Triple-negative breast cancer: new perspectives for targeted therapies

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Abstract:
Breast cancer is a heterogeneous disease, encompassing a large number of entities showing different morphological features and having clinical behaviors. It has become apparent that this diversity may be justified by distinct patterns of genetic, epigenetic, and transcriptomic aberrations. The identification of gene-expression microarray-based characteristics has led to the identification of at least five breast cancer subgroups: luminal A, luminal B, normal breast-like, human epidermal growth factor receptor 2, and basal-like. Triple-negative breast cancer is a complex disease diagnosed by immunohistochemistry, and it is characterized by malignant cells not expressing estrogen receptors or progesterone receptors at all, and human epidermal growth factor receptor 2. Along with this knowledge, recent data show that triple-negative breast cancer has specific molecular features that could be possible targets for new biological targeted drugs. The aim of this article is to explore the use of new drugs in this particular setting, which is still associated with poor prognosis and high risk of distant recurrence and death.

Keywords: basal-like breast cancer, estrogen–progesterone receptors, gene-expression microarray, human epidermal growth factor receptor 2, chemotherapy, target therapy

Introduction
In 2014, about 235,030 new cases of breast cancer (BC) are expected in the USA, with 40,430 cancer-related deaths.\textsuperscript{1} In 2009, approximately 170,000 BC cases were of the triple-negative (TN) phenotype,\textsuperscript{2} accounting for 10%–20% of invasive BC.\textsuperscript{1–3}

Triple-negative breast cancer (TNBC) is defined by its lack of estrogen-receptor (ER) and progesterone-receptor (PR) expression, along with the absence of human epidermal growth factor receptor 2 (HER2) overexpression or gene amplification.\textsuperscript{2} Nowadays, TNBC is one of the most attractive areas of research in oncology. Reasons for this scientific interest are the lack of a recognized target for molecular-oriented therapy, which makes TNBC a new orphan disease. Furthermore, TNBC is a biologically aggressive neoplasm with a strong association with distant recurrence, visceral metastases, and death when compared with other BC types.\textsuperscript{4} Recurrence often occurs within 3 years of diagnosis, while 5-year mortality rates appear to be increased after initial diagnosis.\textsuperscript{4} Moreover, metastatic setting implies a much shorter median time from relapse to death.\textsuperscript{4,5} Particularly, the median survival of advanced TNBC is at best 12 months, much less than the median duration of survival observed in other advanced BC subtypes.\textsuperscript{5}

Considering the gene-expression analysis, it appears very clear that TNBC is a heterogeneous BC form, which shows only partial overlapping features with so-called basal-like (BL) breast cancer (BLBC).\textsuperscript{6–8} In fact, not all TN tumors are identified as...
BL by gene expression, and not all BL tumors are TN; the discordance rate between the two definitions is 20%–30%.9 More recently, subtyping of three large clinical trials (GECAM/9906, MA.12, and MA.5) using the PAM50 quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)-based assay revealed that approximately 30% of tumors identified as TN by central pathology review do not fall into the BL subtype category.10 Therefore, significant biological heterogeneity exists within the group of patients diagnosed with TN disease, with the BL subtype being undoubtedly the most frequently observed (about 75%).11

Very recently, Lehmann et al12 analyzed gene-expression profiles from 21 BC datasets and confirmed the concept that a significant heterogeneity within TNBC and BC tumors occurs. According to this analysis, there are six different TNBC subtypes: two BL (BL1 and BL2), an immunomodulatory, a mesenchymal (M), a mesenchymal stem-like, and a luminal androgen receptor.12

In this regard, investigators identified BC cell lines representative of each of these molecular subtypes with different sensitivities to various targeted therapies currently under clinical investigation, providing an attractive platform for future drug development in TNBC.13

These fascinating results constitute the basis for future approaches in TNBC, using both cytotoxic drugs not classically used in BC (platinum salts) or alternative schedules (dose dense), and new drugs (poly-ADP-ribose-plymerase-1, agents targeting the epidermal growth factor receptor (EGFR), multi-tyrosine-kinase inhibitors, or antiangiogenic agents).

The aim of this paper is, therefore, to discuss targeted agents with a possible activity against TNBC.

**Perspectives in treatment of TNBC: targeting the molecular feature Hormonal therapy**

Recent guidelines dictate that a hormonal receptorial threshold <1% should be used;14 about this, the St Gallen expert group recommends adjuvant endocrine therapy for any detectable hormone receptor.15

Recent findings show that other isoforms of ERs could be present that are not detected by the current techniques; in this way BCs expressing some form of ERs are labeled “hormone-receptor negative” BCs.

Three forms of ERs, ERα, ERβ, and G-protein-coupled receptors (GPCRs), are currently known; they are co-expressed in a large fraction of normal and BC tissues. Particularly the first two, the classical steroid receptors, are localized not only in the plasma membrane,16 but also in the cytosol and/or nucleus; traditionally, they exert their effects at the genomic level. In recent years, however, a large number of reports has described membrane-associated ERs, such as GPCRs.17,18 Recent studies showed that rapid effects are mediated by these novel transmembrane Estrogen receptors, also known as G-protein coupled receptor 30 (GPR 30).19,21 Anyhow, ERs play important roles in the initiation and progression of estrogen-related BC.22

ERα in several studies seems to be responsible for governing estrogen-dependent growth, response to endocrine therapy,23 and prognosis in ERα-positive BC; ERβ seems to be an antagonist against ERα effects.24-28 According to some data, it seems that ERβ functions are different when it is co-expressed with ERα than when it is expressed alone.29,30 In the same way, GPR30 plays a role in the regulation of cellular growth, proliferation, and apoptosis.31 Moreover, effects mediated by GPR30 are maintained when classic ERs are absent or blocked. In addition, GPR30 is involved in drug resistance, implying that GPR30 may be an important therapeutic target for estrogen-related tumors. Blocking both GPR30 and classic ERs may be a better strategy for the treatment of estrogen-related tumors. However, despite the indications that GPR30 may represent a novel estrogen-binding receptor, a description of its ligand-binding properties appears to be lacking in the scientific literature.32 Interestingly, ER antagonists/selective estrogen-receptor modulators, such as tamoxifen and ICI182,780, have also been shown to bind GPR30, which is consistent with previous functional studies showing that these same compounds are agonists for GPR30.32,33 Moreover, with the recent report of a highly selective, nonsteroidal GPR30 agonist, future studies of GPR30 function should be greatly facilitated by this novel reagent.34 However, some breast cells express only ERα, whereas other breast cells express only ERβ. There are also breast cells that express neither ERα nor ERβ.35

Anyhow, the ER status in BC patients has been traditionally defined by the presence or absence of ERα; in fact, the ER-negative status of TNBC patients is regularly negative for ERα but not necessarily negative for ERβ.36 Indeed, several studies have reported expression of ERβ in a substantial fraction of ERα-negative and TNBC patients. Gruberger-Saal et al examined ERα and ERβ expression in patients treated with Tamoxifen (TAM) for 2 years, observing that ERβ was significantly associated with increased distant disease-free survival (DFS); furthermore, ERβ was an independent marker within the ERα-negative tumors.37 Importantly, ERβ1 positivity was significantly associated with better survival...
in patients with ERα-PR-negative or triple-negative (TN)-
tumors. On this basis, ERβ1 and ERβ variants may play some
roles in estrogen signaling and the pathogenesis of TNBC;
however, this hypothesis was not confirmed by clinical and
histopathological parameters.38

Furthermore, approximately 75% of all BCs are ERα
positive, which is a good predictor of treatment response.39–41
The ER-α gene is composed of six functional domains
encoded by eight exons that commonly produce a 66.2 kDa
protein (ER-α66).42 Furthermore, ER-α66 is the isoform
detected in clinical practice. There are at least two different
isoforms of ER-α66 described in human BC:43 ER-α46 and
ER-α36.44,45 The importance of these isoforms lies in the
possibility that some TNBCs are expressing some truncated
ERα receptors that could be a target for anti-estrogen therapy.
Particularly, ER-α36 is a new isoform of ERs without the
transcriptional activation domains of the classical ER-α66.46
ER-α36 has been shown to transduce the membrane-initiated
steroid signaling cascade, and acts as a dominant-negative
effector of estrogen-dependent and -independent transactiva-
tion, mediated by ER-α66.47,48 ER-α66 expression can also
be detected in the cytoplasm of BC cells after long-term
treatment with tamoxifen, coinciding with resistance to
the drug.48

Moreover, Shi et al indicated the importance of ER-α36
in the development of endocrine resistance in a subgroup
of invasive BC that exhibits co-expression of ER-α66 and
ER-α36. The functional importance of ER-α36 is related to
its non-genomic ER activities; according to this hypothesis,
activation of the mitogen-activated protein kinase/extracellular
signal-regulated kinase (MAPK/ERK) signaling pathway
plays a major role. The MAPK/ERK signaling pathway is ac-
tivated in response to both estrogens and antiestrogens, which
might be of particular importance for ER-α66-negative BC,
since this subgroup might still respond to antiestrogen-based
therapy.49 Previous experiments demonstrated that antiestro-
genins induce a stronger and more prolonged activation of the
MAPK/ERK signaling pathway than the estrogens.45

The androgen cluster is a particular kind of BC showing
the androgen receptor (AR) in a very similar way to ER +
PgR + BC.49 Until 1970, androgen therapy was one of the
strategies used to manage BC. Moreover, the presence of
the AR has been well known for a long time; even while the
clinical use of this knowledge has been abandoned,
experimental studies have continued, showing that the AR
is the most commonly expressed nuclear hormone recep-
tor in BC.50–52 Recent studies suggest that 10%–35% of all
TNBC presents an AR-positive gene-expression profile;
moresover, these TNBCs are frequently apocrine BC51,
even if some other experiences show no significantly
different histopathological features between AR-positive
and AR-negative BC.53

Anyhow, AR-targeting has been introduced recently as a
novel therapeutic option in TNBC,54 and a Phase II trial of
nonsteroidal antiandrogen treatment is ongoing in women
with advanced AR-positive, ER-negative, PR-negative BC.
The preliminary results of this trial suggest attractive benefit.55
On this basis, other trials are investigating the use of androgen
inhibition in TNBC.

Poly (adenosine diphosphate-ribose)
polymerase inhibitors

Genomic integrity and cell survival are critically related
to coordinated pathways of DNA repair.56 Poly (adenosine
diphosphate-ribose) polymerase (PARP) enzymes –
particularly the most abundant isoform, PARP1 – play a key
role in these pathways by mediating the repair of single-strand
DNA breaks via base-excision repair.57 Consequently, loss
of PARP activity results in the accumulation of single-strand
breaks, which are normally repaired by double-strand homolo-
gous recombination pathways that include the important
tumor-suppressor proteins BRCA1 and BRCA2.58 It is well
known that germ-line BRCA1 and BRCA2 mutations are
associated with a high risk of oncogenesis for breast and ovar-
ian cancers.59 TNBC shares clinical and pathological features
with hereditary BRCA1-related BC; in sporadic TNBC, dys-
regulation of BRCA1 has been frequently observed together
with other defects in homologous recombination pathways.60,61
A preclinical study showed that TNBC cells are more sensitive
to PARP1 inhibitors when compared with non-TNBC cells,
and that PARP inhibition acts synergically in association
with gemcitabine and cisplatin in TN cells but not in lumi-
nal cancers.62 All this evidence provides a strong rationale
for developing a new therapeutic approach to TNBC based
on targeting the DNA-repair defects via PARP inhibition.
Several PARP inhibitors, such as olaparib, veliparib, iniparib,
niraparib, andrucaparib, are currently under early develop-
ment in TNBC or BRCA-mutated BC.

Establishing which subtypes of BC are most indicated
for PARP inhibitor treatment, and how to identify these,
is a matter of strategic importance for further clinical
development of this class of anticancer agents. Despite
their extremely high therapeutic potential, more research
on the biology of the numerous members of the PARP
families and on their role in the molecular pathogenesis
of BRCA1- and BRCA2-mutated is necessary before we
can claim to understand the utility of the PARP inhibitors in clinical oncology.

**Olaparib**

Olaparib, an oral PARP inhibitor, demonstrated its antitumor activity in patients who were carriers of the BRCA1 or BRCA2 mutation in a Phase I trial. Moreover, olaparib has been investigated as a single agent in BRCA1- and BRCA2-mutated women with advanced BC in a Phase II trial. In this nonrandomized trial, patients were assigned to one of two sequential cohorts; in the first cohort oral olaparib at 400 mg twice daily was given continuously, while in the second one a lower dose of 100 mg twice daily was used. The primary endpoint was objective response rate (RR). All patients had received previous chemotherapy. The objective RR was confirmed in 11 of 26 patients in cohort 1 and six of 24 in cohort 2. The clinical benefit rate (CBR) was 52% for cohort 1 and 26% for cohort 2. Median progression-free survival (mPFS) was 5.7 months for cohort 1 and 3.8 months for cohort 2. Furthermore, olaparib toxicity was generally mild and manageable, with the most frequent adverse events (AEs) occurring in cohort 1 (fatigue, nausea, vomiting, and anemia). The results of this study provide an important proof-of-concept for PARP inhibition in BRCA-deficient BC; selecting patients with TNBC who are also BRCA deficient could isolate a patient population who may be more receptive to treatment with PARP inhibitors. It is interesting to observe that there is no reported activity for olaparib against non-BRCA-mutant or unreported BRCA status.

In another Phase II, multicenter, open-label, non-randomized study, women with advanced high-grade serous and/or undifferentiated ovarian carcinoma or TNBC were enrolled and received olaparib 400 mg twice a day. Patients were stratified according to whether they had a BRCA1 or BRCA2 mutation or not. The primary endpoint was objective RR. In the TNBC cohort, no RECIST (Response Evaluation Criteria In Solid Tumors) validated responses were reported at the first stage, so this cohort was closed.

A Phase I study evaluated the safety, tolerability, and efficacy of olaparib in combination with paclitaxel in patients with metastatic TNBC (mTNBC). Patients who had received ≥1 prior cytotoxic regimens for mTNBC were treated continuously with olaparib plus weekly paclitaxel. Nineteen patients received treatment. Patients in cohort 2 received olaparib and paclitaxel at the same doses and schedule as patients in cohort 1. For patients in cohort 2, upon first occurrence of grade ≥2 neutropenia, the paclitaxel dose was omitted or delayed; olaparib dosing was continued. Objective RR in cohort 1 was 3/9 patients and in cohort 2 was 4/10 patients.

This agent is currently undergoing clinical evaluation in combination with paclitaxel in a Phase II single-arm trial and in combination with carboplatin and/or paclitaxel in a Phase I study – both trials are in patients with mTNBC. Olaparib is also undergoing investigation in combination with cisplatin in a Phase I single-arm trial in neoadjuvant approach for locally advanced TNBC.

**Veliparib**

Veliparib is an oral PARP1 and PARP2 inhibitor. In a single-arm Phase II trial, the activity of veliparib and temozolomide (TMZ) was tested in 41 metastatic BC patients (15 TNBC patients). TMZ has minimal activity in BC, which is probably due to the methylated DNA-repair adducts by the base-excision repair pathway. The primary endpoint in the study was overall RR. Best responses for 24 patients evaluable at the time of abstract submission included one complete response (CR), two partial responses (PRs), seven stable diseases (SDs), and 14 progressive diseases (PDs); 17 patients were not yet evaluable for response. Therefore, veliparib and TMZ may be a promising new strategy in mTNBC.

In a subsequent expansion cohort, first-line therapy for metastatic BC and prior PARP inhibitor therapy were allowed, and eligible patients were required to have a known deleterious BRCA1 or BRCA2 mutation identified from prior clinical testing. All patients received veliparib and TMZ. Twenty eligible patients were enrolled. Best response for the 20 patients evaluable at the time of abstract submission included three PRs, six SDs, eleven PDs, and a clinical benefit rate (CBR) of 45%. Combined with the initial cohort of eight known carriers from the original 41 patients, the total RR was 25% (7/28) and CBR was 50%. The RR was 40% (6/15) in patients without prior platinum treatment, and 9% (1/11) in patients with prior platinum treatment. The mPFS for the 20 BRCA carriers was 85 days, and among patients with prior platinum treatment compared with no prior platinum, the mPFS was 70 and 179 days, respectively.

**Iniparib**

Iniparib was initially developed as a PARP inhibitor, but recent data suggest that it does not show characteristics typical of this class and its exact mechanism of action remains to be elucidated. The cellular effects of iniparib include the induction of γ-H2AX foci (a marker of DNA damage) and cell-cycle arrest in the G2/M phase in tumor cell lines. Iniparib also potentiates the cell-cycle effects of DNA damaging
<table>
<thead>
<tr>
<th>Trial</th>
<th>Line of treatment</th>
<th>Schedule</th>
<th>Patients, n</th>
<th>Median age, years</th>
<th>ORR (%)</th>
<th>Median PFS (months), HR</th>
<th>Median OS (months), HR</th>
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<td><strong>PARP inhibitors</strong></td>
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<tr>
<td>O’Shaughnessy et al</td>
<td>First-line +</td>
<td>Gem, carbo, ini</td>
<td>61</td>
<td>56</td>
<td>52 (P=0.02)</td>
<td>5.9; 0.59 (P=0.01)</td>
<td>12.3; 0.57 (P=0.01)</td>
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<td>Phase II</td>
<td>(0–3 prior regimens)</td>
<td>Gem, carbo</td>
<td>62</td>
<td>53</td>
<td>32</td>
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<td>First-line +</td>
<td>Gem, carbo, ini</td>
<td>261</td>
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<td>34</td>
<td>5.1; 0.79 (P=0.027)</td>
<td>11.8; 0.88 (P=0.28)</td>
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<td>Gem, carbo</td>
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<td>54</td>
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<td>4.1</td>
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<td><strong>Anti-VEGF/VEGFR monoclonal antibody</strong></td>
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<tr>
<td>Miller et al</td>
<td>First-line</td>
<td>Padi + beva</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>10.6; 0.49</td>
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<td>E2100 – Phase III, subgroup</td>
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<td>Miles et al</td>
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<td>Padi</td>
<td>53</td>
<td>56</td>
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<td>AVADO – Phase III</td>
<td>Doce, beva (15 mg/kg)</td>
<td>96</td>
<td>52</td>
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<tr>
<td>Robert et al</td>
<td>(Tax/Anthra) first-line</td>
<td>Tax- or anthra-based + beva</td>
<td>46</td>
<td>NR</td>
<td>NR</td>
<td>6.5; 0.78</td>
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<td>RIBBON-I – Phase III</td>
<td>Doce, placebo</td>
<td>87</td>
<td>62</td>
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<td>(Cape) first-line</td>
<td>Cape-based + beva</td>
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<td>NR</td>
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<td>Brufsky et al</td>
<td>Second-line</td>
<td>Cape-, tax-, gem-, vino-based + beva</td>
<td>112</td>
<td>55</td>
<td>41 (0.0078)</td>
<td>6.0; 0.494 (P=0.0006)</td>
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<td>Phase III – subgroup</td>
<td>Cape-, tax-, gem-, vino-based + placebo</td>
<td>47</td>
<td>49</td>
<td>18</td>
<td>27</td>
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<td>Cameron et al</td>
<td>Adjuvant</td>
<td>Anthra – tax-based + beva</td>
<td>1,301</td>
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<td>Anthra – tax-based</td>
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<td><strong>Anti-VEGFR tyrosine kinase inhibitors</strong></td>
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<td>Curigliano et al</td>
<td>Second-line +</td>
<td>Sun 37.5 mg, continuous daily dosing</td>
<td>113</td>
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<td>9</td>
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<td>Phase II</td>
<td>(≥ 1 prior regimen)</td>
<td>Standard of care</td>
<td>104</td>
<td>52</td>
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<td>10.5; 1.22 (P=0.892)</td>
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<td>Bergh et al</td>
<td>First-line</td>
<td>Doce, sun</td>
<td>58</td>
<td>NR</td>
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<td>Baselga et al</td>
<td>First- or second-line</td>
<td>Cape, sora</td>
<td>20</td>
<td>NR</td>
<td>NR</td>
<td>4.3; 0.596 (0.3–1.1)</td>
<td>17.5; 0.98 (0.50–1.89)</td>
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<td>2.5</td>
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<td>Gradishar et al</td>
<td>First-line</td>
<td>Padi, sora</td>
<td>48</td>
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<td>NR</td>
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<td>Baselga et al</td>
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<td>Cis, cetu</td>
<td>115</td>
<td>NR</td>
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<td>10</td>
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<td>Carey et al</td>
<td>First-line +</td>
<td>Cetu, carbo</td>
<td>71</td>
<td>52</td>
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<td>33</td>
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Results of a Phase II trial (Table 1) in patients with mTNBC showed that adding iniparib to gemcitabine and carboplatin significantly improved CBR and progression-free survival (PFS) ($P=0.01$) compared with chemotherapy alone.$^{76}$ The median overall survival (mOS) was also significantly improved in the arm containing iniparib ($P=0.01$). There were essentially no differences in AE incidence between the two treatment groups, suggesting that iniparib was very well tolerated.

Results of a multicenter Phase III trial assessing the same iniparib combination in advanced TNBC failed to meet the primary study endpoints (Table 1).$^{77}$

Ongoing clinical trials will further evaluate the benefits of weekly or biweekly iniparib in combination with gemcitabine and carboplatin in the advanced setting,$^{78}$ iniparib and PF-01367338 in neoadjuvant and adjuvant settings, respectively, as well as other promising PARP inhibitors.$^{79,80}$

### Anti-EGFR

Different studies have indicated that EGFR is frequently overexpressed in TNBC and it is a negative prognostic factor,$^{81,82}$ suggesting a potential role for EGFR-targeted therapies in this subset of patients. There are randomized data on EGFR inhibitors, including the monoclonal antibody cetuximab and the tyrosine-kinase inhibitors (TKIs) erlotinib and lapatinib in the management of TNBC.

In a large randomized Phase II trial, 173 patients with mTNBC, who had received no more than one previous line of chemotherapy for metastatic disease, received cisplatin, with or without cetuximab (Table 1).$^{83}$ The objective RR was 20% with cisplatin plus cetuximab and 10% with cisplatin alone ($P=0.11$). Cisplatin plus cetuximab resulted in a longer mPFS ($P=0.032$) and an approximately the same mOS ($P=0.31$) compared with cisplatin alone. Although the RR was doubled in the combination arm, it did not meet the prespecified primary endpoint of the study and was not found to be statistically significant (Table 1). Efforts are underway to identify those patients with mTNBC who benefit from cetuximab treatment, which may be correlated with lower expression of the $\alpha$-crystallin B chain (encoded by the CRYAB gene), higher expression of phosphatase and tensin homologue (PTEN) and lack of KRAS expression in patients with BLBC.

In another randomized Phase II trial, 102 patients with mTNBC received cetuximab alone (arm 1) or cetuximab associated with carboplatin (arm 2).$^{84}$ Two of 31 patients in arm 1 modalities in tumor cell lines, and antiproliferative activity has been demonstrated in a TNBC-related cell line.$^{75}$
everolimus in nine different TNBC cell lines. The study suggested that everolimus is a promising agent for the treatment of TNBCs, especially BLBC. Basal markers (EGFR and CK5/6) or cancer stem-cell markers (E-cadherin, snail, or twist) may be predictive markers of the response to everolimus in TNBCs.

Rapamycin potentiates the cytotoxicity of paclitaxel in cell lines with phosphoinositide 3-kinase (PI3K)/PTEN/Akt aberrations, suggesting that combination therapy may be effective in these tumors. In this light, a Phase II study investigated the role of everolimus added to standard neoadjuvant chemotherapy in patients with TNBC. Fifty patients were randomly assigned to 5-FU, epirubicin, and cyclophosphamide with docetaxel (FEC-T), or to FEC-T with everolimus 30 mg weekly. Twelve-week RR evaluated by ultrasounds were 29.6% vs 47.8%, respectively (P=0.075). There were no differences in RR at 24 weeks (P=0.27). The pCR rates after chemotherapy completion were 25.9% for FEC-T and 30.4% for FEC-T plus everolimus (P=0.76). In conclusion, adding everolimus to neoadjuvant paclitaxel showed a trend toward an improvement in the 12-week RR, but it did not improve the pCR rate in TNBC.

Another study evaluated the effects of co-inhibition of mTOR (using rapamycin) and EGFR (using lapatinib) in TNBC cell lines and nude mice models, showing that the combination therapy results in synergistic effects in TNBC models in vitro and suppresses TN tumor growth in vivo.

Anti-vascular endothelial growth factor/Vascular endothelial growth factor-receptor monoclonal antibodies

TNBC is a highly proliferative neoplasm that needs constant angiogenesis throughout all the phases of its development, invasion, and metastasis. Intra-tumoral expression of the vascular endothelial growth factor (VEGF) is significantly higher in TNBC than in non-TNBC, providing a biological rationale for targeting this pathway in the treatment of TNBC patients. The anti-VEGF monoclonal antibody bevacizumab (BV) has been shown to increase the RR and PFS of patients with metastatic BC when added to first-line chemotherapy in three randomized Phase III trials. A meta-analysis of TNBC subgroup data from these first-line Phase III trials revealed a 35% reduction in the risk of progression (hazard ratio [HR]=0.65) and a benefit in mPFS of 2.7 months (P<0.0001) when BV was added to chemotherapy regimens, compared with chemotherapy alone.

An open-label, randomized, Phase III trial compared the efficacy and safety of paclitaxel with or without BV, as initial
treatment for metastatic BC. Particularly, a retrospective subgroup analysis of the E2100 trial suggested that the addition of BV to paclitaxel reduced the risk of progression in first-line TNBC patients by 51% and doubled the mPFS.

The efficacy and safety of combining BV with docetaxel as first-line therapy for HER2-negative, locally recurrent, or metastatic BC was investigated in a three-arm, placebo-controlled, Phase III trial. In this study, patients were randomly assigned to docetaxel plus either placebo or BV 7.5 or 15 mg/kg every 3 weeks. In the TNBC subgroup, mPFS was 8.2 months in the docetaxel in combination with BV 15 mg/kg arm, and it was 6.2 and 5.4 months in the BV 7.5 mg/kg and placebo arms, respectively. The combination of BV with docetaxel had no major impact on the toxicity profile of docetaxel. Grade 3 to 4 AEs of special interest were more common in the BV arms than the placebo group, and most of the differences were attributable to hypertension, neutropenia, and febrile neutropenia. This randomized, double-blind study confirmed the clinical benefit of combining BV with a taxane, as previously reported in trial E2100. Taken together, these studies suggest that the combination of BV with taxane-based chemotherapy should be considered as an option for the first-line treatment of HER2-negative metastatic BC.

Moreover, Regimens in Bevacizumab for Breast Oncology (RIBBON)-1, a Phase III, randomized, and placebo-controlled trial, was designed to evaluate the efficacy and safety of BV in combination with chemotherapy regimens, including non-taxane, such as capecitabine, used as initial chemotherapy-based treatment of metastatic BC. In the capecitabine cohort, mPFS increased from 4.2 months in the placebo arm to 6.1 months in the BV arm. In the taxane/anthracyclines cohort, mPFS was 6.2 months in the placebo arm and 6.5 months in the BV arm. The results of the RIBBON-1 trial indicated no clear benefit for TNBC patients who added BV to chemotherapy.

Regarding the second line of treatment, the RIBBON-2 trial (Table 1) was designed to evaluate the efficacy and safety of combining BV with chemotherapeutic schedules commonly used for patients affected by metastatic BC who had received one previous cytotoxic regimen in the metastatic setting. In patients with TNBC, BV combined with chemotherapy resulted in a 3.3-month improvement in mPFS (P=0.0006), with a trend toward improved OS (P=0.0534).

BV has also been investigated in the neoadjuvant setting, and data from two large randomized clinical trials in patients with HER2-negative operable BC have been presented. The first, the GeparQuinto trial, evaluated the rate of pCR after neoadjuvant epirubicin, cyclophosphamide, and docetaxel chemotherapy with and without the addition of BV in patients with TNBC. The pCR rates were 27.9% without and 39.3% with BV (P=0.003).

In the second trial, the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-40 study, patients were randomly assigned to receive neoadjuvant therapy consisting of docetaxel, docetaxel plus capecitabine, or docetaxel plus gemcitabine for four cycles, with all regimens followed by treatment with doxorubicin-cyclophosphamide for four cycles. Patients were also randomly assigned to receive or not to receive BV for the first six cycles of chemotherapy. The addition of BV significantly increased the rate of pCR; when this rate was examined according to hormone-receptor status, the effect of BV was more pronounced in the hormone-receptor-positive subset (15.1% without BV vs 23.2% with BV [P=0.007]), with a weaker effect in the hormone-receptor-negative subset (47.1% without BV vs 51.5% with BV [P=0.34]).

The differences in the results from the two trials could be attributed to the differences in the definition of HER2 negativity in the patients and to the different regimens that they received. Moreover, in the Phase 2 Cancer and Leukemia Group B (CALGB)/Alliance 40603 trial, a total of 454 women with operable stage II or III TNBC were randomly assigned to receive different schedules of treatment: standard neoadjuvant paclitaxel chemotherapy, including doxorubicin and cyclophosphamide, standard neoadjuvant therapy plus carboplatin, standard neoadjuvant therapy plus BV, or standard neoadjuvant therapy plus both carboplatin and BV. The pCR rate in breast tissue with or without neoadjuvant carboplatin was 60% versus 46% (odds ratio [OR] =1.76; P=0.0018); in the BV arm, pCR was 59% compared with 48% in the non-BV arm (OR =1.58; P=0.0089). When carboplatin and BV were used in combination against the chemotherapy backbone, the highest pCR rate was achieved, 67%, but the P-value for the carboplatin/BV interaction was 0.52, indicating the lack of a synergistic effect.

In an adjuvant setting, the BEATRICE study, a randomized Phase III trial, assessed the addition of BV to chemotherapy for women with TNBC. Almost two-thirds of patients in both groups had node-negative disease. There was no difference between the chemotherapy-alone group and BV group in DFS (HR =0.87; 3-year invasive DFS =82.7% in the chemotherapy group versus 83.7% in the BV group; P=0.18). All secondary efficacy endpoints seemed to favor BV, but none was statistically significant.

While BV binds specifically to the ligand, vascular endothelial growth factor-A (VEGF-A), and all its isoforms, it has...
limited binding affinity for other vascular endothelial growth factor-receptor (VEGFR) ligands, VEGF-B, and VEGF-C. Newer antiangiogenic agents bind to VEGFR-2 and thus prevent all ligand binding to this target. Therefore, targeting VEGFR in this way could potentially lead to a more complete target inhibition and a more effective angiogenesis block. In this regard, the monoclonal antibody ramucirumab (IMC-1121B, ImClone) targeting VEGFR-2 is currently under investigation in combination with docetaxel in a Phase III clinical trial in patients with HER2-negative metastatic BC.107

Anti-VEGFR TKIs

Cell surface-receptor tyrosine kinases, including VEGFRs, are critical to angiogenesis, and the effects of two TKIs on endothelial cell proliferation have been evaluated in TNBC.

The anti-VEGFR TKIs sunitinib and sorafenib have shown some interesting degrees of activity in BC in clinical studies with substantial TNBC populations.

Sunitinib

Sunitinib was investigated in a preliminary Phase II trial as a single agent in patients with metastatic BC who had received prior anthracycline and taxane therapy. Neither steroid receptor nor HER2 status appeared to correlate with clinical response. Among the patients with TNBC, the RR was 15%, and 11% in the total population.108

Another randomized (Table 1), open-label Phase II trial was designed to test the hypothesis that the PFS of patients receiving sunitinib monotherapy (37.5 mg/day) on a continuous-daily-dosing schedule would be superior to that obtained with single-agent standard-of-care (SOC) chemotherapy in patients with previously treated TNBC. Almost all patients had received prior treatment containing an anthracycline and a taxane; approximately three-quarters of patients had received prior chemotherapy for advanced disease. Thirteen percent of patients received prior BV therapy in both arms. The mPFS was 2.0 months with sunitinib versus 2.7 months with SOC (P=0.888). Moreover, the mOS was not prolonged with sunitinib treatment compared with SOC (P=0.839).109

In a prospective and randomized Phase III study, patients with advanced BC were randomly assigned to open-label combination therapy (sunitinib and docetaxel) or monotherapy (docetaxel). There was no difference in mPFS (P=0.265) and mOS (P=0.904) between the two arms. The overall response rate (ORR) was significantly higher with the combination than with monotherapy (P=0.001). Duration of response was similar in both arms.110

Sorafenib

Sorafenib is a multi-kinase inhibitor with antiangiogenic/antiproliferative activity. Bianchi et al described the results of single-agent sorafenib in metastatic BC.111 Their study evaluated 52 patients demonstrating a 2% RR and a 13% SD at 6 months. The authors concluded that further investigation of single-agent sorafenib in this patient population is not recommended.

A randomized, double-blind, placebo-controlled Phase II trial assessed sorafenib with capecitabine for locally advanced or metastatic HER2-negative BC. Patients were randomly assigned to first- or second-line capecitabine with sorafenib or placebo.112 The results suggest a role for the combination of sorafenib and capecitabine in BC and supported a multinational, double-blind, randomized, placebo-controlled Phase III trial assessing the addition of sorafenib to first- or second-line capecitabine in advanced HER2-negative BC.113

In the NU07B1 trial, a multinational, double-blind, placebo-controlled Phase II screening trial, patients were randomized to paclitaxel in combination with placebo or sorafenib. There was no significant difference in OS between treatment arms (P=0.904), but the addition of sorafenib versus placebo to paclitaxel significantly increased median time to progression (8.1 vs 5.6 months, respectively; P=0.0343) and ORR (67% vs 54%, respectively; P=0.0468).114

Another double-blind, placebo-controlled Phase II study enrolled patients with locally advanced or metastatic HER2-negative BC and prior BV treatment. Patients were randomized to chemotherapy with sorafenib or placebo. The combination of sorafenib plus gemcitabine/capecitabine provided a clinically small but statistically significant improvement in PFS compared with placebo plus gemcitabine/capecitabine (HR =0.65; P=0.02). The mOS was 13.4 versus 11.4 months, respectively (HR=1.01; P=0.95).115

New small-molecule receptor TKIs such as apatinib116 and cediranib (in combination with olaparib)117 have reached Phase II clinical development in patients with TNBC.

Other target agents

7-hydroxyxauroporine

The majority of TNBCs carry mutations in TP53, a gene that encodes the tumor-suppressor protein p53 which is required for G1 checkpoint regulation in the presence of genotoxic stress.118,119

7-hydroxyxauroporine (UCN-01) was the first checkpoint kinase 1 inhibitor introduced in clinic, although it also inhibits several other serine-threonine protein kinases.120,121
In a Phase I study of UCN-01 and irinotecan in patients with refractory advanced solid tumor malignancies, two PR were observed in women with TNBC.122 Both tumors were deficient for p53. Therefore, it was hypothesized that UCN-01 and irinotecan would be an effective regimen in mTNBC. The goal of the Phase II trial was to further evaluate the association between irinotecan and UCN-1 in patients with mTNBC previously treated with anthracyclines and taxanes.123 A total of 25 patients were enrolled, and 22 were evaluated for response. No CRs were observed. One PR, lasting 24 weeks, occurred in a patient with liver and lung metastases. Tumor from this patient was found to be BL carrying a TP53 mutation. Two patients had prolonged SD that lasted for 37 and 28 weeks, respectively. The CBR and the OS were 12% and 11.3 months, respectively. Unfortunately, the study did not meet the predefined efficacy criteria for further evaluation. Among the 15 patients with sufficient tumor samples for TP53 mutation analysis, eight carried mutations in TP53. In this limited dataset, a significantly worse OS was observed in patients with mutations in TP53. In particular, the median OS was 5.5 months and 20.3 months in patients with and without TP53 mutation, respectively (\(P=0.004\)).

Bortezomib
Bortezomib is the first proteasome inhibitor to be approved for treatment for multiple myeloma and mantle-cell lymphoma.124,125 Particularly, bortezomib has been shown to block proteasome degradation of IxB, an inhibitor of nuclear factor-kappa B, and demonstrated remarkable antitumor activity against these hematological malignancies.126

Furthermore, preclinical studies have demonstrated an in vitro antitumor effect of bortezomib in BC models.127,128

Moreover, Tseng et al demonstrated that bortezomib induced significant apoptosis in TNBC cell lines but not in hormone-receptor-positive or HER2-overexpressing cells.129 This study reveals a novel mechanism by which bortezomib induces apoptosis in TNBC cells, that is CIP2A-dependent p-Akt downregulation. The data showed that 50/57 (87.7%) of tumor samples from TNBC patients presented variable CIP2A expression, and, as previously demonstrated, CIP2A expression has been shown to correlate with disease aggressiveness in BC.130

Dinaciclib
Dinaciclib (MK-7965, formerly SCH727965) is a novel, potent, small-molecule inhibitor of cyclin-dependent kinase (CDK) 1, CDK2, CDK5, and CDK9.

Previously, two Phase I dose-escalation trials of dinaciclib administered intravenously, weekly or every 3 weeks, were conducted in patients with advanced malignancies and showed some early evidence of clinical activity131,132

A Phase II trial was designed to assess the efficacy and safety of dinaciclib133 compared with standard doses of capecitabine in women with advanced BC. The median time to progression was 2.73 months with dinaciclib, compared with 4.17 months with capecitabine. The HR (dinaciclib/capecitabine) was 1.6. The estimated ORR following initial treatment with dinaciclib was 8% based on one confirmed PR among the 12 evaluable patients. Among the patients randomized to capecitabine, one patient achieved a PR for an estimated ORR of 7% among 14 evaluable patients. Although this trial did not select subpopulations of patients with BC on the basis of either histological or molecular markers, the confirmed and unconfirmed PRs achieved with dinaciclib occurred both in patients with ER-positive/HER2-negative disease.

Panobinostat
Histone deacetylase inhibitors (HDACis) have emerged as a promising new class of multifunctional anticancer agents.134,135 HDACis have been linked to several downstream effects in tumor cell lines, such as cell-cycle arrest, induction of apoptosis, inhibition of angiogenesis, activation or inactivation of tumor-suppressor genes or oncogenes, and decreased invasion and metastases.134–136

Panobinostat (LBH589) is a potent pan-deacetylase inhibitor that can block multiple cancer-related pathways.137 HDACs can be subdivided into two groups: zinc-dependent (Class I, II, and IV) and zinc-independent (Class III).138 Panobinostat is a potent inhibitor with activity against Class I, II, and IV HDACi enzymes, suggesting true pan-HDAC activity.137

To date, panobinostat has demonstrated favorable clinical responses, with limited toxicity.139–141

In their study, Tate et al utilized four TNBC cell lines as models of TNBC growth and progression.142 In confirmation of other preclinical research,143–145 they found that panobinostat induced hyper-acetylation of histones H3 and H4, decreased proliferation and survival, and induced apoptosis and G2/M cell-cycle arrest.142 The panobinostat-induced effects on cell proliferation and survival appear to be TNBC cell specific as the ER-positive cell lines tested were unaffected at all doses tested; this result was contrary to previously published work, which reported that panobinostat significantly inhibited cell survival and induced cell death in ER-positive and ER-negative BC cell lines, though at a different time point.144,146
Dasatinib
Dasatinib, a potent oral inhibitor of the Src family of kinases, is approved for treating patients with Philadelphia chromosome-positive leukemias. Preclinical studies showed growth inhibition of BLBC cells by single-agent dasatinib more frequently than other phenotypes. Preclinical TNBC models also showed synergistic or additive dasatinib activity with chemotherapy, suggesting that dasatinib may provide clinical benefit in TNBC.

A prospective, open-label Phase II trial investigated the role of dasatinib in patients with locally advanced or mTNBC that had progressed after prior chemotherapy for advanced disease. Single-agent dasatinib showed limited efficacy in patients with TNBC. Overall, two of 43 patients (5%) achieved a RECIST PR, both occurring in the lymph nodes and lasting for 14 and 58 weeks, respectively. In addition, 11 of 43 patients (26%) experienced SD, including two lasting for 25 and 33 weeks. The mPFS was 8.3 weeks.

Another study demonstrated a significant reduction in cell viability and induction of apoptosis in TNBC cell lines using the three-drug combination of dasatinib, cetuximab, and cisplatin compared with the dual combination of cetuximab and cisplatin.

Wnt/Frizzled
Wnt signaling is a key oncogenic pathway in multiple cancers and regulates cell proliferation, migration, and differentiation. Wnt proteins, which are secreted glycoproteins, bind to the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) and Frizzled (FZD), a seven-pass transmembrane receptor protein, to activate the Wnt/β-catenin signaling pathway. In the absence of Wnts, β-catenin is sequestered in a complex, which results in the ubiquitination and the subsequent degradation of β-catenin by the 26S proteasome. Conversely, when Wnt proteins are secreted from cells, they form a ternary complex with the FZD and the LRP5/6 receptors, which results in the stabilization of cytosolic β-catenin. The β-catenin then translocates into the nucleus where it induces the expression of downstream target genes that regulate cell cycle, growth, and progression. Recently, two studies demonstrated that Wnt/β-catenin signaling activation is preferentially found in a subgroup of invasive TNBCs and is associated with a poor clinical outcome. This suggests that the Wnt/β-catenin signaling pathway plays an important role in TNBC development and progression.

Multiple attempts to develop antibodies against key Wnt co-receptors, including LRP6 and FZD, have been reported with varying degrees of success.

A O-acyltransferase Porcupine (PORCN) inhibitor, IWP2, has been reported to show good potency and specificity in inhibiting Wnt signaling in vitro. During the biosynthesis of Wnt ligands, Wnt undergoes posttranslational acylation (palmitoylation) that is mediated by PORCN, a membrane-bound oacyltransferase. PORCN is specific and dedicated to Wnt posttranslational acylation, which is required for subsequent Wnt secretion. Loss of PORCN leads to inhibition of Wnt ligand-driven signaling activities in knockout mouse models.

A Phase I dose-escalation study of oral LGK974 is currently ongoing. The primary purpose of this study is to find the recommended dose of LGK974 that can be safely given to adult patients with malignancies (also patients with TNBC) dependent on Wnt ligands for whom no effective standard treatment is available.

Discussion
TNBC is a heterogeneous disease at molecular, pathologic, and clinical levels. Stratification of TNBC into subclasses, using new markers, will identify new screening methods, prognostic factors, methodologies, and perhaps targets for personalized therapies. A lot of new targeted therapies are actually under study, but the efforts are not reaching the hoped results.

Anyhow, bevacizumab showed encouraging results in several trials, as suggested by the meta-analysis of O’Shaughnessy et al. Unfortunately, mPFS advantage was not confirmed also by an advantage in mOS; this may be due to the several currently available post-progression therapies. However, bevacizumab is a well-known drug with a manageable toxicity profile, so it could be useful to further evaluate its action in this difficult patient setting. Meanwhile other anti-VEGF therapies, like TKIs, which have good results in other diseases, have shown all their limits in TNBC; therefore, because TNBC is only a part of a larger BC population, results have been limited to subgroup analysis in the majority of clinical trials.

Regarding the anti-EGFR therapies, there was a strong rationale for their use in TNBC treatment, particularly because of the correlation between EGFR hyper-expression and negative prognosis in this disease. Furthermore, in some trials cetuximab showed a positive trend to an OS advantage in this setting, even if those trials enrolled only a small number of patients. From these results, it seems that the subgroup population of TNBC appears to be more sensitive to these therapies; moreover, many efforts are under way to identify TNBC patients who benefit from cetuximab treatment, which may be correlated with lower expression of alpha-crystallin B.
chain (encoded by the CRYAB gene), higher expression of PTEN homologue, and lack of KRAS expression in patients with BLBC.

In addition, erlotinib and gefitinib showed some actions in TNBC, but the experiences are too small to consider these results conclusive at all; furthermore, it seems that BRCA-mutated TNBC achieves a better response with erlotinib. The mTOR inhibitor everolimus also needs further evaluation to clarify its real action in this clinical setting.

On the basis of preclinical studies, there was a lot expected from PARP inhibitors. Until now, olaparib has not been studied in a Phase III trial, while iniparib activity, which in a Phase II study seemed very promising, was not confirmed in the Phase III study. However, the BRCA-mutated population seems to be the target of this drug family, so future clinical trials should be based on this class of TNBC.

Other target agents, even if based on fascinating theories, at the moment have not shown impressive achievement in the Phase I and Phase II trials in which they have been evaluated. Further investigations are needed, above all in translational studies involving the evaluation of predictive response biomarkers.

Anyhow, expression profiling and genomic studies are changing our view about the molecular biology of BC, which is currently considered as a group of distinct diseases from the molecular point of view. Indeed, although the important role of genomic studies – for example the assessment of p53 status, HER2 amplification, BRCA1 and 2 deletions, and epigenetic and chromosomal modifications – most of the studies regarding the molecular signature of BC are actually related to the expression profile of tumors (ie, the messenger RNA or protein level). The morphologic features of tumor cells have long been validated for the clinical classification of BC and are regularly used as a “gold standard” to ascertain prognostic outcome in patients. Identification of molecular markers, such as expression of the receptors for estrogen and progesterone and HER2, has played an important role in determining targets for the development of efficacious drugs for treatment, and has also offered additional predictive value for the therapeutic assessment of patients with BC. Currently, no routine specific diagnostic procedure exists for this subtype, and the patients’ management is similar to that of other subtypes regarding prevention, prognostic assessment, and treatment.

However, the detailed molecular characterization of TNBC is ongoing, both to better understand the different biology and clinical outcome, and to identify specific diagnostic, prognostic, and therapeutic targets. In particular, one important goal is, therefore, the identification of prognostic factors and markers to reliably select high- and low-risk subsets of patients with TN disease, in order to explore different biologically based treatments and to tailor the therapeutic approaches to the single patient.

The more we learn about TNBC, the more we can improve therapies; in recent years, medical research has been fully oriented toward the targeted therapies; we hope to reach also with TNBC what happened with HER2 in BC and with more uncommon tumors, like kidney cancer and gastrointestinal stromal tumor. To achieve the best for targeted therapy, it seems necessary to study in detail the molecular subtype present in TNBC in a such way as to avoid that a therapy against a particular molecular feature is studied in a population in which that trait is absent.

However, TNBC is clearly a complex disease; indeed the genetic heterogeneity is present not only in differently affected individuals, but also among tumors occurring at different sites within the same patient. As such, it is likely that its biology involves multiple redundancies and pathway cross-talk. If only one pathway is selectively inhibited, the efficacy of the therapeutic strategy would likely be undermined by activation of a compensatory pathway. Therefore, it is not surprising that, to date, not a single targeted therapy has been approved for the treatment of TNBC, for which cytotoxic chemotherapy remains the standard treatment. Combining two or more targeted agents may be required for a more rational and optimal approach to TNBC treatment. Alternatively, one technique that may show promise is gene therapy; in particular, gene transfer should correct abnormal genetic functions in cancer cells. Furthermore, these potential improvements are burdened with technical difficulties, such as insufficient infectivity or inadequately broad biodistribution of the transfer vector.

**Conclusion**

Important results have been achieved with antiangiogenic drugs, although much still needs to be improved. Perspectives on new therapeutic molecules are most interesting, in particular the PARP inhibitors. The overall impact of these agents on patient survival has not been as great as expected, probably because the molecular basis of this illness needs to be better understood so that treatment can be more appropriately tailored.

At the current time, we believe that the systematic evaluation of the predictive value of the genomic alterations is critically important for further progress in this field; for this reason, there is the need for tissue collection
in the post-neoadjuvant and metastatic settings for a better understanding of the relevant pathways that are associated with TNBC pathogenesis and therapeutic resistance.

Disclosure
The authors declare no conflicts of interest in this work.

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