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Review

Novel insights into breast cancer progression and metastasis: A multidisciplinary opportunity to transition from biology to clinical oncology

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ABSTRACT

According to the most recent epidemiological studies, breast cancer shows the highest incidence and the second leading cause of death in women. Cancer progression and metastasis are the main events related to poor survival of breast cancer patients. This can be explained by the presence of highly resistant to chemo- and radiotherapy stem cells in many breast tumor tissues. In this context, numerous studies highlighted the possible involvement of epithelial to mesenchymal transition phenomenon as biological program to generate cancer stem cells, and thus participate to both metastatic and drug resistance process. Therefore, the comprehension of mechanisms (both cellular and molecular) involved in breast cancer occurrence and progression can lay the foundation for the development of new diagnostic and therapeutical protocols. In this review, we reported the most important findings in the field of breast cancer highlighting the most recent data concerning breast tumor biology, diagnosis and therapy.

1. Introduction

According to the most recent epidemiological data, breast cancer shows the highest incidence and is the second leading cause of death in women [1,2]. Cancer progression and metastasis are the main events related to poor survival of breast cancer patients. This can be explained by the presence of cancer stem cells (CSCs) which are highly resistant to chemo- and radiotherapy in a number of breast tumor tissues [3]. In this context, numerous studies highlighted the possible involvement of the epithelial to mesenchymal transition (EMT) phenomenon as biological 'pathway' to the generation of CSCs, which may therefore participate in the formation of both metastatic cancer and drug resistance. Recently, the EMT phenomenon has been linked to the formation of breast microcalcifications [4]. Specifically, a novel breast cancer cell type, the Breast Osteoblast-Like Cell (BOLC), originated from breast epithelium undergo mesenchymal transformation, has recently been proposed. BOLCs show the ability to both produce microcalcification made of hydroxyapatite (HA) and form bone metastatic lesions [6,7]. In this context, the comprehension of cellular and molecular mechanisms of breast cancer occurrence and progression can lay the foundation for the development of new diagnostic and therapeutically protocols. The collaboration between diagnostic imaging and anatomic pathology departments therefore represents a promising strategy to meet this challenge. Sharing data, expertise and skills between these disciplines could create a common platform where new discoveries and synergies concerning the biology of breast cancer are used to improve the

Abbreviation: ALDH1, aldehyde dehydrogenase 1; BCSC, breast cancer stem cell; BMPs, bone morphogenetics proteins; BOLCs, breast osteoblast-like cells; CD, cluster of differentiation; CO, calcium oxalate; CSC, cancer stem cells; CT, computed tomography; EMT, epithelial to mesenchymal transition; ER, estrogen receptor; HA, hydroxyapatite; HER2, human epidermal growth factor receptor 2; MR, magnetic resonance; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; PET, positron emission tomography; PR, progesterone receptor; SPECT, single photon emission computed tomography; WSI, whole slide imaging

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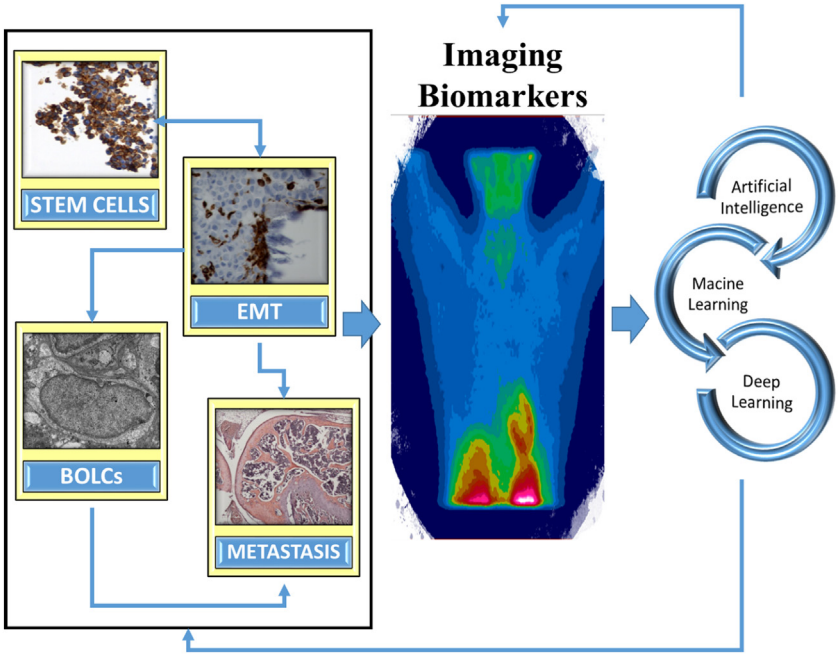

Fig. 1. The scheme displays the connection among the issues discussed in the review providing the key to the reading for correct interpretation of the review.

management of breast cancer patients.

2. Aim and study design

In this review, we highlighted the recent biological findings in the field of breast cancer tumor biology that could provide the scientific rationale for the development of innovative diagnostic and therapeutical approaches. In particular, we focused our review on the biological processes linked to both breast cancer occurrence and progression such as "breast cancer stem cells (BCSCs)", "EMT" and origin and activities of the BOLCs (Fig. 1A). As discussed below, and showed in Fig. 1, these biological processes are closely interlinked among them and associated to the formation of metastatic lesions and therapy resistance (Fig. 1B). In addition, to investigate the potential translational prospectives of "cancer stem cells", "EMT" and BOLCs we discussed the most recent papers reporting the molecular and histological biomarkers involved in these processes. Data reported in the selected papers allowed us to identify and propose the most promising biomarkers for both in vivo (imaging diagnostic) and ex vivo (pathology) analysis. In this scenario, we hypostasized that a synergic collaboration between diagnostic imaging and pathology, in conjunction of artificial intelligence-based tools, could represent an extraordinary opportunity to elaborate data from basic research with the aim to improve the management of breast cancer patients.

3. Prognostic and predictive biomarkers of breast cancer

Breast Cancer is the most diagnosed non-skin cancer in women. Over the last 20 years, improvements in both breast cancer diagnosis and treatment have led to a significant reduction of cancer-related mortality and an increase of patient quality of life [8]. The most prominent prognostic markers used for the stadiation of breast cancer include tumor size, nodal status, presence of metastatic foci, histological tumor type, grading, age, and lymphovascular invasion [9]. Despite the recent introduction of new and promising genetic and molecular methods for the characterization of breast cancer, morphological classification continues routinely employed. In this context, estrogen receptor (ER), progesterone receptor (PR), Ki67 and Human epidermal growth factor receptor 2 (HER2) status are currently the only prognostic markers which can be considered predictive of therapy response [9].

Based on these genetically determined expressions of tumor cells, five molecular subtypes of breast cancer have been classified (St. Gallen International Expert Consensus, 2011). These subtypes can be detected through immunohistochemistry and differ in terms of prognosis [10–13]. In detail, the classification includes the following subtypes: lumina A (ER+, PR+, HER- and Ki67 $<$ 14%), Luminal B with her-2 negative (ER+, PR+, HER- and Ki67 $\geq$ 14%), Luminal B with her-2 positive (ER+, PR+, HER+, any Ki67), Her-2 enriched (ER-, PR-, HER+, any Ki67) and Basal-like (triple negative) (ER-, PR-, HER-, Ck5/6+ and/or egfr+) [10–13]. Such analyses are generally carried out by immunohistochemistry (Fig. 2), fluorescence in situ hybridization, or chromogenic in situ hybridization as well as Silver in situ hybridization techniques [9].

Specifically, the expression of ER, PR and Ki67 for the evaluation of receptor status is carried out by immunohistochemistry. The oncogene HER2 is a gene of the epidermal growth factor receptor family located on chromosome 17q21. HER2 gene amplification is observed in about 20% of breast cancer lesions and represents a negative prognostic factor. The overexpression of the transmembrane protein related to the amplification of HER2 gene is associated to the development of high-grade tumors, lymph node metastases and higher rate of mortality. On note, HER2 status is a biomarker able to predict response to therapy. Indeed, the overexpression of transmembrane protein or HER2 gene amplification predicts the response to trastuzumab [14]. It is known that trastuzumab monotherapy in metastatic breast cancer with HER2-overexpression is associated with 23–26% pathological complete response [15,16], whereas combining chemotherapy with trastuzumab has been reported to yield better results [17]. Accordingly, designing a therapeutic protocol which includes pertuzumab, trastuzumab and chemotherapy has been seen to significantly progression-free survival (12.4 months to 18.7 months) [18]. Also, the use of antibody-drug conjugate trastuzumab-emtansine (T-DM1) can further improve pathological complete response rates to 43.6% [19]. However, even with combination treatments, HER2-targeted therapy fails in 50–70% of patients with HER2-enriched breast cancer [20], and the biological mechanisms underlying this widespread resistance are not completely understood.

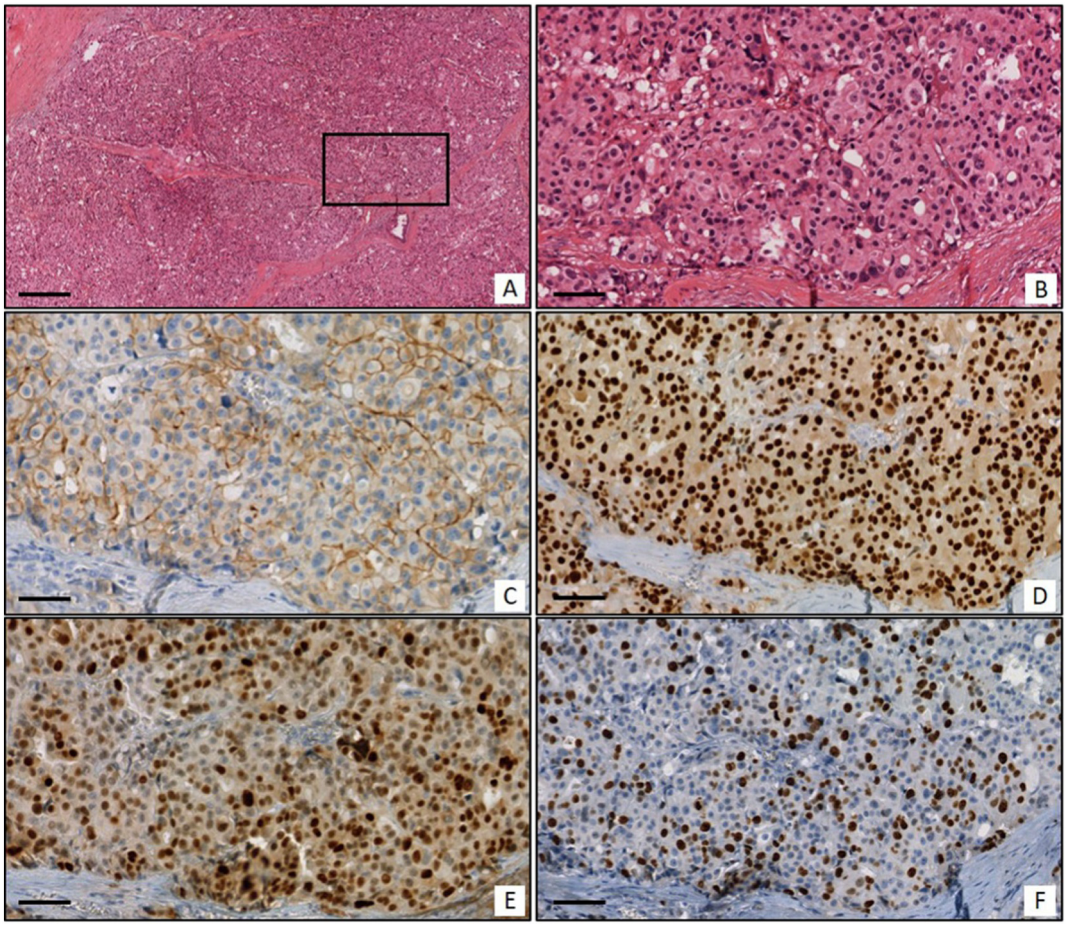

Fig. 2. Immunohistochemical evaluation of the main prognostic and predictive breast biomarkers. A) Hematoxylin & eosin staining of infiltrating breast carcinoma. B) High magnification of area highlighted in the square of panel A. C) HER2 expression in breast cancer (score 2). D) ER expression in breast cancer. E) PR expression in breast cancer lesions. F) Ki67 expression in breast cancer lesions. Panel A scale bar represents 100 μm. Panels B to F scale bars represent 40 μm.

4. Breast cancer stem cells: biological, diagnostic and therapeutic perspectives

The breast is a tissue that completes its maturation only during pregnancy and lactation. In these phases, epithelial cells undergo terminal differentiation in order to acquire the ability for the production and secretion of milk and its delivery [21]. This support the hypothesis that the massive proliferation and self-renewal observed during pregnancy are orchestrated by stem cells in breast tissue, i.e. BCSCs. In an experimental model, Tiede and Kang demonstrated that BCSCs are directly involved in tissue remodeling observable in the mammary gland during lactation. This may be based on their ability to differentiate in both cell types of the breast epithelium (luminal cells and myoepithelial cells [22]). Of note, a recent study showed mammary stem/progenitor characteristics in cell cultures derived from human breast milk [23,24]. This study candidates breast milk-derived cultures as an exceptional model to investigate the role of BCSCs in normal tissue development and breast cancer progression, which also represents a non-invasive and easily accessible source. As concern malignant breast lesions, numerous experimental studies support the hypothesis that a subpopulation of CSCs can participate to both early and late phases of breast carcinogenesis [21]. In line with these findings, it was found that both endogenous and exogenous exposure to sex hormones, mainly estrogen and progesterone, induce malignant transformation of the mammary epithelium [25,26]. A vast effort has been poured into elucidating the molecular mechanisms involved in the connection between sex hormones exposure and breast cancer development. The main findings indicate a different susceptibility to sex hormones according to the type of cell present in the mammary gland. Specifically, breast cells of epithelial origin, both luminal and myoepithelial, acquire somatic mutations with different frequencies when exposed to estrogen and/or progesteron. Interestingly, BCSCs are highly responsive to both estrogen and progesterone (although no expression of their specific receptors has been reported) [27], and the use of hormone receptor antagonists able to inhibit BCSCs activation further suggests that these cells play a role in breast carcinogenesis [27]. In fact, inhibiting the interaction between BCSCs and sex hormones significantly reduced the occurrence of Luminal A-like tumors (ER positive - PR positive – HER2 negative) in mouse model of breast cancer [27]. Thus, the presence of BCSCs could represents a further clue in breast carcinogens related to the exposure to sex hormones.

The ever more incontrovertible evidence of a connection between CSCs and cancer progression allowed to propose a new model for breast carcinogenesis based on the activation and development of BCSCs. Several investigations have evidenced that tumor progression can be triggered by CSCs., and it is well-known that CSCs are able to use several lines of self-defense against cancer therapy based on the intake of chemotherapeutic molecules and/or the use ionization therapies [28]. While several researchers are engaged in the study of the molecular mechanisms involved in the phenomenon of chemoresistance of breast cancer lesions, this process is not yet fully understood. The main activity of numerous chemotherapy drugs is directed against cells undergoing proliferation [29]. Thus, BCSCs that are mainly in a resting G0 phase of the cell cycle, display an intrinsic resistance to the actions of all drugs that target cells with an overactive cell cycle [30]. In addition, from a molecular point of view, BCSCs can activate several mechanisms

that elude the effect of chemotherapy drugs. It is known that the activity of chemotoxic molecules induces both an overexpression of insulin-like growth factor (IGF) type 1 receptor and the secretion of IGF1 by BCSCs [23]. In the non-proliferative phase of the cell cycle, the expression of IGF1 blocks PI3K-AKT signaling and activates molecules that induce both the reducing of the cell cycle and cell self-renewal [31].

Still, BCSCs frequently express ALDH1, a protein of the NADP+ dependent super family of detoxifying enzymes involved in CSCs self-defense. This molecule converts the aldehydes accumulated in cells subjected to chemotherapy, ionizing radiation, and/or any substance involved in oxidative stress, to harmless substances such as carboxylic acids [32]. The co-adjuvant activity of retinoic acid in chemotherapy treatments is a further confirmation of these processes. Indeed, retinoic acid augments the effect of chemotherapy by reducing the expression of ALDH1 [33]. Another mechanism of BCSCs chemoresistance is associated with ABC transporter activation of ATP-dependent chemotoxin efflux. Despite targeting of ABC transporters could lead severe side effects sice this system plays an essential role in many physiological processes, the use of molecules capable to inhibit ABC represents a promising strategy to elude chemoresistance. It is also known that this therapeutic approach activates mechanisms involved in re-sensitizing BCSCs by inhibiting pathways related to drug resistance [33]. The search for new reliable biomarkers of BCSCs is therefore a prominent scientific goal of translational research (The main surface markers of BCSCs are summarized in Table 1).

The identification of BCSCs surface biomarkers of the can represent an opportunity to detect BCSCs by several in vivo and ex vivo diagnostic techniques such as flow cytometry, immunohistochemistry, immuno-Positron Emission Tomography (PET) [34]. Among these biomarkers, clusters of differentiation (CD) 44 and CD24 are the most studied for the detection of BCSCs. Currently, the simultaneous expression of CD44 and CD24 on the surface of breast cells is considered the *conditio* sine qua non to define BCSCs. Given the above, these markers have been the object of numerous studies concerning the development of molecules able to intercept, recognize and block the BCSCs, making them susceptible to drugs. Still, pioneering studies by Saha and colleagues showed a subpopulation of CSCs expressing $CD44+/CD24-/^{low}/ESA+/Lin-$ capable to develop both mammospheres and breast cancer lesions if inoculated in nude mice. An analysis of solid tumors demonstrated the presence of both stem and non-stem cells, supporting the idea that both self-renewal and differentiation capability were present in the cells before inoculation in mice [35]. Also, additional BCSCs biomarkers associated with chemotherapy and radiotherapy breast cancer resistance have been recently described: aldehyde dehydrogenase 1 (ALDH1), CD133, and CD49f. A complete immunophenotipical characterization of BCSCs can provide a key element in the development of second line therapeutic strategies for breast cancer patients showing multidrug resistance. Indeed, targeted therapies against BCSCs could restore a normal tumor sensitivity to chemo- and radiation therapy. A convincing demonstration is provided by the use of monoclonal antibodies (mAbs)-conjugated polymeric nanoparticles directed against the antigen CD133. Loading of chemotherapeutic drugs on anti-CD133 mAbs resulted to be an excellent strategy to inhibit tumor growth in an MDA-MB-231 xenograft model [36]. If confirmed in large-cohort studies on human subjects, the double-targeted drug delivery system against BCSCs could represent an innovative therapeutic approach able to revolutionize the current breast cancer patient management protocol.

Notably, immunotherapies based on the development of antibodies directed against BCSCs surface markers currently show the most promising prospects. It is important to note that highest expression of BCSCs biomarkers has been found in poor differentiated infiltrated breast carcinomas, mainly triple negative subtypes (ER negative, PR Negative, HER2 negative). Specifically, Maisel et al. showed that $CD44+/CD24^{lo}$ triple negative lesions are related with poor survival [37]. Numerous anti-CD44 antibodies have also been tested in terms of their capability to prevent breast cancer tumor progression. In addition, several studies highlighted the capability of CD44 antibodies to target BCSCs [38]. Recently, a link between anti-CD44 antibodies and CD44 positive BCSCs trigger immuno-mediated phagocytosis of the breast malignant cells by antibody-dependent macrophage reaction was demonstrated. [39]. Despite the remarkable progress in the design anti-BCSCs immunotherapies, the most investigated therapeutic approach remains the use of inhibitors of signaling pathways of BCSCs, possibly due to the well-known intrinsic drug resistance of these cells. This therapeutic approach is associated to both minor side effects and reduced associated drug resistance as compared to the use of mAbs. Another characteristic associated to the presence of BCSCs in breast tumors is the ability of cancer cells to elude the surveillance of immune system cells or to induce a condition of immunosuppression in the tumor microenvironment. Accordantly, Sultan described the immunomodulatory activity of several breast cancer cell lines characterized by a subpopulation showing the $CD44+CD24-$ immunophenotype [40]. In particular, immunomodulatory activity of BCSCs was related to the expression of ALDH and to the consequent reduction of the expression of immune co-stimulatory molecules such as CD80. [40]. Thus, expression of ALDH in BCSCs can make them resistant to T cell-mediated attack [40]. Moreover, Sultan demonstrated that $ALDH^+CD44^+CD24^-$ cells subpopulations in MCF-7, SK-BR-3, and MDA-MB-231 lines also overexpressed CD73, a membrane molecule implicated in cancer immune evasion [41]. In agreement with this, the altered expression of CD73 in breast cancer tumor cells further confirms that BCSCs can play an essential role in the complex mechanism of cancer immune evasion. Consequently, the presence of CD73+ BCSC could explain the occurrence of recurrent cancer able to elude immune system activity. In fact, the expression of CD73 on the membrane of BCSCs induce the inactivation of T-cells due to presence of extracellular CD73-related adenosine, that also interferes with the activity of several co-stimulatory molecules such as PD-L1, PD, and indoleamine 2,3-dioxygenase.

The identification and characterization of BCSCs represent a great challenge for breast cancer patient management. Therefore, the identification of any biological phenomena linked to BCSCs generation, such as e.g. EMT, can extend the knowledge about the mechanisms underlying BCSCs-related multidrug resistance.

Table 1
Main surface markers of BCSCs.

Marker	Function	Role in breast cancer	Reference
ALDH	Adhesion, migration, and invasion	Metastasis, drug-resistance	[32,33]
CD133	Self-renewal	Invasion, drug-resistance	[32,37]
CD24	Cell-cell and cell-matrix interactions	Cell adhesion and metastasis	[38–40]
CD271	Cell proliferation, motility	Metastasis	[36]
CD44	Cell–cell interactions, migration	Metastatic diffusion, drug-resistance	[38–40]
CD73	Cell survival, proliferation, migration	Markers of cancer-initiating cells	[41]
CXCR4	Cell survival, proliferation, migration	Metastasis formation	[38–40]

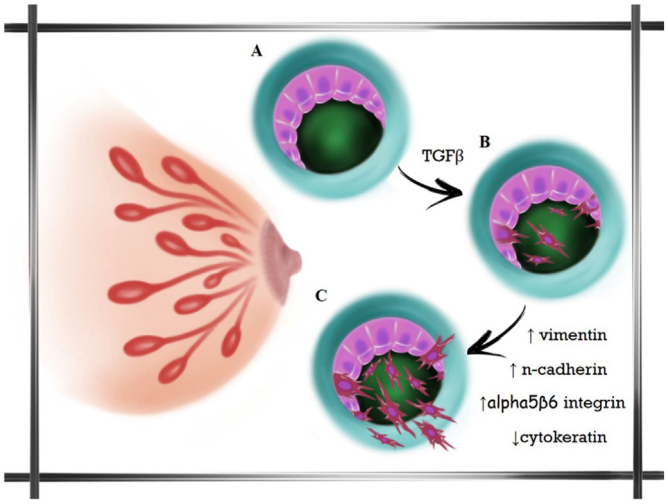

Fig. 3. A schematic model of EMT in breast cancer. A) Normal breast epithelial duct. B) Under the stimulus of EMT-inducing molecules (TGFβ, NFkB, BMPs) breast epithelial cells lose their cell-cell contacts (↓e-cadherin; ↑n-cadherin; ↑ α5β6integrin) and re-arrange the cytoskeleton (↑ vimentin; ↓ cytokeratin). C) Breast cancer cells that acquire mesenchymal phenotype can migrate, invade the neighboring tissue and metastasize to distant organs.

5. Role of EMT in breast cancer progression

The EMT is a biological phenomenon discovered for the first time in a model of chick primitive streak formation [42]. EMT is determined by a quick and often transient modifications of cell phenotype (Fig. 3A) consisting of a) the loss of cell to cell adhesions structures (i.e. adherens junctions and desmosomes) b) modulation of cell polarity and c) re-arrangements of the cytoskeleton (switch from intermediate cytokeratins to vimentin) (Fig. 3B). Following these changes, epithelial cells appear isolated, spindly, motile and resistant to death by apoptosis [42]. The genesis of the EMT phenomenon is related to known genetic and epigenetic changes that occur in epithelial cells. Specifically, the changes concern genes involved in cell proliferation, motility, drug resistance and the development of localized tumors (Fig. 3C). Epithelial cancer cells that acquired mesenchymal characteristics by EMT can invade surrounding tissue and metastasize in distant sites generating clinical manifestations associated to reduction of the patient's quality of life and finally to the patient's death (Fig. 3C).

Notably, epithelial cells can acquire an EMTs-related mesenchymal phenotype with different rates. Indeed, when observing the epithelial cell population during the EMT phenomenon, we could find cells retaining epithelial characteristics while acquiring of mesenchymal phenotype and other cells showing only the mesenchymal profile [5,6]. Nevertheless, the molecules and signaling pathways involved in the early phases of EMT are still under investigation. From a molecular point of view, the EMT phenomenon is characterized by well-defined cellular processes. As concerns the adherent junctions, their structure disassembles and the cytoskeleton, rich in actin and cytokeratin, reorganizes from an epithelial cortical alignment linked with cell-cell junctions into actin stress fibers that are anchored to focal adhesion structures. The main molecular event of the reorganization of the cytoskeleton is probably the loss of E-cadherin from the surface of epithelial cells. Historically, loss of E-cadherin was considered the *conditio* sine qua non to trigger a series of signaling events that lead to EMT. However, experimental studies showed that constitutive block of E-cadherin expression induced by RNA silencing does not induce a full EMT [42]. Also, Hollestelle et al. have demonstrated in an in vitro study that the loss of E-cadherin expression is not a necessity for EMT of breast cancer cell lines [43].

After, or concomitantly, with both the loss of adherent junction complexes and cytoskeleton reorganization, epithelial cells subjected to EMT acquire a mesenchymal identity. The most prominent characteristics of mesenchymal phenotype are the expression of vimentin as the prevalent cytoskeletal protein and an increase in the assembly and secretion of extracellular matrix molecules such as fibronectin and collagens. Notably, the presence of these molecules in the extracellular matrix can stimulate molecular pathways related to the activity of integrins, thus favoring cell migration by the formation of focal adhesion structures [44]. The frequent loss of E-cadherin on the plasma membrane in cells undergoing mesenchymal transition further induces the formation of focal adhesion complexes by the activation of adhesion kinases. Taken together, these cellular modifications are responsible for the loss of apical-basal polarity that is fundamental for the preservation of the typical morphology of an epithelial cell and, more in general, for the function of the epithelial sheet [45]. The modifications in cell polarity play an essential role in the EMT phenomenon. The switch from apical-basal polarity to front-back polarity allows cell to cells to move and migrate in the surrounding tissues and/or in distant organs. Moreover, the ability of mesenchymal cells to synthesize extracellular proteases, such as matrix metalloproteinases, facilitates the migration through tissue by inducing the degradation of extracellular matrix proteins. Therefore, the modifications in the expression of extracellular proteins represent the last step in which epithelial cancer cells acquire an invasive behavior [45].

In breast cancer, the EMT phenomenon is frequently observed in histological/molecular subtypes called basal-like and claudin-low. Conversely, its incidence in luminal A/B subtypes is less evident [46]. The frequent presence of cancer cells undergoing to EMT in basal and claudin-low breast malignant lesions can explain the poor prognosis associated with these cancer subtypes. In fact, as reported above, the EMT generates the cell population capable to form metastasis in distant organs. In this scenario, it has been shown that the inhibition of molecules involved in the activation of EMT, such as Zeb, Snail and Twist, is an efficient strategy to block metastasis of breast cancer cells in an experimental mouse model [45]. This is frequently accomplished by the mesenchymal-epithelial transition (MET), which represents the opposite biological process to the initial EMT at the primary tumor and is hypothesized to contribute to the colonization of circulating cancer cells into metastatic sites [46]. Indeed, these dynamic EMT/MET transitions can play a key role during the development of breast cancer metastasis. Although numerous in vitro and in vivo studies have reported solid data about the molecular mechanisms related to both EMT/MET occurrence and progression, the involvement of EMT/MET in human breast cancer progression (and especially its role in the metastatic process) remains controversial. This is mainly due to the lack of a detailed histological characterization of the process in human tumor samples and related metastatic sites [47]. Thus, in situ characterization of cells subjected to EMT in histological specimens of breast tumors is an essential to confirm in human samples the presence of molecular alterations reported in vitro or in animal models of breast carcinoma. An intrinsic problem associated to the histological characterization of the EMT is the absence of specific in situ biomarkers able to capture the degree to which epithelial cells are engaged in EMT. The analysis of cytokeratin filaments by immunohistochemistry is not particularly sensitive. In addition, analysis of vimentin expression in cytoplasm of epithelial cells is difficult to distinguish due to the presence of numerous vimentin positive stromal cells [48]. In our recent studies, we showed the correlation between EMT occurrence and the expression of new breast cancer prognostic markers related to bone metabolism, such as PTX3 [49] and nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) [50]. Specifically, we found numerous vimentin-positive cancer cells in poorly differentiated breast carcinomas characterized by the expression of these markers. Also, the EMT phenomenon was linked to the presence of breast microcalcification made of HA [51]. In this context, several investigations displayed a positive correlation between the expression of bone markers such as Bone

Morphogenetics Proteins (BMPs), EMT occurrence and bone metastasis formation [52]. In a very recent study, Zhang and colleagues demonstrated that BMP-2 can trigger both the EMT phenomenon and the cancer stemness CD44 signaling pathway in breast cancer cell lines [52,53]. The relationship between EMT and the formation of $CD44^+$ cancer cells also suggests a role of this phenomenon in breast cancer plasticity related to the presence of intra-tumoral CSCs [52]. Due to the importance of EMT in breast cancer development and metastasis formation, the identification of reliable prognostic and predictive biomarkers is needed. Understanding of EMT processes of breast cancer will further lead to new diagnostic and treatment options.

6. Breast osteoblast-like cells in breast cancer and metastasis

Recent studies showed that breast cells frequently express molecules involved in bone metabolism [6–51]. These evidences lay the basis for the research of the biological/molecular mechanisms underlying the connection between breast and bone tissue. The presence of cells with molecular and morphological aspects of osteoblasts in breast tumors with microcalcifications made of HA further confirmed this biological connection. It is important to note that BOLCs are frequently found in poor differentiated breast carcinomas where EMT occurrence was demonstrated [5,43]. In our previous investigation, we associated the presence of BOLCs with the expression of EMT biomarkers such as β-catenin, TNFα and vimentin [6–51]. The localization of such cancer cells around microcalcifications led us to hypothesize that breast cells, in presence of EMT inducer molecules, can acquire mesenchymal phenotype transforming themselves into osteoblast-like cells capable of contributing to the production of ectopic calcifications [5]. However, the biological mechanisms involved in breast cancer cells osteotropism have not been clarified yet. From clinical point of view, the bone metastasis formation can be considered the main manifestation of breast osteotropism. Also, breast cancer cells capable to colonize bone tissue possess unique features, enabling them to utilize the bone microenvironment to their advantage. Communication between cancer cells and bone cells, such as osteoblasts and osteoclasts, is believed to be critical for the development and progression of bone metastases [52]. At the same time, the bone matrix offers an appropriate microenvironment that aids the growth of these cells, and recent data provide further evidences of breast osteotropism showing that BOLCs have a higher likelihood of metastasizing to bone [6].

In particular, BOLCs in primary lesions displayed the expression of bone biomarkers as Vitamin D Receptor, RANKL and RUNX2. Interestingly, we found the expression of molecules capable to sustain osteoblastic proliferation and differentiation (e.g. BMPs) in the breast cancer micro-environment [5,6,53,54]. This evidence supports the idea that cancer micro-environments could represent a “primordial soup” for the development of BOLCs. As concerns the molecular mechanisms of BOLCs generation and activity, pioneering in vitro studies about osteomimicry of mammary cells have been performed by Maria Morgan and colleagues [55], who demonstrated that bioengineered 3D scaffolds made of collagen glycosaminoglycan supporting the growth and mineralization of mammary cell line due to their capability to simulate the bone microenvironment [56].

By combining ex vivo and in vitro evidences about the generation and activity of BOLCs, our group developed a method which involves culturing breast cells with calcium oxalate (CO) and human monocytes to induce breast cells differentiation in BOLCs by mimicking microenvironment of breast lesion with microcalcifications made of CO [57]. Specifically, our data supports the hypothesis that the formation of BOLCs can be triggered by the activation and M2 polarization of macrophages induced by CO (Fig. 4). These experimental results allow to speculate that in vivo, microcalcifications made of CO, generally associated with the formation of breast benign lesions, can trigger the mechanisms involved in the BOLCs formation. This suggests an active role of microcalcifications in breast cancer occurrence and development.

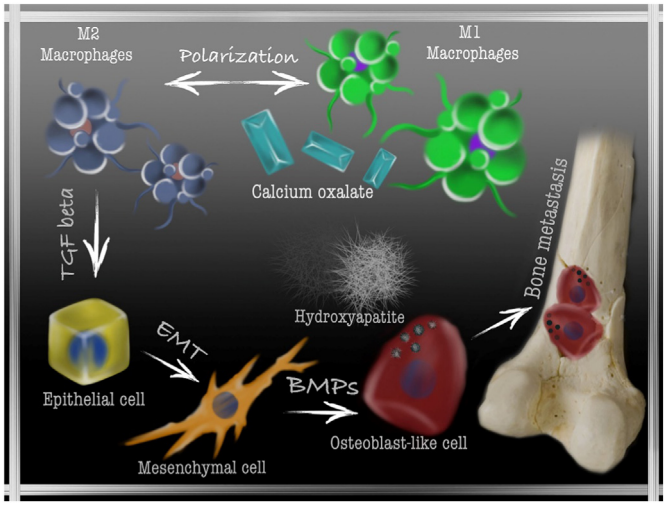

Fig. 4. A schematic model of BOLCs origin and bone metastasis development. The scheme depicts our hypothesis of a possible mechanism of BOLCs origin and bone metastasis formation. Calcium oxalate attracts macrophages in breast lesions inducing their M2 polarization. M2 macrophages in breast lesion produce and secrete EMT-inducing factors such as TGFβ. TGFβ enriched microenvironment drives epithelial cells to acquire mesenchymal characteristics. Then, under BMPs-induction, mesenchymal-like cells could assume an osteoblast-like phenotype and behave as a producer of complex forms of calcification that are secreted in breast cancer microenvironment. Finally, BOLCs detach from the primary tumor site and colonize the bone surface triggering bone resorption through the interaction with resident osteoclasts via the RANK/RANKL pathway.

In conclusion, the identification of breast carcinomas with high propensity to form bone metastases through the presence of cancer stem cells clones or through the presence of BOLCs can provide a scientific rationale for development of new diagnostic tools or therapeutic strategies. Should the role of BOLCs in the formation of breast cancer metastasis to bone should be verified, the presence of such cells in primary breast lesions could become a reliable target for anti-bone metastases therapies. In this context and under this hypothesis, preemptive administration of bone resorption inhibitors (e.g. bisphosphonates) or molecules with anti-osteoblastic effects (e.g. non-steroidal anti-inflammatory drugs) could possibly avoid the onset of the metastatic process.

7. Molecular imaging of breast cancer

Recent development in the fields of diagnostic imaging and image analysis are offering opportunities in medical research in general and oncology in particular. In particular, high-throughput approaches that may process and correlate multiple imaging parameters with omics data, both molecular and genomics, are considered the main elements of the most important revolution of the modern medicine. Part of such data is becoming more and more accessible also due to the advent of hybrid scanners such as positron-emission tomography (PET)-computed tomography (CT) or magnetic resonance (MR)-PET [58], whose use extends well beyond the neurological domain. In this context, radiogenomics represents a new strategy that associates imaging characteristics of breast cancer with gene expression patterns, gene mutations, protein expression and other proteome/genome-related characteristics [58]. Radiogenomics is not only considered the technological development capable of associating different medical disciplines such as imaging diagnostic and pathology (from the anatomical–histological level to the biomolecular level) - it is also an essential step toward exhaustive disease characterization. On note, the introduction of modern analytical software tools, often based on artificial intelligence,

allowed to identify new imaging biomarkers offering knowledge essential insights into the complexity of tumor mechanisms. In general, manually or automatically extracted multiple imaging features are analyzed jointly with histological and molecular/genetic data. This association is able to offer useful bidirectional information: data extracted from images may be used to predict cancer genotypes, and imaging features may be deduced from gene signatures. In the context of breast cancer, Woodard and colleagues studied the possible correlation between features extracted from both BI-RADS mammography and magnetic resonance (MR) and breast cancer recurrence in patients with ER positive lesions by using the OncotypeDx assay [59]. The authors reported a significant association between indistinct mass margins and fine linear branching calcifications with the recurrence score, whereas breast density was inversely related to the recurrence score. Also, Wu et al. a performed a prospective cohort study focused on analysis of mammograms in 498 invasive breast cancer patients [60]. Mammographic tumor appearance was an independent predictor of risk of breast cancer death when conventional tumor attributes and treatment modalities were controlled. Interesting, the presence of casting type calcifications and architectural distortions were associated with a significant increase of risks of breast cancer death.

The basal phenotype independently conferred a 2.68-fold risk compared with nonbasal phenotype. The observed deaths did not differ significantly from expected deaths in the validation cohort. The application of imaging biomarkers together with other predictors classified twelve categories of risk for breast cancer death.

These findings highlighted the potential of radiogenomics in the identification of multiple breast cancer imaging biomarkers capable to predict recurrence risk, although larger-cohort investigations are needed to corroborate these preliminary results. In line with this consideration, several studies have shown that dynamic contrast-enhanced MR can predict the molecular composition of breast carcinomas. For example, Saha and colleagues found a strict association between PR expression and MR image features, suggesting a differential expression in the imaging phenotype for PR status [61]. Conversely, lower levels of association were found between MR image features and the expression of HER 2 and Ki-67 in breast carcinomas. However, the existence of a moderate correlation between MR features and HER2 positive breast cancer subtypes could indicate that HER2 is part of a potential composite biomarker which may be augmented by including other clinical variables related to tumor genomic characteristics. In this context, several research groups are working to both identify and develop radiolabelled molecules that can improve diagnosis and therapy of breast cancer. Among these, superparamagnetic iron oxide nanoparticles (SPIONs), a group of MRI-compatible nanoparticles, represent a very promising novel contrast agent [62]. In this context, Hajiramezanali et al. reported the highly sensitivity of to DOTA-BN-TMC-MNPs labeled with 68Ga (68Ga-DOTA-BN-TMC-MNPs) in detecting small breast cancer lesions by using PET/MRI analysis [62]. Another interesting new molecule was tested in a pilot study concerning the use of PET/CT Computed Tomography (CT) in the assessment of breast cancer. Preliminary results published by Zang et al. show that Ga-NOTA-RM26 PET/CT analysis correlate with ER expression and the menstrual status of patients [63–65], hence candidating Ga-NOTA-RM26 PET as a valuable prognostic and predictive marker for breast cancer. Also, Zang et al. published a pilot study in which investigated the value of an antagonist targeting gastrin-releasing peptide receptor in breast cancer [63]. Although preliminary, the results of this study indicate a potential value in augmenting diagnostic accuracy in breast cancer, hence potentially providing the type of essential prognostic and predictive information that generally is a prerogative of histological and immunohistochemical analysis. Results of these studies, if confirmed on a large-cohort selection of human breast cancer patients, could be used to develop new diagnostic tools capable to guide clinical decision making in those patients with highly heterogeneous lesions. In fact, to date, the survival rate in breast cancer patients subjected to HER2-targeted therapy is strongly depended on patient selection criteria. The latter are currently is based on immunohistochemistry analysis of bioptic materials. Nevertheless, immunohistochemical assessment of HER2 status is negatively affected by cancer heterogeneity as well as by the variability of assay results. To overcome the limitations of the immunohistochemical exam, in vivo protocols for the detection of HER2 positive breast cancer cells have been developed. In this field, the main application concerns the use of ^{89}Zr-trastuzumab for PET examination. ^{89}Zr-trastuzumab PET/CT analysis showed the ability to assess the HER2 status of the full tumor burden in breast cancer patients, thus reducing the necessity of multiple tissue sampling to evaluate the variation of HER2 status during tumor progression or pharmacological treatment [63]. New approaches to the design of HER2-targeted molecules are fueling the development of better neoadjuvant therapy for patients affected by HER2-positive breast cancer lesions [66]. The most promising anti-HER2 molecules currently available are the tyrosine kinase inhibitor lapatinib (LPNB) or monoclonal antibody PZMB, the antibody-drug conjugate trastuzumab-DM1 (T-DM1), and neratinib, an HER2, HER4, and EGFR tyrosine kinase inhibitor. LPNB is a tyrosine kinase inhibitor of the HER1 and HER2 receptors that blocks the intracellular pathway involved in MAPK/Erk1/2 and P13K/Akt signaling [67]. PZMB is a fully humanized monoclonal antibody that constrains dimerization of HER2 with other HER receptors [68]. In 2013, the outcome of the CLEOPATRA trial suggested that the combination of TZMB and PZMB with taxane could become a new first-line standard treatment for HER2-positive advanced breast cancer patients [18]. Currently, these molecules are under investigation for their possible use in new theranostic approaches [69,70]. Still, the main diagnostic challenge in the field of oncological molecular imaging is the development of new methods for early in vivo detection of breast cancers with high metastatic potential [71–73]. In this context, ^{99}mTc sestamibi scintimammography with high resolution, dedicated gamma cameras could offer an additional opportunity in revealing both primary breast malignant lesions and associated bone metastatic sites [74]. According to its chemical and physical nature ^{99}mTc sestamibi appears particularly suitable for in vivo detection of these tumors. In fact, after spreading inside cancer cells, the positive charge of the sestamibi molecule drives it into mitochondria (due to their negative plasma membrane potential) [74]. Also, this phenomenon could increase in cancer cells actively involved in metabolic processes related to the increase of both the number and the negative plasma membrane potential of mitochondria [74–76]. Of note, in vitro examination of osteoblast cell lines treated with mineralization inducing factors, such as estrogen, showed an increase of the amounts of high-transmembrane-potential mitochondria during the production of calcium crystals [77]. According to these data, and in conjunction with current evidence of the presence of numerous mitochondria in the BOLCs [78], we can speculate that ^{99}mTc sestamibi scintimammography could be a suitable approach for the identification of breast carcinomas with high propensity to form bone metastatic lesions characterized by a high percentage of BOLCs. Based on this hypothesis, we performed a pilot study to investigate the uptake ^{99}mTc sestamibi in a small cohort of breast cancer patients. Our preliminary data displayed a correlation between sestamibi uptake and the number of BOLCs in patients affected by breast infiltrating carcinomas [78] (Fig. 5).

The recent discovery of cells and molecular pathways involved in the metastatic process of breast cancer could provide the scientific rationale for setting-up novel diagnostic and therapeutic protocols based on molecular imaging analysis. The early in vivo detection of BOLCs in primary breast lesions can provide a first response to this challenge.

8. Synergies between diagnostic imaging and pathology in breast cancer patient management

The currently most sought-after goal in biomedical research is precision medicine [79], and the development of custom therapeutic

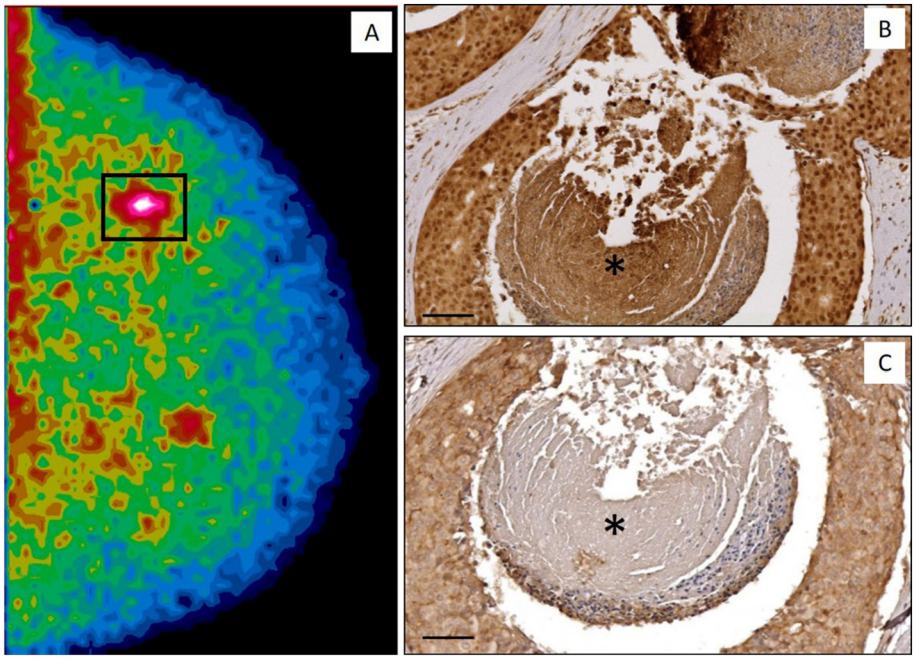

Fig. 5. Sestamibi uptake in breast lesions with BOLCs. A) 99mTc sestamibi high-resolution SPECT image. Square highlight a small breast lesions with high uptake of sestamibi (SUV max 150 SUV average 100.7). B) Image displays several RUNX2-positive breast cancer cells next to microcalcification (asterisk) in the lesions detected by 99mTc sestamibi high-resolution SPECT. C) Image shows numerous RANKL-positive breast cancer cells close to microcalcification (asterisk) in the lesions detected by 99mTc sestamibi high-resolution SPECT. Scale bars represent 40 μm.

protocols needs synergistic and transdisciplinary competencies [80–82]. Currently, in most cases the cancer cells' ability to undergo molecular, morphological and genetic changes as well as the individual within-patient variabilities are not taken into account in experimental therapies. For example, digital pathology is a relatively new arena where novel computer vision technologies are applied on histological samples to conduct multidisciplinary studies on the basis of digital imaging analysis [83–85]. Digital pathology can eventually lead to successful collaboration between e.g. nuclear medicine and anatomic pathology. Indeed, both these disciplines are characterized by image processing followed by analysis and interpretation at the time of diagnosis. In the past two decades, radiology as well as nuclear medicine units experienced a tremendous transformation due to the development of digital imaging techniques that led to enhanced image quality, safety and innovation in analysis and manipulation of diagnostic images [86–88]. However, in spite of its large potential, the interest toward applying the digital pathology techniques in clinical and research practice is still low. However, we hypothesize that diagnostic pathology practice will be revolutionized when WSI (Whole Slide Imaging) and complete digitization will be introduced [89–91]. Currently, digital pathology is predominantly utilized to assess the prognostic and predictive immunohistochemical markers of breast cancer [92,93]. At present, image analysis software is widely used (and has been approved by the US Food and Drug Administration) to quantify nuclear markers such as ER or cell membrane markers such as HER2 [94–96]. Given that treatment decisions are made on the basis of immunohistochemical analysis of ER, PR, Ki67 and HER2 [97], such applications may become essential to manage breast cancer patients. While there is a reasonably good concordance between response to therapy and expression of prognostic breast biomarkers, it is not possible for the immunohistochemical analysis to capture the known spatial heterogeneity of breast cancer tissues. While one can possibly evaluate the temporal heterogeneity of breast cancer in sequential biopsies, this would greatly (and sometimes unacceptably) increase invasivity.

According to recent works [98,99], quantified immunohistochemical data can be utilized to design novel PET-CT diagnostic or therapeutic protocols that have the potential to enhance quality of life among patients through reduced biopsies, increase the accuracy rate of diagnosis and find real-time molecular transformation of cancerous cells. If radiolabeled molecules were able to quantify the expression of ER (i.e. 16α-18F-fluoro-17β-estradiol) or c-erb2 (i.e. radiolabelled anti-HER2 antibodies) by PET/CT, noninvasive imaging could become an attractive alternative in the management of breast cancer patients [100–104]. Additionally, histological techniques can be used to characterize the ultrastructural and elemental composition of breast cancer tissues. TEM and SEM (Transmission Electron Microscopy and Scanning Electron Microscopy, respectively) are able to identify sub-cellular level proteins (immunogold labelling) and chemical elements (microanalysis) [105–108]. This can be leveraged in analyzing bioptic samples from patients who have undergone PET-CT investigation to obtain data about the density of PET targets or, possibly, the precise location of the radiolabelled molecules. For instance, when microanalysis is performed, it can detect the site of the link of radiolabelled molecules in tissues by isolating e.g. gallium or technetium. By combining these data, the bio-distribution of radiolabelled molecules withing cells and the biomechanisms involved in unspecific PET signals could be inferred and/or better understood. Also, the results from molecular imaging analysis can aid in improving the efficacy of immunotherapeutic strategies which are originally developed on the basis of histological evidence about the existence of PD-L1 positive cells among highly aggressive human epithelial cancers [109].

9. Artificial intelligence for patient data integration and building predictive models for disease stratification and evolution

As outlined above, there is much to be gained in the opportunity to combine multi-domain and multiparametric patient data in across disciplinary boundaries, especially in terms of creating more finely tuned patient strata and more powerful and data-driven predictive models. For example, a particle swarm-optimized wavelet neural network was used to diagnose breast cancer based on mammographic images only [110], delivering sensitivity and accuracy of 94% and 92%, respectively. The characterization of breast cancer masses (e.g. through the detection of microcalcifications) can also greatly benefit from techniques rooted in computer vision [111]. Support Vector Machines (SVM) and Fuzzy SVMs have been successfully employed, along with radiomics features, in breast cancer classification [112,113]. Other algorithms which have been able to tackle whole-image processing of breast cancer data include Feed-forward back-propagation artificial neural networks [114] and genetic algorithms [115]. In this context, the

disruptive innovation brought about by the advent of deep learning in general and deep neural networks (DNN) in particular has unprecedented potential. Of note, DNNs have had extremely high success rates in the field of oncology in general, and breast cancer in particular [116]. For example, breast lesions have been classified on the basis of nuclear features only [98], and metastases have been identified using deep learning methods [100]. The abilities of DNN to decompose the information contained in an image though a number of operation impossible to the human eye like repeated filtering, decomposition and re-aggregation into high-order features will most likely continue to push the boundaries of sensitivity and specificity in diagnostic imaging of any kind. DNNs can e.g. also be trained to predicting genetic changes based on the imaging data to generate one image modality from the other. Given the joint potential of nuclear imaging along with WSI to produce a wealth of data whose minute details cannot be integrated by a human observer in a systematic way, deep learning offers a unique opportunity to a) maximise synergic performance in the patient's interest, and b) generate financial savings in e.g. molecular testing for called precision oncology. Pathology work could also be significantly enhanced by training DNN to emphasize and highlight morphological signs whose identification is time-consuming to the naked eye but may result in diagnostically actionable items. In this context, DNN could also be trained to predict response to specific treatments and could be of help in devising patient stratification strategies for the optimization of clinical trials. Similarly, training DNNs with images from known responders and non-responders to specific treatments (e.g., immunotherapy) may help to better stratify patients for appropriate future personalized and precision-based clinical trials. The main issue with the use of DNN is the need for a large number of manually curated cases in order to properly tune the hyperparameters of the network. Unification of criteria in multicenter data collection, as is underway (for example) in large world-wide efforts is therefore an important next step also in the field of joining medical imaging pathology.

With the support of these multicenter and multidisciplinary efforts [117], it is likely that AI-based image processing and, successively, tissue classification algorithms will be integrated more and more in the decision-making process in breast cancer. Validated algorithms and models are likely to be streamlined in to Computer-aided detection and well as diagnosis (CAD) [118] systems able to offer objective support with quantifiable performance to the clinician in charge. In detail, the potential of AI techniques to seamlessly integrate large amounts of multi-modal and multidomain data (see above) could provide an important complement to a) the radiologist's interpretation of multimodal images by automatic contouring, fusion, identification of lesions and image enhancement, and b) the clinician's experience and reasoning by providing probability scores and diagnostic and prognostic suggestions potentially based on a virtually unlimited number of prior cases which may have been only partially archived and/or taken into account.

10. Conclusion

Thanks to recent progress in imaging and screening, advances in the early diagnosis of breast lesions can potentially decrease mortality rates and disease burden in the general population. Nevertheless, in the United States only, invasive breast cancers resulted in about 40,610 deaths in 2017. Therefore, the improvement of knowledge of breast cancer biology is crucial to design new reliable diagnostic and/or therapeutic targets. In this scenario, the synergic collaboration between anatomic pathology and nuclear medicine can represents an opportunity to develop personalized medicine protocols capable to improve the management of breast cancer patients.

Declaration of Competing Interests

The authors have declared no conflicts of interest.

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