Heterogeneity of triple-negative breast cancer: understanding the Daedalian labyrinth and how it could reveal new drug targets

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ABSTRACT

Introduction: Triple-negative breast cancer (TNBC) is considered the most aggressive breast cancer subtype with the least favorable outcomes. However, recent research efforts have generated an enhanced knowledge of the biology of the disease and have provided a new, more comprehensive understanding of the multifaceted ecosystem that underpins TNBC.

Areas covered: In this review, the authors illustrate the principal biological characteristics of TNBC, the molecular driver alterations, targetable genes, and the biomarkers of immune engagement that have been identified across the subgroups of TNBC. Accordingly, the authors summarize the landscape of the innovative and investigative biomarker-driven therapeutic options in TNBC that emerge from the unique biological basis of the disease.

Expert opinion: The therapeutic setting of TNBC is rapidly evolving. An enriched understanding of the tumor spatial and temporal heterogeneity and the surrounding microenvironment of this complex disease can effectively support the development of novel and tailored opportunities of treatment.

1. Introduction

Triple-negative breast cancer (TNBC) is a heterogeneous subtype of tumor defined clinically by the lack of expression of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). Patients with TNBC have a relatively poorer outcome compared to patients affected by other BC subtypes, because of the typically aggressive cancer behavior and the absence of recognized targets for specific therapies [1]. Indeed, until a few years ago, the gold standard systemic therapy for TNBC was cytotoxic chemotherapy, which was associated with limited clinical benefit. In the paucity of effective options, in the last decade considerable efforts have been made to better understand the biological characteristics of TNBC to derive clinical useful information and improve the overall survival of TNBC patients. Herein, we describe the current understanding of the heterogeneous landscape of TNBC and report the molecular and biological characteristics that emerge as possible actionable targets for the treatment of TNBC.

2. Tumor heterogeneity of TNBC

Established evidence clearly showed that TNBC is a unique disease, encompassing multiple entities characterized by histopathological, transcriptomic, and (epi)genomic heterogeneity. From an histopathological point of view, the majority of TNBCs are classified as invasive mammary carcinomas (typically invasive ductal carcinomas), prevailing the poor tumor differentiation and the presence of stromal lymphocytes along with metaplastic elements [2]. Notwithstanding these main characteristics, the TNBCs also recognize rare cases of low-grade neoplasms, including the triple-negative (TN) breast neoplasia (atypical or not microglandular adenosis and acinic cell carcinoma) and the salivary gland-like tumors of the breast as the mucoepidermoid carcinoma, the polymorphous low-grade adenocarcinoma, the adenoid cystic carcinoma, and the secretory carcinoma) [3]. Notably, both the adenoid cystic carcinoma and the secretory carcinoma constitute two rare but unique TN subtypes with pathognomonic genetic alterations of MYB-NFIB and ETV6-NTRK3 fusion genes, respectively [4,5].

With the advent of the genomic era, an additional transcriptomic classification has been developed with the identification of five molecular subtypes, i.e. Luminal A, Luminal B, HER2-enriched, Basal-like, and Claudin-low subtypes. Often Basal-like BC (BLBC) and TNBC have been used as synonyms although this is not formally correct, since these two subtypes do not perfectly overlap.

Several attempts have been done to define TNBC subtypes in order to help clinicians in providing better prognosis and in proposing therapeutic approaches based on molecular peculiarities. A seminal work in this direction has been performed

Article highlights

- TNBC is a heterogeneous disease comprising several subtypes characterized by histopathological, transcriptomic, and (epi)genomic features, which could represent potential actionable molecular targets.
- BRCAness and germline BRCA1/2 mutations are predictive factors for platinum salts and PARP-inhibitors effectiveness
- The combination of immune checkpoint inhibitors and chemotherapy is a significant therapeutic option in selected TNBC, both in the early and metastatic setting
- The antibody–drug conjugates sacituzumab govitecan, trastuzumab deruxtecan (in HER2-low TNBC) and datopotamab deruxtecan are promising therapeutic agents for metastatic TNBC
- Despite preclinical suggestions, neither anti-androgens for AR-expressing TNBC nor inhibitors targeting the PI3K/AKT/mTOR or the MAPK pathways have currently robust data to support their role in the clinical practice.
- Novel targeted, signaling-based therapies are urgently needed in the management of TNBC, exploiting its biological heterogeneity.

in the laboratory of J.A. Pietenpol [6] that in 2011, on the base of TNBC gene expression dataset, was able to identify seven TNBC subtypes (TNBCtype), namely Basal-like 1 (BL1), Basal-like 2 (BL2), Immunomodulatory (IM), Mesenchymal (M), Mesenchymal stem-like (MSL), Luminal androgen receptor (LAR), and unstable (UNS). This work paved the way for the exploitation of specific molecular portraits to design therapeutic approaches for TNBC. The TNBCtype has been revisited by the same research group [7] in 2016 ending up with a simplified TNBCtype-4 (Basal-like 1, Basal-like 2, Mesenchymal-like, and Luminal AR) where the Immunomodulatory and the Mesenchymal Stem-like subtypes were eventually excluded for unstable clustering. Interestingly, in a retrospective analysis, the stratification of TNBC patients with the TNBCtype-4 showed that patients classified as Basal-Like 1 turned out to have a higher pathologic complete response (pCR) with respect to those classified as Basal-Like 2.

In 2013, Aulmann and collaborators [8] based on immunohistochemical analyses of 13 different biomarkers (cytokeratin-19, -7, -18, -5/6, -14, EGFR, Bcl-2, CD117, Vim, WT-1, p53, p16, and Ki67) were able to cluster TNBC into four subtypes (Basal A, Basal B, Basoluminal, and Luminal). Using this subdivision in a retrospective analysis, patients classified as Basal A and Basal B turned out to have a better overall survival probability with respect to those classified as Basoluminal and Luminal, evidencing the prognostic efficacy of TNBC subtyping.

Finally, in 2015 Burstein at al. subdivided TNBCs into four subtypes: Luminal-Androgen receptor (LAR), Mesenchymal (MES), Basal-like immune-suppressed (BLIS), and Basal-like immune-activated (BLIA). The same authors indicated that each of these subtypes was characterized by the expression of potential therapeutic targets, such as the case of LAR subtype expressing the androgen receptor that could benefit from androgen receptor antagonists. Moreover, they showed that different TNBC subtypes were associated with different prognoses. In 2016, Liu et al. defined four subtypes (Immunomodulatory – IM, Luminal-AR – LAR, Mesenchymal-like – MES, and the Basal-like and immune-suppressed – BLIS) on the base of a combination of mRNAs and lncRNAs expression data and also in this case the different subtypes displayed different prognostic outcome [9].

Along with transcriptional heterogeneity, TNBC is also characterized by intricate genome alterations, coupled with high genetic instability, copy number variation, and chromosomal rearrangements (Figure 1). The TP53 somatic mutation is the most frequent alteration reported in TNBC and is more common in basal-like (62–80%) than in non-basal TNBC (43%) [10,11]. Conversely, driver alterations in genes of the phosphoinositide-3-kinase (PI3K)/AKT pathway, including PIK3CA mutations, have been described in 10% of cases [12], notably more frequent in LAR TNBCs (46.2%) than in the other subtypes (average 4.5%) [13]. The LAR subtype is also characterized by higher mutations of AKT1 and CDH1 genes, as described by Bareche et al. [14] in a recent comprehensive genomic analysis of alterations observed in each TNBC molecular stable subtype. Accordingly, the BL1 tumors have high rate of chromosomal instability, TP53 mutations (92%), copy-number variation (gains) and amplifications of PI3KCA and AKT2, and deletions in genes involved in DNA repair machinery. Mesenchymal and MSL subtypes are associated with higher angiogenetic signatures. On the contrary, the IM subtype showed high expression of immune response-associated signatures and checkpoint inhibitor genes, including cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1), and PD-ligand 1 (PD-L1) and associated with a better prognosis [14].

Overall, all other genomic mutations in TNBC occur at a low (1–5%) or very low frequency (<1%) and some of them are actionable (i.e. ERBB2, BRAFV600E) with available target therapies [11]. Finally, the reported TNBC defects' enrichment in double-stranded DNA repair mechanisms (i.e. somatic and germline BRCA1/2 mutations) represents a relevant druggable molecular target [15].

Despite the different criteria used to molecularly dissect the TNBC population, there is an overall general concordance in the subtyping processing that has provided insights on some relevant dysregulated pathways in TNBC, helping to focus on the potential actionable molecular targets, such as BRCA mutations in BL1 subtype, checkpoint inhibitor genes in IM one, and PIK3CA in LAR one (Figure 1).

3. Innovative biomarkers-driven therapeutic approaches

3.1 The BRCAness context

TNBC is enriched in abnormalities of the DNA repair machinery, including the Homologous Recombination process [16]. Mutations of genes controlling the homologous recombination repair pathway, as BRCA1, BRCA2, PALB2, ATM, CHEK2, MUTYH, MSH2, and RAD51C, are especially involved in the TNBC cancerogenesis and are considered as an ensemble entity, named homologous recombination deficiency (HRD), characterized by peculiar clinical-pathological and genomic features. Accordingly, the concept of BRCAness defines the defects in homologous recombination repair, mimicking BRCA1 or BRCA2 loss even in the absence of germline BRCA mutations (gBRCAm) and recapitulates different alterations in

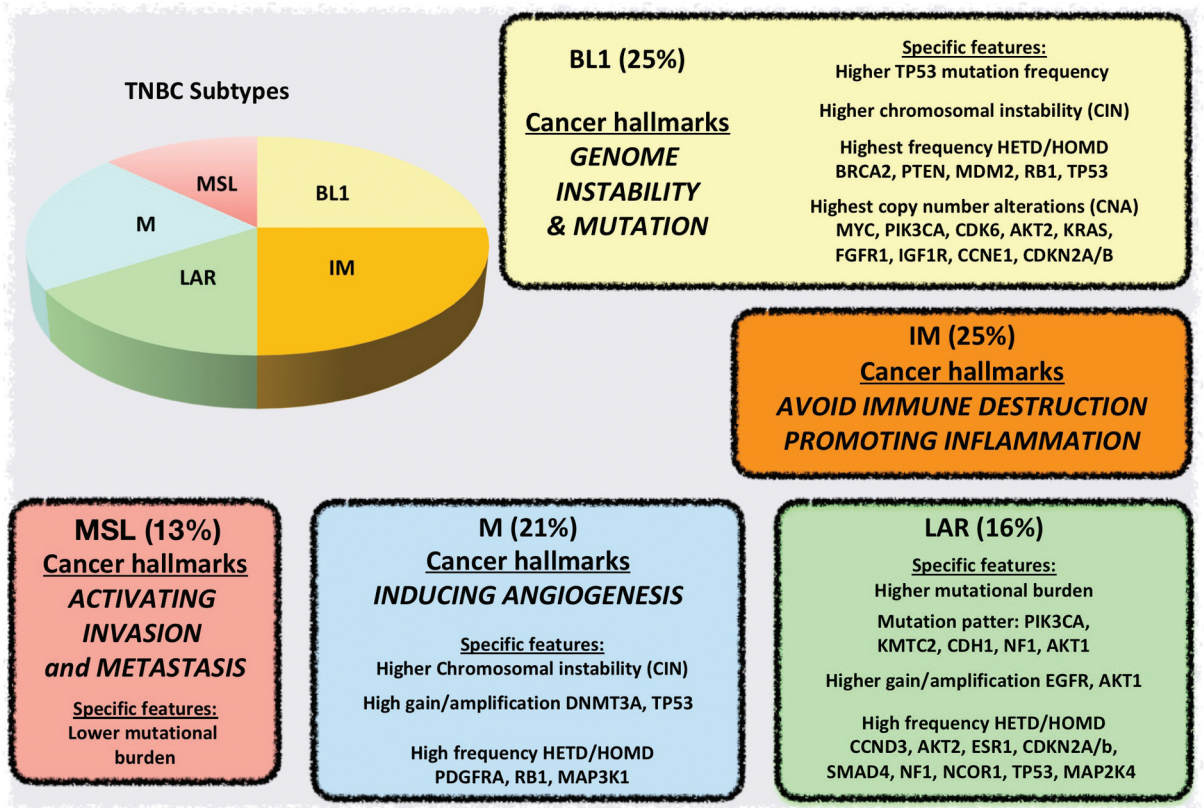

Figure 1. **Schematic representation of the principal features of TNBC subtypes**. The different TNBC subtypes Basal-like 1 (BL1), Immunomodulatory (IM), Luminal Androgen receptor (LAR), Mesenchymal (M), and Mesenchymal stem-like (MSL) are indicated together with their specific features according to Bareche et al. [14].

the DNA repair machinery. These genomic "scars" and mutation signatures have been demonstrated to act as predictive factors of response to the drugs interfering with DNA repair [17] including alkylators (i.e. cyclophosphamide, platinum salts) and topoisomerase inhibitors (i.e. anthracyclines) [18]. Actually, the gBRCA1/2 mutations are crucial predictive factors for platinum and PARP-inhibitors.

In the TNT phase III trial, carboplatin had double the objective response rate (ORR) of docetaxel in metastatic TNBC (mTNBC) patients bearing gBRCA1/2 but not in the overall unselected population [19]. The poly(ADP-ribose) polymerase (PARP) inhibitors have demonstrated to exert a dramatic antitumor activity through the synthetic lethality effect in gBRCA1/2 mTNBC and in selected cases of BRCAness, including somatic BRCA mutation and HRD [20] (Figure 2). Indeed, two phase III trials with olaparib and talazoparib PARP inhibitors in gBRCAm BC have recently led to the approval of these two agents in HER2 negative BC harboring a gBRCA1/2 mutations. In the EMBRACA trial, 431 patients with pre-treated locally advanced or metastatic HER2- BC and a gBRCA1/2 mutation were enrolled. The study randomized patients to receive 2:1 talazoparib 1 mg orally q.d. versus treatment of physician choice (TPC) (capecitabine, eribulin, gemcitabine or vinorelbine). Median progression-free survival (PFS) was 8.6 vs 5.6 months in favor of patients treated with talazoparib *vs.* chemotherapy (hazard ratio (HR) 0.54; 95% confidence interval (CI): 0.41-0.71; $p < 0.0001$), without improvement in overall survival (OS) (19.3 vs 19.5 months; HR 0.848, 95% CI 0.670–1.073; $p = 0.17$). Most frequently adverse events observed among patients treated with talazoparib in the EMBRACA trial were haematological and gastrointestinal (mainly nausea), followed by fatigue and headache [21,22]. Similarly, the Phase III OlympiAD trial enrolled 302 patients with pre-treated metastatic HER2-negative BC and a gBRCA1/2 mutation to receive 2:1 olaparib 300 mg po twice daily versus TPC (capecitabine, eribulin, or vinorelbine). Median PFS was 7.0 versus 4.2 months in olaparib *vs.* chemotherapy arm (HR 0.58, 95% CI 0.43–0.80; $p < 0.001$) without improvement in OS (19.3 vs 17.1 months; HR 0.90; 95% CI 0.66–1.23; $p = 0.513$). Main adverse events were nausea and anemia [23–25]. Moreover, it has been demonstrated that olaparib is active in other germline DNA repair defect mutations, showing 82% of partial response (PR) with a PALB2 germline mutation, and a 50% of PR in patients with somatic BRCA1/2 mutations [26]. The phase III BRAVO trial had a similar study design testing niraparib versus TPC in pretreated gBRCAm mTNBC but discordance between local and central PFS assessment resulted in informative censoring, thus limiting the accurate assessment of the drug activity [27]. Another relevant study on the use of PARP inhibitors in TNBC is BROCADE3 [28], a phase III trial that enrolled 431 patients with pre-treated HER2-negative mBC with a gBRCAm to receive 2:1 veliparib, carboplatin, and paclitaxel (arm 1) or placebo, carboplatin, and paclitaxel (arm 2). Median PFS was 14.5 months in the veliparib group *vs.* 12.6 months in the control group (HR 0.71; 95% CI 0.57–0.88; $p = 0.0016$), confirming the survival advantage in gBRCA mutation (as already

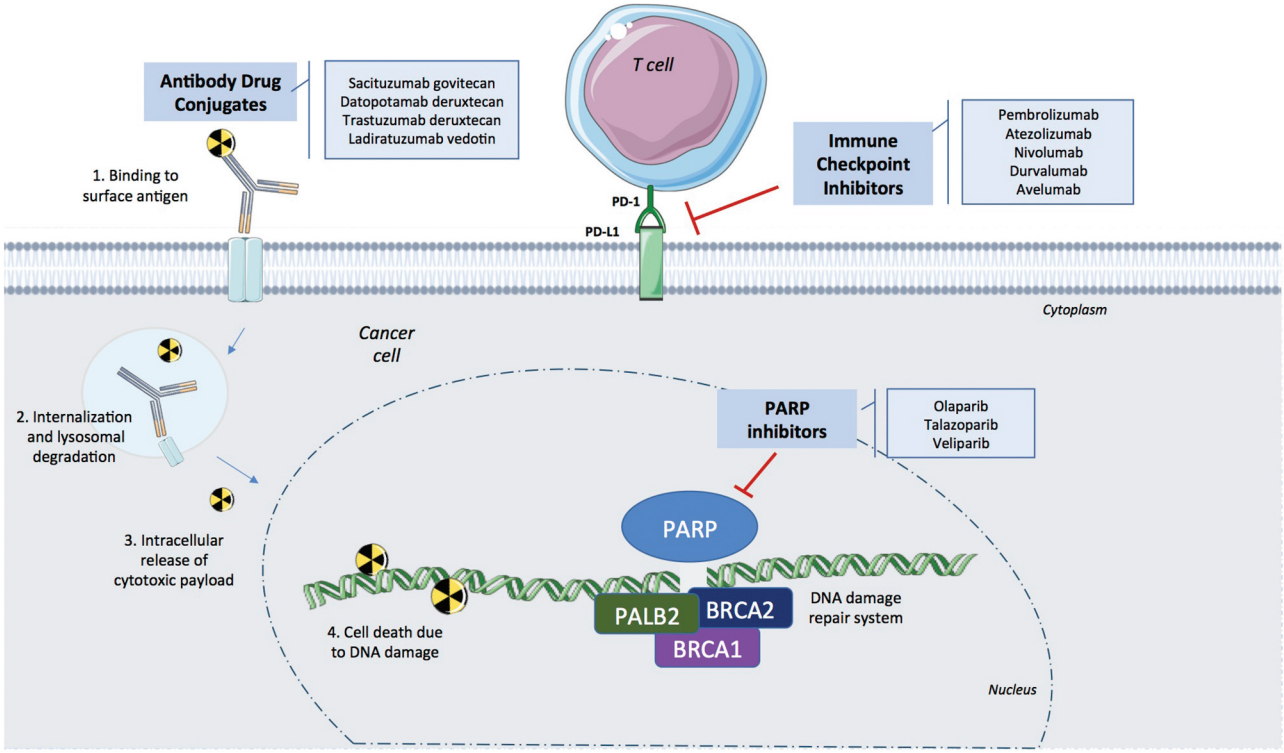

Figure 2. Simplified representation of the mechanisms of action of antibody–drug conjugates, immune-checkpoint inhibitors, and PARP inhibitors in triple-negative breast cancer.

reported in the previous phase II study BROCADE2 [29] but not in the general unselected population of TNBC [30].

Accordingly, a broad genetic testing is crucial in the management of TNBC, extending the treatment opportunity while increasing the chances of variants of uncertain significance, with an overall complexity of patient management [31]. Recent data have shown that Next-Generation Sequencing-based multi-gene panel testing in BC patients with a strong personal and/or family history of cancer, even if BRCA1/2 wild type, could show up to 15% of patients harboring mutations of other HRR genes [32]. HRDetect is a lasso logistic regression model that identifies six distinguishing mutational signatures predictive of BRCA1/BRCA2 deficiency. This validated method reveals a larger proportion of patients harboring BRCA1/BRCA2 deficiency than single nucleotide variants (up to 22% vs 1–5%) [33,34].

Furthermore, new approaches are being explored in mBC and the focus for new agents has largely been in the HRD subset, such as G4 DNA ligands (CX-5461 [35], a phase I/II trial is ongoing), lurbinectedin [36], polymerase theta inhibitors [37], and other druggable enzymes driving Micro-homology Mediated End Joining [38].

Based on the encouraging data in the metastatic setting, several studies have been conducted in the adjuvant setting. The pivotal one is the OlympiA trial, which randomized gBRCA1/2 mutated, HER2-negative high-risk eBC patients to receive 1 year of oral olaparib 300 mg versus placebo, after the completion of local therapies and neoadjuvant/adjuvant chemotherapy. The primary endpoint was iDFS and it was singnificantly improved in the olaparib arm (3-year iDFS 85.9% vs 77.1%; HR 0.58; $p < 0.001$). The result was also confirmed in terms of distant DFS (3-year distant DFS 87.5% vs 80.4%; HR 0.57; 99.5% CI 0.39 to 0.83; $p < 0.001$). Of note, olaparib was well tolerated as adjuvant therapy with no clinically meaningful increase in fatigue during treatment or significant impact on quality of life [39].

3.2 The immunotherapy option

TNBC has been shown to be an ideal target for immunotherapy than the other BC subtypes, possibly because of its high mutational load [10,40], high T-cell infiltration [6,41,42] and higher rates of PD-L1 expression [43] (Figure 2). When using single-agent immunotherapy, low response rates have been reported (around 10%, or higher in case of high TILs levels and PD-L1 positivity) [44–50]. Subsequent studies have combined chemotherapy with immunotherapy, since chemotherapy induces multiple immunomodulatory changes in the tumor microenvironment that may influence the effectiveness of immunotherapy (i.e. increased antigen release, upregulation of PD-L1, and upregulation of immunogenic cell surface markers) [51].

The first phase III trial on a combination of chemotherapy and immune checkpoint inhibitors (ICIs) in mTNBC in the first-line setting was IMpassion130. This trial randomized mTNBC patients to receive atezolizumab + nab-paclitaxel versus placebo+ nab-paclitaxel. The trial enrolled patients regardless PDL1 status but stratified for the PDL1 status (according to IC scoring system per SP1542 assay) for the final analysis. The co-primary endpoints were PFS and OS hierarchically tested in the "intention-to-treat" (ITT) population and in PD-L1 IC+ patients. The primary PFS analysis in the ITT population

showed an improvement of 1.7 months in median PFS (7.2 vs 5.5 months; HR 0.80, 95% CI 0.69–0.92, $p = 0.002$) and such a benefit was confirmed in the PD-L1 IC+ subgroup population (2.5 months benefit; 7.5 vs 5.0 months; HR 0.62, 95% CI 0.49–0.78, $p < 0.001$) [52]. Conversely, while the improvement in median OS was not statistically significant in ITT population (21.0 *vs*. 18.7 months; HR: 0.87; 95% CI 0.75–1.02; $p = 0.077$), an exploratory analysis in the PD-L1 IC+ population showed an absolute OS advantage of 7.5 months in favor of atezolizumab [53]. Even if formally negative, these results led to an first accelerated FDA approval of atezolizumab + nab-paclitaxel in the PD-L1-positive population (pending a confirmatory trial) and this combination is currently recommended for patients with PD-L1 IC+ mTNBC in the European Society of Medical Oncology (ESMO) guidelines [54].

The next and confirmatory trial, IMpassion131, randomized mTNBC patients in the first line setting to receive 2:1 weekly paclitaxel + atezolizumab or placebo. The study population was the same as the IMpassion130 but no improvement in PFS and OS was observed, neither in the ITT nor in the PDL1+ population [55]. As a consequence of both the negative results of the confirmatory trial IMpassion131 and the preliminary positive results of the Keynote 355 trial with the competitive ICI pembrolizumab, in August 2021 Roche announced the decision to voluntarily withdraw the US accelerated approval for atezolizumab in combination with nab-paclitaxel for the treatment of PDL1+ mTNBC patients and the option atezolizumab + nab-paclitaxel has been removed from NCCN guidelines [56].

Differences in results of IMpassion130 versus IMpassion131 raise the question on the potential underlying causes, including the TNBC heterogeneity, the use of corticosteroids for solvent-based paclitaxel (but not for nab-paclitaxel) and the potential unknown confounders (e.g. antibiotic use, microbiome). A comparative analysis of the 2 trials coherently suggests the benefit of immunotherapy is restricted to molecular subtypes enriched in immune-signatures [57].

The Keynote 355 trial randomized mTNBC patients to receive, in a 2:1 fashion, pembrolizumab or placebo in combination with several chemotherapeutic agents consisting of taxanes (paclitaxel or nab-paclitaxel) or gemcitabine + carboplatin. Stratification factors included chemotherapy on study, PD-L1 tumor expression (based on CPS scoring system per 22C3 assay), and prior treatment with the same chemotherapeutic class in the neoadjuvant/adjuvant setting. Briefly, it has been reported a statistically significant PFS improvement in the PD-L1+ population with a CPS ≥10 treated with pembrolizumab (corresponding to the 38% of the ITT population) with a median PFS improved of 4.1 months (9.7 vs 5.6 months; HR 0.65, 95% CI 0.49–0.86, pre-specified p value boundary of 0.00411 met) [58]. In July 2021, a press release reported that the phase III trial met also the co-primary endpoint of OS in patients with mTNBC whose tumors expressed PD-L1 (CPS ≥10). Based on these results of OS and PFS, pembrolizumab received the formal FDA approval.

Because of a growing body of evidence have suggested a superior efficacy of ICIs in TNBC when administered early in the course of the disease, possibly for a less pronounced immune-escape mechanisms [59], several randomized clinical trials investigated the role of ICIs in the early setting, especially as a primary treatment, with preliminary encouraging results. Indeed, of five randomized trials with ICIs added to neoadjuvant chemotherapy [60–64], three showed an improvement in pCR rate with immunotherapy [60–62]. Table 1 summarizes the main results of these studies.

In particular, the recent event-free survival (EFS) results from the KEYNOTE-522 [65] demonstrated that the addition of ICI (i.e. pembrolizumab) in the early stage setting improve long-term outcomes. KEYNOTE-522 was a phase 3 trial in which 1174 stage II–III TNBC patients were randomized to neoadjuvant chemotherapy with paclitaxel-carboplatin followed by doxorubicin-cyclophosphamide, with or without the addition of pembrolizumab (continuing ICI after surgery for up to nine cycles). With 37 months of median follow-up, the trial was positive and met the 2 co-primary endpoints of pCR and EFS in the intention-to-treat (ITT) population, with a pCR rate of 64.8% vs 51.2% (95% CI 5.4–21.8; $p < 0.001$) and with an EFS-event rate of 15.7% vs 23.8% (HR = 0.63, $p = 0.0003$), both in favor of pembrolizumab arm. The significant clinical advantage observed is accompanied by an increase in immune-related adverse events (irAEs), with a rate of grade 3–5 irAEs of 14.9% (vs 2.1% in the control arm) and 10.9% of the events leading to any drug discontinuation (vs 2.6% in the control arm) [65]. Based on these results, on July 26, 2021, the FDA approved pembrolizumab for high-risk, early-stage TNBC in combination with chemotherapy as neoadjuvant therapy (then continuing ICI as adjuvant single-agent treatment after surgery) [66].

Table 1. Five randomized trials with ICIs added to NACT in early TNBC.

Study	Treatment	Phase	Population	pCR results
KEYNOTE-522 [60,65]	Paclitaxel–carboplatin followed by doxorubicin-cyclophosphamide ± pembrolizumab	III	Stage II–III TNBC	pCR 64.8% vs 51.2% (95% CI, 5.4–21.8; $p < 0.001$)
IMpassion031 [61]	Atezolizumab or placebo in combination with weekly nab-paclitaxel followed by doxorubicin and cyclophosphamide	III	Stage II–III TNBC	58% vs 41%; $p = 0.0044$
I-SPY2 [62]	Pembrolizumab or placebo in combination with taxane- and anthracycline-based chemotherapy	II	High-risk stage II–III BC	60% vs 22% in TNBC cohort (29 pts)
GeparNuevo [63]	Durvalumab or placebo in combination with nab-paclitaxel followed by standard epirubicin and cyclophosphamide	II	Stage II–III TNBC	53.4% vs 44.2%; $p = 0.287$
NeoTRIPaPDL1 [64]	Atezolizumab or placebo in combination with carboplatin + nab-paclitaxel	II	Stage II–III TNBC	48.6% vs 44.4%; $p = 0.48$

Abbreviations: ICI, immune checkpoint inhibitors; NACT, neoadjuvant chemotherapy; pCR, pathological complete response.

3.3 Trophoblast cell surface antigen 2 (TROP-2) and antibody–drug conjugates (ADC)

One of the most innovative and promising therapeutic agents for mTNBC is sacituzumab govitecan (Figure 2). It is an antibody drug–conjugate (ADC) constituted by a humanized anti-Trop2 monoclonal antibody linked to the active metabolite of irinotecan SN-38. Trop2 is the Trophoblast cell-surface antigen 2 (a glycoprotein initially identified in a trophoblast cancer cell line [67] and is expressed in about 88% of TNBC but rarely in healthy cells [68]. Therefore, sacituzumab recognizes TNBC cells, delivers and releases SN-38, which acts as a potent inhibitor of topoisomerase I (Topo1), thus preventing repair of DNA damage and leading to apoptosis and cell death.

The Phase III ASCENT trial enrolled 529 mTNBC patients having received at least two prior lines of chemotherapy. Patients were randomized to receive sacituzumab govitecan versus TPC including eribulin, vinorelbine, gemcitabine, or capecitabine. Sacituzumab govitecan significantly improved PFS (5.6 vs 1.7 months; HR 0.41, 95% CI 0.32–0.52) and nearly doubled median OS (12.1 vs 6.7 months; HR 0.48, 95% CI 0.38–0.59) compared with conventional chemotherapy [69]. These efficacy results were observed regardless Trop2 expression levels, albeit with greater efficacy in patients with a medium or high Trop2 score [70]. These data have raised doubts on its intrinsic mechanism of action. Pharmacokinetic analysis supported the hypothesis that sacituzumab govitecan mainly acts as an SN-38 prodrug, beyond the conventional ADC activity [71]. Indeed, the rapid hydrolysis of the linker attaching SN-38 to the mAb leads to the release of high concentrations of SN-38 systemically [72].

Another ADC that could potentially revolutionize the classical therapeutic strategies of mBC, not only mTNBC, is trastuzumab deruxtecan (T-DXd). It is composed of an antiHER2 (human epidermal growth factor receptor 2) mAb linked to a topoisomerase I inhibitor, an exatecan derivative. In HER2-low BC (HER2 IHC score 1+ or 2+ and FISH test negative, corresponding to the HER2-negative disease as defined by ASCO/CAP guidelines), its peculiar mechanism of action is mainly based on the bystander effect. In detail, T-DXd binds to HER2 expressed on the surface of HER2-positive tumor cells (even HER2-low), then it is internalized and DXd is released into the cytoplasm, thus inducing apoptosis. DXd is then transferred to and induces apoptosis in neighboring HER2-negative cells. Indeed, T-DXd showed an ORR of 14% in patients with HER2-low TNBC in a phase I trial. Its toxicity profile is well known and mainly characterized by nausea and the rarer interstitial lung disease [73,74] More data are expected from the DESTINY Breast 04 phase III trial, which enrolled mTNBC HER2 low to receive T-DXd or TPC; the enrollment has been recently completed (NCT03734029).

A novel anti-TROP2 ADC, datopotamab deruxtecan (Dato-DXd) has been recently developed and its activity and safety have been tested in preclinical trials [75]. In a phase I trial on 21 patients the Overall Response Rate (ORR) was 43% and the primary toxicities were stomatitis and skin rash [76]. Future clinical trials are awaited to test Dato-DXd in patients with TROP2-expressing tumors. A phase III trial is planned in HR-positive, HER2-negative BC (NCT05104866).

Ladiratuzumab vedotin is a new ADC targeting LIV1a (involved in immunogenic cell death mechanisms) showed an impressive ORR of 35% in a phase II trial on 63 patients even if complicated by neuropathy and neutropenia [77]. This drug is now being tested on a weekly schedule in order to improve the toxicity profile] and also in combination with pembrolizumab (see Table 2).

Patritumab deruxtecan (U3-1402) is an ERBB3-directed ADC with the topoisomerase I inhibitor, DXd (the same of T-DXd), that in a phase I trial showed an ORR of 16% in 31 patients with TNBC overexpressing ERBB3. Its toxicity profile comprises nausea, cytopenia, and pneumonitis [78]. Data need to be confirmed in larger trials (see Table 2).

A further class of promising ADCs is that constituted by trastuzumab conjugated with duocarmycins, a class of DNA minor-groove-binding alkylating molecules [79]. In phase I trials, one of them, SYD985 has shown encouraging ORR (32%) not only in HER2 positive disease but also in HER2 low. Safety profile was acceptable (mainly fatigue and conjunctivitis in one-third of the population) [80].

New directions are combinations of ADC with immunotherapy (Sacituzumab + pembrolizumab or avelumab; trastuzumab deruxtecan + durvalumab or datopotamab + durvalumab; ladiratuzumab vedotin + pembrolizumab; see Tables 2 and 3).

4. Investigative biomarkers-driven therapeutic approaches

4.1 The androgen pathway

Approximately 24% of TNBC are characterized by androgen receptor (AR) positivity [86]. ARs belong to the family of steroid hormonal nuclear receptors, together with estrogen, progesterone, glucocorticoid, and mineralocorticoid receptors, and are involved in several cellular processes, including cell proliferation and apoptosis [87] (Figure 3). Although some findings support a key role of AR and of its downstream pathway in BC, its predictive and prognostic role in TNBC remains debated. In a large meta-analysis including 2826 TNBC patients, AR-positive tumors were associated with lower tumor grade (p <0.001), and with prolonged disease free survival (HR 0.81, 95% CI 0.66–0.99, $p < 0.05$), although no significant differences were observed in terms of OS (HR 1.27, 95% CI 0.90–1.78, $p = 0.17$) [86]. Similarly, smaller cohorts of patients with TNBC showed a significant association between AR and low clinical stage, low grade, and low proliferation index [88–90]. Regarding the predictive role of AR, AR-positive TNBC seems to be associated with a lower responsiveness to chemotherapy. In the GeparTRIO study, pCR rate was 12.8% in AR-positive tumors, compared to 25.4% in AR-negative tumors ($p < 0.0001$) [91]. A retrospective analysis led by Masuda et al. of 146 patients with TNBC treated with neoadjuvant chemotherapy, and classified according to their gene expression profile, showed that AR-positive tumors had a lower pCR rate, as none of them ($n = 20$) achieved pCR after neoadjuvant chemotherapy [92].

The good prognosis and the lower responsiveness to chemotherapy strongly suggest that AR-driven TNBC may

Table 2. Ongoing trials on antibody–drug conjugates, immune checkpoint inhibitors, PARP inhibitors, and anti-androgens in neoadjuvant/post-neoadjuvant and/or adjuvant setting in TNBC.

Agent(s)	Target(s)	Phase	Primary endpoint(s)	Estimated study completion	ClinicalTrials.gov Identifier
Sacituzumab govitecan + Atezolizumab	TROP2, PD-L1	II	Rate of undetectable circulating tumor cfDNA	December 30, 2025	NCT04434040
AMG 386, Ganitumab, MK-2206, Ganetespib, Veliparib, PLX3397, Pembrolizumab, Talazoparib, Patritumab, SGN-LIV1A, Durvalumab, Olaparib, SD-101, Cemiplimab, REGN3767, Trilaciclib, Encequidar, Dostarlimab (adaptive trial)	Ang1&Ang2, IGF-IR, Akt1/2/3, Hsp90, PARP, CSF-1R, c-Kit& FLT3, PD-1, ERBB3/HER3, LIV-1, PD-L1, TLR9, LAG3, CDK4/6, P-gp	II	pCR	December 2031	NCT01042379
Pembrolizumab+ Docetaxel + IL-12 gene therapy + L-NMMA	PD-1, IL-12	II	pCR	August 2024	NCT04095689
Nivolumab + Ipilimumab vs CT	PD-1, CTLA-4	II	DFS	December 1, 2022	NCT03818685
Camrelizumab + CT	PD-1	II	pCR	February 28, 2024	NCT04676997
Tislelizumab + Anlotinib + CT	PD-1, VEGFR2, PDGFRβ and FGFR1	II	pCR	July 31, 2023	NCT04914390
Pembrolizumab + CT	PD-1	II	pCR	November 2024	NCT03639948
Nivolumab + Cabiralizumab + CT	PD-1, CSF1R	I/II	Safety, %change in TILS and TAM	February 28, 2024	NCT04331067
Lenvatinib + Pembrolizumab	VEGFR, FGFR, RET, KIT & PDGFR, PD-1	I	Presence of a T-cell inflamed TME	July 2026	NCT04427293
Niraparib + RT	PARP	I	MTD	December 31, 2026	NCT03945721
Durvalumab + CT	PD-L1	I/II	AEs, pCR	January 2022	NCT03356860
Sintilimab + CT	PD-1	II	pCR	March 2024	NCT04809779
Sintilimab+ Anlotinib + CT	PD-1, VEGFR2, PDGFRβ and FGFR1	II	pCR	December 31, 2024	NCT04877821
Mono Atezolizumab Window -> atezolizumab + CT	PD-L1	II	pCR	January 1, 2026	NCT04770272
Camrelizumab + RT	PD-1	I/II	iDFS	October 1, 2026	NCT04481763
Atezolizumab + Capecitabine	PD-L1	II	IDFS	January 31, 2027	NCT03756298
Rucaparib + RT	PARP	I	MTD	May 2023	NCT03542175
AZD6738 + Olaparib+ Durvalumab	ATR, PARP, PD-L1	II	ORR, AEs	December 2025	NCT03740893
Cemiplimab + CT	PD-1	II	pCR	March 15, 2023	NCT04243616
Eganelisib + Atezolizumab + Bevacizumab + CT	PI3K-γ, PD-L1, VEGF	II	pCR	August 1, 2022	NCT03961698
Atezolizumab + Sacituzumab govitecan	PD-L1, TROP2	II	Rate of undetectable circulating tumor cfDNA	December 30, 2025	NCT04434040
Olaparib + CT	PARP	II/III	AEs, pCR	January 2032	NCT03150576
Sintilimab + Apatinib + CT	PD-1, VEGFR2	II	pCR	January 31, 2023	NCT04722718
Pembrolizumab + RT	PD-1	I/II	%change in TILs	February 21, 2022	NCT03366844
Nivolumab + Ipilimumab	PD-1, CTLA-4	II	Immune activation (tumor-associated CD8)	January 7, 2025	NCT03815890
Sacituzumab govitecan	TROP2	III	iDFS	December 1, 2028	NCT04595565
HLX10 + chemotherapy	PD-1	III	pCR	April 9, 2027	NCT04301739
Atezolizumab + CT	PD-L1	III	iDFS	August 31, 2025	NCT03498716
Niraparib	PARP	III	DFS	August 24, 2029	NCT04915755
Sacituzumab govitecan + CT	TROP2	III	iDFS	December 1, 2028	NCT04595565
Enzalutamide	AR	II	Feasibility	May 2022	NCT02750358

Abbreviations: TROP2, trophoblast cell surface antigen 2; PD-L1, programmed death-ligand 1; Ang1&Ang2, angiopoeitins 1&2; IGF-IR, insulin-like growth factor-type I receptor; Akt1/2/3, protein kinase B 1/2/3; Hsp90, heat shock protein 90; PARP, poly(ADP-ribose) polymerase; CSF-1R, colony-stimulating factor 1 receptor; c-Kit, receptor tyrosine kinase (also referred to as stem cell factor receptor or CD117); FLT3, Fms-like tyrosine kinase 3; PD-1, programmed death-1; HER, human epidermal growth factor receptor; LIV-1, zinc transporter; TLR9, Toll-like receptor 9; LAG3, lymphocyte activation gene 3; CDK4/6, cyclin-dependent kinase 4 and 6; P-gp, P-glycoprotein; IL-12, Interleukin-12; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; VEGFR, vascular endothelial growth factor receptor; PDGFRβ, platelet-derived growth factor receptor beta; FGFR1, fibroblast growth factor receptor 1; CSF1R, colony-stimulating factor 1 receptor; RET, rearranged during transfection gene; ATR, ataxia telangiectasia and Rad3-related protein; PI3K-γ, phospholnositide 3-kinases gamma; AR, androgen receptors.

represent a distinct subtype, among TNBCs (93). In the molecular classifications of TNBC by Lehmann [6] and Burstein (94), AR-positive tumors largely overlap with the LAR subtype, where specific biomarkers like ARs, MUC-1, and several estrogen-regulated genes were described as potential therapeutic targets. Anti-androgens like bicalutamide and enzalutamide have been tested in phase II trials and showed proof-of-efficacy in AR-positive TNBC patients (95,96). However, despite preliminary promising data on the use of anti-androgens for AR-positive TNBC, more mature data failed to show a meaningful benefit of these agents in monotherapy (clinical benefit rate (CBR) ranging from 19% to 33%) (95–97), thus leading to the development of several trials testing the combination of anti-androgens with other treatments (e.g. chemotherapy, PI3K inhibitors, and CDK4-6 inhibitors). Main ongoing and published studies targeting the androgen pathway in AR-positive TNBC are reported in Tables 2-4, respectively. Enzalutamide was being evaluated in a phase III trial, both as single agent and combined with paclitaxel *versus* paclitaxel monotherapy in patients selected by a genomic signature for AR-driven disease (98). However,

Table 3. Ongoing trials on antibody–drug conjugates, immune checkpoint inhibitors, PARP inhibitors, and anti-androgens in the metastatic setting in TNBC.

Agent(s)	Target(s)	Phase	Primary endpoint(s)	Estimated study completion	ClinicalTrials.gov Identifier
Ladiratuzumab vedotin ± trastuzumab	LIV-1	I	DLT, safety	June 30, 2023	NCT01969643
Trastuzumab deruxtecan + durvalumab and paclitaxel/ capivasertib/capecitabin / endocrine therapy	HER-2 low, PD-L1,AKT	I	AEs	NCT04556773	NCT04556773
Tiragolumab + Atezolizumab + CT	TIGIT, PD-L1	I	AEs, ORR	March 15, 2022	NCT04584112
Spartalizumab, LAG525, NIR178, capmatinib, MCS110, canakinumab	PD-1,LAG-3, adenosine A2a, MET, CSF-1, IL-1 beta	I	AEs, DLT	January 17, 2022	NCT03742349
Palbociclib + Avelumab	CDK4/6, PD-L1	I	MTD, ORR	July 1, 2024	NCT04360941
Ruxolitinib Phosphate + Pembrolizumab	JAK1&JAK2, PD-1	I	MTD, AEs	March 1, 2022	NCT03012230
Mirvetuximab soravtansine + CT	Folate receptor α	I	AEs	February 22, 2023	NCT02996825
ASTX660 + Pembrolizumab	IAP, PD-1	I	MTD, RP2D, ORR	March 16, 2026	NCT05082259
DS-1062a	TROP2	I	DLT, AEs	January 1, 2024	NCT03401385
MK-5890 ± Pembrolizumab	CD27, PD-1	I	DLT, AEs	October 25, 2024	NCT03396445
Niraparib + MGD013	PARP, PD-1&LAG3	I	DLT, MTD, ORR	December 30, 2024	NCT04178460
MGC018 ± MGA012	B7-H3, PD-1	I	AEs, MTD	May 2025	NCT03729596
Budigalimab ± ABBV-927	PD-1, CD40	I	ORR, RP2D	November 27, 2023	NCT03893955
NZV930 ± spartalizumab ± NIR178	CD73, PD-1, adenosine A2a receptor	I	AEs	April 30, 2023	NCT03549000
XmAb®22841 ± Pembrolizumab	CTLA-4 & LAG-3, PD-1	I	AEs	November 2025	NCT03849469
SO-C101 ± pembrolizumab	IL-15, PD-1	I	AEs, DLT	December 2023	NCT04234113
Sasanlimab	PD-1	I	AEs, DLT	March 31, 2023	NCT04254107
Ladiratuzumab vedotin + pembrolizumab	LIV-1, PD-1	I/II	DLT, safety, ORR	December 30, 2023	NCT03310957
U3-1402	HER-3	I/II	AEs, ORR	December 2022	NCT02980341
Trastuzumab deruxtecan + durvalumab, or datopotamab deruxtecan + durvalumab, or durvalumab + paclitaxel + oleclumab, durvalumab + paclitaxel + capivasertib, or durvalumab + paclitaxel	HER-2 low, PD-L1, TROP2, CD73, AKT	I/II	AEs	February 13, 2023	NCT03742102
Sacituzumab govitecan+ chemoimmunotherapy (cyclophosphamide, N-803, and PD-L1 t-haNK)	TROP-2, IL-15, NK cells	I/II	MTD, safety, ORR	October 2028	NCT04927884
Multiple immunotherapy-based treatment combinations comprising atezolizumab + sacituzumab govitecan	TROP2, PD-L1	I/II	ORR, AEs	January 3, 2023	NCT03424005
Durvalumab in combination with novel therapies (capivasertib, oleclumab, trastuzumab deruxtecan, datopotamab deruxtecan) with or without paclitaxel and durvalumab + paclitaxel	PD-L1, AKT, CD73, HER2 low, TROP2	I/II	Safety, ORR	February 13, 2023	NCT03742102
Leronlimab+ carboplatin	CCR5	I/II	MTD, PFS	January 2022	NCT03838367
Fruquintinib + Tislelizumab	VEGFR, PD-1	I/II	AEs, RP2D, ORR	August 2022	NCT04577963
N-803 + PD-L1 t-haNK + Sacituzumab govitecan + CT	IL-15, NK cells, TROP2	I/II	MTD, safety, ORR	October 2028	NCT04927884
Talazoparib + Selinexor	PARP, XPO1	I/II	Safety	November 2025	NCT05035745
Durvalumab + Oleclumab+CT	PD-L1, CD73	I/II	AEs, CB	October 2023	NCT03616886
GX-I7 + Pembrolizumab+CT	IL7, PD-1	I/II	DLT, AEs, ORR	December 2021	NCT03752723
Gedatolisib + Talazoparib	PI3K &mTOR, PARP	I/II	MTD, ORR	December 2022	NCT03911973
Sarilumab + CT	IL-6R	I/II	MTD, DLT, % negativization bone marrow DTC	September 26, 2029	NCT04333706
Ladiratuzumab vedotin + Pembrolizumab	LIV-1, PD-1	I/II		December 30, 2023	NCT03310957
Pembrolizumab + Binimetinib	PD-1, MEK	I/II	MTD, ORR	July 15, 2022	NCT03106415
NBE-002	ROR1	I/II	RP2D, ORR	December 2025	NCT04441099
CAB-ROR2-ADC	ROR2	I/II	AEs, ORR	June 30, 2023	NCT03504488
NT-I7 + Pembrolizumab	IL-7, PD-1	I/II	MTD, RP2D, ORR	April 30, 2023	NCT04332653
Atezolizumab + genetically engineered cells	PD-L1, MAGE-A1 TCR	I/II	AEs, ORR	December 1, 2024	NCT04639245
GEN1046 + CT	PD-L1	I/II	DLT, AEs	December 2022	NCT03917381
KY1044 + Atezolizumab	ICOS, PD-L1	I/II	AEs, DLT, ORR	May 2023	NCT03829501
CYT-0851	RAD51	I/II	DLT, ORR	October 2022	NCT03997968
Durvalumab ± Olaparib or Cediranib	PD-L1, PARP, VEGF	I/II	RP2D,ORR	December 29, 2022	NCT02484404
Pamiparib + Temozolomide	PARP	I/II	DLT, AEs, ORR	June 30, 2023	NCT03150810
U3-1402	HER-3	II	ORR	June 11, 2026	NCT04965766
U3-1402	HER-3	II	ORR, PFS-6	November 30, 2023	NCT04699630
Sacituzumab govitecan + Trilaciclib	TROP2, CDK4/6	II	PFS	July 2024	NCT05113966
Sacituzumab govitecan + Sabizabulin	TROP2, tubulin	II	rPFS	June 30, 2023	NCT05008510
Sacituzumab govitecan ± Pembrolizumab	TROP2,PD-1	II	PFS	June 1, 2026	NCT04468061
Avelumab+ Sacituzumab govitecan vs avelumab, binimetinib, liposomal doxorubicin vs avelumab, liposomal doxorubicin	PD-L1, TROP2, MEK	II	Best ORR	July 30, 2023	NCT03971409
SG001 + nab-paclitaxel	PD-1	II	ORR	October 1, 2023	NCT05068141
Niraparib + Dostarlimab + RT	PARP, PD-1	II	ORR	December 1, 2029	NCT04837209
Tislelizumab + eribulin	PD-1	II	ORR	June 1, 2023	NCT04913571
Talazoparib maintenance	PARP	II	PFS	March 1, 2024	NCT04755868
Talazoparib + Atezolizumab + RT	PARP, PD-L1	II	ORR	apr-23	NCT04690855
Pembrolizumab + Olaparib+ RT	PD-1, PARP	II	ORR	January 2025	NCT04683679

(*Continued*)

Table 3. (Continued).

Agent(s)	Target(s)	Phase	Primary endpoint(s)	Estimated study completion	ClinicalTrials.gov Identifier
Atezolizumab + Bevacizumab + CT	PD-L1, VEGF	II	PFS	September 30, 2025	NCT04739670
Tavokinogene telseplasmid + Pembrolizumab + CT	IL-12, PD-1	II	ORR	August 2024	NCT03567720
Olinvacimab + Pembrolizumab	PD-1	II	ORR	August 30, 2026	NCT04986852
Sabizabulin ± Sacituzumab govitecan	Tubulin, TROP2	II	PFS	June 30, 2023	NCT05008510
Sitravatinib + Tislelizumab	TAM, PD-1	II	ORR, AEs	January 30, 2023	NCT04734262
Temozolomide ± Olaparib	PARP	II	DCR	December 1, 2027	NCT05128734
Pembrolizumab +CT	PD-1	II	ORR, AEs	apr-25	NCT02755272
Durvalumab + Olaparib	PD-L1, PARP	II	ORR	December 31, 2026	NCT03801369
Atezolizumab + Bevacizumab+CT	PD-L1, VEGF	II	PFS	apr-23	NCT04408118
Nivolumab + CT	PD-1	II	PFS	December 15, 2026	NCT04159818
CFI-400945 + Durvalumab + CT	PLK4, PD-L1	II	ORR	December 31, 2022	NCT04176848
Talazoparib	PARP	II	ORR	December 2023	NCT02401347
Atezolizumab ± CT	PD-L1	II	PFS prediction	December 2030	NCT01898117
CX-2009 ± CX-072	CD166, PD-L1	II	ORR	March 10, 2023	NCT04596150
Atezolizumab + BDB001 + RT	PD-L1, TLR 7/8	II	ORR	March 2025	NCT03915678
NIR178+spartalizumab	Adenosine A2a receptor, PD-1	II	ORR	June 17, 2022	NCT03207867
Pembrolizumab/Vibostolimab Co-Formulation, lenvatinib ± CT	PD-1, TIGIT, VEGFR,FGFR, RET, KIT, PDGFR	II	ORR, PFS	February 19, 2025	NCT05007106
Spartalizumab	PD-1	II	ORR	December 11, 2024	NCT04802876
Atezolizumab + chemotherapy	PD-L1	III	OS	March 30, 2024	NCT03371017
TQB2450 + Anlotinib + CT	PD-L1, VEGFR2, PDGFRβ and FGFR1	III	PFS	July 1, 2022	NCT04405505
Toripalimab + CT	PD-1	III	PFS	February 28, 2022	NCT04085276
Carelizumab + Nab-paclitaxel + Apatinib	CD20, VEGFR2	III	PFS	January 1, 2024	NCT04335006
Trastuzumab deruxtecan	HER-2 low	III	PFS	January 1, 2023	NCT03734029
Bicalutamide and ribociclib	AR, CDK4/6i	I/II	MDT, CBR, ORR	September 2024	NCT03090165
Bicalutamide	AR	II	CBR, PFS	May 2017	NCT02353988

Abbreviations: TROP2, trophoblast cell surface antigen 2; PD-L1, programmed death-ligand 1; Akt1/2/3, protein kinase B 1/2/3; PARP, poly(ADP-ribose) polymerase; CSF-1R, colony-stimulating factor 1 receptor; c-Kit, receptor tyrosine kinase (also referred to as stem cell factor receptor or CD117); PD-1, programmed death-1; HER, human epidermal growth factor receptor; LIV-1, zinc transporter; LAG3, lymphocyte activation gene 3; CDK4/6, cyclin-dependent kinase 4 and 6; IL-12, interleukin-12; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; VEGFR, vascular endothelial growth factor receptor; PDGFRβ, platelet-derived growth factor receptor beta; FGFR1, fibroblast growth factor receptor 1; CSF1R, colony-stimulating factor 1 receptor; RET rearranged during transfection gene; PI3K-γ, phospholnositide 3-kinases gamma; AR, androgen receptors; TIGIT T, cell immunoreceptor with Ig and ITIM domains; CSF-1, colony-stimulating factor 1; IL-1 beta, Interleukin beta; JAK1&JAK2, janus kinase 1 and 2; IAP, inhibitor of apoptosis proteins; B7-H3, B7 homolog 3 protein; NK, natural killer; CCR5, C-C chemokine receptor type 5; XPO1, exportin 1; PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; IL-6R, interleukin 6 receptor; MEK, MAPK/ERK Kinase; ROR, receptor tyrosine kinase like orphan receptor; MAGE-A1, melanoma-associated antigen 1; TCR, T cell receptor; ICOS, inducible co-stimulator; RAD51, recombinase in DNA repair; TAM, TYRO3/AXL/MERTK pathway; PLK4, polo-like kinase 4; pCR, pathological complete response; DFS, disease-free survival; TILs, tumor-infiltrating lymphocytes; TAM, tumor-associated macrophages; TME, tumor microenvironment; MTD, maximum tolerated dose; AEs, adverse events; IDFS, invasive disease-free survival; ORR, overall response rate; cfDNA, cell free DNA; DLT, dose-limiting toxicity; RP2D, recommended phase II dose; CB, clinical benefit; DTC, disseminated tumor cells; PFS, progression-free survival; PFS-6, progression-free survival at 6 months; rPFS, radiographic progression-free survival.

the study has been temporarily withdrawn after the request of further understanding about the role of androgen signaling in TNBC.

Interestingly, the expression of retinoblastoma protein (Rb), the product of the RB tumor suppressor gene, tends to be associated with AR expression in TNBC [99]. This observation suggests a luminal-like biology despite the TNBC subtype, and support the hypothesis of increased efficacy by combining anti-androgens with CDK4-6 inhibitors for these tumors [99]. A phase II study evaluating the combination of bicalutamide with palbociclib for AR-positive TNBC showed that this dual treatment was safe and active, with 33% of patients (11/33) progression-free at 6 months [100]. A phase II study (NCT03090165) is evaluating the combination of bicalutamide and ribociclib in AR-positive advanced TNBC. Moreover, the combination of the anti-androgen blockade with the inhibition of PI3K pathway is another promising strategy. The addition of taselisib to enzalutamide has been tested in a phase I/II study and showed higher benefit in patients with LAR TNBC compared to those with non-LAR disease (CBR: 75% and 12.5%, respectively, $p = 0.06$; PFS 4.6 vs. 2.0 months, respectively, $p = 0.082$) [101]. A phase I study testing another PI3K-inhibitor, alpelisib, in combination with enzalutamide in patients with AR and PTEN-positive metastatic BC is ongoing (NCT03207529).

Thus far, the benefit of anti-androgen treatment for AR-expressing BCs has been the topic of an intense academic discussion, and according to the international guidelines recently released by the ESMO for patients with mBC, there is no data to support anti-androgen therapy for routine use outside a clinical trial in patients with advanced TNBC [54].

4.2 The PI3K-AKT-mTOR pathway

Alterations in the PI3K-AKT-mTOR pathway are common genomic aberrations occurring in BC, across various subtypes (10,102). In TNBC, approximately 35% of all cancers harbor *PIK3CA/AKT1/PTEN* alterations. The activation of this signaling pathway is stimulated by tyrosine kinase receptors, which trigger PI3K activation and, subsequently, the phosphorylation of AKT and mTOR complex (Figure 3). In TNBC, the constitutive activation of this pathway can occur as the result of an overexpression of upstream regulators (e.g. epidermal growth factor receptor (EGFR)), or of

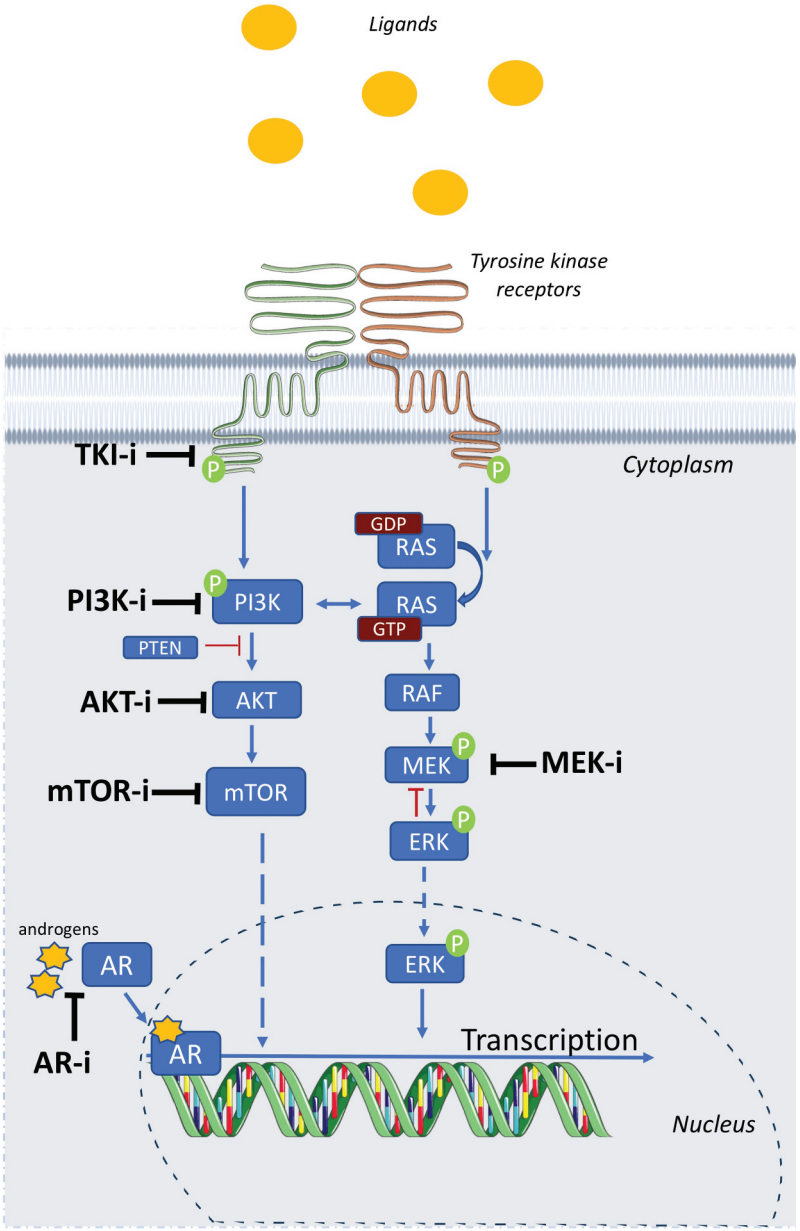

Figure 3. Simplified representation of the PI3K-AKT-mTOR, MAPK, and AR signaling pathways. The activation of PI3K-AKT-mTOR pathway is stimulated by tyrosine kinase receptor, which triggers PI3K activation and, subsequently, the phosphorylation of AKT and mTOR complex. The activation of the tyrosine kinases receptor also stimulates the activation of RAS; RAS activates RAF intracellular kinases, thus starting a downstream signaling involving MEK and ERK. Both signaling pathways trigger nuclear transcription factors that regulate, among others, cell growth, proliferation, survival, angiogenesis, and migration. The PI3K-AKT-mTOR and MAPK pathways are strictly interconnected to each other. Moreover, several other oncogenic pathways (e.g. FR, cMET) released by the inactivation of P53 may activate the PI3K-AKT-mTOR pathway. The AR is a nuclear receptor that is activated by binding of an androgen in the cytoplasm and then translocates into the nucleus regulating gene transcription.

activating mutations of PI3K catalytic subunit α (PIK3CA) and/or of AKT1, or the loss of function of downregulators of PI3K (e.g. PTEN) [103]. Moreover, other oncogenic pathways (e.g. FGFR cMET) released by the inactivation of P53 can activate the PI3K-AKT-mTOR pathway [104]. Several PI3K/AKT/mTOR-targeted therapies for TNBC are currently under investigation. The combination of alpelisib, a PI3Kα-selective inhibitor, and nab-paclitaxel has been tested in a phase I/II study and demonstrated encouraging antitumor activity along with a manageable toxicity profile in patients with HER2-negative mBC (ORR 59%, median PFS 8.7 months) (Table 5). The improvement was even more pronounced in patients with tumor and/or circulating tumor DNA (ctDNA) *PIK3CA* mutations, compared to those without mutation (PFS 11.9 vs. 7.5 months; HR 0.44; $p = 0.027$) [105]. Capivasertib is a pan-AKT inhibitor that has been evaluated, in combination with paclitaxel, as first-line treatment for patients with mTNBC, in a phase II randomized trial [106]. The addition of capivasertib has resulted in a significant improvement of PFS and OS, compared to placebo (HR 0.74, 95% CI 0.50–1.08 and HR 0.61, 95% CI 0.37–0.99, respectively), and in particular in those patients with alterations of *PIK3CA/AKT1/PTEN* [106] (Table 5). Consistently, another AKT inhibitor, ipatasertib, evaluated in a phase II

Table 4. Main studies targeting the androgen pathway in AR-positive TNBC.

Study	Treatment	Phase	Population	Main results
Gucalp et al. [95]	Bicalutamide 150 mg daily	II	AR-positive (AR>10%), ER/PgR-negative advanced breast cancer (n = 452)	CBR (CR, PR, or SD>6 mos): 19% (95% CI 7–39%) mPFS: 12 weeks (95% CI 11–22 weeks)
Traina et al. [96]	Enzalutamide 160 mg daily	II	AR-positive (AR >0%) advanced TNBC (n = 118)	• ITT population: CBR 25%, mPFS 2.9 mos, mOS 12.7 mos • Patients with AR ≥10%: CBR 33% and mPFS 3.3 mos, mOS 17.6 mos
Bonnefoi et al. [97]	Abiraterone acetate 1000 mg once daily + prednisone 5 mg twice daily	II	AR-positive (AR >10%) advanced TNBC (n = 30)	6-month CBR (CR, PR, or SD >6 mos): 20% ORR 6.7% mPFS 2.8 mos
Gucalp et al. [100]	Bicalutamide 100 mg daily + Palbociclib 100 mg daily 3 weeks on and 1 week off	II	AR-positive (AR ≥1%) advanced TNBC (n = 31)	Best response: 11 pts progression-free at 6 mos (10 SD > 6 mos, 1 PR) Median weeks on study: 14 (2–6,6–80,86–105)
Lim et al. 81]	Doxorubicin-based neoadjuvant CT (AC) + enzalutamide 160 or 120 mg daily + paclitaxel 80 mg/m^2 weekly for 12 cycles before surgery	II	AC insensitive AR-positive (AR >10%) stage I-III TNBC (n = 15)	pCR or RCB I: 33.3% (5/15)
Traina et al. [82,83]	Adjuvant enzalutamide for 1 year	II	AR-positive (AR ≥1%) stage I-III TNBC (n = 50)	Preliminary results: 34 pts completed 1 y of treatment. 15 pts are off treatment: PD [3], toxicity (5), noncompliance (4), withdrawal of consent [3].
Vetter et al. [84]	CR1447 (transdermal formulation of 4-OH-Testosterone)	II	Cohort A: ER-positive advanced BC (n= 29) C ohort B: AR-positive advanced TNBC (n = 8)	Disease control at 24 weeks: 0% in cohort B. mPFS: 2.5 mos in cohort B. mOS: 10.8 mos in cohort B.
Yuan et al. 85]	Enobosarm + permbrolizumab	II	AR-positive (AR ≥1%) advanced TNBC (n = 16)	RR: 2 of 16 (13%). CBR at 16 weeks 4 of 16 (25%). mPFS 2.6 mos and mOS 25.5 mos.
Lehmann et al. [101]	Enzalutamide 160 mg daily ± taselisib 4 mg	II	AR-positive (AR ≥10%) advanced TNBC (n = 17)	16-weeks CBR (combination arm): 35.7% mPFS (combination arm): 3.4 mos CBR in LAR vs. non-LAR TNBC: 75.0% vs. 12.5%, p = 0.06. PFS in LAR vs. non-LAR TNBC: 4.6 vs. 2.0 mos, p = 0.082.

Abbreviations: AR, androgen receptors; ER, estrogen receptors; PgR, progesterone receptors; CBR, clinical benefit rate; CR, complete response; PR, partial response; SD: stable disease; mos, months; PFS, progression-free survival; TNBC, triple-negative breast cancer; ITT, intention-to-treat; OS, overall survival; ORR, overall response rate; CT, chemotherapy; AC, doxorubicin–cyclophosphamide; pCR, pathological complete response; RCB, residual cancer burden; RR, response rate, LAR, luminal androgen receptor; y, years.

study (LOTUS) in combination with paclitaxel as first-line treatment for mTNBC, showed an increase in median PFS (from 4.9 to 6.2 months in the ITT population, and from 3.7 to 6.2 months in patients with *PTEN*-low tumors) and a trend toward improved OS [107,108] (Table 5). Disappointingly, despite the heels of positive phase II data, the phase III study of ipatasertib plus paclitaxel in advanced TNBC (IPATunity130 [NCT03337724]) failed to improve PFS (median PFS 7.4 months in the ipatasertib arm versus 6.1 months in the placebo arm, HR 1.02, 95% CI 0.71–1.45) [109] (Table 5). The phase III studies testing capivasertib (CAPItello-290 [NCT03997123]) and alpelisib (EPIK-B3 [NCT04251533]) in addition to (nab)-paclitaxel for mTNBC are ongoing.

Ipatasertib has also been evaluated in the neaoadjuvant setting. In a phase II trial, the combination of ipatasertib with 12 cycles of weekly paclitaxel did not increase pCR rates compared to placebo/paclitaxel [110]. Nevertheless, MRI-assessed responses, a secondary endpoint, favored ipatasertib/paclitaxel, especially in the *PIK3CA/AKT1/PTEN*-altered population [110]. Alpelisib is being evaluated in the neoadjuvant setting in a phase II trial in association with nab-paclitaxel in anthracycline refractory TNBC with *PIK3CA* or *PTEN* alterations [111].

Other drugs targeting the PI3K/AKT/mTOR pathway are being investigated in TNBC, as mTOR inhibitors and dual inhibitors, and combinations with immune checkpoint inhibitors (NCT02616848, NCT04177108).

At the moment, as indicated in the recently published ESMO clinical practice guidelines for patients with mBC, there is no data to support inhibitors targeting PI3K or AKT for advanced TNBC, and therefore these cannot be recommended in routine clinical practice outside a clinical trial [54].

4.3 The MAPK pathway

The mitogen-activated protein kinase (MAPK) pathway has been the subject of cancer research for several tumor types, as its aberrant activity is known to have a crucial role in the initiation and progression of cancer [112]. In TNBC, there are controversial data on the role of this signaling pathway as a potential therapeutic target. In a comprehensive analysis of primary breast tumors of The Cancer Genome Atlas, the incidence of RAS and RAF family mutations assessed by next-generation sequencing was reported to be lower than 2% [10], and this rate was confirmed in other subsequent studies [113]. Although one may hypothesize that a low frequency of *RAS/MAPK* mutations implies that cancer cells do not use this pathway as a preferential way for sustained growth and proliferation, some evidence suggests that targeting this pathway might yield therapeutic efficacy. Indeed, the RAS/MAPK pathway is strictly interconnected with other pathways (e.g. PI3K/

Table 5. Main studies targeting the PI3K-AKT-mTOR pathway in patients with advanced TNBC.

Study	Treatment	Phase	Population	Main results
Sharma et al. [105]	Alpelisib + Nab-paclitaxel	I/II	HER2-negative advanced breast cancer (any line of prior therapies) (n = 42)	• All patients: ORR: 59% (complete response, 7% and partial response, 52%); mPFS 8.7 months. • Patients with vs. without tumor/ctDNA *PIK3CA* mutation: PFS 11.9 vs. 7.5 months; HR 0.44; p = 0.027.
PAKT Trial [106]	Capivasertib + Paclitaxel vs. Placebo + Paclitaxel	II	Untreated advanced TNBC (n = 140)	• All patients: mPFS 5.9 months in capivasertib arm vs. 4.2 months in placebo arm (HR 0.74; 95% CI 0.50–1.08; p = 0.06; mOS 19.1 vs. 12.6 months (HR, 0.61; 95% CI, 0.37-0.99; p=0.04). • Patients with *PIK3CA/AKT1/PTEN*-altered tumors: mPFS 9.3 months in capivasertib arm vs. 3.7 months in placebo arm (HR 0.30; 95% CI 0.11–0.79; p = 0.01).
LOTUS Trial [107,108]	Ipatasertib + Paclitaxel vs. Placebo + Paclitaxel	II	Untreated advanced TNBC (n = 124)	• All patients: mPFS 6.2 months in ipatasertib arm vs. 4.9 in placebo arm (HR 0.60, 95% CI 0.37–0.98; p = 0.037); mOS 25.8 months in ipatasertib arm vs. 16.9 in placebo arm (HR 0.80, 95% CI 0.50-1.28) • Patients with PTEN-low tumors: mPFS 6.2 months in ipatasertib arm vs. 3.7 in placebo arm (HR 0.59, 95% CI 0.26–1.32, p = 0.18); mOS 23.1 in ipatasertib arm vs. 15.5 in placebo arm (HR 0.83) • Patients with *PIK3CA/AKT1/PTEN*-altered tumors: mOS 25.8 in ipatasertib arm vs 22.1 in placebo arm (HR 1.13)
IPATunity-130 [109]	Ipatasertib + Paclitaxel vs. Placebo + Paclitaxel	III	Untreated advanced TNBC (n = 255)	mPFS 7.4 months in ipatasertib arm vs. 6.1 in placebo arm (HR 1.02, 95% CI 0.71–1.45; p = 0.92) ORR: 39% in ipatasertib arm vs 35% in placebo arm

Abbreviations: ORR, overall response rate; mPFS, median progression-free survival; ctDNA, circulating tumor DNA; HR, hazard ratio; TNBC, triple-negative breast cancer; CI, confidence interval; mOS, median overall survival.

AKT), and alterations in different, parallel but highly interconnected pathways may result in the aberrant expression of downstream effectors of MAPK signaling as well [112,114]. Consistently, in an analysis of 26 primary breast tumors, active MAPK expression was observed in 48% of samples, and was significantly increased in tumors compared with adjacent normal breast (p = 0.027). Moreover, increased active MAPK expression was observed in concurrent lymph node metastases, compared with primary breast tumors (p = 0.0098), suggesting that MAPK could have a role in the metastatic process [115]. In the absence of straightforward mutations able to predict therapeutic efficacy, an approach targeting a downstream effector of the MAPK pathways, as MEK (Figure 3), has been explored. MEK inhibitors in monotherapy showed only a modest activity in solid tumors in early phase clinical trials [116–118], probably due to compensatory activation of alternative pathways, thus leading to test combination therapies as a more promising strategy. In a phase Ib study, the association of vistusertib (dual mTOR1/2 inhibitor) and selumetinib (MEK1/2 inhibitor) induced stable disease for more than 16 weeks in 7 patients with advanced tumors (including TNBC), with a duration of response ranging up to more than 55 weeks [119]. Conversely, in a phase II study on 33 patients with advanced TNBC, the MEK inhibitor trametinib showed limited efficacy, both alone and in combination with an AKT inhibitor (partial response or stable disease in 3/31 and in 1/16 patients, respectively) [120]. Additionally, the combination strategy was associated with high levels of toxicity, including diarrhea and cutaneous rash [120].

Several hypotheses have been proposed to explain these disappointing results. First, most early phase clinical trials were conducted on patients with mBC. Considering the role of MAPK pathway in the metastatic process, it is possible that the inhibition of MEK does not result in an observable phenotype once metastasis has occurred in the patient [112]. Additionally, it is likely that the poor safety profile of MEK and AKT inhibitors could have negatively affected their efficacy, at least in part. Indeed, due to their toxicity profile, continuous dosing was not possible, and these kinase inhibitors typically required a 3-weeks on and 1-week off administration. The 1-week off could potentially give the cancer cells time to reprogram and/or grow. Translational research projects are ongoing to help in identifying biomarkers of response to better select patients who may benefit from this treatment. An ongoing phase II study (InCITe, NCT03971409) is testing the combination of avelumab with liposomal doxorubicin with or without binimetinib, a MEK-inhibitor, in patients with advanced TNBC.

The extracellular signal-regulated kinase 1/2 (ERK1/2) is another target under investigation in TNBC. Thus far, only preclinical evidence is available, showing that the combination ERK1/2 inhibitors with PD-1/PD-L1 inhibitors can actively reduce the proliferation of TNBC cell lines [121].

5. Expert opinion

5.1 TNBC: an evolving treatment landscape

TNBC has traditionally been considered as a “targetless” subtype, where chemotherapy was the only available treatment strategy. Currently, several non-chemotherapeutic treatments entered the clinic, enriching the therapeutic armamentarium and the strategies of cure. In particular, the anthracycline/taxane-based chemotherapy still represents the standard adjuvant treatment in stage II-III TNBC, but post-operative olaparib plays a role in germline BRCA mutation [39]. Moreover, the standard neoadjuvant chemotherapy treatment takes now advantage of the addition of platinum and perioperative pembrolizumab [65]. Whether chemotherapy is often used in stage IV disease, the first-line treatment clearly benefits of the

adding of the immune checkpoint inhibitors in the PD-L1 positive tumors [54,58] and the use of single-agent PARP-I is an attracting option both in the first and subsequent lines of treatment in germline BRCA mutation. In patients progressing after the first-line therapy, the use of the ADC is currently recommended as the new gold standard of treatment [72], leaving the other chemotherapy options for more advanced lines of therapies, if clinically indicated.

5.2 Open challenges and key developments

The main characteristic of TNBC is the huge biological diversity, observed both among different tumors (intertumor heterogeneity) and individual tumor (intratumor heterogeneity), and actually expressed as spatial and temporal heterogeneity. While the spatial heterogeneity describes the biological differences of distinct areas of tumor, the temporal heterogeneity refers to variations occurring overtime during tumor progression because of intrinsic (epi)genetic instability and clonal evolution under therapeutic pressure. Deciphering the TNBC temporal heterogeneity is of paramount importance because it is able to offer a new dimensional cancer measurement not otherwise captured.

Moreover, tumor microenvironment has a close relationship with cancer cells, and it can play a crucial role in terms of cancer development and response to therapies. Hence, both intrinsic tumor heterogeneity and extrinsic characteristics of the microenvironment should be considered to explain potential tumor resistance to treatments.

5.3 Future directions

Several new therapeutic options are under investigation for patients with TNBC. Beyond those already described above in the manuscript, the oral CDK7 inhibitor samuraciclib seems another promising option, that has been granted fast track designation by FDA in August 2021 in combination with chemotherapy for patients with metastatic TNBC. RSK (p90 ribosomal S6 kinase 2) is a novel target kinase for TNBC; PMD-026 is an oral inhibitor of RSK that showed some preliminary data in monotherapy in metastatic TNBC patients whose disease had progressed on standard therapy [122].

Treatments targeting epigenetic can represent another promising strategy. Epigenetic regulators are essential for the temporal maintenance of cell identity overtime, with alterations including DNA methylation, histone, and chromatin modification, and non-coding RNA interference [123]. Since epigenome dysregulation is pervasive in cancer, significant progresses have been made in developing drugs targeting epigenetics [124,125] including DNA hypomethylating agents (DNA methyltransferase inhibitors) and the histone deacetylase (HDAC) inhibitors [126,127].

DNA methyltrasferase 1 (DNMT1) is the most crucial enzyme in the DNMTs family in humans and it is highly expressed in TNBC compared to other subtypes [128]. Notably, a preclinical study suggests that DNMT inhibitors such as 5-azacytidine can increase the efficacy of PARP inhibitors in BC cells with wild-type BRCA1 [129]. Moreover, hypomethylating agents might play a role in selected cases of breast cancer with sporadic abnormally BRCA1 gene promoter methylation [130]. The HDACs are a class of enzymes that deacetylate histones leading to chromatin condensation, repressing transcription. Therefore, HDAC inhibitors could induce tumor cell apoptosis, inhibit cell migration and invasion and sensitize cancer cells to chemotherapy [131]. Preclinical and clinical studies with HDAC inhibitors in combination with other drugs showed promising results in TNBC [125]. Another relevant candidate target, which is involved in chromatin regulation, is the architectural chromatin family of high mobility group A proteins (HMGA). Indeed, HMGA are overexpressed in cancers, regulate chromatin plasticity and, in TNBC, can act as master regulators of genes involved in epithelial-to-mesenchymal transition, migration, invasion, and angiogenesis [132,133].

While the TNBC epigenome portraits deserve further investigations, a comprehensive and unified (epi)genetic understanding of the disease would potentially support the emerging of novel therapeutic approaches to extend the clinical benefit for the patient.

6. Conclusion

The optimal treatment strategy for TNBC is still an unmet medical need. The better understanding of the biological diversity of the disease, through the accurate pathological and molecular dissection, could pave the way for the development of novel signaling-based therapies, with the potential to improve the outcomes of patients with TNBC, fulfilling the promises of the precision oncology.

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List of abbreviations

ADC, antibody drug–conjugate; AR, androgen receptor; BC, breast cancer; BL, Basal-like: CBR, clinical benefit rate; CI, confidence interval; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; ctDNA, circulating tumor DNA; EGFR, epidermal growth factor receptor; ER, estrogen receptor; gBRCAm, germline BRCA mutations; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; HRD, homologous recombination deficiency; ICI, immune checkpoint inhibitor; IM, immunomodulatory; ITT, intention to treat; LAR, luminal androgen receptor; M, mesenchymal; MAPK, mitogen-activated protein kinase; MSL, mesenchymal stem-like; mTNBC, metastatic TNBC; ORR, overall response rate; PARP, poly(ADP-ribose) polymerase; pCR, pathologic complete response; PD-1, cell death protein-1; PD-L1, PD-ligand 1; PFS, progression-free survival; PgR, progesterone receptor; po, oral administration; PI3K, phosphoinositide-3-kinase; q.d., once a day; TNBC, triple-negative breast cancer; TN, triple-negative; Topo1, topoisomerase I; TPC, treatment of physician choice.

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