also in presence of other kinds of neoplasms, from breast adenocarcinomas to lung, gastric, pancreatic, and ovarian cancers [5]. Moreover, CA15-3 augmented serum levels can also be the result of chronic hepatitis, hypothyroidism, or liver cirrhosis [5, 6]. With the exception of the European Group on Tumor Markers (EGTM) and, partially, of the

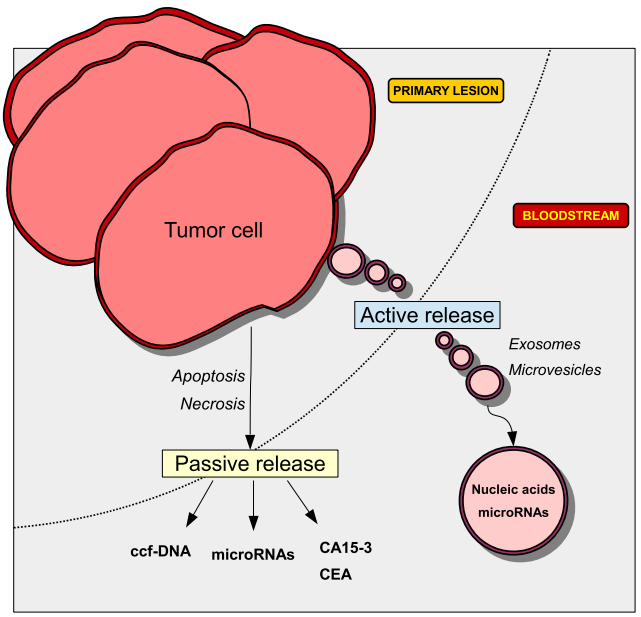

Fig. 1 Representation of the mechanisms driving the release of soluble indicators with potential or demonstrated clinical utility in terms of a liquid biopsy

Table 2 Current levels of validation of circulating biomarkers

Circulating biomarker	Analytical validity	Clinical validity	Clinical utility	Molecular characterization/clinical impact
CEA/CA 15-3	Y	Y	Y	NA
HER2-ECD	Y	N	N	N
Circulating tumor cells				
CellSearch®	Y	Y	Y	Y
Gilupi™	Y	NE	NE	NE
DEPArray	Y	NE	NE	NE
ISET	Y	NE	NE	NE
Circulating cell-free DNA	Y	NE	NE	Y
Exosomes/microvesicles	NE	NE	NE	NE
microRNA	Y	NE	NE	NE
HLA-G	Y	NE	NE	NA

Y yes, *N* no, *NA* not applicable, *NE* not evaluable

National Academy of Clinical Biochemistry (NACB), serial measurements of CA15-3 are currently not recommended by ASCO, ESMO, and NCCN expert panels [5, 7]. However, this marker has clearly been seen to possess a moderate prognostic value in the setting of advanced diseases, especially regarding metastatic BC: increased CA15-3 serum levels preoperatively are predictive of worse outcomes in terms of disease-free survival (DFS) and overall survival (OS) in a way which is independent from tumor size and from lymph-node involvement; so, a possible utilization of CA15-3 has been proposed to be in the early detection of micrometastases [8, 9]. A more reliable utility of CA15-3 has been stated in the context of advanced BC in terms of management of patient's response to systemic therapies. In this regards, CA15-3 can help to indicate whether an individual is a good responder or not, especially if conventional procedures are not able to evaluate the disease status. Expert panels, however, recommend serial measurements of this marker only in association with conventional diagnostic and prognostic procedures; the exception is given again by EGTM guidelines, which state that CA15-3 may be of clinical use during follow-up of advanced BC, and the levels of this marker should be measured preoperatively and every 3 months in the patient who is undergoing endocrine-therapy [10]. A 50 % reduction of CA15-3 serum level might imply the success of a treatment, and hence, patient's responsiveness [5].

Carcinoembryonic antigen (CEA) is one of the most famous soluble biomarkers in the setting of BC, although its usefulness is officially controversial. Increased preoperative serum values of this marker have been generally associated with poorer prognosis in many papers almost 15 years ago, usually in combination with the values of CA15-3 [11]. A study operated on 1046 primary BC cases analyzed the correlation between CEA and patient's outcome after surgery; the comparison between preoperative and post-operative CEA levels in an univariate analysis showed that a reduction of more than 33 % after surgery was predictive of higher risk of relapse, and the confirmation of the same finding in a multivariate analysis conferred to CEA the title of independent prognostic factor [12]. The serum levels of CEA and CA15-3 were measured in 1681 patients with BC through univariate and multivariate analysis. Incremented preoperative values were detected in respectively 10.5 and 7.8 % of patients and clearly associated with poorer clinical outcomes and overall worst cancer status in terms of tumor burden, lymph-node metastasis, and tumor stage. On the counterpart, non-augmented serum CEA and CA15-3 levels were predictive of overall better outcomes; it is worth noting the most interesting results and the more reliable prediction power have been obtained with the combination of the two biomarkers, whereas when measured singularly, both sensitivity and specificity values were drastically decreased. As a result, the association of the two markers may be a useful independent tool in the follow-up of BC patients [13].

More recent studies are focused on the prognostic value of CEA in the setting of metastatic diseases. Lee et al. documented that increased serum levels of CEA and CA15-3 were independent prognostic factors for the diagnosis of metastatic BC; CA15-3 incrementation was seen to be more evident in younger patients, whereas CEA had a better prognostic importance in older individuals and in those with ER-negative disease. Moreover, patients without any augmentation of the two markers exhibited significantly better clinical outcomes in terms of OS; comprehensively, the study proposed that both CEA and CA15-3 may be indicative of survival in metastatic BC and may provide information on eventual recurrence [14]. Conversely, however, CEA and CA15.3 do not improve the prognostication of metastatic BC, when added to full clinicopathological predictive models, as recently reported by Bidard and coll. [15].

Classic markers then, although in some circumstances demonstrated to be of clinical utility, do not objectively represent a reliable panel in the context of a liquid biopsy.

HER2/ECD

Human epidermal growth factor receptor 2 (HER2) is expressed on the 20–30 % of BCs, and its tissue status is routinely analyzed. Although the positivity of one patient for this receptor indicates increased tumor aggressiveness, it also represents a therapeutic opportunity since HER2 is the target of trastuzumab and pertuzumab, two monoclonal antibodies with demonstrated clinical efficacy for the treatment of HER2 positive BC [16]. Recently, it has been observed that the shed form of HER2—specifically, its extracellular domain (ECD)—can be detected in the sera of BC patients and may serve as a source of information about tumor status. The determination of serum ECD can be useful as a means of improving the HER2 quantitative determination, which as of today does not take in consideration many borderline cases such as those HER2 negative BC cases which are seen to respond to trastuzumab-based therapies (apparently) without explanation [17]. The quantitative evaluation of serum HER2 in the setting of primary BC may therefore increase the sensitivity of its tissue-determination tests, which currently are based on immunohistochemical (IHC) and fluorescence in-situ hybridization (FISH) methods [18]. Recently, the levels of serum ECD have been collected from 241 early BC patients and compared to the grade of HER2 tissue overexpression. Also, ECD levels were combined with the measurement of serum CA15-3 and CEA. The results of this work showed that ECD levels are significantly correlated with tissue HER2 overexpression and with postmenopausal status and that increased ECD and CA15-3 serum levels can be considered as better independent prognostic factors than tissue HER2 determination. However, the authors conclude highlighting the necessity of a prospective validation [19].

The utility of ECD measurements in the setting of advanced BC is instead slightly more immediately appealing: a recent work shows how serum HER2-ECD levels are linked to the various molecular BC subtypes and how a lowering of serum HER2-ECD after a trastuzumab-based treatment is started can be a real-time indicator of the success of the therapy [20]. Also, the increased levels of serum ECD have been clearly associated with tissue HER2 status, presence and number of metastases, and CA15-3 and CEA levels [20]. A large study carried out on 2862 primary BC patients recently evaluated the correlation between ECD levels and tissue HER2 overexpression since its serial measurement is not currently suggested by official ASCO guidelines but may be clinically helpful [21]. The results of this study showed that 15 % of tissue HER2 positive patients showed contemporary augmented ECD serum levels, with an interesting linear correlation with the increased aggressiveness of tumors. Furthermore, the multivariate analysis brought the authors to indicate the increased ECD serum levels as an independent prognostic factor for worse distant metastasis-free survival and BC-specific survival with more emphasis to HER2-positive cases [21].

Although no clinical validation has been obtained so far, the shed form of HER2 can nonetheless be considered as a reliable source of information in the setting of HER2 positive BC cases, where the measurement of the levels of both tissue and serum HER2 is of primary importance. Moreover, HER2-ECD may serve to better understand the phenomenon of responsiveness to trastuzumab in the absence of tissue HER2. On behalf of these rationales, further clinical validations would be worthwhile [22].

Quantification of circulating tumor cells in the prognosis of metastatic diseases

Circulating tumor cells (CTCs), as the name itself suggests, are tumor cells which typically circulate in the bloodstream of cancer patients at extremely low levels (typically one CTC every one billion of blood cells) and which represent a particular novel class of tumor markers. When found inside the bone marrow, tumor cells are referred to as disseminated-tumor cells (DTCs). The phenotype of CTCs is nowadays thought to result from a process referred to as epithelial-mesenchymal transition (EMT), which is a characteristic of the metastatization phenomena; in addition to the EMT phenotype, which is predominantly expressed and detected in the bloodstream, a portion of CTCs is seen to exhibit some of the characteristics of stem-cells that may explain the intrinsic high resistance to systemic therapies and the increased recurrence phenomena in the context of the most severe forms of BC [23, 24].

Today, the main prognostic value of CTCs resides on their quantitative evaluation in the setting of metastatic carcinomas. The presence of circulating and disseminated-tumor cells is not documented for healthy individuals, whereas large numbers of malignancies are commonly observed to release these cells in the bloodstream (e.g., breast, ovarian, colorectal, and prostate carcinomas) [25]. Hence, the detection of CTCs is a very specific indicator of overall worse outcomes. The detection and quantification of CTCs is commonly performed through CellSearch® technology, which is based on the initial enrichment and the subsequent enumeration of these cells [26]. The technique, which has been approved by the Food and Drug Administration in recent times, exploits the principle that CTCs specifically express characteristic surface molecules such as epithelial cell adhesion molecule (EpCAM) and cytokeratin 8/18/19 (CK-8/18/19). Also, CTCs are

characterized by being negative for CD45 staining (CD45-ve). Basing therefore the quantification on the detection of this phenotype, it is today accepted that a CTC count equal to or higher than 5 per 7.5 mL of whole peripheral blood is a consolidated prognostic factor, meaning a strong metastatization potential and an unfavorable clinical outcome (Fig. 2) [27]. The prognostic value of the detection of 5 or more CTCs in one patient's bloodstream has been confirmed by several evaluations: in a study carried out on 492 advanced BC patients, Giuliano et al. demonstrated that the detection of a number of CTCs higher than 5 is associated with increased baseline levels of metastatic niches, whereas values lower than 5 indicate reduced probabilities of metastatic processes. Moreover, the same authors showed that 5 or more CTCs detected prior to treatment initiation are also predictive of increased risk of relapse [28]. A retrospective study performed by Mego et al. in 2014 aimed to evaluate the correlation between CTC count and clinical outcome on a total of 147 inflammatory breast cancer (IBC) patients, involving both stage III IBCs and metastatic cases. The authors confirmed that the presence of 5 or more CTCs in newly-diagnosed IBC patients was more likely to be detected in metastatic cases and that CTC enumeration is a prognostic factor for newly diagnosed IBC [29]. Moreover, the results from a recent pooled analysis operated in the setting of metastatic BC confirmed the independent prognostic value of CTC quantification since a count of 5 or more CTCs per 7.5 mL of peripheral blood has been seen in this study to be clearly associated with poorer outcomes in terms of progression-free survival and overall survival [15].

A 2013 study introduced human mammaglobin (hMAM) as an additional CTC-specific surface molecule, with the aim of improving the detection of CTCs [30]. The results showed

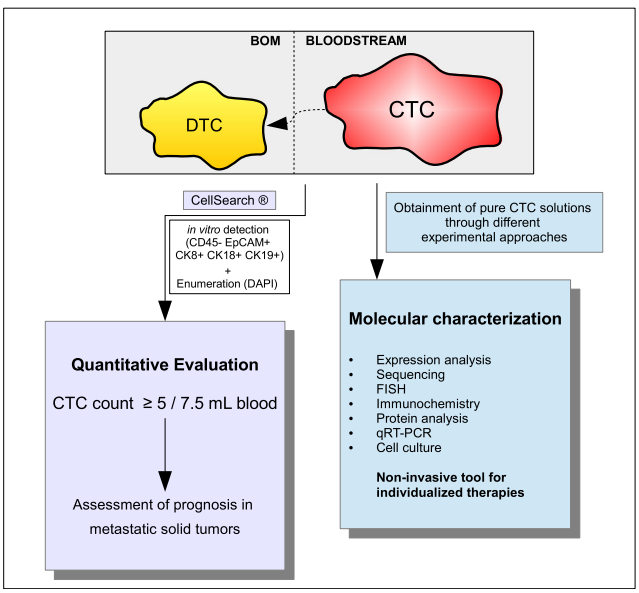

Fig. 2 Schematic representation of the diverse usages of CTCs in clinically proven (CellSearch®) or experimental settings

that the co-presence of the three markers—hMAM, CK19, and EpCAM—was clearly associated with poorer prognosis in terms of lymph node metastasis and tumor grade with an intriguing 100 % specificity. The results were also matched with increased CA15-3 and CEA serum levels [30]. Therefore, the authors suggested that an EpCAM, CK19, and hMAM comprehensive expression assay could improve the already good prognostic and predictive value of CTC count [30].

The CellSearch®-based quantification of CTCs is as of today the panel with the highest level of clinical validation, as it represents a reliable source of information about ongoing metastatization phenomena with regards to cell counting and molecular characterization.

CTCs and novel technologies

Although the field of molecular medicine already allows for the set-up of personalized treatments by genetically analyzing tumor tissue, the phenomenon of recurrence, even after years, is still characterized by a major unpredictable component also in those patients who perfectly responded to systemic therapies. It has been documented that the molecular and genetic features of primary lesions are often more diverse than those observed on metastases, and these are usually impossible to reach for a biopsy. Since at least a portion of CTCs is thought to be actively implicated in the formation of metastatic niches, the molecular analysis of these cells can represent a powerful tool to retrieve information about the mutational status of metastases, allowing the set-up of individualized treatments as a result. Hence, in spite of the heterogeneous and still partially unknown nature of CTCs, some of these cells may be referred to as liquid biopsies for metastases.

As of today, many methodologies allow to separate specific types of cells, including cancer cells, in order to obtain purified solutions and even single cells. Among these, isolation by size of epithelial tumors (ISET) technology allows to separate specific types of cells by size and to obtain purified solutions containing the desired cells only [31]. Recently, Buim et al. showed that this method would be a possible approach in isolating CTCs in order to proceed with mutation analysis required for addressing the proper therapy in cancer treatment. Moreover, this method highlights again that liquid biopsy is a fast and feasible approach providing important information to clinical management of cancer patients, especially when tumor tissue is not available [32].

Then, the DEParray system is a technology which offers the possibility of obtaining single-cell solutions. The technology exploits the principle of dielectrophoresis (DEP) to create an electric field which traps suspended cells in dielectrophoretic cages. A microelectrode-based array then forces trapped cells to move towards in a programmed

direction to ultimately obtain a solution containing an individual cell. It is then possible to perform molecular and genomic analysis on the selected cell and, considering the case of CTCs, to retrieve information about the mutational status of metastases (Fig. 2).

In practice, obtaining single-CTCs by using the DEParray technology has been validated by several studies, involving breast, colon, lung, and neuroblastoma carcinomas [33–36]. The mutation of p53 renders cells are more likely to survive and be resistant to treatments and strongly contributes to increase the aggressiveness of the tumor. TNBC and IBC are the most severe forms of BC, and their development is typically accompanied by a TP53 mutation. Thanks to the combination of CellSearch® with DEParray technology, it has been possible to observe that some CTCs obtained from triple-negative BC patients were likely to carry the same TP53 mutation of the primary lesion, whereas others were seen to express the wild type allele [37]. This study provides a "proof-of-concept" about the heterogeneity of CTCs but also validates the DEParray technology as a powerful procedure to explore novel individualized approaches for the treatment of highly aggressive metastatic diseases.

GILUPI CellColector™ has been recently developed as a novel method for in vivo CTC isolation. The method has been tested previously in vitro and is able to collect CTCs through the use of EpCAM-directed chimeric monoclonal antibodies to enrich the desired cells. This procedure has been successfully used to isolate CTCs from the blood of BC and non-small cell lung carcinoma (NSCLC) patients and represents a novel opportunity in the field of CTC capture in view of subsequent molecular characterization [38].

Comprehensively, novel separation technologies are posing the bases for a new way to perform molecular analyses and to subsequently improve the setting-up of individualized treatments. However, novel technologies such as DEParray and GILUPI do not offer the same level of validation as CellSearch®-based analysis, and therefore, their clinical utility is still confined in an experimental setting.

Circulating cell-free DNA

The presence of generic circulating cell-free DNA (ccf-DNA) in physiological conditions has been noticed for the first time almost 75 years ago [39]; about 55 years later, a correlation between increased levels of circulating DNA and cancer has been definitely proven [40]. Nowadays, it is universally accepted that bloodstream concentration of ccf-DNA is likely to increase in presence of solid and hematologic malignancies, but it is also known that the same finding can be the result of a great variety of other conditions, such as arthritis [41], generic traumas [42], and inflammation, and even after intensive physical exercise [43]. Nonetheless, the source of ccf-DNA is still unclear, although evidences suggest that it may result from apoptotic release, which may also explain the presence of minimal amounts of ccf-DNA in healthy individuals [44]. Other authors, instead, propose CTCs as the main source of circulating DNA [45].

At present, analysis of ccf-DNA retrieved promising results in the management of metastatic BC, a condition which requires a careful follow-up. In an interesting analysis, Dawson et al. performed serial measurements of circulating tumor DNA and compared the values obtained with serum levels of CA 15-3 and CTCs [46]. Cancer-related somatic genomic aberrations were detected through targeted or whole-genome sequencing, whereas the quantification of ccf-DNA was performed through personalized assays. In the results, the authors underlined a prognostic advantage of ccf-DNA over CTCs and classical markers, especially with regard to the management of metastatic BC therapies [46]; however, this statement has been subsequently refuted by Cristofanilli et al. through a comment resizing the predictive utility of ccf-DNA, stating that "circulating tumor DNA provides a complementary method in the assessment of patients with detectable mutations and should be more appropriately used to select and monitor molecularly targeted therapies. Combined diagnostic methods will provide a more effective approach than each method alone to the implementation of precision medicine and improved clinical outcomes" [47]. The debate is still open.

Analysis of ccf-DNA mutations as novel source of information

Since neoplastic cells are known to typically harbor somatic mutations such as deletions, single-base substitutions, insertions, or translocations, the analysis of the genomic alterations of tumor-derived ccf-DNA is theoretically one of the most powerful instruments to retrieve individualized information after a simple withdrawal of peripheral blood [48, 49].

Given the rationale that the genetic aberrations typical of BC cells might be reflected also in the respective circulating nucleic acids, the validity of this statement has been recently proven for TP53 mutation in the setting of primary BC, whereas similar results have been achieved for KRAS mutations in the setting of pancreatic and colorectal carcinomas about 15 years ago [50, 51]. Moreover, in the same years, ccf-DNA has been demonstrated to bear also eventual microsatellite alterations, such as loss of heterozygosity and microsatellite instability. These last observations were obtained from NSCLC and renal cell carcinoma with moderate sensitivity [52, 53]. Also, BC-derived soluble nucleic acids have been seen to carry also epigenetic mutations such as DNA hypermethylation in an apparently age-dependent fashion [54].

The analysis of mutational status of ccf-DNA has been also proposed as a novel instrument to predict relapse in primary

BC-affected patients who underwent a neoadjuvant treatment before surgery. The results of this abstract, presented at the 2014 ASCO meeting, showed an impressive correlation between the presence of tumor-specific mutations at baseline and increased risk of recurrence: ccf-DNA tumor-specific mutations have been detected in 9 of 12 patients, and among these 9 relapsed. Intriguingly, none of the patients who did not experience relapse was found to carry the same genomic alterations at baseline [55].

Moreover, since phosphoinositide 3 kinase (PI3K) inhibitors represent a novel therapeutic option for BC treatment and considering that up to 40 % of breast lesions bear somatic mutations for PI3K, a 2010 analysis evaluated the mutational status of this gene on ccf-DNA of both metastatic and localized BC patients [56]. Of the total 76 diseased patients, 41 showed a concurrence between the presence of PI3K mutation on plasma and tissue, suggesting a possible role of this assay in improving prognosis.

Ccf-DNA can therefore be seen as a potentially powerful tool to retrieve information about one patient's prognosis. Further validations are however necessary to fully estimate the clinical utility of this class of markers.

MicroRNAs

MicroRNAs (miRs) are short (20–22 nucleotides), non-coding, single-stranded nucleic acids which negatively regulate post-transcriptional gene expression in a wide range of biological and pathological processes, including cancer. Since the discovery of the first miR in 1993, the regulatory function of these molecules have been demonstrated during the last two decades with growing interest [57]; it is nowadays clear that miRs regulate gene expression through targeting specific binding-sites—the 3' or the 5' UTRs—on messenger-RNAs (mRNAs), resulting in their cleavage which leads to a post-transcriptional gene-silencing effect [58]. In addition, it has been recently demonstrated that miRs may, in certain conditions, up-regulate mRNA translation and regulate gene transcription in a positive fashion [59].

The role of miRs has been proven to be paramount in virtually every physiological process, from cell-cycle and apoptosis control to immune system regulation, neuronal development, and heat and shock protein modulation [60]. However, miRs exert important functions also in the etiopathogenesis of several diseases, including cancer: since they can directly influence the expression of oncogenes and tumor-suppressor genes [61], miRs are properly defined as anti-oncomiRs and oncomiRs with regard to a great number of malignancies. In addition, many neoplasms have been shown to possess distinct miR-based tissue profiles, which may be a useful option for tumor characterization since they are representative of diseased conditions only. Interestingly, some of the same cancer-specific miRs can also be found and detected in both serum and plasma of mammalians as a consequence of their shedding from lesions and, interestingly, their profiles vary according to the type of malignancy: therefore, beyond their proposed therapeutic role as targets, miRs represent more realistically an intriguing potential non-invasive class of cancer biomarkers as of today [62]. In a milestone paper, Chen et al. demonstrated that circulating miRs are detectable in sera and plasma of humans, mice, and other mammalians in a stable, reproducible fashion and that their profiles allow to discriminate between different kinds of solid tumors such as breast, lung, colon, gastric, urothelial, and hepatocellular carcinomas [62]. It is not clear whether the shedding of miRs from tumor cells may be due to an active secretion or due to a passive release consequent to apoptosis or necrosis ongoing phenomena (Fig. 1). In addition, some miRs are not released from cancer cells at all, and they can be found only within the original site of the tumor [63]. When released, miRs are often included into microvesicles and exosomes, types of "molecular shields" that confer to nucleic acids a relatively high resistance to degradation. Moreover, the association of miRs with argonaute 2 proteins further increases the overall stability of these markers [64].

Circulating MicroRNAs as diagnostic tools for BC

In the specific case of BC, the presence of miRs has been noticed in bloodstream, urine, milk, and several other human secretions [65]. The first analysis on circulating miRs has been performed to eventually discriminate between diseased and non-diseased patients, finding that the expression levels of circulating miR-29a and miR-21 were increased only in the presence of breast malignancies [66]. Another study confirmed the same findings with regard to circulating miR-21 and suggested miR-146a as another potential diagnostic biomarker [67]. Another study found that the expression levels of circulating miR-589 and let-7c were impaired only in BC patients, with an up-regulation for miR-589 and a down-regulation for let-7c [68]. The results obtained by a cohort study comprising 132 BC patients and 101 controls highlighted miR-1, miR-92a, miR-133a, and miR-133b as four valid diagnostic circulating biomarkers characterized by ROC curves with high AUCs (0.90 to 0.91) [63]. Another study, then, indicates the combination between miR-145 and miR-451 as a very reliable diagnostic tool for BC. These two miRs can successfully allow for the identification of BC-affected individuals from healthy donors and patients affected by other kinds of neoplasms with high positive (88 %) and negative (92 %) predictive values [69].

Circulating microRNAs as prognostic tools for BC

Interestingly, a series of studies revealed a strict association between the expression of certain miRs and the clinicopathological characteristics of BC [70]. The results from a study on 68 BC patients showed that circulating levels of miR-21, miR-106a, and miR-155 are likely to linearly increase proportionally to the tumor histological grade. Moreover, augmented serum levels of the same nucleic acids showed a clear association with the absence of hormone-receptors (ER and PgR), whereas a decrease of miR-126, miR-199a, and miR-335 has been seen to be related to the same clinicopathological characteristics [71]. In another study, the levels of circulating miR-10b and miR-373 have been observed to increase by more than four-folds in presence of lymph-node metastases, and the combination of the two miRs showed a further improvement in terms of specificity (94.3 %) and sensitivity (72 %) [72].

A recent study on 173 both tissue and plasma primary BC samples indicates miR-106b as a promising prognostic circulating biomarker, being linearly associated with tumor size, proliferation, and lymph-node involvement. Also, increased miR-106b concentrations have been related to a reduced OS and a worst disease-free survival [73]. Circulating miRs have been demonstrated to be useful also in the post-management of surgical procedures and prediction of relapse after mastectomy. In particular, Igglezou et al. noticed that the serum levels of miR-155 typically increase 3 days after mastectomy and then dramatically decrease about 1 month later, probably due to a potential role of this miR in surgery-induced angiogenesis. Moreover, the same authors observe that high post-operative levels of circulating miR-195 are clearly linked to early tumor relapse and hence that increased serum values of both miR-155 and miR-195 may potentially be a reliable tool in the post-operative management of BC [74]. Recently, a study conducted on 63 early BC patients and 21 healthy donors analyzed the expression profiles of serum miR-155, miR-19a, miR-181b, and miR-24 and compared the values obtained to the currently used clinical prognostic factors tissue HER2 and Ki-67 during diverse disease phases. The results showed that at the time of diagnosis of early BC, the serum values of these miRs are augmented in diseased individuals and strongly augmented in the high-risk BC subgroups; the levels of miR-155, miR-181b, and miR-24 clearly tend to decrease after surgery, and miR-19a, instead, has been seen to decrease after therapy initiation [75].

MicroRNAs reflect breast cancer molecular subtypes

Luminal-A Analysis on systemic miR-1280—an atypical tRNA-derived microRNA—revealed a potential new tool for the management of luminal-A disease, a BC variant characterized by the presence of ER, the absence of HER2 and relatively more favorable clinical outcomes. Serum levels of miR-1280 in BC patients are typically subject to an incrementation which is directly proportional to the severity of the disease: therefore, lower miR-1280 serum values can be index of early stage breast lesions, while increased levels would be more typical of advanced disease or could reveal the presence of metastases [76]. Intriguingly, a correlation between serum miR-1280 and the success of a systemic treatment has also been noticed for metastatic BC, since serum miR-1280 levels have been seen to linearly decrease as therapies retrieved good clinical efficacy. Hence, serial measurements of serum miR-1280 could help clinicians in monitoring therapies and be an early signal of resistance to particular treatments [76].

Triple-negative In a recent paper, a subset of high-risk TNBC signature has been characterized according to the expression profile of four miRs with elevated prognostic potential (miR-18b, miR-103, miR-107, and miR-652). This finding has been performed on a group of 60 TNBC patients and further confirmed by a following group of 70. The results showed that the increased serum levels of these four miRs are independently associated with strong probabilities of recurrence and with overall reduced OS values, consequently introducing a novel potential prognostic tool for triple-negative breast lesions [77]. Strictly associated to the latter work, the results from a study on 169 invasive BC patients evidenced that incremented serum levels of exosomal miR-373 are associated with to triple-negative genotype and generally to an increased aggressiveness [78]. Also, it has been observed that circulating miR-101 and the latter, when not included inside microvesicles or exosomes, may be helpful to discriminate between breast benign lesions and carcinomas [78]. In a study operated on 113 BC cases, miR-21 and miR-10b have been observed to be specifically increased in the serum of HER2 positive BC patients, whereas this incrementation was not observed in HER2-ve and healthy donors [79].

A comparison between triple-negative cell cultures MDA-231 and hormono-responsive MCF-7 cell lines showed that the triple-negative phenotype was more likely to secrete higher amounts of miR-130a, a molecule which has been implicated with the cancerogenesis of colon cancer, and of miR-328, which is linked to increased metastatic and angiogenetic potential [80–82]. Instead, MCF-7 luminal BC cells were seen to secrete higher amounts of exosomal miR-301a, a characteristic negative prognostic factor for invasive ductal breast carcinoma. Moreover, the same study evidenced that the same cells also secreted increased levels of exosomal miR-106b, an acid which has been recently implicated in the promotion of TGF-beta-induced EMT transition [82, 83].

Inflammatory phenotype Increased levels of serum miR-19a have been found to be associated with the development of an

inflammatory BC phenotype, a rare form of BC characterized by increased aggressiveness and a higher metastatization potential [79]. This study, operated by Anfossi et al., also highlighted a link between serum miR-19a and improved clinical outcomes in the setting of HER2 positive BC, thus revealing a potential role of this marker in predicting the effectiveness of trastuzumab-based therapies [79].

Overall, miRs offer the possibility of obtaining real-time information about virtually any kind of condition, including cancer. The multitude of different molecules becoming available every year represents a great advantage over other types of cancer soluble biomarkers, since the diverse combinations can be exploited in view of the high heterogeneity of BC. However, experimental miR-based liquid biopsies will require further validations in order to become clinically available.

Microvesicles and exosomes

The tumor microenvironment, or stroma, has been gradually discovered to play an essential role in cancer development, being able to mediate tumor-suppressive or tumor promotion effects [84], to actively shape the immune system [85] and to give rise to pharmaco-resistance phenomena through complex interactions [86]. All these processes are possible only thanks to the existence of specific mechanisms that keep tumor cells constantly interconnected to stromal cells and that can be seen as a proper cell-communication system: the message has to be specifically delivered to the right cell and, likewise, it must mediate a specific effect in the receiving-cell without undergoing degradation along the way. In addition to the more classical direct cell-to-cell contact and cytokyne-based parakryne signals, the structural basis of cancer cells communication also involves microvesicles (100–1000 nm) and exosomes (50–100 nm), two lyposomic systems constituted by an external lipophilic barrier with an enclosed message to be delivered. The biosynthesis of an exosome involves the initial formation of a multivesicular body, a kind of intracellular vesicle which is originated by the invagination of the plasmatic membrane and which further contents multiple microvesicles [87]. Slightly differently, circulating microvesicles are membrane-derived systems that are directly released by the cell through a pinching-off mechanism of secretion [88].

Exosomes and microvesicles are both released into the extracellular space and eventually into the bloodstream, and they can mediate cell-to-cell communication in a very distant fashion. Since these systems are originated inside cancer cells, and as their membrane and content were once part of that cancer cell, the molecular characteristics of exosomes and microvesicles can be seen as a reflection of the properties of the originating cell and therefore be an important source of information for researchers and clinicians. Exosomes and microvesicles can be collected from a great variety of body fluids, including blood, urine, saliva, and milk, representing a very easy accessible source of information in terms of liquid biopsy [89]. The molecules included inside these liposomic systems are usually tumor-specific molecules such as proteins and nucleic acids (mostly miRs), and the molecular patterns of the plasma membrane are characteristics of the tumor tissue [89].

The possibility of exploiting exosomes and microvesicles in order to obtain information about virtually any biological process, including cancer, is therefore emerging. However, an objective clinical validation is still required for this class of biomarkers, which potentially would represent some of the most elegant soluble indicators so far.

Immune system status

In the light of the recent development of immune-based therapies for solid tumors, this brief paragraph is intended to introduce a biomarker which may be potentially useful in defining one patient's overall immune status: indeed, today it is clear that the immune system can play a primary role in the early eradication of transforming cells since it successfully recognizes aberrations and provides to kill malignant cells [90]. However, after decades of debate, it is commonly accepted that tumor cells become able to proliferate in spite of immune-mediate recognition and elimination in a process referred to as immunoediting. The proliferation of cells, such as FOXP3 T-regulatory cells (T-regs) and myeloid-derived suppressor cells (MDSCs), is the result of an active selection mediated by the tumor cells thanks to the overexploitation of physiological inhibitory interactions such as PD1/PDL1 or CTLA-4 immune checkpoints [91].

Therefore, the status of the immune system of a given patient could be a parameter from which would be possible to define the entity of an eventual immunosuppression predominance, with the aim of evaluating the possibility of adopting novel immune-based therapeutic strategies. Indeed, the efficacy of novel immune-based therapies such as immune checkpoint blockers increased proportionally to the extent of immunosuppressive conditions. Therefore, a soluble marker of immunosuppression would be ideally extremely useful in this setting. For instance, soluble HLA-G has emerged as a reliable biomarker in recent times since it has potential as an immunoediting indicator. HLA-G is a member of the human leukocyte antigen (HLA) class I molecule family, but it differs from the classical ones since its expression is physiologically limited to fetal tissues and to other so-called "immune-privileged" organs (thymus, pancreas, erythroid, and endothelial precursors). Its natural function is to protect apparently "non-self organs", as for example the fetus, from immune-mediated attack through the inhibition of peripheral natural

killer cells (NKs) and the promotion of immunosuppressive cells such as T-regs, but major roles have been observed also in the context of transplantation and autoimmune diseases [92–94]. It has been observed that HLA-G expression is increased in association with the insurgence of BC, given its immunogenicity, and to also be related to worse clinical outcomes [95, 96]. The soluble isoform of HLA-G antigens can be found in the bloodstream as a consequence of the secretion operated by bone marrow mesenchymal stromal cells, usually in response to IL-10 stimulation. In an interesting paper, the immunosuppressive effect of soluble HLA-G molecules on peripheral blood mononuclear cells has been evaluated and confirmed [97]. Moreover, since HLA-G is thought to be a direct promoter of immunosuppression, some authors hypothesize its eventual therapeutic targeting [93]. In consideration of these findings, and taking into account the recent advances in immunotherapy for solid tumors, HLA-G may be a useful tool to retrieve information about the grade of immunosuppression of a particular patient, and hence, may help clinicians to select treatments and eventually decide whether an immune-based therapy would be effective or not.

Conclusion

Besides multiplex genotyping technologies and high-throughput genomic profile assays, which represent the latest improvements for tissue-based cancer characterization, the hypothesis of a perfect liquid biopsy would imply a considerable step forward in the fields of cancer diagnosis, prognosis, and especially in the context of the definition of individualized therapies. As of today, the best achievements are still provided by CTC count, which has been demonstrated clearly to be independently related to prognosis in the setting of metastatic diseases. Some authors sustain that the clinical usefulness of the early-detection of metastases through CTC count may not be translated in an improvement for the patient but only in an incrementation of the number of diagnoses of metastatic disease since therapies hardly eradicate metastases [32]. However, thanks to the continuous work of researchers and clinicians, novel therapeutic possibilities become available almost every year, with many of them directed against metastatic cells with documented efficacy. In this perspective view, the early-detection of metastases and micrometastases with the help of a liquid biopsy would be a big advantage for the patient. Moreover, some authors tend to consider soluble biomarkers separately, as if singularly they would represent revolutionary novel indicators of disease status; as demonstrated by many other authors, the strength of a liquid biopsy would be based on the association of multiple biomarkers since the unavoidable limits and the drawbacks of the ones may be counterbalanced by the other and vice versa. The field of BC liquid biopsy is, however, characterized by the discovery of new candidate biomarkers almost every year and represents a blooming research field. Novel soluble markers, and their associations, could offer overall good predictive values and are particularly required alongside with already-assessed procedures such as radiographic imaging or tomographic assays. The great number of possible circulating indicators also offers new insights for surgery-free cancer characterization, but, realistically, the utilization of primary tumor tissue-based samples represents now and in the near future an indispensable passage for setting-up effective genomic-based clinical considerations. It must be emphasized, however, that the amount of circulating cancer biomarkers is growing almost exponentially and that profiles of some of them, such as miRs, demonstrate an intriguing ability in discriminating among different molecular cancer subtypes. In the case of the breast, this finding deserves particular attention in the light of the high heterogeneity of carcinomas, which are known to greatly vary in terms of molecular profiles and, on reflection, in terms of aggressiveness. The most severe variants of breast carcinomas, such as triple-receptor negative cases and inflammatory phenotypes, may benefit much more from a panel of circulating indicators than others, also in consideration of the lack of well-defined therapeutic targets and the higher metastatization potential typical of these diseases. Lastly, in the light of the recent advances in immunotherapy for solid tumors and in consideration of the documented high-immunogenicity of the most aggressive BC subvariants, a circulating biomarker which might be able to provide information on the immune-profile of a given cancer patient would be, to our knowledge, extremely useful. To this aim, the proposed role of immune indicator for HLA-G may be a possible solution, which deserves at least a deeper evaluation. In view of the multitude of soluble indicators, the evaluation of the union of diverse circulating cancer biomarkers may represent the strength of a liquid biopsy: “united we stand, divided we fall.”

Conflicts of interest None.

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