Critical Reviews in Oncology / Hematology 145 (2020) 102855

Contents lists available at ScienceDirect

Critical Reviews in Oncology / Hematology

journal homepage: www.elsevier.com/locate/critrevonc

Triple negative breast cancer: A thorough review of biomarkers

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ARTICLE INFO

Keywords:
Triple-negative breast cancer
Biomarkers
Tumor-infiltrating lymphocytes
PD-L1
BRCA mutation
Molecular target therapy

ABSTRACT

Triple-negative breast cancer (TNBC) is defined as a type of breast cancer with lack of expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 protein. The tumorigenesis is not likely to be driven by hormonal or HER2 pathway. In comparison to other types of breast cancer, TNBC stands out for its aggressive behavior, more prone to early recurrence. Historically, TNBC has been considered a disease with poor response to molecular target therapy, requiring better validation of biomarkers. Recent issues related to tumor heterogeneity have been widely discussed suggesting the subdivision of TNBC into different molecular subtypes. Through a complete research on the main published trials databases and platforms of ongoing clinical studies, the current manuscript was carried out in order to present a critical view of the role of immunohistochemical and molecular biomarkers for the prognosis and response prediction of TNBC to traditional therapy and new molecular target agents.

1. Introduction

Within the spectrum of breast cancer, triple negative breast cancer (TNBC) is known as a type of breast cancer in which there is a lack of expression of estrogen receptor (ER), progesterone receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) (Ryu et al., 2011). According to Globocan, the estimated incidence of breast cancer for 2018 was 2,088,849 new cases worldwide, considered the most incident tumor among women, excluding non-melanoma skin cancer (Changavi et al., 2015; Bray et al., 2018).

More common among specific ethnicities, such as Latin, African and African American women, TNBC accounts for approximately 10%–15% of all breast cancers (Jo et al., 2009). These tumors are also characterized by aggressive behavior, with trend to early relapse and metastatic spread to the lung, liver and central nervous system, as well as poorer survival. At diagnosis, patients usually present with clinically positive axillary lymph node, larger primary tumor size, pushing borders and poorer Nottingham prognostic index, calculated using pathological criteria and used to determine prognosis following surgery for breast cancer (Dent et al., 2007).

Tumor heterogeneity of TNBC has been widely pointed as the reason for different clinical outcomes, with diverse response rates either to traditional treatments or to new targeted therapies, often leading to discrepant times of survival. Some authors (Prat et al., 2010; Sørlie et al., 2001; Burstein et al., 2015; Lehmann et al., 2011) have used genomic expression profile (GEP) assays for molecular characterization of TNBC subgroups, defining their "molecular fingerprints". As shown in Fig. 1, intersections may occur between the proposed subclassification groups.

The most comprehensive and used subclassification was proposed by Lehmann et al. Lehmann et al. (2011), through a division of TNBCs into 7 molecular subtypes: immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor (LAR), unstable (UNS) subtype and two basal-like subtypes (BL1 and BL2). Thereafter, a subclassification refinement was performed to be defined in only 4 groups (BL1, BL2, M and LAR) based on a retrospective analysis of some clinical trials dataset (Lehmann et al., 2016). Based on this cluster analysis from both GEP and some additional information of immunohistochemistry (IHC), this subclassification can be used as prognostic and predictive tool and determine specifications for proof of concept that involves discovery of new drugs and design of clinical trials with better patient selection for personalized treatment to

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https://doi.org/10.1016/j.critrevonc.2019.102855
Received 14 August 2019; Received in revised form 1 December 2019; Accepted 2 December 2019

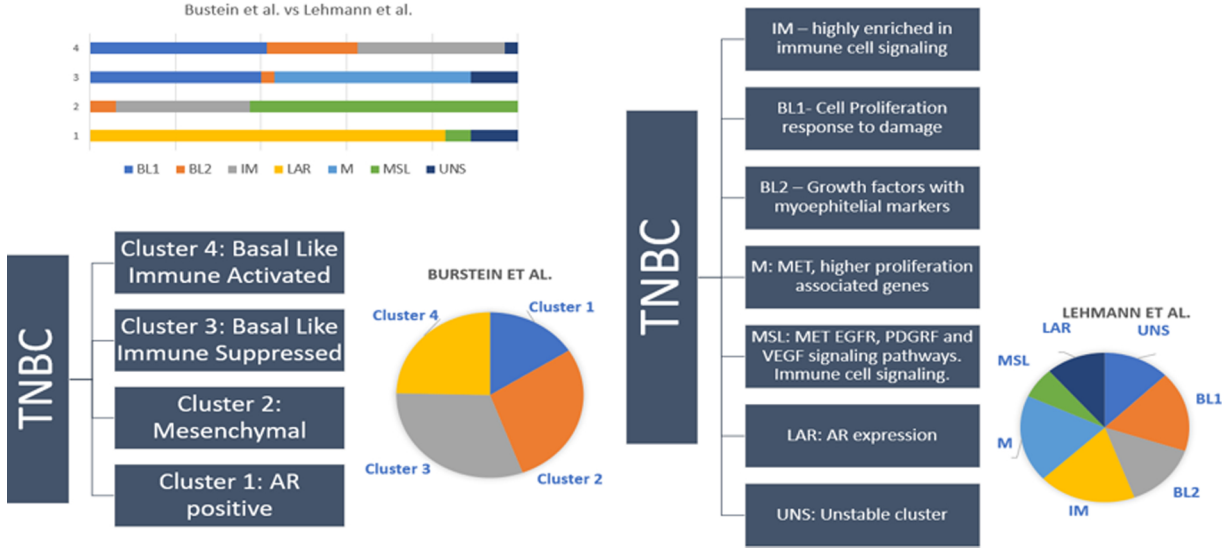

Fig. 1. Intersections of the subclassifications of Lehmann et al. and Burstein et al.
Lehmann et al. in 2011 proposed a division of TNBCs into 7 molecular subtypes: immunomodulatory (IM), mesenchymal (NI), mesenchymal stem-like {MSL): luminal androgen receptor {LAIR.). unstable (UNS) subtype and two basal-like subtypes (BL1 and BL2). in 2015: Burstein et al. used DNA profiling to identify TNBC subtypes: Cluster 1: luminal AR (AI), cluster 2: mesenchymal (MES), cluster 3: basal-like immunosuppressed (BLIS), and cluster 4: basal-like immune-activated (B LIA). Comparing the two classifications, cluster 1 contains all of Lehmann's LAR tumors and cluster 2 contains most of Lehmann's mesenchymal stem—like. Lehmann's basal-like 1 and basal-like 2 tumors are split between clusters 3 and 4, mesenchymal tumors reside in cluster 3, whereas the immunomodulatory tumors are distributed across clusters 2 and 4: which express common signaling pathways]

improve response and survival outcomes.

Biomarkers are defined as reproducibly quantifiable biological variables. In clinical oncology practice, they can be measured as parameters to predict survival or even response to a therapeutic intervention, as defined by the National Institutes of Health (FDA-NIH Biomarker Working Group, 2016). When used in translational research discussions, they can also refer to factors used for early diagnosis, monitoring of treatment, as well as to provide "personalized" drug information. The integration of biomarkers into clinical practice depends on laboratory and clinical validations through well-designed clinical trials.

A complete search of relevant literature was undertaken to identify suitable published papers from peer-reviewed journals that assessed reliable predictive and prognostic biomarkers in TNBC. From immunohistochemical testing to attempts to genomic expression profiling, several recent studies involving TNBC patients have identified new biological tumor factors as potential biomarkers, some with promising results while others with conflicting data. The present paper provides a critical overview of the vast universe of these biomarkers and their subclassifications.

1.1. TP53 gene, p53 protein and Ki-67

The tumor protein 53 gene (*TP53*) is located on chromosome 17 (17p13.1) and encodes the p53 protein, a transcription factor that suppresses tumor growth and is essential in the process of cellular response to DNA damage. When DNA damage occurs, there is increase in p53 transcription, causing cell cycle arrest and DNA repair or cell death. This is effectively established by interaction with targets such as p21, cycling dependent kinase (CDK), repair proteins (PARP, BRCA) and PTEN (Girardini et al., 2011; Walerych et al., 2012), (Di Agostino et al., 2006).

Literature data suggest that *TP53* mutation is most frequently found in malignant tumors, occurring in approximately 30% of cases of breast cancer and in 75%–80% of TNBCs, according to the Cancer Genome Atlas project (Cancer Genome Atlas Network, 2012). The mutant p53 protein may exert aberrant functions, interacting differently with downstream targets, upregulating CDK-1 and PI3K/AKT/mTOR pathway, downregulating tumor suppressing proteins such as p63 and p73, stimulating cell proliferation and thereafter evading apoptosis (Turner et al., 2013). The p53 protein expression in TNBC tumor tissues may vary according to the type of mutation occurring in the *TP53* gene: patients with missense mutations tend to show high p53 protein expression, since they normally produce a more stable full-length protein, in contrast to patients with deletion mutations, which do not express the protein (Yemelyanova et al., 2011).

Several studies have attempted to determine the impact of *TP53* mutation on TNBC patient prognosis. In a study with 174 TNBC tumors harboring a *TP53* mutation, low mRNA expression was associated with poor prognosis in patients with missense mutation, 5-year distant recurrence-free survival (5-years-DRFS: low vs high, 50.0% vs 87.8%; $p = 0.009$), however no significant association was observed in patients with deletion mutations (Kim et al., 2016). Analyzing p53 protein expression, data from a retrospective Kenyan population study revealed that women with node-negative TNBC with p53 expression by IHC presented significantly higher overall survival (OS) than patients without p53 expression (p53+ vs. p53-; Hazard Ratio [HR] 2.8; 95% confidence interval [CI]: 1.1–7.1, $p = 0.022$) (Breast cancer, 2016).

As an option for personalized therapies, although formerly p53 has been regarded as “undruggable”, recent studies have emerged with compounds that can selectively target the p53 mutant protein and restore its wild-type properties in breast cancer cells, such as PRIMA-1 and PRIMA-1^{Met} (p53 reactivation and induction of massive apoptosis 1) (Synnott et al., 2017). As PRIMA-1 and PRIMA-1^{Met} compounds have shown anticancer activity in TNBC cell lines of animal models and have not demonstrated evidence of major toxicities in a dose-finding phase 1 trial (Lehmann et al., 2012), they may be considered an attractive p53-targeted therapy for evaluation in larger clinical studies of patients with TNBC.

Over the years, research has developed several molecular techniques to measure cell proliferation rates. One of which is the quantification of proliferation-related membrane antigens by IHC. Ki-67, a protein encoded by the *MKI67* gene (marker of proliferation Ki-67 gene), is the most commonly used cell membrane antigen to determine cell proliferation, and is therefore considered a prognostic biomarker in breast cancer, although with an uncertain value in the context of

patients with TNBC (Viale et al., 2008; Blows et al., 2010). These tumors tend to have increased expression of Ki-67, with studies showing prevalence of 44.7%–53.4% of tumors with Ki-67 expression over 20% (Nakagawa et al., 2011).

Recently, results from a retrospective cohort that evaluated 363 women with operable early TBNCs through multivariate analysis have suggested that higher Ki-67 score is an independent risk factor for disease-free survival (DFS; RR 2.83, 95% CI: 1.58–5.06, $p < 0.001$) and OS (RR 3.18, 95% CI: 1.48–6.79, $p = 0.003$) (Wang et al., 2016a). In another cohort, Ki-67 was significantly correlated with the TNBC phenotype. The mean value was 44.7% and 22.2% in TNBC and non-TNBC patients, respectively, and higher scores were also associated with advanced stage ($p = 0.004$) and nodal involvement ($p = 0.033$) (Ilie et al., 2018).

1.2. – Epidermal growth factor receptor, c-KIT and cytokeratins (CK 5/6, CK 14, CK 17, CK 56)

Epidermal growth factor receptor (EGFR) is part of a transmembrane glycoprotein family with a tyrosine kinase domain that activate signal transduction pathways, playing an important role in cell proliferation and apoptosis inhibition (Lehmann et al., 2011). The prevalence of EGFR overexpression in TNBC cases is quite variable among studies, ranging from 13 to 78% (Gluz et al., 2009; Gumuskaya et al., 2010), due to lack of standardized measurement of IHC results and to a wide demographic variation. Although some results suggest a strong association of higher *EGFR* gene copy number with poor survival, higher tumor grade and axillary lymph node metastasis, data from EGFR protein overexpression in triple-negative is controversial and it has not been confirmed as a prognostic biomarker (Park et al., 2014; Nakajima and Ishikawa, 2012; Liu et al., 2012).

In a cohort with 287 patients with TNBC, 36.2% were positive for EGFR, and multivariate analysis indicated that EGFR was a significant independent prognostic factor in terms of DFS ($p = 0.011$) with poorer outcomes in all patients (Liu et al., 2012). Although EGFR overexpression is common in metastatic TNBC, phase 2 studies evaluating the efficacy of EGFR inhibitors such as tyrosine kinase inhibitors (TKIs, gefitinib, afatinib and erlotinib) and monoclonal antibodies (cetuximab and panitumumab) have not shown any effective results (Carey et al., 2012; Albanell et al., 2019; Dickler et al., 2009).

Signaling by c-KIT (CD117) is likely to play an important role in cell transformation and differentiation. The c-KIT protein expression is detected in approximately half of TNBC tumor tissues (Jansson et al., 2014). The aberrant activation of the *c-KIT* gene is part of the process of carcinogenesis and metastatic mechanisms of various human malignancies. Studying hyperactivation and alterations of the c-KIT pathway is highly interesting because it is potentially amenable to TKI treatment with imatinib, which is already traditionally used in the treatment of some onco-hematological diseases such as chronic myeloid leukemia as well as in solid tumors such as Gastrointestinal Stromal Tumors (GISTs) and dermatofibrosarcoma protuberans (Shams and Shams). In a retrospective study of 58 patients with TNBCs, the positive status of c-KIT by IHC was associated with *TP53* missense mutations ($p = 0.031$), vascular invasion, recurrence and higher Ki-67 proliferation index. The presence of c-KIT protein expression and *TP53* missense mutations together in the primary tumors was an independent prognostic factor for worse survival (Luo et al., 2019). In another cohort with 653 TNBCs, c-KIT positive staining was accompanied by decreased OS ($p = 0.036$) (Thike et al., 2010).

Basal like breast cancer is considered an aggressive subtype of TNBC and usually express basal cytokeratins like CK 5/6, CK 14 and CK 17 (Cheang et al., 2008). CK 5/6 expression ranges from 24% to 72% in TNBC (Ryu et al., 2011; Gokoz et al., 2010). Some results suggested that CK 5/6 positive TNBC have poorer prognosis independent of well-known clinical-pathological features (Nielsen et al., 1989; van de Rijn et al., 2002). Other results reported that CK 5/6 also have a positive correlation with nodal metastasis and tumor size (Inanc et al., 2014). On the other hand, a cohort with 150 patients failed to determine a correlation of CK 5/6 expression with ominous clinicopathological features, probably due to the low expression of CK 5/6 in that sample (Hashmi et al., 2018). Overall, the expression of CK 14 and CK 17 overlaps with CK 5/6. The CK 17 alone was not identified as a reliable prognostic biomarker42]. In a cross-sectional study of 150 patients with TNBC, CK 14 expression was positive in 50.8% of the cases(Lesar et al., 2016), whereas in a study with Asian women it was around 39.6% (YANXI et al., 2016).

1.3. - Vascular endothelial growth factor (VEGF)

The signaling of angiogenesis, mediated by vascular endothelial growth factor (VEGF), is crucial in the process of growth and tumor spreading. VEGF comprises a family of 6 proteins: VEGF-A, B, C, D, E and placental growth factor. The alternative splicing of mRNA creates 4 isoforms, the most common being VEGF165. The mediators of gene expression are hypoxia, growth factor, nitric oxide, oncogenes, HER2 and tumor suppressor genes (Holmes and Zachary, 2005; Gerwins et al., 2000). VEGF is highly expressed in around 30–60% of patients with TNBC (Linderholm et al., 2009). Along with VEGF, the scoring of microvascular density by IHC has been used as a prognostic biomarker in TNBC. Conceptually, a high mean vascular density in breast cancer has been associated with dismal prognosis and worsening survival (Chanana et al., 2014; Ali et al., 2011).

In a retrospective cohort evaluating Swedish women with operable breast cancer, the mean serum VEGF level of 87 patients with TNBC was statistically higher than that of non-TNBC patients. Comparing the two groups, 62% of patients with TNBC had a VEGF expression higher than the median value, while only 47% non-TNBC patients had a higher expression of the biomarker ($p = 0.036$) (Linderholm et al., 2009). Likewise, in a small clinical trial, 60 patients with metastatic breast cancer were treated with a low-dose metronomic therapy with capecitabine and cyclophosphamide. In the patients with partial and completed response, the VEGF levels was declined after 2 and 6 months of treatment ($p = 0.001$) (El-Arab et al., 2012). In another study that recruited 41 Egyptian women with metastatic TNBC, the VEGF-A presented higher level in patients with larger tumors compared to those with smaller ones ($p = 0.053$), as well as in cases of progressive disease compared to those who had partial response or stable disease ($p = 0.016$) (Taha et al., 2009).

VEGF has been widely studied as a target for treatment of TNBC in several studies evaluating efficacy of bevacizumab, the humanized monoclonal antibody of VEGF-A. Among 663 patients with triple-negative tumors enrolled in a clinical trial, the addition of bevacizumab to neoadjuvant chemotherapy significantly increased the rate of pathological complete response (pCR) from 27.9%–39.3% ($p = 0.003$) (Bucherini et al., 2012). Likewise, 46 patients with metastatic TNBC enrolled in a phase 2 trial had overall response rate (ORR) of 65.2% (95% CI: 52.9 %–80.4 %) (Saloustros et al., 2018). Despite showing increased progression-free survival (PFS), three phase 3 studies (Miller et al., 2007; Pivot et al., 2010; Robert et al., 2011) failed to show a statistically significant improvement in OS. Also, regarding adjuvant setting, bevacizumab did not show any benefit in survival outcomes (Cameron et al., 2013). Other therapies with monoclonal antibodies or anti-VEGF TKIs have not demonstrated benefit in this setting (Ribatti et al., 2016).

1.4. – Androgen receptor

Androgen receptor (AR) is part of a complex of steroidal hormone receptors that modulate transcription factors, controlling gene expression in different cellular processes, sometimes in a dualistic way. AR can both stimulate proliferation and dedifferentiation and induce apoptosis and cell death, depending on the simultaneously activated

signaling pathways. Although early studies have suggested a negative prognostic effect of AR in TNBC, the latest data have reaffirmed that patients with AR-positive TBNC have a more favorable outcome. The immunohistochemical expression of AR in TNBC may vary widely between 10–90 % according to the cohort (Niemeier et al., 2010; He et al., 2012; Galal et al., 2013; Gucalp and Traina, 2016).

A large systematic review with meta-analysis assessed pooled data from 13 clinical trials that recruited 2826 patients with TNBC from 2007 to 2015. Herein, 24.4% of the cases were AR-positive, and it was associated with low tumor grade (40.8% of patients AR-positive), and post-menopausal status (26.9% of AR-positive patients) and lower risk of nodal involvement (28.8% of AR-positive patients) (Wang et al., 2016b). Likewise, other data suggested that patients with more aggressive TNBC were negative for AR, whereas cases with higher AR expression were associated with early-clinical staging disease, low Ki-67 and low nuclear grade (Gasparini et al., 2014; McNamara et al., 2013; Maeda et al., 2016). As for the pooled analysis of 12 studies grouping 5270 women with TNBC, AR-positive group had 48% reduction of risk of progression or death compared to AR-negative patients (DFS HR 0.52; 95% CI: 0.43-0.64) (Qu et al., 2013). Similarly, in other meta-analysis with 521 TNBC patients, the odds ratio for DFS was 0.44 ($p = 0.002$) (Kim et al., 2015). However, there was no correlation of AR status with OS outcome in any of these studies.

Regarding the predictive value of AR status in TNBC, there are some evidence that AR-positive patients are more likely to be chemo-resistant than AR-negative patients. Analysis of 637 core biopsy samples from primary tumors of patients enrolled in Gepartrio trial showed that pCR was 12.8% in AR-positive breast cancer compared to 25.4% in AR-negative ones ($p < 0.0001$) (Hilborn et al., 2016). Similar results were observed in a Japanese retrospective cohort, in which AR-positive TNBCs presented lower rate of pCR than AR-negative in a univariate analysis (HR 5.26; 95% CI: 1.39–19.86, $p = 0.014$) (Asano et al., 2016).

Some clinical trials have evaluated the efficacy and safety of anti-androgens in patients with advanced or metastatic TNBC. A single-arm study with bicalutamide as monotherapy in heavily treated patients showed a clinical benefit rate (CBR) of 19% with a median PFS of 12 weeks (range 6.25–57.5 months) (Gucalp and Traina, 2016). Furthermore, a phase 2 trial with patients treated with single-agent enzalutamide AR-positive TNBC showed a CBR at 16 and 24 weeks of 35% and 29%, respectively (Traina et al., 2018). And finally, in a phase 2 study with monotherapy abiraterone, the CBR was 20% and median PFS was 2.8 months with manageable adverse events (Bonnefoi et al., 2016).

1.5. - Homologous recombination deficiency (HRD) and BRCA 1/2 mutations

All cells in the human body undergo constant external aggressions to the DNA apparatus. However, they rely on efficient DNA damage response (DDR) machinery. Double-strands breaks are severe forms of damage and are repaired by two main pathways: error-free homologous recombination and nonhomologous end-joining (NHEJ) (Jasin and Rothstein, 2013; Anon, 2019a). Initially described in patients with *BRCA1 and BRCA2* gene mutations, homologous recombination deficiency (HRD) can occur in sporadic cancers through genetic and epigenetic inactivation of other components (PALB2, BARD1, BRIP1, RAD51B, RAD51C, RAD51D, ATM, FAAP20, CHEK2, FAN1, FANCE, FANCM, and POLQ), a condition defined as “BRCAness” (Lord and Ashworth, 2016). Homologous recombination-deficient (HRd) tumors is likely to be more sensitive to platinum chemotherapy as well as to inhibitors of the DNA repair enzyme poly-ADP ribose polymerase 1 (PARP1) (Tan et al., 2008; Underhill et al., 2011).

Germline *BRCA 1/2* mutations are present in approximately 14–20 % of TNBCs, but a larger proportion of patients have been reported to harbor HRD (Couch et al., 2015; Sharma et al., 2014; Telli et al., 2016). In a retrospective study of 45 patients with TNBC, among *BRCA1/2* wild-type patients, HRd patients were more likely to achieve pCR (OR 16; 95% CI: 1.65–160.41, $p = 0.0041$) compared with non-HRd patients (Telli et al., 2018). Likewise, in a cohort of 77 patients with TNBC, 19.5% were *BRCA* mutated (*BRCA*m), and the 5-year recurrence-free survival (RFS) estimates were 51.7% for *BRCA* wild-type (*BRCA*wt) versus 86.2% for *BRCA*m, ($p = 0.031$) and 5-year OS estimates were 52.8% and 73.3% ($p = 0.225$) for *BRCA*wt and *BRCA*m, respectively (Gonzalez-Angulo et al., 2011).

A phase 3 study evaluated the role of the addition of the PARP inhibitor veliparib plus carboplatin to standard neoadjuvant chemotherapy in patients with TNBC. The pCR rate was significantly higher in the paclitaxel, carboplatin, and veliparib group than in patients receiving paclitaxel alone (53% of 316 patients vs 31% of 158, $p < 00,001$), but not when compared with the population that receiving paclitaxel plus carboplatin without the PARP inhibitor (58% of 160 patients, $p = 0,36$). (Loibl et al., 2018). Recently, a phase 3 trial compared olaparib monotherapy with standard therapy in patients with germline *BRCA*m HER2-negative metastatic breast cancer previously treated with two or more chemotherapy regimens. Of the 150 patients with TNBC, the HR for PFS was 0.43 (95% CI: 0.29-0.63) in favor of olaparib monotherapy (Robson et al., 2017).

In addition to deleterious mutations of BRCA and HRD profile, the genomic instability has also been assessed by techniques such as loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large-scale state transitions (LST) (Abkevich et al., 2012; Birkbak et al., 2012; Sastre-Garau et al., 2012). A phase 2 trial evaluated the efficacy of gemcitabine, carboplatin, and iniparib as neoadjuvant therapy for BRCAm patients with TNBC. The overall pCR rate in the intention-to-treat population ($n = 80$) was 36% (90% CI: 27–46) and the mean HRD-LOH scores were higher in responders compared with non-responders ($p = 0.02$) (Telli et al., 2015).

1.6. - Tumor-infiltrating lymphocytes and PD-L1/PD-1 expression

More recent studies have focused on the tumor microenvironment as a determinant of survival, invasiveness and metastasis in cases of TNBC. Normal breast tissue generally does not contain immune cells, but breast tumor tissue and surrounding stroma may contain higher levels of immune cell infiltrates (Degnim et al., 2014). There is growing evidence of the role of tumor lymphocytic immune infiltrates in TNBC. The immunoassay concept hypothesizes that host immunity, depending on peritumoral and intra-tumoral composition, can either stimulate tumor growth or eradicate the disease, grounding the definition of immune evasion and immunogenicity, respectively. Following this idea, tumor cells are initially rejected by the immune system, then remaining surviving tumor cells persist in a state of dormancy and, after upregulating pro-survival pathways, express molecules that promote immune suppression and angiogenesis. Herein, elimination, equilibrium and escape phases make up the three stages of immunoediting (Cancer Immunoediting, 2019).

CD4 and CD8 T-helper lymphocytes are part of the pro-inflammatory complex of type 1 immunity needed to eliminate tumor cells. Both innate immune system (neutrophils, monocytes, macrophages, antigen-presenting cells) and adaptive system cells (B and T-lymphocytes) are critical to prompt recognition and response to pathogens as well as non-self-cells or tumor antigens. Many antigens present in the cell membrane of breast cancer can activate and stimulate T-cells, inducing regulatory immune response. However, the ability of immune suppression is critical for the survival of normal cells (Society and Clinical, 2015).

Tumor-infiltrating lymphocytes (TILs) are highly expressed in approximately 20% of TNBC cases. Some studies have suggested that TILs in breast cancer may be a surrogate biomarker for adaptive immune response, especially for the TNBC subtype, considered to be one of the most immunogenic. There is a consensus that the cytotoxic effect of chemotherapy is partially influenced by the immune reaction against

tumor cells. In the same way, chemotherapy may provide a better immune response by modifying the microenvironment, as well as increasing tumor immunogenicity, leading to tumor shrinkage (DeNardo and Coussens, 2007; Schmidt et al., 2008).

In a neoadjuvant anthracycline and taxane-based studies, the pCR rates in lymphocyte-predominant breast cancer were greater than in tumors without any TILs, 42% and 3%, respectively (Denkert et al., 2010). Also in a meta-analysis that included neoadjuvant chemotherapy studies, TNBC with higher CD8+ and lower FoxP3+ T-lymphocyte levels was associated with better pCR rates (OR 2.49; 95% CI: 1.16–3.83) (Mao et al., 2014). For 278 cases of TNBC operated on with residual disease following neoadjuvant treatment evaluated in a multicenter restrictive cohort, the 5-year OS rate was 91% (95% CI: 68 %–97 %) for high-TIL patients (n = 27) and 55% (95% CI: 48 %–61 %) for low-TIL patients (HR 0.19; 95% CI: 0.06-0.61, p = 0.0017) (Dieci et al., 2014). Similarly, in a pooled analysis of the TNBC cases of two phase III randomized adjuvant breast cancer trials (ECOG 2197 and ECOG 1199), for every 10% increase in TILs, a 14% reduction of risk of recurrence or death (p = 0.02), 18% reduction of risk of distant recurrence (p = 0.04), and 19% reduction of risk of death (p = 0.01) were observed (Adams et al., 2014).

The anti-tumor activity of checkpoint immune inhibitors has been extensively studied in TNBC. Programmed cell death protein 1 (PD-1) is an immune checkpoint receptor that limits the action of T-cell effector within tissues, playing a crucial role in the tumor immune evasion process. The two ligands of PD-1, with distinct expression profiles in tumor types, are PD-L1 and PD-L2 (Ishida et al., 1992). The regulation of PD-L1 can occur by several processes: response to IFN-gamma action, oncogenic signaling, deletion or silencing of PTEN with consequent overexpression of the PI3K pathway (Castaneda et al., 2016a).

Immunotherapy with PD-1 and PD-L1 inhibitors results in the activation of T-cells, restoring the host's anti-tumor immune activity, demonstrating long-lasting activity and increased survival in selected tumors. Due to differences in the methods of detection, sampling and tumor size, the expression rate of PD-1/PD-L1 is variable in several studies (Ghebeh et al., 2006). Through analysis by IHC in patients with TNBC, PD-L1 have been reported between 15.8% and 30% of cases (Ghebeh et al., 2006; Castaneda et al., 2016b; Beckers et al., 2016). In this setting, PD-L1 scoring has been strongly used as a predictive biomarker and can be measured on tumor cells (TC) or on tumor infiltrating immune cells (IC). The in situ mRNA hybridization has been detected in PD-L1 mRNA in 55%–60 % of tissue microarrays (Schalper et al., 2014).

In a large retrospective cohort involving patients with breast cancer, PD-L1 upregulation, detected through mRNA analysis, was associated with poor prognostic features (large tumor size, ER-negative, PR-negative, HER2-positive status, high proliferation, basal and HER2-enriched subtypes), with a higher pCR (50% vs 21%) and a 5-year metastasis-free survival rate of 61% (95% CI: 0.58-0.64) (Sabatier et al., 2015). Similarly, another retrospective study showed a positive significant association between the PD-L1 ≥ 25% status and the achievement of pCR (p = 0.024) (Cerbelli et al., 2017).

In a phase 1 trial of patients with TNBC treated with single-agent atezolizumab, the overall response rate (ORR) for patients with PD-L1 ≥ 5% of IC (as also called IC2/3) were 17% versus 8% for PD-L1 < 5% (defined as IC0/1). The group with over 10% of TILs score or with more CD8 + cells in primary tumor was also more likely to have higher ORR and longer OS (Schmid et al., 2017). Recently, the combination atezolizumab and nab-paclitaxel was evaluated as first-line therapy for TNBC in a large phase 3 study. Among patients with PD-L1 positive tumors (defined as PD-L1 ≥ 1% on IC), the median PFS was 7.5 months vs 5 months in PD-L1 negative (HR 0.62; 95% CI: 0.49-0.78; p < 0.001) and the median OS was 25.5 and 15.5 months, respectively (HR 0.62; 95% CI: 0.45-0.86) (Emens et al., 2018; IMpassion130 Substudy, 20192019). Table 1 presents the main studies with TNBC immunotherapy.

Table 1
Summary of phase 3 trials of immunotherapy in TNBC.

Trial name	Line of therapy and population	Intervention	Control	Comments
IMpassion 130	First-line therapy - previous (neo)adjuvant chemotherapy allowed only if treatment was completed ≥12 months (mo) before randomization	Nab-paclitaxel plus atezolizumab	Nab-paclitaxel plus placebo	Published 2018 – median PFS ITT pts 7.2 mo vs 5.5 mo (HR 0.8, p = 0.002). In the PD-L1 + pts: 7.5 mo vs 5.0 mo (HR 0.62, p < 0.001). ASCO 2019 update: median OS in PD-L1 + pts with atezolizumab + nP 25.0 mo vs placebo + nP 18.0 mo (HR 0.71, 95% CI: 0.54 - 0.93) (IMpassion130 Substudy, 2019; van Schooneveld et al., 2015).
IMpassion 131	First Line treatment	Paclitaxel plus atezolizumab	Paclitaxel plus placebo	Estimated Completion date 2021. (*NCT 03125902*)
IMpassion 132	First Line early recurrence <12mo after curative treatment	Chemotherapy plus atezolizumab	Chemotherapy plus placebo	Estimated completion data 2021. (*NCT 03371017*)
KEYNOTE 119	Previously treated second or third line	Pembrolizumab	Physician's choice	Estimated completion data 2019. (*NCT 02555657*)
KEYNOTE 355	First Line	Pembrolizumab plus chemotherapy	Placebo plus Chemotherapy	Estimated completion data 2019. (*NCT 02819518*)

1.7. – microRNAs and long non-coding RNAs

MicroRNA (miRNA; miR) expression signatures have been studied for both early diagnosis and treatment monitoring, through intense search for reliable early-stage blood biomarkers in TNBC. Defined as small noncoding RNA molecules of 17–27 nucleotides in length, miRNAs exhibit regulatory function in the expression of multiple genes through the stimulation or degradation of mRNA targets. Noncoding RNAs are functional RNA molecules that are transcribed from the DNA, but not classified as protein-coding due to the lack of the long open reading frames (ORFs), and these include the miRNAs, the small interfering RNAs (siRNAs), the piwi-interacting RNAs (piRNAs) and the long non-coding RNAs (lncRNAs). Due to its stability, miRNAs may be advantageously studied in non-invasive samples, such as blood, serum and urine (van Schooneveld et al., 2015).

Specific miRNAs could serve as potential prognostic biomarkers in TNBC. In a recent meta-analysis with 21 relevant studies, reduced expression of miR-155 and higher miR-21 expression was associated with poorer OS (crude HR 1.49; 95% CI: 1.26–1.72 and crude HR 2.50; 95% CI: 1.56–4.01, respectively) (Lü et al., 2017). A pilot study with blood-borne miRNA signatures from 21 basal-like TNBC cases treated with neoadjuvant therapy highlighted 321 miRNAs that were deregulated when comparing expressions pre and post-treatment (among them, miR-34a, with p-value <00,001), and also found that complete responders showed a tendency to have higher miRNA levels after platinum-based neoadjuvant chemotherapy, suggesting that changes in miRNA expression during treatment may have predictive value in pCR (Kahraman et al., 2018).

The miR-34 family members miR-34a, miR-34b and miR-34c have been among the most studied miRNAs in TNBC and have shown multiple roles as biomarkers (Malla et al., 2019). Mir-34a have been associated with attenuation of tumor growth in TNBC and miR-34c with worse prognosis (Adams et al., 2016; Anon, 2019b). As several studies have shown that miR-34a acts as a tumor suppressor, inducing cell cycle arrest, apoptosis and senescence in cancer cell lines, multiple authors have investigated its potential as a target for therapy and, recently, a liposomal miR-34a mimic formulation called MRX34 have been evaluated in patients with advanced solid tumors (Imani et al., 2018). By comprehensive analyses of miRNAs expression data from TCGA database, a study suggested that higher levels of miR-135b-5p, miR-9-3p and miR-135b-3p have favorable prognosis, despite high expression of miR- 455-5p exhibited poor prognosis in TNBC (Bao et al., 2019).

LncRNAs are transcripts with lengths exceeding 200 nucleotides that may not be translated into proteins. They modulate transcription of protein-coding genes by association with proteins to regulate their functions and control RNA maturation and transport (Zhang et al., 2017). Like miRNAs, they also perform regulatory functions in various hallmarks of cancer biology. LncRNAs are disordered in many cancer types, including TNBC (Matouk et al., 2009). Considered as presumed biomarkers, a set of lncRNA are pointed out in some recent studies as consistently aberrantly expressed in TNBC, and may be involved in both carcinogenic process and progression (Augoff et al., 2012).

A lncRNA, known as long non-coding RNA in non-homologous end joining pathway 1 (LINP1), was found to be overexpressed in TNBC. LINP1 enhances double-strand DNA break repair by serving as a scaffold to form a synaptic complex joining the broken DNA ends and promoting its repair. LINP1 blockade may therefore increase the sensitivity of TNBC to radiotherapy (Sakthianandeswaren et al., 2018). Another lncRNA, termed long intergenic noncoding RNA for regulator of reprogramming (lincRNA-RoR), is overexpressed in TNBC and serves as a competitive endogenous RNA for miR-145, a regulator of embryonic stem cell renewal, suggesting that lincRNA-RoR may be a critical factor for TNBC metastasis and could serve as a biomarkers or therapeutic target for TNBC therapy, as well as miR-145 (Eades et al., 2014). The lncRNA known as highly up-regulated in liver cancer (HULC) is also upregulated in TNBC tissues and cell lines and correlates with poorer clinical outcomes and might be a potential therapeutic target in TNBC (Shi et al., 2016).

The metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a highly conserved lncRNA, known to regulate gene expression by modulating transcription and posttranscriptional RNA processing. Recent pre-clinical studies have pointed out MALAT1 as a potential biomarker in TNBC, helping to predict prognosis and metastasis (Zhao et al., 2014). Long intergenic noncoding RNA for kinase activation (LINK-A) is critical for growth factor-induced normoxic HIF1α signaling pathway, also being a promising therapeutic target in TNBC (Lin et al., 2016). The HOX transcript antisense intergenic lncRNA (HOTAIR) has significant role in tumorigenesis with increased expression in TNBC. Combination treatment with lapatinib and imatinib repressed HOTAIR expression in TNBC cells through inhibition of β-catenin and inhibited cell growth (Wang et al., 2015).

1.8. Promising molecular biomarkers

Tumor heterogeneity, biopsy sampling, tissue processing and storage are serious limitations of the histopathological analysis by IHC that may impair the subtyping and even the diagnostic accuracy, leading up to poorer outcomes. Therefore, some molecular biomarkers have been explored to guide treatment and improve the approach of patients with TNBC (Andreopoulou et al., 2017; Srinivasan et al., 2002). Plasma circulating tumor DNA (ctDNA), which is obtained through "liquid

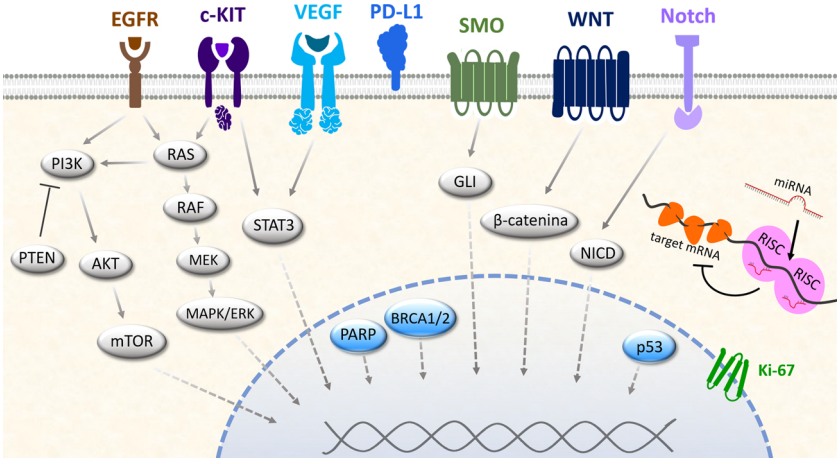

Fig. 2. Key signal transduction mechanisms in TNBC tumorigenesis.

Table 2
Summary of prognostic biomarkers in triple negative breast cancer.

Molecular Biomarker	% of TNBC with expression/mutations	Main Function	Prognostic significance	Targeted therapies	References
TP53 gene	75-80% (somatic mutations)	Apoptosis	Low gene expression in *TP53* missense mutations correlate with poor prognosis (worst DFS, but conflicting data).	NA	Kim et al., 2016; (Kim et al., 2016) ; Synnott et al., 2017; (Synnott et al., 2017)
Ki-67	45-53% (high expression, > 20%)	Cell proliferation	High index and high expression correlate with shorter DFS and OS.	NA	Wang et al., 2016a,2016b;(Wang et al., 2016a) ; Ilie et al., 2018; (Ilie et al., 2018)
EGFR	13–78 %	Cellular growth	Increased expression associates with worst DFS.	Erlotinib, gefitinib, afatinib	Gumuskaya et al., 2016 (Gumuskaya et al., 2010); Gluz et al., 2009; (Porta et al., 2014)[p]
c-KIT	50%	Cell transformation and differentiation	Predictor of poor cancer-specific survival in patients with TNBC	Imatinib	Kashiwagi et al., 2013 (Sørlie et al., 2001); Jansson et al., 2014; (Jansson et al., 2014)
VEGF	32 %−62 %	Angiogenesis	High levels associate with disease progression and metastasis rates.	Bevacizumab	Linderholm et al., 2009; (Ali et al., 2011); Ali et al., 2011; (El-Arab et al., 2012)
Androgen receptor	10-55%	Cell proliferation and dedifferentiation	Positive expression correlates with higher DFS. May be associated with chemo-resistance.	Bicalutamide, enzalutamide, abiraterone	Niemeier et al., 2010; (He et al., 2012)[p]; He et al., 2012; (Galal et al., 2013); Gucalp and Traina, 2016; (Wang et al., 2016b)
BRCA1 and *BRCA2* genes	14–20 % (germline mutations)	DNA-double strand break repair	Mutated status correlates with increased DFS.	PARP inhibitors - Olaparib	Gonzalez-Ângulo et al., 2012; (Loibl et al., 2018); Robson et al., 2019 (Abkevich et al., 2012)
PD-L1 protein	15-30%	Tumor immune evasion process	High expression correlates with a higher survival rates in trials with checkpoint inhibitors	Immune Checkpoint inhibitor - atezolizumab	Castaneda et al., 2016a,2016b (Ghebeh et al., 2006); Ghebeh et al., 2006; (Castaneda et al., 2016b); Beckers et al., 2016; (Schalper et al., 2014);
Notch pathway	~10%	Cell Proliferation and differentiation	Potential target under development	AL101	Speiser et al., 2013(Mathe et al., 2016); Broner et al., 2019; (Pineda et al., 2018)
PI3-kinase pathway	~25%	Cell Proliferation	Multiple genomic alterations lead to activated PI3-Kinase pathway, including activation in PIK3CA, AKT and mTOR or inactivation in tumor suppressor genes such as PTEN,	PI3K inhibitor - alpelisib AKT Inhibitors – ipatasertib, capivasertib	Baselga, 2011 (Speiser et al., 2013); Pascual and Turner, 2019(Baselga, 2011); Kim et al., 2017 (Porta et al., 2014)

NA: not applicable; DFS: disease free survival; OS: overall survival.

biopsy" by blood sampling, provides genetic information not only from the primary tumor but also from the metastatic disease (Diaz and Bardelli, 2014). Besides being a non-invasive method, the ctDNA can potentially provide timely and comprehensively information, laying the groundwork for real-time disease monitoring, enabling more accurate prognostics evaluation and even early therapy modification (Olsson et al., 2015; Iqbal et al., 2015).

The prognostic value of ctDNA is still under evaluation in solid tumors and its validation in TNBC is urgently needed. In a small study of 36 TNBC patients using digital PCR for detection of *TP53* mutations by liquid biopsy, one patient with rising ctDNA levels experienced tumor progression during neoadjuvant chemotherapy (Riva et al., 2017). Next-generation sequencing was used in matched tumors in other study of 38 patients with early-stage TNBC. Patients with detectable ctDNA had poorer disease free survival (Chen et al., 2017). Other study was negative in proving the prognostic and predictive value of ctDNA for metastatic TNBC (J. M et al., 2015).

The prognostic utility of circulating tumor cells (CTC) for stratification of patients with stage metastatic disease was evaluated in the large multicenter cohort with 358 TNBCs. Patients with score <5 CTC/7.5 ml had longer median overall survival than those with score ≥ 5 CTC/7.5 ml (23.8 months vs. 9.0 months, $p < 0.0001$) (Cristofanilli et al., 2019). CTC resistant to anoikis, a type of apoptosis in which triggering occurs after the cell detaches from the native extracellular matrix, can predispose to metastasis, particularly in TNBC. Some studies point to the manipulation of some genes and molecules such as miRs may advantageously influence the sensitivity to anoikis (Tajbakhsh et al., 2019).

DNA methylation loci is another strong candidate to biomarker for TNBC, since they are more stable than RNA and is readily detectable in tissue samples and blood (How Kit et al., 2012). Many patterns of methylation have been assessed in different subtypes of breast cancer. In a small cohort of 39 patients, the presence of the BRCA1 promoter methylation was associated with poorer OS and RFS in TNBC (Sharma et al., 2014). Using whole genome DNA methylation analysis, another study proposed signatures that divided TNBC into three prognostic subgroups with different methylated regions (DMRs) (Stirzaker et al., 2015). Other cohort with 23 primary TNBC samples highlighted the important role that DNA methylation plays in the altered gene expression of TNBC-specific genes and lymph node metastases (Mathe et al., 2016). Using pre-treatment samples in an attempt to identify an epigenomic signature that could predict higher pCR rates after neoadjuvant chemotherapy in patients with TNBC, one study validated a hypermethylation pattern of 2 specific genes that accurately can predict response in TNBC (Pineda et al., 2018). However, further validation in prospective trials is warranted.

Notch signaling may be a potential target for the treatment of breast cancer. In the mammalian system, there are four Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4). Notch activates many genes associated with differentiation and/or survival, including, transcription factors, cyclin D1 and c-Myc. Notch-1 and Notch-4 expression has particularly been associated with the triple negative subtype of breast cancer (Speiser et al., 2013). This motivated the development of Notch inhibitors, including AL101, a pan-Notch gamma secretase inhibitor, with preclinical data with interesting results supporting the design of clinical trials of AL101 as a targeted therapy for TNBC with a hyperactivated Notch pathway (Broner et al., 2019).

The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR)-dependent pathway is one of the most important pathways associated with cell metabolism, proliferation, differentiation, and survival. When deregulated, they play a crucial role during the tumorigenesis process, interacting with other signaling processes, also causing resistance to therapies. These pathways may be aberrantly expressed in breast cancer, and mutations may occur in up to 25% of cases (Baselga, 2011). Phosphatase and tensin homologate (PTEN), Inositol polyphosphate 4-phosphatase type II (INPP4B) and others phosphatases are key regulators of this pathway (Porta et al., 2014). Multiple genomic alterations can potentially lead up to hyperactivated PI3K pathway, including activating events in the oncogenes *PIK3CA, AKT* and *MTOR* or inactivating events in tumor suppressor genes such as PTEN (Pascual and Turner, 2019).

Different subtypes of TNBC have specific PI3K pathway mutations/alterations. For example, PIK3CA and AKT1 mutations are more likely to be found in androgen receptor-positive TNBC and INPP4B is frequently inactivated in basal-like TNBC[146(p)]. Some data have suggested that the AKT activation can be used as a potential predictive biomarker to AKT inhibitors. There is evidence from a phase 2 trial suggesting that this pathway may be successfully targeted in TNBC. The addition of ipatasertib, an AKT inhibitor, to paclitaxel as first-line therapy for metastatic TNBC improved progression-free survival over placebo (stratified HR 0.59; 95% CI: 0.26–1.32, $p = 0.018$) (Kim et al., 2017). Phase III trials with PI3K inhibitors are ongoing.

Fig. 2 summarizes the main signal transduction pathways that play a fundamental role in tumorigenesis processes as well as in the mechanisms of therapeutic resistance in cases of TNBC. And Table 2 shows the main biomarkers with their impacts on prognosis or survival.

2. Conclusion

Undoubtedly, recent advances have been made in understanding TNBC as a disease with intrinsic molecular and immunological heterogeneity, recognizing the variety of clinical phenotypes. This new scenario demands an urgent comprehensive subclassification that incorporate immune-molecular signatures for a more targeted and effective treatment. Although PARP inhibitors and checkpoint inhibitors have been recently incorporated in some settings, cytotoxic chemotherapy remains as the mainstay of therapy against TNBC, resulting in different outcomes for patients with similar clinicopathologic features.

The role of a more complete accessible panel of immunohistochemical biomarkers has improved decisions in the treatment of TNBC. Additionally, new biomarkers have been proposed to predict survival and response to chemotherapy in many cases, allowing for personalized approaches such as the need for dose escalation as well as incorporation of new antitumor agents into the standard regimen. On the other hand, more modern NGS-based biomarkers still need to be better validated through reliable prospective studies and to become more accessible to daily practice.

Formerly considered an unattainable disease by molecular therapy, the TNBC has recently been the center of successful investigations for the incorporation of new targeted therapy, due to improvements in response predictions. Considering the proposed subtypes with their molecular variations as defined by specific biomarkers, the incorporation of platinum agents, checkpoint inhibitors and PARP inhibitors, great advances have been achieved in both neoadjuvant treatment and in the metastatic disease approach.

Authorship statement

All authors have actively contributed to the conception, design, execution or interpretation of the current review.

Funding source

This paper has no funding.

Declaration of Competing Interest

No potential conflicts of interest were disclosed by the authors.

Acknowledgment

Authors would like to thank Lucas Zanetti for editing the figures of this manuscript.

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