Published in final edited form as:

Crit Rev Oncog. 2016 ; 21(5-6): 333–351. doi:10.1615/CritRevOncog.2017021307.

Review of Triple Negative Breast Cancer and the Impact of Inducible Nitric Oxide Synthase on Tumor Biology and Patient Outcomes

Elaine M. Walsh^{a,b}, Maccon M. Keane^b, David A. Wink^c, Grace Callagy^a, and Sharon A. Glynn^{a,*}

^aDiscipline of Pathology, Lambe Institute for Translational Research, School of Medicine, National University of Ireland Galway, Galway, Ireland; ^bMedical Oncology, Galway University Hospitals, Galway, Ireland; ^cCancer and Inflammation Program, National Cancer Institute-Frederick, Frederick, Maryland, USA

Abstract

Triple negative breast cancers (TNBCs), which are defined as estrogen-receptor, progesterone-receptor, and HER2-receptor negative, account for 10–20% of breast cancers, and they are associated with early metastasis, chemotherapeutic resistance, and poor survival rates. One aspect of TNBC that complicates its prognosis and the development of new molecular therapeutic targets is its clinical and molecular heterogeneity. Herein we compare TNBC and basal cytokeratin-positive breast cancers. We examine the different TNBC molecular subtypes, based on gene expression profiling, which include basal-like, mesenchymal, and luminal androgen receptors, in the context of their biology and impact on TNBC prognosis. We explore the potential role of inducible nitric oxide synthase (iNOS) in TNBC tumor biology and treatment responses. iNOS has been shown to induce p53 mutation accumulation, basal-like gene signature enrichment, and transactivation of the epidermal growth factor receptor (EGFR) via S-nitrosylation, all of which are key components of TNBC biology. Moreover, iNOS predicts poor outcome in TNBC, and iNOS inhibitors show efficacy against TNBC when used in combination with chemotherapy. We discuss molecular targeted approaches, including EGFR, PARP, and VEGF inhibitors and immunotherapeutics, that are under consideration for the treatment of TNBC and what role, if any, iNOS may play in their success.

Keywords

triple negative breast cancer; TNBC; nitric oxide; molecular targets; EGFR; chemotherapy; platinum; taxanes

* Address all correspondence to: Sharon Glynn, Discipline of Pathology, Lambe Institute for Translational Research, School of Medicine, National University of Ireland Galway, Galway, H91 V4AY Ireland, sharon.glynn@nuigalway.ie.

I. Introduction

Breast cancer is the most common female cancer worldwide, with greater than 1.3 million cases and 450,000 deaths each year.^{1,2} Breast cancer is a heterogeneous disease and encompasses a wide variety of entities, each with distinct morphological features and clinical behaviors. Triple negative breast cancer (TNBC) is a subtype, accounting for 10–20% of all breast cancers, which shows pathological, molecular, and clinical heterogeneity and is associated with challenging biological features.³ TNBC is defined by a lack of expression of estrogen receptor alpha (ER) and progesterone receptors (PR), and a lack of amplification or overexpression of human epidermal growth factor receptor 2 (HER2).^{4,5} In view of the fact that TNBCs lack these receptors and are unresponsive to endocrine and HER2-targeting therapies, cytotoxic chemotherapy remains the mainstay of treatment.^{5–7}

Because improvements in targeted therapies are an urgent unmet medical need in the TNBC patient population,⁸ it is necessary to increase our understanding of the complexity of this disease at the genomic, molecular, and biological levels so that therapeutic targets might be identified and more effective treatments developed.⁹ One potential target is inducible nitric oxide synthase (iNOS), which we had previously demonstrated to be associated with poor survival in ER-negative breast cancer patients and enhanced expression of basal-like gene signatures.¹⁰ Furthermore, Granados-Principal et al. demonstrated an association between high levels of iNOS and decreased relapse-free survival in TNBC, indicating iNOS's potential role in this disease.¹¹ In a large cohort of 209 patients with TNBC,¹² we subsequently demonstrated that iNOS predicts increased risk of distant metastasis and decreased overall survival, validating iNOS as a predictor of poor outcome in patients from different geographic regions (the United States and Ireland). In this review, we examine the clinical and pathological features of TNBC, the potential role of iNOS in TNBC tumor biology (Figure 1), and the potential benefits of iNOS-targeted therapy for TNBC patients.

II. Clinical Outcomes in TNBC

Although TNBC accounts for only 10–20% of all breast cancer subtypes, it accounts for a disproportionate share of breast cancer mortality.² TNBCs are distinctly aggressive and are associated with higher rates of relapse and shorter survival, stage-for-stage, than ER/PR-positive and HER2-positive diseases.^{2,4,7,13,14} In view of the hematogenous pattern of dissemination, distant recurrence in TNBC is rarely preceded by local recurrence.^{15,16} The average time to recurrence is shorter in TNBCs compared to other breast cancer subtypes, and the incidence of distant recurrence peaks between the first and third years after the primary TNBC diagnosis.^{17,18} After the first development of metastatic disease, patients with TNBC have significantly shorter survival rates compared to patients with other disease subtypes.^{17,19}

Despite improvements in systemic chemotherapy, virtually all women with metastatic TNBC will die.⁷ The poor prognosis and increased mortality observed in TNBCs have been shown to persist even after adjustment for other predictive variables such as age, race, grade, tumor size, nodal status, and chemotherapy. There are several explanations for the unfavorable prognosis associated with TNBCs. The “TNBC paradox” describes the fact that TNBCs

have higher initial response rates to neoadjuvant chemotherapy (NACT), but overall show poorer survival than other subtypes.² This overall poor prognosis is influenced by the presence of a subset of TNBCs with intrinsic chemoresistance while other subsets may be chemosensitive, with higher pathological complete response (pCR) rates and better overall outcomes.² In addition, the well-described heterogeneous and aggressive biological nature of the disease as well as the absence of molecular targets, which could potentially form the basis for targeted therapy, contribute to the poor prognosis associated with TNBCs⁹. Understanding the mechanisms of this intrinsic aggressiveness of TNBC is key.

A. Establishing a Role for iNOS in Poor Outcomes Associated with TNBC

In 1995, Thomsen et al. reported that breast tumors exhibited increased expression of iNOS when compared to normal breast tissue, in particular tumors with high-grade disease.²⁰ This was in contrast to a later report by Tschugguel et al., who found an inverse correlation between iNOS and tumor grade.²¹ Vakkala et al. further explored the role of iNOS in breast cancer, recapitulating the findings of Thomsen et al. and demonstrating iNOS's association with high-grade disease, also adding increased angio-genesis and apoptotic index. However, Vakkala et al. did not find a significant association with patient outcome.²²

In 2005, two separate studies found that iNOS is associated with poor survival, but again varied in their reports of association with tumor grade and nodal status.^{23,24} As mentioned previously, breast cancer's heterogeneous nature led us to examine the role of iNOS in the context of hormonal status. We found that iNOS is predictive of poor outcome in ER-negative but not ER-positive breast cancer.¹⁰ Taking hormonal status a step further, Granados-Principal et al. demonstrated an association between high levels of iNOS and decreased relapse-free survival in TNBC, indicating its potential role in this disease,¹¹ which we have now validated in an Irish TNBC population of patients, showing that iNOS is not only prognostic for breast cancer-specific survival but is also prognostic for the development of metastatic disease.¹² Table 1 summarizes historical studies of the role of iNOS in breast cancer tumor biology and patient outcomes. The vast majority of cell lines used in these studies belong to the TNBC subtype (Table 2).

III. Clinicopathologic Features of TNBC

TNBCs show distinct features in terms of clinical presentations, histological features, chemotherapy response, patterns of relapse, and outcomes.²⁵ A higher proportion of patients with TNBC develops distant metastases when compared to patients with other breast cancer subtypes.^{17,19} TNBCs less frequently metastasize to the bone and lymph nodes and are more likely to disseminate to the viscera, such as the lungs and brain.^{17,18} This pattern of metastases suggests that TNBC disseminates by a hematogenous route rather than by lymphatic spread.^{16,17}

Histologically, the majority (> 90%) of TNBCs are invasive ductal carcinomas. They tend to be high-grade tumors with high proliferation indices, a conspicuous lymphocytic infiltrate, necrosis, and pushing margins.^{2,17} Almost all medullary or atypical medullary carcinomas are TNBCs. Other, less common histological types of breast cancer can have a triple negative phenotype, including meta-plastic carcinomas and some pleomorphic lobular and

apocrine carcinomas. While most TNBCs are high grade, the rare special types of secretory carcinoma and adenoid cystic carcinoma are inherently low grade and are associated with a more favorable clinical outcome than other TNBCs. These are recognized by their characteristic morphology and have associated cytogenetic aberrations. However, although most TNBCs are histologically similar, they can show marked differences in clinical behavior due, in a large part, to molecular differences between tumors.²⁶

A. Defining Basal-Like Breast Cancer vs. TNBC and the Contribution of iNOS to the Basal-Like Phenotype

TNBCs display a large overlap with basal-like cancers, with many expressing genes that are more frequently found in normal breast basal or myoepithelial cells.^{18,27} Both cancer types are more prevalent in younger, premenopausal African American women and in breast cancer 1 gene (BRCA1) mutation carriers.^{2,14,17,18,28,29} Both basal-like tumors and TNBC are higher grade, have higher mitotic indexes, and high rates of TP53 mutations (up to 85%), and they frequently express the epidermal growth factor receptor (EGFR) and the KIT proto-oncogene receptor tyrosine kinase.^{9,28,30,31}

Although there are many similarities between basal-like breast cancer and TNBCs, these phenotypes are not synonymous, as has been proven by microarray, immunohistochemical, and clinical data.^{17,18} Basal-like breast tumors are defined as expressing positivity for the basal markers cytokeratin 5/6 (CK5/6) and/or EGFR. Although the majority of tumors with basal-like gene expression are also triple negative by immunohistochemistry, not all TNBCs are basal-like breast cancers.³² Between 15 and 45% of basal-like cancers express ER and a further 14% overexpress HER2. Conversely, 16–44% of TNBCs are negative for the basal markers CK5/6 and CK14. A study by Cheang et al. showed that, while 17% of all breast cancers are TNBC, only 9% are truly basal-like.³³

There are significant clinical differences between TNBCs that express basal markers by immunohistochemistry and TNBCs that do not. It has been suggested that among TNBCs poor outcomes are almost exclusively seen among the subset that is basal-like.³³ Cheang et al.³³ showed that basal-like TNBCs are associated with a higher grade among younger patients when compared to TNBCs that do not express basal markers. In addition, basal-like TNBCs are associated with a worse prognosis, with a 5- and 10- year breast cancer-specific survival rate of 68 and 62%, respectively, compared to 79 and 72% among non-basal TNBCs. With these findings in mind, TNBC and basal-like breast cancers should not be considered synonymous and should continue to be separate for both clinical and research purposes.⁸

We previously demonstrated that high levels of iNOS expression in ER-negative breast tumors are associated with increased expression of basal-like gene expression signatures.¹⁰ Furthermore, treatment of the TNBC breast cell lines MDA-MB-231 and MDA-MB-468 increased the expression of the basal-like marker P-cadherin and the stem-like markers CD44 and interleukin-8 (IL-8). Examining the ER-negative tumors positive for CK5/6 or EGFR (i.e., Basal-like), we found that iNOS is predictive of poor outcome in this subset. iNOS also correlated with increased EGFR phosphorylation, further indicating a role for iNOS in basal-like breast cancer.^{10,34} Importantly, iNOS is associated with increased

accumulation of p53 mutations,¹⁰ which is synonymous with both TNBC and basal-like tumors as described previously.^{9,28,30,31}

B. Increase in TNBC Complexity due to Its Molecular Subtypes

In 2011, Lehmann et al. subtyped TNBCs on the basis of differential gene expression. These “TNBC type” subtypes are labeled as follows: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory, mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). The BL1, BL2, and M subtypes express high levels of basal cytokeratins, while LAR tumors express high levels of luminal cytokeratins and other luminal markers, but lack basal cytokeratin expression. The TNBC type subtypes can be classified into three main groups: basal-like (BL1 and BL2), mesenchymal-like (M and MSL), and LAR. Despite the obvious heterogeneity among TNBCs, tumor size and grade do not appear to be significantly different among the TNBC subtypes.⁹ In 2016, Lehmann et al. demonstrated that the previously classified immunomodulatory and MSL subtypes are in fact tumors with substantial tumor-infiltrating lymphocytes (TILs) and tumor-associated mesenchymal cells, respectively. Therefore, the original “TNBC type” has been refined and is now classified as “TNBC type 4,” which describes BL1, BL2, M, and LAR.³⁵

The BL1 subtype is enriched in pathways responsible for cell division and DNA damage repair. BL1 tumors also express genes associated with proliferation, having high levels of Ki67.^{6,9} A combination of proliferation gene enrichment and increased Ki67 expression suggests that BL1 TNBCs preferentially respond to antimitotic agents such as taxanes.⁹ The BL2 subtype expresses genes involved in growth factor signaling, glycolysis, and gluconeogenesis. This subtype is heavily enriched in growth factor receptor gene expression (e.g., EGFR, MET) and shows higher levels of TP63 and membrane metallo-endopeptidase expression.^{6,9} In addition, the BL2 subtype is enriched in PIK3CA and phosphatase and tensin homolog mutations.¹³

The M subtype displays elevated expression of genes involved in epithelial mesenchymal transition (EMT) and growth factor pathways.⁶ This subtype specifically expresses a variety of genes that are enriched in components and pathways involved in cell motility, interaction of extracellular matrix receptors, and cell differentiation pathways.^{6,9} The signaling pathway components differentially expressed in the M subtype share features similar to those seen in metaplastic breast cancers. Metaplastic breast cancers are characterized by mesenchymal/sarcomatoid or squamous features and tend to be chemoresistant.⁹

Finally, the LAR subtype is driven by the androgen receptor and is characterized by luminal gene expression.⁶ Genes representing hormonally regulated pathways, including steroid synthesis and androgen/estrogen metabolism, are heavily enriched in this subtype.^{6,9}

Important clinical differences and treatment implications are associated with each subtype. For example, BL1 tumors are higher grade, while LAR tumors are lower grade compared to other subtypes. Despite being higher grade, BL1 tumors tend to be diagnosed at an earlier stage than BL2 and LAR tumors. In the NACT setting, there is a significant association between TNBC subtypes and a pathological pCR. Despite BL1 and BL2 subtypes having similar biology, they have significant differences in pCR rates. A study by Masuda et al.⁴

showed that the BL1 subtype has the highest pCR rate (52%) while the BL2 and LAR subtypes have the lowest (0 and 10%, respectively). Lehmann et al.³⁵ demonstrated differences in relapse-free survival (RFS) and overall survival (OS) among TNBC subtypes. BL1 patients displayed better RFS than all other TNBC type-4 subtypes combined, with almost 60% survival at 10 years. Similarly, the OS for BL1 patients was significantly better than all other TNBC type-4 subtypes combined.³⁵

Investigating the role of iNOS in the context of BL1 and BL2 subtypes, we examined the effects of the nitric oxide (NO) donor diethylenetriamine NONOate (DETA/NO) on the BL1 cell line MDA-MB-468 and the BL2 cell line HCC1806. Interestingly, the BL2 subtype was more sensitive to NO-induced EGFR signaling. We demonstrated that in BL2 cells, NO activation of the EGFR/ERK signaling pathway plays a direct role in the development of a proinflammatory phenotype that increases tumor cell invasive capacity.¹² These results highlight a novel mechanism that may explain the observation that TNBC patients expressing iNOS receive a worse prognosis. Future studies will examine the role of iNOS in the M and LAR subtypes.

Other groups have identified similar TNBC subtypes. Burstein et al. identified four similar but different TNBC subgroups, each associated with differing clinical outcomes. These subgroups included LAR, mesenchymal (MES), basal-like immunosuppressed (BLIS), and basal-like immune-activated (BLIA). Like Lehmann et al., Burstein et al. found that TNBC patients with tumors exhibiting immune component features have the best outcome.³⁶ There are two major points of interest regarding the Burstein subtypes. One, gene expression profiling reveals the expression of estrogen-regulated genes such as *ESR1*, *PGR*, *FOXA*, *XBPI*, and *GATA3* in the LAR subtype. Despite being ER negative, then, these tumors demonstrate evidence of ER activation and may respond to traditional antiestrogen therapies as well as to antiandrogen therapy.^{9,36} Two, the BLIS subtype displays downregulation of B cell, T cell, and natural killer cell immune-regulating pathways as well as cytokine pathways. In addition, factors controlling antigen presentation, immune cell differentiation, and innate and adaptive immune cell communications are expressed at low levels.

The BLIS subtype has been shown to be associated with the worst disease-free survival and breast cancer-specific survival.³⁶ We previously demonstrated that iNOS-expressing tumors show increased macrophage infiltration. These macrophages are negative for iNOS, indicating that they may be polarized toward a macrophage type 2 (M2) protumorigenic phenotype.¹⁰ M2 macrophages increase angiogenesis, tissue remodeling, and repair of wounded/damaged tissues. They produce arginase, resulting in the generation of ornithine and polyamines,³⁷ and have also been shown to facilitate tumor progression by various mechanisms.³⁸⁻⁴¹ Moreover, they have the capacity to suppress adaptive tumor-specific immune responses.⁴² A key question will be whether iNOS tumor expression in TNBC contributes to the regulation of BLIS or BLIA phenotypes.

C. TILs in TNBC

TILs are frequently found in highly proliferative tumors. They tend to be associated with ductal histology, high histologic grade, high Ki67, ER negativity, HER2 overexpression, larger tumors, and lymph node metastases. As a result, TIL levels tend to be higher in

TNBCs and HER2-positive cancers compared to hormone receptor-positive subtypes.^{43,44} An association between stromal TILs (sTILs) and immunohistochemical basal cell markers, such as CK5, CK14, EGFR, and P-cadherin, has also been observed.⁴⁴ The majority of TNBCs (65–80%) have low to moderate levels of lymphocytes, whereas 15–20% have no lymphocytic infiltration. On the other hand, lymphocyte-predominant breast cancers (LPBCs) account for 5–28% of all TNBCs and are defined as tumors with 50–60% lymphocytes in the stroma; they can be described as highly immunogenic.^{45,46} TNBCs with TILs may represent the immune phenotype that has been observed repeatedly in gene expression profiling, potentially aligning with the molecular subtypes described previously.^{6,9,47}

TIL levels in TNBC may have implications for patient outcomes. TNBCs with high levels have been associated with a greater pathological response to NACT. In the GeparSixto trial, which studied the addition of carboplatin to anthracycline and taxane-based NACT, increased levels of sTILs, and in particular LPBC, predicted pCR in TNBC. The pCR rate was 74% in LPBC TNBCs compared to 46% in non-LPBC TNBCs.⁴⁸ TNBCs with high TIL levels have been reported to have better survival outcomes after either anthracycline-based or platinum-based chemotherapy.^{45,47,49,50} It has been suggested that once an immune response has been stimulated, the overall prognosis is improved, independent of the chemotherapy drugs used and the measured response to treatment.⁵¹

We also examined the percentage of sTILs in the tumor-adjacent stroma and found it to be highest in patients with iNOS-negative tumors and lowest in patients with iNOS-moderate tumors. Given that decreased TIL counts are associated with worse outcomes in TNBC, this indicates the need to determine the impact of iNOS expression on both TIL infiltration and the TIL phenotype in the tumor.⁵² Understanding this may open new avenues for optimizing immunotherapeutic approaches.

A major determinant of therapeutic outcome is the degree of immunosuppression in the tumor. Immune status is shaped by the interaction of cancer cells with T_{reg}, myeloid-derived suppressor cell-like cells, stromal cells (mesenchymal stem cells and cancer-associated fibroblasts), and even vascular cells. The coordination and communication between the cancer cell and these other components of the tumor shape the immune environment, and iNOS plays an important role in this. iNOS has been shown to increase TGF- β activation via nitrosylation of the latency-associated peptide, which inhibits its TGF- β inactivation.⁵³ iNOS induces COX2, leading to PGE2,⁵⁴ which in turn increases IL-10 and VEGF, regulates PD-L1 in myeloid-derived suppressor cells and myeloid cells, and decreases natural killer cell activity.⁵⁵⁻⁵⁸ It also induces IL-10 in T_{reg} cells,⁵⁹ and is associated with an increase in VEGF, which has been shown to inhibit CD8 cells.⁶⁰ In sum, iNOS orchestrates many important immune-suppressive effects that reduce activated lymphocytes essential for eradication of tumors.

D. Genetic Mutation Landscape of TNBC

Nitrites, nitrates, S-nitrosothiols and nitrosamines are metabolites of NO and mediators of its cytotoxic/cytoprotective effects, and they have the potential to induce DNA damage, leading to accumulation of gene mutations.⁶¹ One of the research hypotheses of our group is that in

the case of chronic induction of iNOS in breast epithelial cells—for example, during TNBC carcinogenesis—a proportion of these cells may adapt and become immune to or dependent on high levels of iNOS-related redox, resulting in the accumulation of mutations and genetic instability and in the simultaneous activation of multiple signaling pathways that drive tumor aggression.

TNBCs are known to display mutational heterogeneity, with tumors differing immensely in their clonal frequencies at any given time. Mutational status at diagnosis, for example, may be at different phases of molecular progression, which undoubtedly has treatment implications. For example, some TNBCs have small numbers of involved pathways, with relatively few identifiable mutations. Others have multiple involved pathways and carry significant mutational burdens.⁶² The Cancer Genome Atlas project examined the genomic heterogeneity of TNBCs and found that the most frequent loss-of-function alterations in TNBC involve genes associated with DNA damage repair, such as *TP53*, *BRCA1* function, and retinoblastoma protein 1.¹ It is suggested that loss of TP53 function occurs within most, if not all, TNBCs, bringing with it a combination of high levels of constitutive p53 protein expression and a high frequency of TP53 gene mutations (~ 80%).^{1,63,64}

NO has been implicated in the induction of TP53 mutations in numerous tumor types,⁶⁵ including ER-negative breast tumors.¹⁰ In normal physiology, p53 accumulates post-NO-mediated DNA damage and can lead to apoptosis. Therein lies a negative feedback loop, as this leads to transrepression of iNOS.³⁴ However, the mutation of p53 perturbs this negative feedback loop and there is a constant induction of INOS by mutant p53 activity without a resulting induction in apoptosis.⁶⁶ Indeed, INOS is associated with increased p53 mutant frequencies of both G:C --> A:T transitions at the p53 CpG site of codon 248 and C:G --> T:A transitions at codon 247 in colon lesions from ulcerative colitis cases. These p53 mutations were also detected in sporadic colon cancer tissue and were associated with INOS activity in these tissues.⁶⁶

The most common gain-of-function alterations in TNBC involve genes associated with the phosphatidylinositol 3-kinase (PI3K) signaling pathways.^{1,7} Clinically, TNBC phenotypes are often associated with dysfunction in the *BRCA1* gene, and a diagnosis of TNBC suggests the possibility of a germline *BRCA1* mutation.^{2,18} A meta-analysis by Tun et al. reported that women with TNBC are 5.6 times more likely to harbor a *BRCA1* mutation compared to women with other breast cancer subtypes.⁶⁷ Germline mutations causing *BRCA* loss of function mean that cells are unable to repair DNA damage by homologous recombination; for this reason, they are associated with hereditary breast cancers.^{2,7,68} TNBCs can also be associated with so-called “BRCAness,” meaning that even in the absence of a germline *BRCA1/2* mutation, TNBCs can have pathological characteristics similar to those of cancers that develop in *BRCA1/2* mutation carriers. These characteristics include high tumor grade, lymphocytic infiltration, TP53 mutations, C-MYC amplification, and multiple chromosome abnormalities.⁶⁹

TNBCs have several potentially targetable alterations. Twenty percent of TNBCs contain so-called clinically actionable somatic alterations, including BRAF V600E mutations, EGFR amplifications, and ERBB2 and ERBB3 mutations.⁶² In addition, less common alterations

may potentially be targeted: phosphatase and tensin homolog alterations can be targeted by PI3K inhibitors and AKT (protein kinase B) inhibitors; cyclin-dependent kinase 6, cyclin D1, cyclin D2, and cyclin D3 amplifications can be targeted by cyclin-dependent kinase 4/6 inhibitors.⁷⁰ Amplification of the MYC oncogene coincides with mitogen-activated protein kinase 1 (MEK) activity by gene expression signatures. Therefore, MEK inhibitors can inhibit cells that overexpress MYC and may play a role in MYC-amplified breast cancers.⁷⁰

IV. TNBC Treatment and Clinical Outcomes

A. Treatment Strategies

When diagnosed at an early stage, TNBCs are treated with combination chemotherapy and surgery, with or without radiotherapy. Chemotherapy may be delivered in the neoadjuvant (presurgery) setting or in the adjuvant (postsurgery) setting. The use of NACT increases the possibility of using breast-conserving surgery and, more importantly, can eliminate clinically silent micrometastases and therefore provide important prognostic information. NACT is particularly effective in TNBCs, with up to 30% of patients exhibiting excellent pathological responses.⁶ Currently the best predictor of disease-free and overall survival in TNBC patients receiving NACT is the achievement of a pCR. Intriguingly, patients who attain a pCR after NACT have excellent survival outcomes, comparable to those of their non-TNBC counterparts who attain a pCR.^{18,71,72} This pattern supports the idea of a cohort of TNBCs whose tumors are exquisitely sensitive to chemotherapy.¹⁸ Understanding the tumor biology of these responders will greatly improve our ability to identify those who will most benefit from NACT. Unfortunately, biomarkers that predict the chemo-sensitivity of TNBCs are yet to be identified.

B. Chemotherapeutics in Treatment

The main chemotherapy agents used in the treatment of TNBC are anthracyclines, taxanes, and platinum salts. In the NACT setting, taxanes appear more active in TNBCs than in hormone receptor–positive breast cancers.^{2,26,64} Anthracycline-taxane combination therapies are particularly effective. Blum et al. showed that docetaxel-cyclophosphamide is inferior to anthracycline-taxane chemotherapy, with the largest difference seen in TNBCs.⁷³ Sikov et al. demonstrated that 42% of patients with stage II to III TNBC treated with anthracycline-taxane chemotherapy achieve a pCR in the breast and 39% achieve a pCR in the breast and axilla.⁷⁴ One of the proposed mechanisms of action of paclitaxel-mediated cell death, in addition to microtubule stabilization, is the induction of free radicals including NO,^{75,76} super-oxide, and hydrogen peroxide.⁷⁵

iNOS targeting is a potential strategy to enhance the activity of the taxanes in TNBC. Frederiksen et al. demonstrated that the addition of low levels of NO using glyceryl trinitrate sensitizes the TNBC cell line MDA-MB-231 to paclitaxel and doxorubicin-induced apoptosis in hypoxia but not in normoxia.⁷⁷ In contrast, Switzer et al. showed that the addition of a high level of NO via the NO donor DETA/NO increases the resistance of MDA-MB-231 to paclitaxel and doxorubicin via upregulation of the p-glycoprotein drug resistance pump.⁵⁴ These data suggest that the effects of NO on paclitaxel and doxorubicin are dose dependent, which may mean that in TNBC tumor–expressing iNOS that generates

moderate to high levels of NO, iNOS likely confers resistance to chemotherapy and may account in some part for the intrinsic resistance to chemotherapy often seen in TNBC. It should be noted that TNBC has been found to have higher levels of NO (estimated by nitrite levels) than luminal- or HER2-positive breast tumors.⁷⁸ A key study by Granados-Principal et al. demonstrated that therapy combining docetaxel and the pan-NOS inhibitor NG-monomethyl-L-arginine acetate (L-NMMA) in a xenograft model of MDA-MB-231 increases tumor apoptosis, decreases Ki-67 proliferating cells, and, importantly, reduces the tumor-initiating capacity of residual tumor cells after chemotherapy to a greater degree than docetaxel alone.¹¹ In a follow-on study, Dave et al. examined the potential of L-NMMA to sensitize a rare histological subtype of breast cancer, known as metaplastic, to docetaxel.⁷⁹ Metaplastic breast cancer associates predominantly with TNBC and has a mesenchymal-like phenotype. It is predominantly intrinsically resistant to chemotherapy. Dave et al. showed that 39 of the 40 metaplastic tumors in their study carried a RPL39 A14V mutation, which they had previously found to be associated with enhanced NO activity and stemness. Tumors carrying the RPL39 A14V that were resistant to docetaxel were sensitized in the presence of L-NMMA via inhibition of STAT3 signaling.⁷⁹

Perhaps one of the greatest advances in recent years is the addition of platinum salts to the treatment of TNBCs. Combination chemotherapy with a platinum backbone has been shown to be both effective and well tolerated in the NACT of TNBCs,^{80,81} and also shows some efficacy in the metastatic setting.^{82,83} However, the optimal way to incorporate platinum into the treatment paradigm of TNBCs has not yet been clearly established. Platinum salts induce double-stranded DNA breaks to cause cell death. On the other hand, the BRCA1 and BRCA2 genes play an important role in the repair of double-strand DNA breaks.⁷⁴ As a result, cells that are deficient in homologous recombination repair mechanisms, such as BRCA-mutated cells or TNBCs, can be exquisitely sensitive to platinum agents.^{7,13,30} In particular, tumors with BRCA1 mutations have a reduced capacity for DNA repair and are unable to completely recover from the effects of DNA-damaging agents such as platinum. In view of the high frequency of BRCA1 germline mutations in TNBCs, platinum salts are highly active in this cohort of patients.⁸⁴ Platinum salts have been shown to be beneficial among all TNBCs and not just those associated with BRCA mutation.

A study by Jin et al. examined the impact of iNOS expression on TNBC response to cisplatin-based NACT in 20 patients with TNBC.⁸⁵ Those with high levels of iNOS expression were resistant to cisplatin compared to those with low levels. This is in contrast to findings in ovarian cancer, where Leung et al. showed that cisplatin-sensitive ovarian cancer cell lines have higher basal iNOS levels. They demonstrated that the NO donor SNAP increases p53 protein levels and enhances cisplatin-induced apoptosis in resistant C13(*) cells in a p53-dependent manner, while the iNOS inhibitor 1400W partially blocks cisplatin-induced apoptosis in OV2008 cells.⁸⁶ This suggests that ovarian tumors with high iNOS expression may be a good target for platinum drugs and that tumors with low iNOS expression can be sensitized to cisplatin-based therapies by including NO-donating drugs in the treatment regimen. Given these observations and that of Jin et al., further research is needed to assess the role of iNOS in TNBC platinum chemotherapy responses.

V. Potential TNBC Therapeutic Targets Modulated By iNOS

The combination of inter- and intratumoral heterogeneities has led to the limited success of attempts to identify targetable states in TNBC via genomic profiling.³² The identification of molecular targets is critical if the survival of patients with TNBC is to be improved. So far, however, extensive research has failed to demonstrate one unifying targetable alteration in TNBCs.¹³ Examining signal transduction pathways targeted by iNOS in ER-negative breast cancer and TNBC may provide novel targets for TNBC treatment. Current molecular-targeted therapies under investigation in TNBC include PARP inhibitors, EGFR inhibitors, immunotherapeutic, and VEGF inhibitors.

A. PARP Inhibitors

The poly (ADP-ribose) polymerase (PARP) enzyme is an important regulator of the DNA base-excision repair pathway and is responsible for the repair of single-strand DNA breaks.⁷ As outlined earlier, the BRCA genes play an important role in the repair of double-strand DNA breaks. When there is loss of both BRCA and PARP function, cell death occurs.² Therefore, PARP inhibitors act by inhibiting the repair of DNA strand breaks and are most effective in patients with germline BRCA mutations.^{2,7,68} There are several potential clinical uses for PARP inhibitors: inducing cell kill in patients with BRCA1/2 mutations, enhancing the “BRCA-like” DNA repair defects seen in TNBCs, and increasing sensitization to both chemotherapy and radiotherapy.^{2,15,30}

Early clinical studies using PARP inhibitors in TNBC showed great promise and, as a result, several PARP inhibitors are currently being developed as therapeutic targets for TNBCs.^{2,15,68} It is anticipated that they will be most effective in combination with platinum agents. In the phase II ISPY2 study, the addition of a PARP inhibitor, veliparib, plus carboplatin to an anthracycline-taxane NACT backbone increased the rate of pCR in TNBCs when compared to standard chemotherapy alone (51 vs. 26%, respectively).⁸⁷ Similarly, iniparib in combination with carboplatin and gemcitabine demonstrated a pCR of 36%, rising to 56% in BRCA1/2 mutation carriers.⁸⁸ Currently it is not known what if any impact tumor iNOS expression may have on predicting PARP inhibitor activity. This will be important to address in addition to the potential of iNOS-targeting combination therapies.

B. EGFR Inhibitors

As previously mentioned, TNBC and in particular basal-like tumors frequently express EGFR, which then initiates downstream signaling through the PI3K-AKT-mTOR and RAS-MEK pathways, which in turn promote cell proliferation and survival.⁸⁹ EGFR thus plays a critical role in normal cell proliferation and migration and helps cells to evade apoptosis. EGFR overexpression is seen in approximately 20% of all breast cancers and in up to 80% of TNBCs, and is associated with a poorer prognosis.¹⁵ Although it is not routinely tested in breast cancer, EGFR is a potential target for TNBCs.¹⁵ In 2010 we demonstrated that tumors from ER-negative patients with high levels of iNOS expression display increased EGFR phosphorylation.¹⁰ In the MDA-MB-468 TNBC cell line, the NO donor DETA/NO was able to autophosphorylate the EGFR in the absence of any ligand, indicating that NO provides an alternative mechanism for EGFR activation.¹⁰ Subsequently, we showed that this is via S-

nitrosylation.^{54,90} Further investigation has shown that NO mediates cell migration and invasion, which are hallmarks of EMT, via activation of the EGFR-RAS-MEK-ERK pathway, which can be abrogated with EGFR or MEK inhibition.¹² Other key mechanisms activated in this manner include enhanced cytokine secretions and NF- κ B activation,¹² suggesting a role for EGFR inhibition in TNBC either as a single agent or in combination with chemotherapeutics or iNOS inhibitors.

Both tyrosine kinase inhibitors (TKIs) and monoclonal antibodies can therapeutically target and inhibit EGFR. TKIs (gefitinib) competitively bind to the intracellular domain of EGFR, while monoclonal antibodies (cetuximab and panitumumab) target the extracellular domain.⁹¹ Gefitinib has been shown to enhance the response of both carboplatin and docetaxel in cell line studies, and the combination of gefitinib, carboplatin, and docetaxel has been shown to be synergistic in preclinical work.⁹² Cetuximab has also been shown to increase the efficacy of anthracyclines in preclinical models⁹³ and shows clinical utility in combination with platinum-based therapies in metastatic TNBC patients.^{94,95} Similar efficacy has been reported in clinical trials with panitumumab.^{96,97} On balance, EGFR may prove to be a useful target in the treatment of TNBCs. However, its overexpression does not necessarily predict response to EGFR inhibitors, meaning EGFR inhibition is unlikely to be beneficial in an unselected population of TNBCs.^{15,32,92} Further evidence is necessary before EGFR inhibitors can be routinely used outside of the clinical trial setting.² In future trials, prospectively identifying patients with EGFR amplifications or activating mutations may yield more promising results if those patients most likely to respond to EGFR inhibition are preselected.³²

C. VEGF Inhibitors

TNBCs have been shown to express increased levels of vascular endothelial growth factor (VEGF), which therefore may be considered a potential target for therapeutic strategies in TNBC,² particularly in tumors overexpressing iNOS. iNOS is associated with increased microvessel density and tumor angiogenesis,¹⁰ which represent dysfunctional tumor vascularity.^{98,99} This is a hallmark of poor prognosis due to decreased O₂ and nutrient delivery and an inability to recruit immune cells in the control and eradication of the tumor. NO has been shown to increase VEGF in the TNBC cell line MDA-MB-231,¹⁰⁰ suggesting that iNOS may promote angiogenesis via VEGF induction. Another source of NO that would also be a key player in tumor angiogenesis is endothelial NOS (eNOS), which also correlates with VEGF in breast cancer¹⁰¹ and is a target of the pan-NOS inhibitor L-NMMA, which shows activity in TNBC xenografts, as described previously.¹¹ VEGF supports the growth and maintenance of tumor vasculature, which is necessary for tumor cell survival and metastasis. Bevacizumab, a humanized monoclonal antibody, binds to and inactivates VEGF.⁷⁴ In clinical trials in the NACT setting, it appears to have the greatest activity in TNBC when compared to other subtypes, as measured by pCR,¹⁰² and it has been shown to work particularly well when given in combination with anthracycline-taxane therapies.¹⁰³ Bevacizumab may also have benefit in the metastatic setting when used in combination with paclitaxel.¹⁰⁴

The tumor endothelium has been shown incapable of increasing the essential adhesion molecules ICAM or VCAM for immune cell recruitment in response to immunological stimuli. According to Dewhirst et al., while in normal vascular lipopolysaccharide (LPS) (which stimulates iNOS), treatment can induce leukocyte rolling, adhesion, and tissue extravasation, in tumor vasculature no leukocyte rolling or adhesion is present.¹⁰⁵ In another study, low-dose tumor gamma irradiation induced iNOS in tumor-infiltrating macrophages, which resulted in increased tumor-infiltrating CD8 cell recruitment to tumors. Furthermore, this led to normalization of the aberrant vasculature via increased expression of VCAM-1, which could be blocked using the iNOS inhibitor 1400W.¹⁰⁶ In contrast, Fukumura et al. showed that the use of L-NAME can inhibit angio-genesis and restore normal vasculature, which in turn increases O₂ and recruitment of immune cells to the tumor microenvironment.¹⁰⁷ This suggests that iNOS plays a major role in tumor vascular status, which is a critical component of therapy.

D. Immunotherapy

There are two major factors that determine therapeutic outcome: the efficacy of cancer cell killing and the immune profile. Over the last few decades, a tremendous focus has been on identifying agents that specifically target cancer cell killing and overcome the intrinsic mechanisms that lead to drug resistance. Recently, it was shown that immune response, even in conventional therapy settings, has a predominate effect on the outcome of cancer therapy. In murine models, the elimination of CD8 and CD4 cells dramatically attenuates radiotherapy and chemotherapy, pointing to the importance of the immune response.¹⁰⁸⁻¹¹¹ With the advent of cancer vaccines, immunotherapy, and adopted T cell therapy, the idea has emerged that combinations of conventional and immune checkpoint inhibitors and/or immune response-activating therapies augment the immune response and thus dramatically improve patient outcomes. This will lead to exciting new opportunities for potential approaches to previous intractable diseases like TNBC.

The benefit of immune checkpoint inhibitors has been explored in TNBCs. Polónia et al.⁴⁴ showed that programmed cell death ligand 1 (PD-L1) expression is significantly associated with higher-grade TNBCs and with high expression of Ki67. They also reported a significant association between PD-L1 expression and basal cell markers, such as CK5, CK14, EGFR, and P-cadherin.⁴⁴ Intriguingly, we demonstrated that ER-negative tumors with high levels of INOS expression have increased levels of CK5, CK14, EGFR, and P-cadherin.¹⁰ Additionally, we recently found that TNBC patients with high levels of INOS have reduced sTIL levels.¹² Whether INOS regulates PD-L1 expression in TNBC remains to be determined.

Pembrolizumab, a monoclonal anti-programmed cell death 1 (PD-1) antibody, was assessed in combination with an anthracycline-taxane NACT combination. It demonstrated a pCR rate increase from 20 to 60% among TNBCs.¹¹² While immunotherapy remains investigational at the present time, it is highly likely that anti-PD-1 and anti-PD-L1 antibodies will join the TNBC treatment paradigm in the not too distant future.

VI. Conclusions

TNBC is a complex disease with many subtypes that are regulated by various signal transduction pathways. Tumor iNOS expression is associated with poorer outcomes and increased rates of distant metastasis, indicating that iNOS may be a prognostic indicator for TNBC. Moreover, therapies combining paclitaxel and iNOS inhibitors show efficacy in reducing tumor volumes in xenograft mouse models of TNBC, particularly against the chemoresistant metaplastic breast cancer subtype. This begs the question as to the appropriate use of NOS inhibitors. The clinically available NOS inhibitors include L-NMMA and L-NAME as pan-NOS inhibitors or iNOS-specific inhibitors such as GW274150 and aminoguanidine.¹¹³⁻¹¹⁵ Ongoing clinical studies of TNBC and pan-NOS inhibitors will provide important insight into this treatment strategy (clinical trial number: NCT02834403).

Future studies should focus on the timing of iNOS inhibitor delivery. iNOS inhibition in the initial stages of treatment may be important in reshaping tumor vasculature and decreasing immune-suppressive responses, while iNOS inhibition in the later stages may be useful in the eradication of the tumor and sensitizing to chemotherapeutics. Altogether, the emerging data suggest that iNOS is a potential molecular target for the treatment of TNBC.

Acknowledgments

Sharon Glynn is funded by Breast Cancer Now (2013MayPR019 and 2015NovPhD643). Elaine Walsh is the recipient of a NUI Galway School of Medicine PhD scholarship.

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Abbreviations

AKT	protein kinase B
BL1	basal-like 1
BL2	basal-like 2
CK5/6	cytokeratin 5/6
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	estrogen receptor alpha
HER2	human epidermal growth factor receptor 2
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
LAR	luminal androgen receptor
L-NMMA	NG-monomethyl-L-arginine acetate
LPBC	lymphocyte-predominant breast cancers
M	mesenchymal
M2	macrophage type 2
MEK	mitogen-activated protein kinase kinase 1
MSL	mesenchymal stem-like
NACT	neoadjuvant chemotherapy
NO	nitric oxide
PARP	poly (ADP-ribose) polymerase
pCR	pathological complete response

PD-L1	programmed cell death ligand 1
PI3K	phosphatidylinositol 3-kinase
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
TILs	tumor-infiltrating lymphocytes
TNBC	triple negative breast cancer
VEGF	vascular endothelial growth factor

Fig. 1. Impact of iNOS expression on TNBC

Table 1
Summary of findings from studies on iNOS and breast cancer

Publication	Breast cancer type	Impact of iNOS on patient outcome	iNOS mechanisms of action	Therapeutic impact of iNOS targeting
Thomsen et al., 1995 ²⁰	All breast cancers combined	Not tested	Associated with high-grade disease	Not tested
Tschugguel et al., 1999 ²¹	All breast cancers combined	Not tested	Associated with low-grade disease	Not tested
Vakkala et al., 2000 ²²	All breast cancers combined	Not associated with poor survival	Associated with high-grade disease, increased microvessel density, and increased apoptotic index	Not tested
Loibl et al., 2005 ²³	All breast cancers combined	Associated with poor survival	Associated with increased tumor size and high-grade disease	Not tested
Bulut et al., 2005 ²⁴	All breast cancers combined	Associated with poor survival	No association with tumor grade or node status	Not tested
Glynn et al., 2010 ¹⁰	All breast cancers combined and ER status stratification; MDA-MB-231 & MDA-MB-468 TNBC cell lines; MCF-7 and T47D ER-positive cell lines	Associated with poor survival in ER-negative tumors only	Induction of basal-like signature, increased IL-8, S100A8, and P-cadherin, increased EGFR phosphorylation, and cell migration and invasion	Not tested
Ridnour et al., 2012 ¹¹⁶	All breast cancers combined; MDA-MB-231 TNBC cell line	Patients with high levels of TIMP-1 have increased poor outcome in iNOS-expressing tumors	TIMP-1 mediates NO induction of AKT and Bad; phosphorylation of iNOS/NO increases phospho-Akt and phospho-Bad via TIMP1/ CD63 complexes, which activate PI3K signaling	Not tested
Switzer, et al., 2012 ⁵⁴	MDA-MB-231 and MDA-MB-468 TNBC cell lines	Not applicable	EGFR and Src activation via S-nitrosylation of EGFR, Src, and STAT3; NO activates β -catenin signaling, induces EMT, and inhibits PP2A tumor suppressor activity	NO increases MDA-MB-231 resistance to doxorubicin and paclitaxel
Switzer, et al., 2012 ¹¹⁷	MDA-MB-231 and MDA-MB-468 TNBC cell lines	Not applicable	NO induces ETS-mediated basal-like gene signatures via Ras/ MEK/ERK signaling and mediated by Ras S-nitrosylation	Not tested
Heinecke et al., 2014 ¹¹⁸	MDA-MB-231 and MDA-MB-468 TNBC cell lines; MCF-7 ER positive cell line; MDA-MB-231 xenograft (nude mice)	Not applicable	iNOS inhibition reduces COX2, TLR4, S100A8, CD44, IL-6, and IL-8 in MDA-MB-231 xenografts IFN- γ induction of TLR4, S100A8, IL-6, and IL-8 is iNOS-mediated	iNOS inhibition reduced tumor growth and brain metastasis in MDA-MB-231 ER-negative breast cancer xenografts
Granados-Principal et al., 2015 ¹¹	All breast cancers combined and TNBC status stratification; MDA-MB-231 and SUM159 TNBC cell lines	Associated with poor outcome in TNBC	iNOS inhibition reduces Snail, Slug, Twist1, and Zeb1 markers of EMT; iNOS inhibition reduces Hif1, IRE1, ATF4, and SMAD 2/3 markers of hypoxia and endoplasmic reticulum stress	iNOS inhibition reduces tumor proliferation and reduces number of cells with tumor-initiating capacity; iNOS inhibition increases response to paclitaxel
Dave et al., 2017 ⁷⁹	Metaplastic breast cancer; Hs578T and BT549 metaplastic TNBC breast cell lines	Associated with poor outcome in metaplastic breast cancer	iNOS inhibitors reduce cell proliferation and invasion; RPL39 mediates its effects through iNOS	iNOS inhibitors reduce metaplastic patient-derived xenograft proliferation
Garrido et al., 2017 ¹²	TNBC patients; HCC1806 and MDA-MB-468 TNBC cell	Associated with increased distant metastasis and poor	NO-mediated EGFR & ERK activation induces proinflammatory cytokine secretion, NF κ B activation,	Not assessed

Publication	Breast cancer type	Impact of iNOS on patient outcome	iNOS mechanisms of action	Therapeutic impact of iNOS targeting
	lines; MCF-10A normal basal immortalized breast cells	outcome in TNBC; associated with reduced TILs	and cell migration and invasion in basal-like breast cancer	

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Table 2
Designation of cell lines commonly used in NOS2 breast cancer studies

Cell line	ER status	TNBC	Histological subtype	Lehmann subtype
MCF-7	Positive	No	Adenocarcinoma	Not applicable
T47D	Positive	No	Infiltrating ductal carcinoma	Not applicable
MDA-MB-231	Negative	Yes	Adenocarcinoma	MSL
MDA-MB-468	Negative	Yes	Adenocarcinoma	BL1
Hs578T	Negative	Yes	Carcinosarcoma/metaplastic	MSL
BT549	Negative	Yes	Ductal carcinoma, papillary	M
SUM159	Negative	Yes	Anaplastic	MSL
HCC1806	Negative	Yes	Primary acantholytic squamous cell carcinoma	BL2
MCF-10A	Negative	Yes	Normal immortalized	Not applicable