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Know your enemy: genetics, aging, exposomic and inflammation in the war against triple negative breast cancer

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Abstract

Triple negative breast cancer (TNBC) is one of the most biologically aggressive and very often lethal breast disease. It is one of the most puzzling women malignancies, and it currently appears not to be a good candidate to a standardized, unanimously accepted and sufficiently active therapeutic strategy. Fast proliferating and poorly differentiated, it is histopathologically heterogeneous, and even more ambiguous at the molecular level, offering few recurrent actionable targets to the clinicians. It is a formidable and vicious enemy that requires a huge investigational effort to find its vital weak spots. Here, we provide a broad review of "old but gold" biological aspects that taken together may help in finding new TNBC management strategies. A better and updated knowledge of the origins, war-like tactics, refueling mechanisms and escape routes of TNBC, will help in moving the decisive steps towards its final defeat.

Introduction: enemy in sight

Breast cancer (BC) is the most common cancer in women [1] and around 15-20% of them belong to the triple negative breast cancer (TNBC) subtype. TNBC is defined in case of lack of expression of the estrogen receptor (ER) and progesterone receptor (PR), and of the human epidermal growth factor receptor 2 (HER2) [2]. It is prognostically unfavorable, diagnosed at younger age, likely recurs during the first 3 years, and aggressive due to the lack of response to targeted therapies, i.e. hormonal or HER2 receptor-targeted approaches. Currently, chemotherapy is the only feasible and overall approved therapeutic strategy in the adjuvant or metastatic setting for TNBC [3]. Some potential targeted therapies have been studied, such as anti-VEGF, EGFR, FGFR, mTOR, PARP1, AR, NOTCH, CDK, PI3K, MET. However, their efficacy has still to be validated in clinical trials. There is a huge need to find and new test

and/or clinically useful line of attack. Risk factors of TNBC are several. Despite, genetics, aging, chronic inflammation, reproduction-related factors, e.g. menopausal status or parity, and exogenous hormones, are some of the most important signals in BC onset and development, their role in TNBC is not completely clear [4].

The battlefield: starting conditions, and allies

Genetics

TNBC displays a full spectrum of mutations and clonal evolution. Some cancers show a few coding somatic aberrations, others contain hundreds of alterations within a limited number of molecular pathways, whereas others exhibit considerable additional mutational involvement. The clonal heterogeneity of these cancers is also a continuum, with some patients presenting with low-clonality cancers and other cases exhibiting more extensive clonal evolution.

Nowadays, in general, the knowledge regarding molecular alterations, in particular age-specific, that characterize the overall TNBC patient population is not complete. Mainly, studies aimed at exploring BC genetic and genomic modifications as a function of age based and clinico-pathological data. One of the most studied, of course, is BRCA. In particular, some authors reported that 15% to 20% of TNBC carrying *BRCA1/2* germline mutations in contrast to 10% in the general BC population [5]. It has been shown that at least 10–30% of *BRCA1* mutations were found in TNBC patients before age 50 years, despite lacking family history. Furthermore, it has been observed that 20% of TNBC that do not own the somatic *BRCA1* mutation, may have issues in DNA repair mechanisms due to other irregularities in the BRCA-related pathway. In contrast, other authors reported that up to 75% of *BRCA1*-mutation-carrier cancer patients are either TNBC or basal-like, or both. These differences could depend from ethnical dissimilarities in the examined case series, but are still fairly high and therefore ask for further investigation [6]. In agreement, it has been observed that BRCA1 mutated genotype may predispose to the basal tumor subtype [7]. As already reported, at diagnosis, TNBC present a wide spectrum of genomic alterations: some cancers show a few coding somatic aberrations in a handful of pathways, whereas others contain hundreds of coding somatic mutations. p53, PIK3CA, PTEN, WNT signalling, integrin signalling, HER2 signalling, hypoxia and PI3K modifications are some of the most easily detectable mutations (higher median clonal frequencies) and appear to be clonally dominant compared to other genes. Mutations in genes involved in cytoskeletal, cell shape and motility pathways (myosins, laminins, collagens and

integrins) occurred with lower clonal incidence, leading to the hypothesis that they appeared later during tumour progression [8].

Other researchers observed copy number alteration (CNA) events in tumour suppressors and oncogenes such as *PARK2* (intragenic deletion), *RB1*, *PTEN*, and *EGFR*. Few recurrent structural rearrangements and individual fusion events involving known oncogenes or tumour suppressors (e.g., *KRAS*, *RB1*, *IDH1*, *ETV6*) were reported too [9]. Other researchers identified a recurrent MAGI3–AKT3 fusion enriched in TNBC. The MAGI3–AKT3 fusion leads to AKT kinase constitutive activation, combined with the loss of function of a tumour suppressor gene (*PTEN*) and activation of an oncogene (*AKT3*) [10]. Metastatic TNBC showed an increase in the frequency of somatic biallelic loss-of-function mutations in genes related to homologous recombination DNA repair, compared to early TNBCs (7% versus 2%) [12].

Hence, in general, since at the time of primary diagnosis TNBC might be at different phases of molecular progression, with many levels of clonality, TNBC may yield a higher mutational burden, and in turn a higher neo-antigen expression [8, 9]. It is easy to understand that, during progression, the already high mutational burden of TNBC can increase, even more, making TNBC a good target for therapeutic approaches tuned to hit cells with a greater mutational load.

Finally, it has to be kept in mind that cancer is strictly interrelated with aging processes TNBC are influenced by factors such as epigenetic DNA modifications, gene expression and microRNA (miRNA) level variations. Epigenetic DNA modifications are frequently used as indicator of "biological age" and DNA methylation-based measures of biological age may be important predictors of cancer risk [13]. Approaches aimed at reverting this epi-modifications are under investigation. Indeed, some epigenetic-based therapeutic approaches were tested on TNBC cell lines. Compounds such as resveratrol and pterostilbene have been shown to alter genetic and epigenetic profiles of tumor cells. A therapeutic combination of resveratrol and pterostilbene seems to cause a synergistic growth inhibition of TNBCs, a down-regulation of SIRT1, a type III histone deacetylase (HDAC) and of DNA methyltransferases (DNMT) enzymes, a significant decrease of γ-H2AX and telomerase expression. These data suggest a potential role of this class of drugs as a new option worthy of being studied in settings where therapeutic approaches are still limited [14].

Comprehensive analyses like gene-expression signatures (GES) were used to describe a functional annotation of BC in function of age. Although, there is still a few evidences about TNBC subtype and age, studies in the general BC population suggest a potential correlation between cancer aggressiveness and age that should investigated in TNBC also. In particular, data obtained from twenty-five GES (five for molecular subtyping, seven for immune response,

three for metabolism, seven for critical pathways in cancer and three for prognosis), showed that, in contrast to tumours of young patients, tumours of elderly patients had a favourable GES scores (high oestrogen receptors and mitochondrial oxidative phosphorylation, low proliferation, chromosomal instability, iron metabolism). Therefore, it is possible to deduce that age is related with the incidence of molecular subtypes, showing a decreasing aggressiveness of cancers in function of age [15].

Also non-coding RNA, such as miRNA, could have a significant impact on the preliminary battle-field of TNBC. miRNAs, approximately 22-nt-long nucleotide sequences, control gene activity interacting with RNA. A single miRNA can have multiple targets in normal and pathological processes. For example, a potential link between BC and aging through NF-kB pathways could be deeply influenced by specific circulating miRNAs (miR-21, miR-34, miR-126, miR-195, let-7a, miR-146a, and miR-155). These miRNAs are promising therapeutic targets, opening the possibility to different newstrategies against BC and, more specifically, TNBC [16].

The battlefield, starting conditions, and allies - part 2

Aging

Aging is a general process, characterized by significant variability among organisms in terms of specific components and of the rate at which it proceeds. No two individuals age at the same rate and biological aging mechanisms remain far to be fully clarified. However, it is quite well recognized that aging potentially occurs through the gradual accumulation of unrepaired mutations and loss of tissue homeostasis [17]. From these mirroring points of view, a relationship with cancer is clear. Indeed, age is considered the single most significant risk factor for many chronic conditions including the majority of malignancies. Malignancy and aging may be even regarded as the two sides of the same cellular and molecular processes [18].

There are few reports directly regarding TNBC and aging. Mostly, TNBC appears to be more frequent among younger patients, despite a not trivial percentage of older patients (≈ 15%) still presents at diagnosis with this tumor type. Moreover, in advanced age, BC has been associated with a little increase in the probability of a favourable tumour biology. Following this reasoning, some studies reported that older TNBC patients may have a better outcome when compared with their younger counterparts. For example, younger age, was found to be strongly correlated with shorter disease free survival (DFS) and overall survival (OS) in a population of

TNBC, but they did not condidered BRCA mutations and this could be a bias in younger patients [19]. Differences in prognosis have yet to be fully understood, but may be due to age-related biological variations, more or less unknown [20]. Nevertheless, currently, there is no direct evidence of a causal link between young age and TNBC development.

Interestingly, some evidence of molecular cross-linking involves NF-κB. This transcription factor is a hallmark of inflammatory responses and there are reports that suggest the sustained transcriptional activity of NF-κB in different tissues with aging. It has been shown that the inhibition of NF-κB in TNBC cells can decrease their proliferation and invasiveness [21]. Keeping in mind these results, it can be intuitively suggested that in the TNBC younger patient population there is a precocious and/or high expression of elements related to inflammation and aging, in addition to mechanism of evasion from immuno-surveillance [22]. This starting condition might offer to cancer cells a suitable microenvironment and prepare the way to early tumor invasion. In agreement, studies reported the notion that secretions from aging stromal cells support pre-cancer cells and that aging potentiates pro-inflammatory pathways providing selective advantages to cancer cells [21, 23]. Age is related to an increase in number and importance of general metabolic defects or impairments, e.g. metabolic syndrome. These disorders have been directly associated with TNBC risk for women during their whole life.

Irrespectively of age, it has also to be always kept in mind that therapeutic approaches may trigger cell aging, potentially fueling the vicious circle cancer-inflammation-aging [24,25].

Aging & the ruler inside: the role of telomerase

Telomeres are nucleoprotein structures that protect chromosome ends from degradation and recombination. Telomerase has an important role in genome stability, immortality, aging, and cancer. Cancers often have critically shortened telomeres, contributing to genomic instability. Many of these tumors activate telomerase to stabilize telomeric ends and achieve the capacity for unlimited replication. Telomere shortening has been reported in *in situ* and invasive carcinomas, including breast, and has been associated with disease recurrence after surgical resection. Liu and colleagues reported that telomere length was shorter in more aggressive cancer subtypes, such as TNBC. This data suggested that tumor telomere length might have a utility as a prognostic and/or predictive marker [26].

Telomerase lack would limit the growth of tumors by causing continually dividing cells to shorten their telomeres and to die before starting to spread. For this reason, telomerase inhibitors seem to be promising approaches to eliminate cancer cells. As already mentioned, Kala and colleagues studied the effects of SIRT1 knockdown combining resveratrol and

pterostilbene in TNBC. They demonstrated the induction of SIRT1 down-regulation through inhibition of both telomerase activity and γ-H2AX expression in HCC1806 BC cells [14].
Finally, Storci and colleagues underpinned that telomeric sequences are major components of the cy/cfDNA payload. Telomere shortening causes the depletion of telomeric sequences in cy/cfDNA pool, thus unleashing their potential to exert an age-related activation of the innate immune system [27].

Tumor war-like tactics & refueling mechanisms - part 1

Inflammaging and Nucleic acid garbage

Inflamm-aging describe the age-related increase in the systemic pro-inflammatory status of humans. Although not fully understood, it is associated with the progressive activation of the innate immune system that accompanies human aging. Its role as a disease-predisposing condition has emerged since it has been proposed to be a major contributor to the increase in cancer incidence and progression in aged people [27]. A potential mechanism suggests that upon stress, nuclear and mitochondrial genomes are released into the cytoplasmic and extracellular compartments [27]. The misplacement of nuclear and mtDNA into cytoplasm lead to the activation of innate immunity, i.e. inflammation and type I interferon response. Cytoplasmic (cy) and cell-free (cf) DNA pools trigger inflammation and innate immunity at local and systemic level. In particular, cyDNA plays a crucial role in the phenomenon of cell senescence and in the cognate pro-inflammatory secretome [27]. Changes in a variety of biochemical "tastes" of cy- and cf-DNA (e.g. the amount of 8-oxo-deoxy-guanosine and 5-methyl-deoxy-cytosine, the proportion of DNA hybridized with RNA) potentially affect the capability of these DNA pools to ignite the innate immune system. Various sources of DNA (extracellular vesicles, the commensal metagenome and food) contribute to the cy/cfDNA payloads. Fragility of nuclear DNA and DNA-Damage Response (DDR) fuel cyDNA formation. On the other hands, DNA molecules produced by DDR may contribute to the cy DNA pool such as DNA molecules hybridized with cognate RNA strands called RNA:DNA hybrids. These molecules have been defined as molecular garbage and act as pro-inflammatory stimulus [27].
Recent studies of tumor lymphocytic immune infiltrates have suggested an improved prognosis associated with increasing levels of tumor-infiltrating lymphocytes (TILs) [28]. Among the BC spectrum, TNBC has the greatest incidence of patients with a robust tumor immune infiltrate, although it is still a minority of patients. Elevated levels of either intratumoral or stromal T

cells are associated with an improved overall survival (OS) and disease-free survival (DFS) in TNBC as compared to other BC subtypes. TNBC may be immunogenic for several reasons [29, 30]. TNBC subtypes have a significant number of genetic mutations, and the immune system may recognize the aberrant proteins encoded by these mutations as foreign, and immunogenic. Antibodies secreted by B cells may bind to tumor antigens and amplify the adaptive immune response that has already been initiated in the tumor [31]. New immunomodulatory agents, including immune checkpoint inhibitors, have demonstrated activity in immunogenic tumors such as bladder cancer and melanoma and have recently been tested in TNBC [32,33].

These datalay the foundation for the development of immune-based therapies.

Macrophages are thought to be a major cellular player in inflamm-aging [27]. Their role as tumor-associated macrophages (TAMs) in the cancer immune landscape has been investigated. Their potential as treatment targets or modulators of response to treatment are gaining increasing interest. TAMs display high molecular and functional complexity. Usually, macrophages are classified into M1 (classic) and M2 (alternative) subtypes [34]. M1 macrophages have anti-tumor activities while M2 macrophages stimulate the tumor tissue repair and growth. CD68 and CD163 have been considered as surrogate markers to investigate macrophage polarity. In TNBC, TAMs promote tumor growth and progression by several mechanisms including the secretion of inhibitory cytokines, the reduction of effector functions of TILs and the promotion of regulatory T cell (Treg) [35]. Interestingly, TAMs have been shown to directly and indirectly modulate PD-1/PD-L1 expression in tumor environment [36]. In this scenario, several TAM-centered strategies have been proposed, such as the suppression of TAM recruitment, the depletion of their number, the switch of M2-TAMs into antitumor M1 phenotype and the inhibition of TAM-associated molecules. Some authors studied their prognostic role. Zhang and colleagues demonstrated that TAMs correlate with the phenomenon of epithelial-mesenchymal transition and contribute to poor prognosis in TNBC patients [37]. In a recent paper by Pelekanou and colleagues CD68, CD163, and matrix metalloproteinase 9 (MMP-9) co-localization in breast tumor microenvironment predicts survival differently in ER-positive and -negative cancers [38]. High expression of CD163 protein in TAMs was associated with improved OS in ER- cases but not in ER+ cancers, suggesting TNBC could benefit from investigating CD163 with a diagnostic and/or therapeutic intent. It is interesting to note that basal-like BC cells induce phenotypic and genomic changes in macrophages, and present specific differentially expressed cytokines in their microenvironments, suggesting plausible

targets for modulating immune responses. These observations could be valid for TNBC also and are worthy of further studies [39].

Tumor war-like tactics & refueling mechanisms - part 2

Inflammation and immunosenescence

Rudolph Virchow hypothesized for the first time in 1863 a connection between inflammation and cancer, but only in the last two decades researchers have produced striking evidences on the role played by the inflammatory process in promoting cancer [40].

Cancer can arise on sites of chronic inflammation and, in turn, a pro-inflammatory microenvironment, inflammatory cells and mediators, are essential components of cancer [41-43].

During chronic inflammation, in fact, a few but important key molecular players such as prostaglandins, cytokines, NF-κB, cytokines, chemokines and angiogenic factors, predispose the inflamed tissues to malignant transformation [23]. In this context, as already mentioned, upon stress, nuclear and mitochondrial genomes may be released into the cytoplasmic and extracellular compartments. Cy/cfDNA pools trigger inflammation and innate immunity, playing a crucial role in the phenomenon of cell senescence also. The misplacement of nuclear and mtDNA into cytoplasm elicits a powerful activation of innate immunity, i.e. inflammation and type I interferon response that resemble viral infection and/or intracellular pathogen invasion pathway [44, 45]. In addition, recent literature conveys that DNA-Damage Response (DDR) also may incite cyDNA formation [46-48].

Immunosenescence refers to a number of deleterious alterations of innate and acquired immunity often occurring in the the elderly population. It is a complex process involving multiple changes, rather than simple unidirectional decline of complete immune function. Some immunological parameters are commonly reduced in the elderly, and good function is tightly correlated to health status. In addition, while innate immunity is relatively well preserved in elderly, acquired immunity is more susceptible due to both the functional decline associated with the passage of time, and to the antigen burden to which an individual has been exposed during lifetime. This chronic antigenic stress, which affects the immune system throughout life with a progressive activation of macrophages and related cells, contributes to determine an inflammatory status. Our immune system is quite capable in fighting acute infections in young people, but not particularly efficient in responding to chronic stimuli,

especially when they occur late in life. This leads to an increased production of inflammatory mediators associated with the presence of chronic infections [43,49,50].

As well as the immunosenescence, also the cellular senescence is involved in the vicious circle inflammation-aging-cancer. Cellular senescence leads to a state of permanent cell-cycle arrest caused by exposure to stressful stimuli such as telomere erosion, oncogene activation, oxygen free radicals (ROS), chemicals and ionizing radiation. It has been widely considered a tumor suppressing mechanism, but growing evidences link this process to hyperplastic and degenerative diseases through chronic inflammation [51-55]. The "senescence-associated secretory phenotype" (SASP) is considered one of the key processes for understanding the link between cellular senescence, inflammation and cancer development and progression [52,56]. Merging these aspects, Brouwers et al., in a small case series of TNBC patients, found that an older age at diagnosis was associated with a in term of up-regulation of several senescence genes in the tromal micro-environment. The SASP and the presence of autophagy appear to be important age-induced stromal features [57].

In addition to senescence processes, accumulating data have demonstrated that cancer-associated fibroblasts (CAFs) are regarded as senescent cells and contribute to cancer progression in various human cancers. several researchers have gradually clarified the origins, features, and roles of CAFs, a major component of the cancer stroma [58]. CAFs release cytokines and stimulate the growth of preneoplastic and malignant epithelial cells and the migration and invasion of immortalized or premalignant epithelial cells Interleukin-6 (IL-6), as an example, a multifunctional cytokine, plays a central role in regulating inflammatory and immune responses, and important roles in the progression, including proliferation, migration, and angiogenesis, of several cancers. CAFs are an important IL-6 source and can contribute to drug resistance acquisition in cancer cells [58].

Moreover, it has been recently reported CAFs are highly heterogeneous in BC and then their relation with macrophages and cancer cells need a wider characterization also in the TNBC [59]. This will open the possibility to treat TNBC by targeting CAFs. CAF-based or mesenchymal stem cell (MSC)-based cellular therapies can be used to deliver anticancer drugs (such as oncolytic adenoviruses, TNF-related apoptosis-inducing ligand (TRAIL) or type I interferon (IFN)). CAF-derived extracellular matrix (ECM) proteins and associated signalling can be targeted to induce stromal depletion. Finally, CAFs can be directly depleted by either transgenic technologies or immunotherapies [60].

Tumor war-like tactics & refueling mechanisms - part 3

The exposome and immunogenic aptitude

More than ten years ago, the exposome was described as the overall environmental complement to the genome in determining risk of disease. Wild defined the exposome as the totality of exposures throughout the lifespan [61]. Hence, in general, it can be inferred that, in addition to genetic predisposition, every molecule that can prompt tumor onset and growth can be seen as exposome, or, more definitely, cancer exposome. Following this reasoning, also inflammation-related molecules, known to be connected to tumor development and progression too, may be included in this definition.

Since it has been hypothesized that various sources of DNA (e.g., extracellular vesicles, commensal metagenome, and food), contribute to the cy/cfDNA payloads, they might be considered cancer exposome also. Aging is another pivotal factor linked to the exposome. It is associated with a progressive accumulation of damaged macromolecules and cells (self-debris) due to increased production and/or inadequate elimination, e.g. the molecular garbage already discussed. These waste products derived from cellular and metabolic processes and are released as a consequence of cell/organelle injury. Self-debris can mimic bacterial products and can activate innate immunity and inflammation [62].

Nutrition and aging have a strict correlation to exposome also. Aging has been associated with an increase in visceral fat that leads to obesity along with insulin resistance. In turn, visceral fat has been related to a higher BC risk [63]. Moreover, epidemiological data suggest a significant association between increased body mass index and post-menopausal breast and other cancers [64,65]. It is known that obesity is responsible for a chronic inflammatory state even though the molecular links between obesity and cancer are not yet completely clear [66]. An important feature of obese inflammation is that it originates from metabolic signals and within metabolic cells such as the adipocyte. Indeed, the exposure to excessive levels of nutrients, in particular of glucose and free fatty acids, induces a stress activation that in turn triggers inflammatory intracellular signalling pathways [66].

Generally, BC was not highly responsive to immunotherapy as compared to melanoma, lung cancer, renal cancer, lymphoma, bladder cancer, or head and neck cancer [67,68].

Suggesting the existence of variable immunogenic activity in BC subtypes, some authors have identified subtypes of BC more immunogenic than others, *e.g.* TNBC [29,30].

In agreement, an analysis from The Cancer Genome Atlas (TCGA) on gene expression, DNA copy number, somatic and germline mutations, Safonov et al reported that TNBC and HER2+ BC had high immune gene expression and lower clonal heterogeneity respect to other BC subtypes. A relation between the expression of immunologic signatures and clinical outcomes in TNBC, and elevated expression of HLA-C, HLA-F, HLA-G, and TIGIT, was associated with improved RFS and OS [69]. In addition, p53 status and tumor mutational burden may be associated with immune activities in TNBC. These findings may have important clinical implications for TNBC immunotherapy, and warrant immunotherapeutic options for TNBC. *TP53*-mutated TNBC had significantly higher expression levels of the immune checkpoint gene-set than *TP53*-wild type [26].

2. Tactical pre-battle maneuvers: biomarker detection

2.1 Tissue biomarkers

Among the different methods to test biomarkers both in primary tumor and in metastasis, immunohistochemistry (IHC) is the cheapest method and can be performed routinely in all laboratories. Different way to classify biomarkers have been used as well as different cut off, such as 1%, 10%, 50% and staining intensity 0, 1, 2, 3+. In addition, some authors have use H score (the product of percentage and staining intensity) to define their positivity.

Up to now, the conventional tissue biomarkers (ER, PgR, HER2 and Ki-67) are always evaluated in the clinical practice to define BC patients' prognosis and to predict the response to therapy also in TNBC subtype. Despite the experimental limitations due to the lack of reproducibility of PgR and Ki-67 evaluation intra and inter-laboratories, Zenzola et al. showed the prognostic value of Ki-67 in TNBC, using a cut-off point of 60%, depending on the patients age [70].

Recently, the evaluation of the immune components by using SP142 antibody for PD-L1 detection has been demonstrated to be important to select TNBC patients potentially responsive to immune checkpoint inhibitors [71]. However, the lack of precise guidelines in PD-L1 detection due to the different antibody used and platform lead to bias in patients selection and response to therapy.

Other methods to detect clinically useful biomarkers on tissue are the measure i) of the gene copy number (GCN) by Fluorescence in situ hybridization (FISH), ii) the mutational status by NGS and digital PCR approaches and iii) the in situ evaluation of mRNA by RNA scope. Some studies were conducted on TNBC by using in situ methods to evaluate gene copy number of

cMET [72] and *Topoisomerase II α* [73], but currently, in the clinical practice, FISH test is usually performed to assess the gene copy number of HER2 only.

2.2 Liquid biomarkers

Despite the great importance of the biomarkers detectable in primary and metastatic solid tumor tissues, the dynamic heterogeneity of cancers is a critical issue that impairs their value and should be always kept in mind. Dynamic heterogeneity can be described as the characteristic of the tumor to change genotypically and phenotypically during its own history, since its first stages, spatially and in time [74,75]. This event can yield solid masses composed of many cell clones that may be different in their genetic, transcriptomic, proteomic, and functional makeup. Dynamic heterogeneity, and in particular that of the most aggressive tumors such as TNBC, can be quite impressive. It has been shown that no two single cells from TNBCs have an identical genomic profile [76]. This suggests that studying singly or few biomarkers at few time points, e.g. at first diagnosis and/or relapse, could offer just a very limited actionable vision of the disease. Hence, monitoring tumor progression through a timely and accurate multi-marker detection is a crucial investigation opportunity. Since it is not always possible to accomplish this continuous follow up on solid tissues via conventional biopsy, liquid biopsy (LB) comes to our rescue. LB is primarily made up by circulating tumor cells (CTC) [77,78], circulating tumor DNA (ctDNA), ncRNA [79] and extracellular vesicles (EVs) [80-82]. Samples can be obtained from different sources, e.g., blood, urine, saliva, and at different time points, repeatedly. It has already shown a number of benefits: it is non-invasive, often fast and accurate, and can timely monitor markers during disease progression [83,84]. Liquid biopsy can shed light even on dynamic heterogeneity, enormously supporting tissue biopsy helping clinicians. For example, it can be hypothesized that, in the future, the well-timed detection of the mutational burden of a patient's disease through the analysis of CTCs and/or ctDNA could suggest the activity of an immune-therapeutic approach [85-88]. Liquid biopsy encompasses a broad number of potential applications: screening and monitoring, early diagnosis, tumor heterogeneity, drug resistance, and establishment of targets. Some of them have still to be defined and fully validated; others are already giving great help in the clinics [89]. Due to its intrinsic features, TNBC could be an ideal target to be investigated and monitored through liquid biopsy. Usually larger and with a higher grade than non-TNBC, more biologically aggressive, and with a lack of conventional molecular targets [90]. Probably the most critical issue to identifying actionable targets in this disease tumor solid tissue is the huge disease

heterogeneity both inter- and intra-tumor. So far, CTC and total cfDNA are the two liquid biopsy components that have been studied the most. They have been associated with the clinical outcome in breast cancer patients and in TNBC also, singly or combined [93-96], despite between these biomarkers there are significant differences. ctDNA may be detected more easily than CTCs, due to the higher ctDNA concentration in blood and to higher sensitivity and specificity in analytical methods presently available to study this factor. However, despite being extremely rare, CTC number in metastatic BC, including TNBC, has been correlated with prognosis, whereas baseline ctDNA levels were not [96]. ctDNA could be useful in identifying actionable mutations that could provide therapeutic targets. CTCs, probably in the near future, may perform a double role, as a prognostic and as a disease-characterizing marker. Finally, since different biological pathways release these elements, they may offer different clinically relevant answers and hence they should be utilized in association [97].

Regarding ctDNA, there are still few results in TNBC, and sometimes uneven. As a biomarker for the early detection of TNBC, ctDNA still needs investigation and validation. More specifically, ctDNA fraction and cfDNA total concentration seems to be related to breast cancer stage, significantly lower at earlier stages, and its use for the early diagnosis may be forecast [98]. In metastatic TNBC, the prognostic value of ctDNA was somehow contradictory. Madic et al. [96] found that the baseline ctDNA levels of patients with metastatic TNBC were not predictive of radiological tumor response and were not correlated with time to progression (TTP) or OS duration. On another hand, a high methylation index in cfDNA was associated with shorter median progression free survival (PFS) and OS [99]. Few mutated recurrent genes have been regularly identified in TNBC, e.g. *TP53*, *PIK3CA* [93], but some authors reported that ctDNA truly mirrors the mutational composition of individual CTCs in mBC and could monitor the metastatic burden for clinical decision-making [100]. Notwithstanding this uncertainty and the absence of fully defined guidelines for the use of ctDNA to monitor tumor progression or treatment response in TNBC patients, it is still believed that ctDNA can help in describing heterogeneity and metastasis-specific mutations providing an alternative to tumor tissue profiling [93].

In the face of present issues in analyzing single CTCs, i.e. rarity & heterogeneity among the others, it is believed that CTCs may be utilized to deepen the classification and the molecular heterogeneity of patients with TNBC [78], and in order to find specific actionable mutation. In order to better understand clinical management strategies, MRD monitoring, selection of targeted drugs and drug resistance pathways, the identification, recovery, and analysis of CTCs is becoming more and more feasible, even in early breast cancer [94,101]. CTCs detected in

early TNBCs are phenotypically heterogeneous in terms of hormonal receptors (HRs), EGFR and HER2 expression, pre- and post-adjuvant chemotherapy. HER2-pos CTCs seemed to appear more frequently during disease evolution [102]. Actually, CTCs may shed a light on the progression of cancer. Pestrin et al. [103], already in 2009, demonstrated that the HER2 status between primary tumors and CTCs in advanced breast cancer was concordant in only 68% of cases, shifting from HER2-neg to HER2-pos in the 29% of cases. This data suggests a sub-population of TNBC patients could have CTCs that can revert to a non-TNBC status and hence suitable for a challenge with HER2-targeted drugs. HER2-positive CTCs were detected in ductal in situ carcinoma or M0 BC regardless of the primary tumor HER2-status also by Ignatiadis et al [104]. Hence, probably even TNBC tissues could disseminate HER2-pos cells. Unfortunately, since a negligible percentage of patients with a HER2-negative primary tumor and HER2-positive CTCs received a benefit from an anti-HER2 therapeutic approach, trials designed on the base of CTC HER2-status were not recommended [105]. This negative result could have been due to a lack of sensitivity and specificity of the CTC detection method utilized, prompting a robust update of the technology and of the target investigated on CTCs. Years after these preliminary studies, we are still waiting for such an improvement. NGS techniques could be an excellent starting point to prompt this field. An NGS study of EpCAM-positive CTCs from mBC patients was able to show mutational heterogeneity in *PIK3CA*, *TP53*, *ESR1*, and *KRAS* genes between single CTCs. Corresponding primary tumor tissues did not harbor *ESR1* and *KRAS* mutations, thus implying either the detection of a tumor sub-clone or mutations acquired with disease progression. Such investigations suggest the feasibility of the monitoring of the TNBC metastatic burden and mutational makeup for clinical decision-making utilizing CTCs [100,106,107].

A new frontier of liquid biopsy, not least fascinating and promising, is the field of extracellular vesicles (EVs) and their content, e.g. miRNA and ncRNA. EVs are sub-micrometric particles potentially released from all of the prokaryotic and eukaryotic cells, delimited by a lipid bilayer and presenting various molecules, proteins, nucleic acid, lipid, on their surface or in their lumen. So far, EVs have been recognized as transporters of intercellular signals, thanks to their capacity to transfer the reported molecules from cell to cell. This shuttling activity is able to influence various physiological and pathological functions deeply, and this has prompted the meticulous research of the EV role in cancer. Among the EVs, the smaller ones (< 150 nm) have been defined as exosomes also [108]. TNBC exosomes also have been reported to be involved in cancer cell-to-cell communication. They are able to induce phenotypic traits to secondary cells that reflect those of their cells of origin, and to fostering the ability of TNBC

cells to produce exosomes containing proteins and miRNAs which induce malignant transformation [109,110]. In addition to conventional exosomal markers, such as CD9, CD63, and CD81, TNBC exosomes present some apparently specific antigens, including CD98, CD147, and CD59, and some overexpressed miRNAs (miR-134, miR-21, miR-373, and miR-1246) [111]. Researchers [110] showed that exosomes released by cancer cells could be classified depending on their miRNAs content. In agreement with others, this data suggest that exosomes and their miRNA content can be potentially applied in early diagnosis and staging of patients with TNBC. Other authors confirmed this assumption reporting that serum levels of deregulated exosomal miRNAs can be associated with clinic-pathological parameters [112], a more aggressive phenotype [113], worse prognosis, and with the capability to prompts the metastatic processes by stimulating an ill-fated macrophage polarization [114]. This information stresses the importance of studying exosomes and their content, in particular miRNAs, for a more comprehensive understanding of their potential to act as diagnostic, prognostic and therapeutic targets/agents [115-118].

3. Innovative strategies: the real fight against TNBC

Chemotherapy is currently the standard approach to treatment, and no targeted therapy has been approved yet. New treatments for TNBC are thus being sought. Immunotherapy is one option that is being investigated. TILs may represent an epiphenomenon of the relationship between the tumor and the immune system. An increased number of TILs, an important part of the BC microenvironment, characterizes TNBC. Despite the overall literature on this topic, TILs real role in TNBC evolution is not well known. PD-L1 shows a higher expression in TNBC than in other tumor subtypes (about 50%). In the last few years, some authors have found that PD-L1 expression correlates with hormone receptor-negative and triple-negative status and high levels of TILs [36], but the relationship between PD-L1- expressing TILs, cancer cells and other immunological features of the breast tumor microenvironment remains unclear. It is possible that PD-L1 expression also reflects an association with a TIL-mediated anti tumor inflammatory response rather than only being associated with tumor immune evasion. Wimberly and colleagues concluded that PD-L1 and TILs appear to be capable of predicting response to neoadjuvant chemotherapy in BC patients [36]. Hence, also in TNBCs similar investigation should be further performed to reveal predictive markers and improve overall disease management.

As TILs have been shown to have a prognostic and potentially predictive value, especially in triple-negative and HER2-positive infiltrating BCs [119] a standardized methodology to evaluate TILs has been developed [120]. A key immune modulatory pathway is mediated by Programmed Death receptor Ligand-1 (PD-L1), a surface protein that blocks the function of T lymphocytes. PD-L1 has been shown to be expressed on the tumor cell membrane, in cytoplasm and in immune cells including infiltrating T cells, B cells, macrophages and dendritic cells [28]. Moreover, the presence of PD-L1 in the stroma and cytoplasm has been associated with good prognosis, while no relation has been found between membranous PD-L1 expression and outcome [28]. Stromal immune cell expression of PD-L1 is not well documented in the literature. Treatment involving the use of an anti–PD-L1 could be considered because TNBC is highly mutagenic, producing neoantigens that induce an immune response. PD-L1 expression in tumor cells or its presence in the tumor microenvironment has been recently correlated with the presence of TILs [28,36]. Moreover, TNBCs express higher levels of PD-L1 than other BC subtypes, suggesting that the ligand could potentially represent a new therapeutic target in these tumors [121]. Indeed, immune checkpoint inhibition has been shown to be an effective anticancer strategy. There is ample evidence to support the use of immunotherapy in TNBC. A total of 174 TNBC patients, stratifying by stromal TILs were randomized in phase II double-blind placebo-controlled study randomizing to Durvalumab or placebo given every 4 weeks in addition to nab-paclitaxel followed by standard EC. In both arms, the authors observed a significantly increased pathologic complete response (pCR) rates with higher stromal TILs. There was a trend for increased pCR rates in PD-L1-positive tumors, which was significant for PD-L1-tumour-cell in Durvalumab and for PD-L1-immune cell in the placebo arm. These results suggested that the addition of durvalumab to anthracycline/taxane-based standard neoadjuvant chemotherapy increases pCR rate [122].

More recently, anti-PD-L1 combination therapies, nab-paclitaxel plus Atezolizumab, showed prolonged progression-free survival among patients with metastatic TNBC in both the intention-to-treat population and the PD-L1-positive subgroup [123] and on March 2019 the U.S. Food and Drug Administration (FDA) granted accelerated approval to atezolizumab (Tecentriq) plus nab-paclitaxel (Abraxane) for the treatment of patients with unresectable, locally advanced or metastatic, PD-L1–positive TNBC [71].

Another recent study aimed to explore the landscape of TNBC microenvironment using the largest original multi-omics dataset of TNBC (n = 386). The authors found that TILs and expression of immune checkpoint molecules are potential biomarkers for predicting the

therapeutic efficacy. In particular, they highlighted that immune checkpoint inhibitors might be effective for "immune-inflamed" cluster of TNBC patients [124].

The use of pembrolizumab in metastatic cancers with microsatellite instability or deficiency in DNA mismatch repair highlighted the central role of mutational burden in response to immune-checkpoint blockade. Regarding TNBC, recently, the results of the phase II KEYNOTE-086 study were published. In this study pembrolizumab was evaluated as second or later line of treatment for patients with mTNBC, showing a median PFS of 2.0 months (95% CI, 1.9-2.0), a median OS of 9.0 months (95% CI, 7.6-11.2), and a manageable safety profile [125].

As already reported, considerable heterogeneity exists among TNBCs and, whilst the majority show basal cell gene expression profiles, others resemble luminal tumors with Androgen Receptor (AR)-related gene expression. AR is widely expressed in BC, but its prognostic and predictive significance in invasive tumors is still very much open to debate [126-128]. Moreover, AR has been shown to play an oncogenic or oncosuppressive role in invasive BC, but its prognostic and predictive role in TNBC has not yet been revealed [126,127]. Furthermore, although it has been seen that androgens are involved in regulating the immune/inflammatory response in prostate cancer [129], there are still no conclusive data on the link between the two variables in TNBC. The availability of new anti-AR compounds such as apalutamide could open up new avenues of treatment for AR-positive TNBC. Lastly, Hilborn and colleagues recently reported that AR can predict tamoxifen treatment benefit in patients with ER-negative tumors or TNBC [130].

4. Conclusions

TNBC has a poor prognosis compared to other BC subtypes. So far, chemotherapy is currently the standard approach to treatment, and no targeted therapy has been approved. New treatments and strategies for TNBC are thus extremely necessary. In order to win the war against TNBC, all of the principal key factors and their interactions have to be cleared and followed during disease progression. Genetics, aging, exposome, microenvironment, and inflammation should be taken into consideration, striving to found innovative age- and inflammation-related pathways.

5. References

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