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Maggie Louie
Department of Natural Sciences and Mathematics, Dominican University of California,
maggie.louie@dominican.edu

Mary B. Sevigny
Department of Natural Sciences and Mathematics, Dominican University of California,
mary.sevigny@dominican.edu

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Review Article

Steroid hormone receptors as prognostic markers in breast cancer

Maggie C Louie*, Mary B Sevigny*

*Department of Natural Sciences and Mathematics, Dominican University of California, 50 Acacia Avenue, San Rafael, CA 94901, USA. *Equal contributors.*

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Abstract: Despite the existence of many promising anti-cancer therapies, not all breast cancers are equally treatable, due partly to the fact that focus has been primarily on a few select breast cancer biomarkers- notably ERα, PR and HER2. In cases like triple negative breast cancer ($ER\alpha^-$, PR^-, and $HER2^-$), there is a complete lack of available biomarkers for prognosis and therapeutic purposes. The goal of this review is to determine if other steroid receptors, like ERβ and AR, could play a prognostic and/or therapeutic role. Data from various *in vitro*, *in vivo*, and clinical breast cancer studies were examined to analyze the presence and function of ERβ, PR, and AR in the presence and absence of ERα. Additionally, we focused on studies that examined how expression of the various steroid receptor isoforms affects breast cancer progression. Our findings suggest that while we have a solid understanding of how these receptors work individually, how they interact and behave in the presence and absence of other receptors requires further research. Furthermore, there is an incomplete understanding of how the various steroid receptor isoforms interact and impact receptor function and breast cancer progression, partly due to the difficulty in detecting all the various isoforms. More large-scale clinical studies must be made to analyze systematically the expression of steroid hormone receptors and their respective isoforms in breast cancer patients in order to determine how these receptors interact with each other and in turn affect cancer progression.

Keywords: Breast cancer, steroid hormone receptors, prognostic markers, estrogen, progesterone, androgen

Introduction

Though both estrogen and progesterone receptors are commonly used as prognostic markers for breast cancer, current endocrine therapy primarily targets the estrogen receptor, ERα. Unfortunately, for about 10-15% of breast cancer patients [1-3]- like those diagnosed with triple negative breast cancer, defined as breast tumors lacking the expression of estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2), $ER\alpha^-/PR^-/HER2^-$ - the established endocrine therapies are ineffective, highlighting an urgent need for additional therapeutic targets in breast cancer. Therefore, the goal of the following review is to examine the role of the steroid hormone receptors- ERα, ERβ, PR, and androgen receptor (AR)- in the progression of breast cancer in order to determine their role and utility as prognostic markers and therapeutic targets.

Steroid receptors: an overview

The steroid hormone receptor subfamily, which includes estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), and glucocorticoid receptor (GR), is part of the larger superfamily of nuclear hormone receptors [4]. The members of this superfamily function as ligand-gated transcription factors that modulate the expression of genes [5]. While unbound steroid receptors are typically located in the cytosol, ligand binding induces receptor dimerization and conformational changes which in turn exposes the nuclear localization signal, allowing translocation into the nucleus [6]. Once inside the nucleus, the receptor dimer recognizes and binds specific DNA sequences

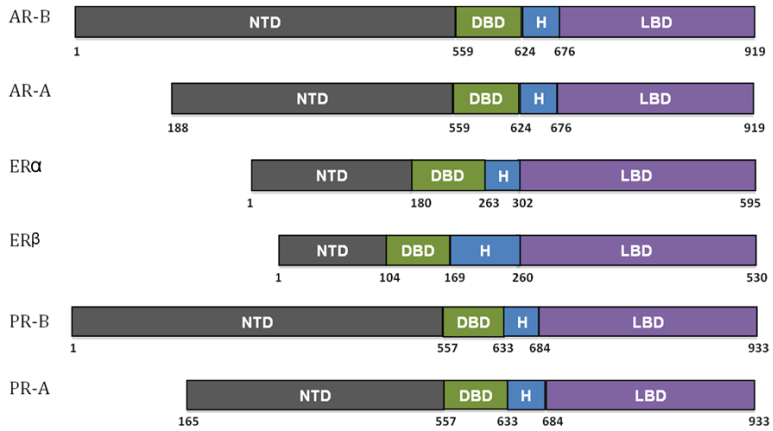

Figure 1. Comparing the functional domains of nuclear hormone receptors. *NTD*, N-terminal A/B hypervariable domain; *DBD*, DNA binding domain; *H*, hinge region; *LBD*, ligand binding domain. Adapted from [196].

that in turn results in enhancing or silencing the transcription of specific target genes regulated by the receptor [7, 8].

As depicted in **Figure 1**, nuclear hormone receptors share common functional domains, such as a DNA binding domain (DBD), a ligand binding domain (LBD), and two transactivation domains (AF-1 and AF-2) [9-12]. DBDs contain two zinc finger motifs that allow them to recognize and bind to specific DNA sequences- often referred to as hormone response elements (HREs)- within the promoter and/or enhancer regions to regulate transcription. Different steroid hormone receptors bind to different response elements, thus allowing the receptors to regulate subsets of genes that are necessary to elicit a physiological response. The LBD is involved in the binding of specific hormones, which induces dimerization and nuclear translocation [9-12]. The two transactivation domains, AF-1 and AF-2, are important for modulating transcription of the target genes. AF-2 is located within the LBD and is involved in ligand-dependent transactivation, while AF-1 is found in the N-terminal A/B hypervariable domain (NTD) and is responsible for ligand-independent transcriptional activation and mediates protein-protein interaction with other transcription factors [13]. AF-1 is also responsive to phosphorylation by kinases that are activated in various signaling pathways, including the epidermal growth factor receptor (EGFR) pathway.

Estrogen receptor

The estrogen receptor (ER) was identified in the 1950s by Dr. Elwood V. Jensen as reviewed in [14, 15]. Eventually, it was determined that three forms of ER exist- ERα, ERβ, and GPR-30 [16-19]. All three ERs are encoded by different genes located on different chromosomes [20]. ERα is encoded by the gene ESR1 located on chromosome 6; ERβ is encoded by gene ESR2 on chromosome 14; and GPR-30 is encoded by the GPER gene on chromosome 7. While ERα and ERβ are nuclear hormone receptors, GPR-30 is not a member of the steroid receptor family but is instead a G-protein coupled receptor that has been shown to bind and respond to estrogen [21-23]. Therefore, since our interest is in the role of steroid hormone receptors in breast cancer, GPR-30 will not be discussed further in this review.

Although ERα and ERβ are both expressed in breast tissue and bind to estrogen with similar affinities [24], studies have shown that only ERα is necessary for normal mammary gland development [25, 26] leading researchers to question the function of ERβ in normal breast tissue. Multiple studies have also reported that ERβ expression actually represses ERα expression and function [27, 28]. In addition to the breast, both nuclear receptors are expressed in many other tissues within the human body, including the endometrium, ovary, testes, cerebral cortex, myocardium, and thyroid [29-32]. However, their expression patterns do differ in certain tissues- for example, ERα is the sole estrogen receptor expressed in the hippocampus, and only ERβ is found in prostate tissue [32].

In addition to full length ERα (as shown in **Figure 1**), there are 2 truncated splice variants of ERα- 46 kDa estrogen receptor (ER46) [33] and 36 kDa estrogen receptor (ER36) [34, 35]. Some of the full length ERα (ER66) along with ER36 and ER46 [36] associate with the plasma

membrane and are referred to as mERs due to their ability to translocate to the plasma membrane through palmitoylation and caveolin-1 association, which in turn allows for rapid estrogen receptor signaling. ER46 does not have an AF-1 transactivation domain, which suggests that ER46 does not mediate any nuclear functions. ER36 lacks both AF-1 and AF-2 transactivation domains, and part of the ligand binding domain is replaced by a 27 amino acid sequence in the C terminus [34]. ER36 has been shown to mediate estrogen stimulation via mitogen-activated protein kinase (MAPK) pathway [35].

As with ERα, ERβ also exists as several isoforms- ERβ1 (**Figure 1**), ERβ1 "short form", ERβ2/cx, ERβ3, ERβ4, and ERβ5 [37-40]. Most are due to the alternative splicing of exons 7 and 8, although the truncated form of ERβ1 is due to proteolysis at the N-terminus [41, 42]. Of all the isoforms, only ERβ1 contains the ligand-binding domain [43]. However, it has been shown that the formation of heterodimers between ERβ1 and the other isoforms increases the transcriptional activity of ERβ1 [37].

Progesterone receptor

The expression of PR is primarily regulated by ERα at the transcriptional level [44, 45]. There are two known isoforms of PR, PR-A and PR-B. PR-A is a truncated version of PR-B, lacking 164 amino acids at the N-terminus (**Figure 1**). The two proteins are transcribed from two different promoters located within the same gene on chromosome 11 [46] and can form homo- or heterodimers. Studies using knockout mice confirmed the functional importance of both PR isoforms [47, 48]. Although animals lacking PR-A did not display significant developmental effects in the mammary glands or thymus, they did display severe dysfunctions in their ovaries and uterus resulting in infertility, suggesting that its primary function is maintaining normal ovarian and uterine functions [49-52]. Conversely, PR-B knockout-mice retained normal ovarian, uterine, and thymic functions but exhibited a significant decrease in mammary ductal morphogenesis [49-51, 53], indicating that PR-B mediates the proliferative effects of progesterone in the mammary gland.

Structural and functional analyses of each PR isoform suggest that they have different transcription activation properties when bound to progesterone [53, 54]. According to Richer et al., approximately 27% of PR-regulated genes are controlled by both PR isoforms. However, this study also indicated that PR-B alone controls the majority of the PR-regulated genes in comparison to PR-A alone (69% versus 4%) [55] which may be in part due to PR-B being intrinsically a stronger transcriptional activator than PR-A. In fact, PR-A has been reported to function as a transcriptional *repressor* under certain cellular conditions [56]. Curiously however, it is PR-A- not PR-B- that is more frequently over-expressed in breast cancer [57]. Some studies have even indicated that it is not so much the expression of either isoform but rather the *ratio* of the two isoforms that are important in breast cancer development. For example, a higher ratio of PR-A/B has been associated with poorer prognosis and response to hormone therapy [58].

Androgen receptor

AR is expressed in all tissues, including testis, prostate, foreskin, cervix, vagina, mammary glands, bone, brain, sebaceous and sweat glands of the skin, and breast [59-61]. The gene that codes for the androgen receptor (AR) is located on the X chromosome [60, 62]. While AR is closely related to PR and ER, one distinctive feature of the AR protein is the presence of glutamine and glycine repeats in the N-terminal activation domain of the receptor which have been linked to certain cancers and chronic neurological diseases in humans [60, 63, 64]. In 1994, Wilson and McPhaul discovered two isoforms of AR in human genital skin fibroblasts that are structurally very similar to PR-A and PR-B [65]. Their formation is due to two distinct translation initiation sites which result in the full-length receptor (110 kDa) and an N-terminally truncated form (87 kDa) known as AR-B and AR-A, respectively (**Figure 1**). Since 1994, several low molecular weight isoforms of AR have been identified, particularly in prostate cancer cell lines and tumors (reviewed in [66]). A few different mechanisms are responsible for these variants, including premature chain termination during translation, proteolysis by calpains, and alternative splicing [66]. Several isoforms, such as AR-V7 (also known as AR3), lack the LBD and are thus capable of transcription activation in the absence of androgen (i.e.

androgen-independent transcription) [67, 68]. Though originally found in prostate cancer cases that have become androgen deprivation therapy (ADT) or castration resistant [67-70], AR-V7 has likewise been found in a substantial number of primary breast tumors (~50%) and most breast cancer cell lines [71]. Another AR variant, Δ3AR, has been found exclusively in some breast tumors and breast cancer cell lines but not in normal breast tissue [72]. Originally discovered in patients suffering from androgen insensitivity syndrome (AIS), the Δ3AR isoform lacks the second zinc finger in the DBD, which potentially could reduce its ability to inhibit cell growth, at least within an ERα$^+$ setting [72, 73].

ERα-positive breast cancer

Prognosis and treatment

In 1896, George Thomas Beatson found that removing the ovaries from patients with advanced stages of breast cancer resulted in significant regression, which later lead to the speculation of estrogen's stimulating effect on breast cancer. Hence, oophorectomy and/or the use of drugs that target the estrogen receptor have become standard therapies for treating estrogen responsive breast cancer. To date, much of what we know about the relationship between ER and breast cancer centers primarily on one particular receptor- ERα. In fact, it is the presence or absence of ERα that determines whether a patient's breast cancer can be classified as either estrogen receptor positive or negative, respectively. ERα-negative breast cancers may express other hormone receptors such as PR, AR, and even ERβ, but they are often non-responsive to estrogen. Of all the breast cancer subtypes, ERα-positive breast cancer is the most prevalent, accounting for approximately 75% of breast cancers diagnosed in women [1].

Studies have shown that patients with ERα-positive breast cancers have a better prognosis because these tumors tend to be lower grade and have less aggressive phenotypes. Even patients with metastatic tumors that expressed ERα often had significantly better survival outcomes in comparison to patients with ERα-negative tumors [74], and this is most likely due to the fact that most patients with ERα-positive tumors also had an increase likelihood of responding to the established endocrine therapies [74]. However, not all ER-positive tumors respond to endocrine therapy, and even those that are initially responsive eventually become resistant as the disease progresses.

Drugs that specifically target ERα- estrogen receptor antagonists- can be used to treat or manage the disease. ER antagonists specifically compete with estrogen and block it from binding to the receptor. There are two types of ER antagonists- 1) selective ER modulators (SERMs), also referred to as partial antagonists, and 2) pure or complete antagonists, also referred to as selective ER down-regulators (SERDs) [75]. Among the most common SERMs are the anti-estrogens tamoxifen and raloxifene. Tamoxifen is effective in antagonizing estrogen-dependent cancer cell growth by binding to ERα and promoting the recruitment of co-repressors rather than co-activators in mediating transcriptional repression of ER target genes [11, 76]. An example of a pure antagonist or SERD is ICI 182, 780, also referred to as faslodex or fulvestrant, which binds to either ERα or β and promotes receptor degradation [77-79]. Ultimately, ERα is considered a good prognostic marker for breast cancer not only because it is vital in both the development and progression of the disease but also because its presence determines whether the cancer will likely respond to anti-estrogen treatment.

Association with progesterone receptors

Of all the ERα$^+$ mammary tumors, about 50-60% are PR$^+$ [80-82]. Yet while the ER status has been well established as a predictive factor for breast cancer prognosis and cancer treatment, less is known about the significance of PR in the presence of ERα. Studies have shown that tumors expressing both receptors tend to be less aggressive and least likely to metastasize [83, 84]. Others have confirmed that the presence of both ERα and PR in tumors often translates to better prognosis [83-86]. Specifically, Dunnwald et al. examined the correlation between PR/ERα expression and mortality risk amongst 155,175 breast cancer patients and found that patients with ERα$^+$/PR$^+$ tumors had lower mortality rates compared to women with ERα$^+$/PR$^-$, ERα$^-$/PR$^+$, and ERα$^-$/PR$^-$. The highest mortality rate was seen in ERα$^-$/PR$^-$

patients. A similar study carried out by Salmen et al. determined that patients with $ER\alpha^-/PR^-$ tumors had worse prognoses than patients with $ER\alpha^-/PR^+$ or $ER\alpha^+/PR^+$ tumors [87]. Furthermore, tumors that have lost PR expression are often more aggressive and have negative prognoses, signifying that PR is an important indicator of the progression of the disease [82].

Compared to $ER\alpha^+/PR^+$ patients, a smaller percentage of $ER\alpha^+/PR^-$ breast cancer patients respond to tamoxifen treatment [85, 88-91], suggesting that PR plays an important role in endocrine therapy response. This could account for the consistent observation that not all $ER\alpha^+$ breast cancer patients respond to endocrine therapies like tamoxifen. As noted earlier, the presence of ERα may not always be sufficient to indicate positive outcome towards endocrine based-therapeutics. This may be due in part to the fact that expression of the ERα protein does not always translate to a functional ERα signaling pathway. Since PR is one of the target genes of ERα, it has long been proposed that the expression of PR may serve as a good indicator of ERα functionality and signaling [92]. Studies have in fact confirmed that the presence PR in $ER\alpha^+$ breast cancer significantly improves the outcome prediction for adjuvant endocrine therapy [85, 91, 93-96]. Specifically, Ferno et al. found that patients with $ER\alpha^+/PR^+$ tumors had significant increase in response to adjuvant tamoxifen therapy compared with patients with $ER\alpha^+/PR^-$ tumors [94, 97]. Other studies have also confirmed that PR status can be a better indicator of tamoxifen response than ERα status alone [96, 98]. We speculate that the presence of ERα without PR expression likely suggests a signaling dysfunction in the ERα pathway that reduces its ability to transcriptionally regulate its target genes; therefore, it is not surprising that the presence of both ERα and PR is a better indicator of endocrine therapy responsiveness.

Studies have also demonstrated that PR is associated with overall survival of cancer patients [85, 99]. Specifically, patients diagnosed as $ER\alpha^+/PR^+$ had less cancer recurrence in comparison to $ER\alpha^+/PR^-$ cancer patients [85]. We acknowledge that this reported decrease in breast cancer recurrence in $ER\alpha^+/PR^+$ patients contradicted earlier studies [86, 100], but this may be attributed to the researchers' use of biochemical assays to measure PR, which lack the sensitivity of other methods such as immunohistochemistry, which is the current practice.

Further complicating the analysis of PR's role in breast cancer prognosis and therapeutic response is the existence of the two PR isoforms mentioned previously- PR-A and PR-B. The ratio of PR-A to PR-B has been shown to change during the development of breast cancer [55, 101], and this may alter prognosis and therapeutic response. Although some breast cancers may express both isoforms, the ratios of the two receptors vary, with PR-A showing a higher expression in most tumors [55, 101]. This ratio of PR-A to B can impact the prognosis and staging of the disease, with an equal to low PR-A:PR-B ratio associated with lower tumor grading (G1 and G2) and a high PR-A:PR-B ratio associated with undifferentiated, higher grade tumors (G3) [101]. Recent microarray analysis also suggests that the PR-A:PR-B ratio is a critical determinant of PR target gene selectivity and response to hormonal stimuli [102]. This indicates the importance of evaluating the interplay between PR-A and PR-B when determining the clinical outcomes and responsiveness to endocrine therapy. However, typical methods used to determine the presence of PR in tumors are either ligand binding assays using tumor extracts or antibody-based assays such as immunohistochemistry [103-106], and neither of these assays is capable of differentiating between the two PR isoforms. In some cases, immunohistochemistry may only detect PR-A and not PR-B in formalin fixed tissue, suggesting that conformational differences between the two may interfere with detection [105]. Ultimately, further analysis of the role of PR-A and PR-B in breast cancer is needed to provide a better understanding of PR in prognosis and therapeutic response.

Association with ERβ

ERβ is not commonly used as a prognostic marker for breast cancer, partially because the presence and function of ERβ in ERα-positive tumors are not well understood. As with PR, the existence of several isoforms of ERβ adds to the confusion and has resulted in conflicting data, since the majority of studies that analyze

the role of ERβ in ERα$^+$ cells do not differentiate between the various ERβ isoforms. Despite this complexity, some researchers have had the foresight to study the specific ERβ isoforms, and several agree that expression of the ligand-binding isoform ERβ1 in ERα$^+$ cells counteracts ERα activity, thereby suppressing cell proliferation and enhancing apoptosis [39, 107, 108]. Two other studies carried out by Ogawa et al. and Peng et al. found that overexpression of the isoform ERβ2/cx inhibited the transcriptional activity of ERα [38, 109], while this same isoform has been shown to *enhance* the transcriptional activity of ERβ1 [37]. An *in vitro* study led by John Hawse concluded that the tamoxifen metabolite endoxifen worked best at inhibiting estrogen-mediated cell proliferation in ERα$^+$ cells if ERβ was also expressed [110]. It was also determined that endoxifen treatment of cells led to ERβ accumulation and subsequent increase in ERα/ERβ heterodimers, providing further evidence that the observed cancer suppressing activity of ERβ is due in part to a direct interaction between ERβ and ERα. However, a clinical study analyzing the effect of a 2-year tamoxifen treatment on 353 patients with stage II primary breast tumors found that patients who were ERα$^-$/ERβ$^+$ had significantly greater distant disease-free survival compared with patients who were ERα$^+$/ERβ$^+$, suggesting a mechanism for ERβ independent of ERα [111]. Since neither the levels of the tamoxifen metabolite endoxifen nor the exact ERβ isoforms were determined in these patients, it is difficult to directly compare this study with that of Hawse's. Yet these seemingly differing conclusions do point to a rather complex role for ERβ in breast cancer, which clearly involves more than just inactivation of ERα.

Association with androgen receptors

AR expression is most commonly associated with prostate cancer, and prostate tumor progression is as dependent on AR activity as breast tumor progression is on ERα activity. In fact, the treatment of prostate cancers usually involves hindering AR function via ligand depletion, treatment with AR antagonists, or both [112]. The role of AR in breast cancer is not quite as clear. AR is frequently expressed in ductal carcinoma in situ (DCIS) and invasive breast carcinoma [113]. In addition, most ERα$^+$ breast cancers also appear to express AR, as exemplified by a study carried out by Hu et al. which found that 88% of 1,164 ER positive breast cancer cases also expressed AR [114]. Another study by Agrawal et al. analyzed the importance of using AR as a prognostic marker in 488 breast cancer patients who underwent radical mastectomies [115]. Data from this study suggest that the presence of AR increased the success of adjuvant therapy and prognosis in patients. Additionally, they found that 50.7% of breast cancer patients who were AR negative had lower 5-year survival rates, indicating poorer prognosis [115]. In yet another study carried out by Qu and colleagues, 109 breast cancer patients in Shanghai were retrospectively analyzed between 2003 and 2008 [116]. Of the 109 patients, 52 were diagnosed AR$^+$. Overall, there were 13 deaths and 15 recurrences but only two of the deaths and three of the recurrences were from the AR$^+$ group, which again indicates that AR could be a good marker for longer overall survival and lower risk of recurrence. Conversely, mammary tumors that do not express AR have been shown to respond poorly to hormone therapy [117]. The absence of AR has also been correlated with higher levels of Ki-67, a cell proliferation marker associated with cancer progression [118], though the molecular mechanism behind this finding has not been elucidated. Furthermore, androgen-activated AR appears to directly bind to the ERβ promoter and enhance transcription of ERβ in both ERα-positive and -negative breast cancer cells [119], suggesting that blocking AR function may subsequently decrease ERβ-dependent gene expression.

Although many studies appear to support the hypothesis that AR helps counteract the tumorigenic effects of ERα, others- such as one carried out by Paliouras and Diamandis- report a synergistic mechanism between the two receptors, resulting in an increase in breast cancer progression [120]. Specifically, the ER-dependent expression of a group of cancer biomarkers called kallikrein (KLKs) genes was shown to be enhanced significantly by the binding of androgen to AR. One noteworthy limitation of this study is that it focused on just one breast cancer cell line (BT474) and therefore may not be indicative of most breast cancer cases. Another study by Liao et al. showed that simultaneous treatment with androgen and estrogen stimulated mammary gland carcino-

mas in 100% of the Noble rats tested [121]. The researchers concluded that high levels of androgen and estrogen together may be an important risk factor for breast cancer and that direct binding of androgens to either AR or PR were involved in this carcinogenic process. However, although the researchers analyzed various isoforms of ER and PR, they only concentrated on the two AR isoforms, AR-A and AR-B, and used only male rats in their study. Additionally-and perhaps most importantly- the authors did not sufficiently rule out the possibility that the results were due to aromatization of androgen to estrogen. A more recent study carried out by Richer and colleagues did determine that treatment of $AR^+/ER\alpha^+$ breast cancer cell lines with the AR inhibitor enzalutamide- a drug currently used to treat metastatic prostate cancer- effectively inhibited cell proliferation both *in vitro* and in animal models, suggesting that AR activity may indeed promote cancer progression in the presence of ERα [122]. Yet again, isoforms of AR were neither analyzed nor even acknowledged in this study.

In contrast to the above studies, several clinical studies have indicated that administration of normal, physiological levels of androgen to women receiving estrogen therapy actually decreases breast cancer risk (reviewed in [59]), but the mechanism behind this- particularly in regards to AR signaling- is not clear. Agrawal and colleagues reported that only in the absence of estrogen will androgens directly bind to ERα and stimulate the proliferation of cancerous cells [115]. Finally, in an excellent, in-depth review of AR in breast cancer, K. M. McNamara and colleagues acknowledge that the role of AR in breast cancer risk and progression depends greatly on the specific disease subtype and the presence or absence of the other steroid receptors, such as ERα [123]. The authors further assert that in the presence of ERα, AR activation appears to counteract disease progression in most breast cancer subtypes. However, a further complication involves the ratio of AR to ER, which also appears to affect progression and efficacy of endocrine treatment. A clinical analysis of 192 $ER\alpha^+$ breast cancer patients carried out by Richer and colleagues revealed that a high AR to ERα ratio correlated positively with increased incidence of tamoxifen failure [122]. Despite all the confusing and contradictory findings, it does appear evident that AR could serve as an important prognostic indicator as long as AR isoforms, ERα status and estrogen levels are also taken into account.

ERα-negative breast cancer

ERβ-positive breast cancer

Although studies have found that both mRNA and protein levels of ERβ are significantly lower in breast tumors compared to normal breast tissue [124, 125], approximately 17% of primary breast cancers are ERα-negative/ERβ-positive [126, 127]. In addition, 47-60% of all ERα-negative tumors have been reported to be ERβ-positive [42, 126]. Most studies agree that the presence of ERβ in these tumors is correlated with a positive prognosis, as the absence of ERβ in ERα-negative patients ($ER\alpha^-/ER\beta^-$) is associated with early relapse [111, 128-130]. It has also been reported that as breast tumors become more malignant, ERβ expression decreases (reviewed in [131]). However, one recent study carried out by Chen et al. contradicts these findings [132]. The researchers found that expression of ERβ actually enhanced cancer progression by inducing the expression of IL-8, which is known to play a role in angiogenesis and metastasis [132, 133].

Predictably, most of the aforementioned studies failed to differentiate between the various isoforms of ERβ, which once again could be responsible for these conflicting results. The small handful of studies that actually have analyzed the expression of specific ERβ isoforms have found that ERβ1, -2/cx, -3, and -5 are expressed at varying degrees in breast tumors and breast tumor cell lines [42, 43, 134, 135]. ERβ2/cx (also designated simply as ERβ2) is the main isoform expressed in the hormone-sensitive breast tumor cell line T47D, whereas ERβ5 is the major isoform in the hormone-insensitive BT20 breast tumor cell line [134]. Increased expression of both ERβ2/cx and ERβ5 relative to the full-length isoform ERβ1 appears to correlate with increased breast cancer progression [135], and yet increased expression of ERβ5 has also been positively correlated with breast cancer survival [136, 137]. A recent clinical study of 95 patients with ERα-negative invasive breast carcinomas demonstrated a correlation between prognosis and the ERβ1 to ERβ2 ratio, associating higher lev-

els of ERβ2 with tumor relapse [138]. Yet despite their apparent statistical significance, the differences between ERβ1, ERβ2 and tumor relapse appeared fairly modest, which could be due to the relatively modest sample size or to the researchers' sole use of immunohistochemistry to detect the individual isoforms. Though the *clinical* significance of this particular study is somewhat questionable, an earlier prostate cancer study carried out by Leung et al. did reveal a correlation between the increased expression of ERβ2 and ERβ5 and enhanced metastasis and poor prognosis [139]. Clearly more studies are needed to better understand the prognostic value of the various ERβ isoforms in breast cancer.

Regardless, the overwhelming consensus that ERβ is a positive prognostic marker is due largely to the fact that a significant number of $ERβ^+$ patients respond well to endocrine therapy, such as tamoxifen. Several clinical studies have shown that patients who are ERα-negative or even triple negative ($ERα^-/PR^-/HER2^-$) but express high levels of ERβ respond well to tamoxifen [111, 128, 130], whereas low levels of ERβ correlate with resistance to tamoxifen treatment [140, 141]. In addition, other chemotherapeutic agents such as doxorubicin and cisplatin have been found to be more effective on breast cancer cell lines that express ERβ5 [142]. Aside from drug response, modification of ERβ has recently been shown to indicate good prognosis as well. Specifically, a clinical study led by Valerie Speirs found that breast cancer patients expressing ERβ phosphorylated at serine 105 had more favorable prognosis [143]. Though the mechanism behind this finding is unknown, it adds yet another level of complexity to an already complicated ERβ narrative.

PR-positive breast cancer

$ERα^-/PR^+$ breast cancers only account for about 2-7% of total breast cancer cases [81, 144-146], although there is controversy over whether or not such cancers actually exist. While many studies have supported the claim that $ERα^-/PR^+$ is a distinct class of breast tumors [81, 94, 144-152], skeptics contend that since PR is an ER target gene, ER expression is a prerequisite for the expression of PR [153-155]. They maintain that the PR positivity in ERα-negative tumors may simply reflect methodological errors in detecting PR and/or ERα, resulting in either a false-negative ERα result or a false-positive PR result [153, 154]. In fact, it is possible that tumors denoted as $ERα^-/PR^+$ may actually have fairly low levels of $ERα^-$ far below the sensitivity of the current assays [153]. Furthermore, results from Iwase et al. have indicated the possibility that the presence of ERα variants may exist in $ERα^-/PR^+$ tumors, specifically a variant with a deletion of exon 5 [156]. Though the presence of this variant in human breast tumors was confirmed in three other studies [157-159], this and other ERα variants may be difficult to detect via the traditional ERα assays used in immunohistochemistry, thus complicating the ERα status of certain mammary tumors. However, ERα aside, the PR promoter has been shown to be regulated by other transcription factors such as AP-1 and SP-1 [160-162]. In fact, an earlier study by Encarnación et al. indicated that while most of the $ERα^+$ tumors converted to an $ERα^-$ phenotype, the PR status remained unchanged, further supporting the existence of an $ERα^-/PR^+$ clinical subtype and the possibility that PR can be regulated by signaling pathways other than ERα [163].

Although the expression of PR in breast cancer cells has been linked to both positive endocrine response and clinical outcome [82], it is unclear how PR affects the outcome in ERα-negative breast cancers. Multiple reports have indicated that $ERα^-/PR^+$ breast tumors constitute a distinct clinicopathological group of cancers that results in outcomes worse than those that are $ERα^+/PR^+$ or $ERα^+/PR^-$, yet results in a better prognosis than double negative tumors ($ERα^-/PR^-$) [144, 152, 164-166]. It is questionable whether this clinical subtype of tumor is responsive to endocrine therapies like tamoxifen. As noted earlier, PR has been shown to be an indicator of responsiveness to endocrine therapy in the presence of ERα, and thus it is conceivable that $ERα^-/PR^+$ tumors may also respond to tamoxifen. On the other hand, since current endocrine therapies are believed to target the ERα signaling pathway, it seems counterintuitive to attempt such therapeutic options on $ERα^-/PR^+$ cancer patients, particularly since the presence of PR in $ERα^+$ tissues merely indicates a functional ERα, the presence of which is believed necessary for tamoxifen to have any

effect. Yet a recent study by Yang et al. found that patients with low-grade ERα$^-$/PR$^+$ tumors did experience an overall survival benefit from adjuvant hormone therapy using tamoxifen; and conversely, no benefit was observed in patients with high-grade ERα$^-$/PR$^+$ tumors [96]. Unfortunately, the scarcity of studies on this particular clinical subtype limits our ability to do a complete evaluation of the effectiveness of tamoxifen.

Theoretically, tumors that are ERα$^-$/PR$^+$ should be treatable with selective progesterone receptor modulators (SPRM), also referred to as anti-progestins. Anti-progestins such as mifepristone (RU-486) have been proposed as a new form of endocrine treatment or as an adjunct to the anti-estrogenic treatments for breast cancer. Multiple *in vitro* studies using cancer cell lines have indicated that low doses of anti-progestins can inhibit PR- and estrogen-mediated cell proliferation [167-169]. Contrary to these observations, other studies have demonstrated that at higher concentrations, mifepristone and other anti-progestins can actually stimulate proliferation of the ER$^+$/PR$^+$ breast cancer cells, T47D and MCF7 [170-172], indicating that the effect of anti-progestins on proliferation is dose-dependent and perhaps even dependent on the level of functional ERα. The few clinical studies that have tested the effectiveness of anti-progestins show limited to minimal efficacy in treating PR$^+$ breast cancer [173-175]. Yet there is evidence that anti-progestins can augment the effects of anti-estrogens like tamoxifen [176-178]. Clearly, further clinical studies are necessary to determine if PR is a viable therapeutic target in ERα$^-$ breast cancer.

AR-positive breast cancer

AR has been found in a significant number of ERα-negative tumors as well, with 22 to 49% of ERα-negative breast tumors expressing AR, depending on the clinical study [114, 118, 179, 180]. The presence of AR in ERα$^-$ breast cancer is often associated with lower tumor grade, smaller tumor size, and significant increase in survival rates. Results inconsistent with these clinical data have been reported by some researchers who have found that androgen-enhanced expression of AR in the ER$^-$/PR$^-$/HER2$^+$ breast cancer cell line MDA-MB-453 increases cell proliferation [181, 182], which can be inhibited by the AR antagonist bicalutamide [182]. Additionally, Richer et al. found that the AR inhibitor enzalutamide inhibited cell proliferation in ERα$^-$/AR$^+$ breast cancer cell lines [122]. Given that isoforms of AR were not taken into consideration in any of these studies, such contradictory findings between clinical and *in vitro* studies are not that surprising.

A fraction of triple-negative breast cancer cases (13-35%) appear to express AR [180, 183, 184]. Although some *in vitro* studies on triple-negative/AR$^+$ cell lines have demonstrated an androgen-induced increase in cell proliferation [182, 185], most clinical studies find that the presence of AR in triple-negative tumors is correlated with a lower recurrence rate, fewer positive lymph node and distant metastases, lower histological grade, and higher overall survival rate compared to triple-negative tumors that are AR-negative [180, 183, 184, 186]. These data support the notion that AR can be used as a positive prognostic factor, not only in ERα-negative but also triple-negative breast cancers.

In addition to its prospective use as a prognostic biomarker for breast cancer, additional studies have indicated that AR may also serve as a potential therapeutic target for those breast cancer patients who traditionally have had very few treatment options, such as those whose tumors are ER$^-$/PR$^-$/AR$^+$. Hardin and colleagues tested the effectiveness of the androgen dehydroepiandrosterone sulfate (DHEAS) on ER$^-$/PR$^-$/AR$^+$ breast cancer cell lines and found that DHEAS stimulation of AR hampered cell growth by enhancing apoptosis [187]. Conflicting studies by Ni et al. and Arce-Salinas et al. showed that inhibiting AR with the antagonist bicalutamide led to growth inhibition and enhanced cell death [182, 188]. Interestingly, AR expression seems to be correlated with the overexpression of the oncogene HER2 via a complicated signaling cascade that involves upregulation of HER2 by AR and transcription factor β-catenin [182]. In addition, expression of the constitutively active AR variant AR-V7 in both ERα$^-$ breast cancer cell lines (e.g. MDA-MB-453) and ERα$^-$ primary tissues is actually enhanced by the AR antagonist enzalutamide, which in turn increases cell growth and, as with more advanced stages of prostate cancer, could result in ADT resistance [71]. Clearly the

use of AR as a therapeutic target is wrought with complications, as both agonists and antagonists of AR have succeeded in either suppressing or promoting tumor progression, and success appears to be highly dependent on AR isoform expression and ERα levels.

Discussion and conclusion

Triple negative breast cancer (i.e. $ER^-/PR^-/HER2^-$) is virtually impossible to treat with the established endocrine-based therapies, as such therapies were originally intended for cancers that are ERα-positive. However, our review illustrates how confining the triple negative designation can be, potentially preventing clinicians from considering other factors that play a role in breast cancer progression. Data obtained from basic and translational research studies indicate that there are indeed many other proteins involved in breast cancer development and progression; but despite these findings, most clinical pathologists continue to follow the standard practice of categorizing breast tumors using only the three established markers- ERα, PR, and HER2. This apparent disconnect between the bench and the clinic is alarming when one considers that 10-15% of breast cancers are diagnosed as triple negative [2, 3] and that even patients diagnosed as ERα-positive do not all respond equally well to anti-estrogen treatment, which further demonstrates the complexity of breast cancer and emphasizes the need for more therapeutic targets.

The goal of this review was to determine if other steroid hormone receptors- most notably ERβ and AR- should also be routinely analyzed and used as additional targets for breast cancer treatment and prognosis. Although a significant number of studies exist describing the presence of ERβ, AR, and PR in breast cancer cells or tissues, obtaining truly accurate expression levels from the literature proved difficult as all three of these receptors exist in at least two isoform states. Most studies did not account for this variability. Therefore, it is probable that many isoforms went undetected, which in turn could have led to an underestimate of a receptor's actual expression level. Yet, quantitative inaccuracies aside, we still found very strong evidence that each of these receptors influences tumor progression, either negatively or positively, depending upon which isoforms are present and the level of ERα expression. For example, $ER\alpha^+/PR^+$ breast tumors are generally found to be more responsive to endocrine treatment than $ER\alpha^+/PR^-$ tumors [85, 91, 93-96], and yet a higher expression level of the PR-A isoform compared to PR-B has been associated with anti-estrogen resistance and subsequently poorer prognosis [102]. The isoforms of ERβ are capable of forming heterodimers with ERα, inhibiting ERα activity and consequently resulting in tumor suppression. However, other studies have shown a positive correlation between tumor progression and increased levels of ERβ2/cx and ERβ5 isoforms relative to ERβ1 [135, 138, 139]. The expression of certain isoforms, such as ERβ5, has also been correlated with positive drug response [142]. Unfortunately, due to the relative scarcity of studies analyzing AR in breast tumors, few clinically significant associations were found between a specific AR isoform and breast tumor progression. However, certain splice variants such as the ligand-independent isoform AR-V7 have been shown to impact tumor development and progression in prostate cancer [67, 68], and a recent study by the Tilley lab strongly suggest that it may have the same impact on breast cancer progression [71]. Additionally, the available data do show that the presence of AR is correlated with good prognosis for both ERα-positive and ERα-negative breast cancer, indicating that anti-androgen therapy might be a viable option in certain breast cancers as it is in prostate cancer.

We acknowledge that more basic research is necessary to elucidate the molecular mechanisms behind the steroid hormone receptors' effects on breast cancer development and progression. In particular, more studies are needed to better understand how ERβ, PR, and AR influence cell proliferation, apoptosis, and metastasis. However, in the interest of saving lives, we feel that the existing data justify collecting more clinical data on a more massive scale. We contend that all breast tumor biopsies should be analyzed not only for ERα, HER2, and overall PR expression but also for each isoform of ERα, PR, ERβ, and AR. Obviously this is an ambitious undertaking that would require developing new techniques and improving current detection methods for each isoform. In

addition, subsequent large-scale analysis of the data will be necessary to determine if consistent patterns emerge linking the various expression levels of these markers and their isoforms with particular grades and stages of breast cancer.

More and more clinical labs are trending towards molecular diagnostic procedures such as next generation sequencing (NGS), RNA sequencing, and high-throughput qRT-PCR, all of which will allow scientists to analyze more genetic markers in a relatively short period of time. Additionally, the isolation and analysis of circulating cell-free DNA (ccfDNA) could allow for initial prognosis using very little sample. In fact, several clinical and translational studies have already shown how NGS and ccfDNA analysis can aid in breast cancer prognosis and tumor classification [189-193]. However, techniques such as NGS are best suited for patients who may have a genetic predisposition for a particular cancer (e.g. *BRCA1* and *BRCA2* in some familial breast cancers); and though RNA sequencing and qRT-PCR do give information regarding transcriptional expression, it is at the protein level that phenotypes are often determined.

Therefore, although the utilization of molecular techniques will enable clinicians to obtain more information about a patient's tumor, these techniques should always be used in conjunction with protein expression analyses- particularly since not all of the steroid receptor isoforms are due to splicing variations but instead are generated post-translationally. In addition, post-translational modifications such as phosphorylation and glycosylation cannot be detected at the DNA or RNA level but only by analyzing the protein expression profiles of tumors. Finally, the heterogeneity of the cell population within an individual tumor must be taken into account when analyzing all the data this new technology will generate. To date, the most common procedures for studying protein expression are immunohistochemistry and western blotting. Though immunohistochemistry provides an important visual of protein expression variations between tissues and even between cells within the tumor, and western blotting is a more stringent and exact method for detecting isoforms of varying molecular weights, both depend on antibody-binding, which is an indirect method of protein detection. Recent advances in multiplexed protein analysis include mass spectrometry immunohistochemistry (MSIHC), in which metal tags of varying masses are used to label antibodies, thus allowing for the simultaneous detection of up to 100 different protein targets [194]. Such a technique has been used successfully in analyzing various markers such as ERα, PR, and HER2 in breast tumor samples [195]. Again, however, such a technique still relies on antibody recognition and binding. Another technique that involves rapid protein isolation and sequencing on a large-scale would need to be developed in future to directly identify the various steroid receptor isoforms expressed in a given tumor. Ultimately, we envision a multipronged approach to breast cancer analysis that will allow detection of a variety of markers, including the many isoforms of ERα, ERβ, PR, and AR, coupled with the pathology of the tumor. The overall goal would be to provide each patient with a more personalized and accurate prognosis and more effective treatment options, and maybe even render obsolete the term "triple negative breast cancer".

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None.

Abbreviations

ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor; HER2, human epidermal growth factor receptor type 2; GR, glucocorticoid receptor; GPR-30, G-protein coupled receptor-30; DBD, DNA binding domain; LBD, ligand binding domain; HRE, hormone response element; NTD, N-terminal domain; SERMs, selective ER modulators; SERDs, selective ER down-regulators.

Address correspondence to: Dr. Mary B Sevigny, Department of Natural Sciences and Mathematics, Dominican University of California, 50 Acacia Avenue, San Rafael, CA 94901, USA. Tel: 415-458-

3743; Fax: 415-482-1972; E-mail: mary.sevigny@dominican.edu

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