REVIEW

Hormone therapy and breast cancer: emerging steroid receptor mechanisms

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Abstract

Although hormone therapy is widely used by millions of women to relieve symptoms of menopause, it has been associated with several side effects such as coronary heart disease, stroke and increased invasive breast cancer risk. These side effects have caused many women to seek alternatives to conventional hormone therapy, including the controversial custom-compounded bioidentical hormone therapy suggested to not increase breast cancer risk. Historically, estrogens and the estrogen receptor were considered the principal factors promoting breast cancer development and progression; however, a role for other members of the steroid receptor family in breast cancer pathogenesis is now evident, with emerging studies revealing an interplay between some steroid receptors. In this review, we discuss examples of hormone therapy used for the relief of menopausal symptoms, highlighting the distinction between conventional hormone therapy and custom-compounded bioidentical hormone therapy. Moreover, we highlight the fact that not all hormones have been evaluated for an association with increased breast cancer risk. We also summarize the current knowledge regarding the role of steroid receptors in mediating the carcinogenic effects of hormones used in menopausal hormone therapy, with special emphasis on the influence of the interplay or crosstalk between steroid receptors. Unraveling the intertwined nature of steroid hormone receptor signaling pathways in breast cancer biology is of utmost importance, considering that breast cancer is the most prevalent cancer among women worldwide. Moreover, understanding these mechanisms may reveal novel prevention or treatment options and lead to the development of new hormone therapies that do not cause increased breast cancer risk.

Key Words

- ▶ breast cancer
- ▶ custom-compounded bioidentical hormones
- ▶ estrogens
- ▶ menopausal hormone therapy
- ▶ progestins
- ▶ steroid receptor crosstalk

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Introduction

Menopause is characterized by the natural, age-related decrease in endogenous estrogen production in women, often leading to a variety of symptoms such as hot flashes, mood swings and night sweats (Johnson 1998, Greendale *et al.* 1999, Marsden 2003). Conventional United States Food and Drug Administration (FDA)-approved hormone therapy (HT) has been used for decades to alleviate these

symptoms and is typically administered as estrogen alone to hysterectomized women or an estrogen-progestin combination to women with a uterus (Johnson 1998, Greendale *et al.* 1999, Marsden 2003, Cuzick 2008). While the estrogen component alleviates the symptoms of menopause by compensating for reduced endogenous estrogen production, the progestin constituent

counteracts the proliferative effects of estrogens on the uterine epithelium (Johnson 1998). Despite the fact that HT is effective in relieving menopausal symptoms, some HT regimens have been associated with several severe side effects including coronary heart disease, stroke and increased invasive breast cancer risk (Writing Group for the Women's Health Initiative Investigators 2002, Million Women Study Collaborators 2003, Krieger *et al.* 2005, Vickers *et al.* 2007, Cuzick 2008, Marjoribanks *et al.* 2017). Considering that breast cancer is the most prevalent cancer among women in developed countries (Ferlay *et al.* 2015, Torre *et al.* 2015, Siegel *et al.* 2016), the association between HT and increased breast cancer risk is of significant concern.

The increased breast cancer risk linked to conventional HT has caused many women and medical professionals to seek various safer HT options, including the use of 'natural' alternatives such as custom-compounded bioidentical HT (bHT) (Curcio *et al.* 2006, Gass *et al.* 2015). Notably, some bioidentical hormones such as bioidentical estradiol (bE₂) or bioidentical progesterone (bP₄) are available in FDA-approved standard-dose prescription medications (Sood *et al.* 2011, 2014). However, unlike these FDA-approved HT products containing bioidentical hormones, custom-compounded bHT formulations are administered in personalized doses and are typically composed of a mixture of up to six hormones (Pinkerton 2012, 2014). Uncertainty remains regarding the efficacy and safety of custom-compounded bHT, especially pertaining to these multiple-hormone combination therapies (Pinkerton 2012, 2014). Although proponents of bHT claim that there is in fact evidence to support the efficacy and safety of custom-compounded bHT in terms of breast cancer risk (Boothby *et al.* 2004, Bosarge & Freeman 2009), these claims are unsubstantiated due to the lack of large-scale, double-blinded clinical trials investigating custom-compounded multiple-hormone bHT regimens at various doses.

Estrogen only and estrogen-progestin combination conventional HT have both been implicated in increased breast cancer risk; however, evidence suggests that estrogen-progestin combination therapies are associated with a greater increased risk than estrogen-alone therapies (Writing Group for the Women's Health Initiative Investigators 2002, Million Women Study Collaborators 2003, Fournier *et al.* 2005, 2008a, Marjoribanks *et al.* 2017). Estrogens predominantly mediate their effects by binding to the estrogen receptor (ER), while progestins are synthetic progestogens (progesterone receptor (PR) ligands) that were designed to mimic the activity of the

natural progestogen, progesterone (P₄), by binding to the PR. However, it is known that some progestins can bind to the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), androgen receptor (AR) (Koubovec *et al.* 2005, Africander *et al.* 2013, 2014, Louw-du Toit *et al.* 2017, reviewed in Schindler *et al.* 2003, Africander *et al.* 2011, Hapgood *et al.* 2014) and/or the ER (Larrea *et al.* 2001, Escande *et al.* 2006, Louw-du Toit *et al.* 2017, reviewed in Africander *et al.* 2011). Whether progestins bind to the ER is contradictory. Some studies suggest that medroxyprogesterone acetate (MPA) and norethisterone (NET) can bind to the ER and elicit estrogenic activity, others suggest that they do not (Larrea *et al.* 2001, Pasapera *et al.* 2002, Escande *et al.* 2006, Lemus *et al.* 2009, Louw-du Toit *et al.* 2017). Interestingly, we recently showed that NET-acetate (NET-A), levonorgestrel (LNG) and gestodene (GES) can bind to ER-A, but not ER-B, while P₄, MPA, nesterone (NES), nomegestrol acetate (NoMAC) and drospirenone (DRSP) do not bind to either ER subtype (Louw-du Toit *et al.* 2017). Furthermore, some studies suggest that it is the progestin metabolites rather than the parent progestin itself that bind to the ER. Although a number of studies have investigated effects of progestins via steroid receptors other than the PR (Stanczyk *et al.* 2013, Hapgood *et al.* 2014), these studies seldom directly compare different progestins in parallel and often use cell lines that endogenously express many steroid receptors to which progestins can bind, which may result in inaccurate results in terms of binding affinities, as well as potencies and efficacies for gene expression. It is thus essential that the pharmacological properties of the progestins for each individual steroid receptor are determined in parallel in a model system expressing only the receptor of interest, as has been done for MPA and NET via the human GR, MR and AR (Koubovec *et al.* 2005, Africander *et al.* 2013, 2014).

The role of the ER, which exists as two subtypes transcribed from two distinct genes (Kuiper *et al.* 1996, Kuiper & Gustafsson 1997, Enmark & Gustafsson 1999), ER-A and ER-B (Ascenzi *et al.* 2006), has been extensively studied in breast cancer cell biology. Traditionally, estrogens and ER-A were thought to be the main etiological factors contributing to breast cancer pathogenesis, while the PR was considered only as an indicator of a functional ER in breast cancer tumors, implying that the cancer should be sensitive to endocrine-targeting therapies (Horwitz & McGuire 1975, Carroll *et al.* 2016, Lim *et al.* 2016). However, recent studies have highlighted novel roles for the PR in breast cancer cell biology (Daniel *et al.* 2011, Giulianelli *et al.* 2012, Mohammed *et al.* 2015, Singhal *et al.* 2016). Two main PR isoforms have been

identified, PR-A and PR-B (Kastner *et al.* 1990), which have been shown to elicit differential effects (Conneely *et al.* 2000, 2003, Jacobsen *et al.* 2002, Richer *et al.* 2002, Mulac-Jericevic *et al.* 2003, Faivre & Lange 2007, Lanari *et al.* 2012, Briskin 2013). A recent study has shown that unliganded PR-B enhances the effects of ER agonists on ER-A-mediated breast cancer cell proliferation and gene expression by forming a complex with ER-A (Daniel *et al.* 2015). Moreover, it has been shown that when PR-B is activated by P₄ or the synthetic PR agonist, promegestone (R5020), it is recruited to the ER-A complex and redirects the complex to different target genes such that the new gene expression profile is associated with a good clinical outcome (Mohammed *et al.* 2015). ER-A has also been shown to be required for PR-mediated increased cell proliferation and the expression of PR-regulated genes induced by the progestin MPA (Giulianelli *et al.* 2012). Interestingly, the presence and critical roles of other steroid receptors in breast cancer cell biology have been highlighted in recent studies. For example, the AR is expressed in 90% of breast cancer tumors (reviewed in Hickey *et al.* 2012), and its expression is associated with either a good or poor prognosis depending on the absence or presence of the ER (McNamara *et al.* 2014). Similarly, GR expression has been associated with a good outcome in ER-A-positive cancers, but is associated with a poor outcome in ER-A-negative cancers (Pan *et al.* 2011, Leehy *et al.* 2016).

Steroid receptor signaling pathways have often been studied in isolation; however, it is becoming increasingly clear that these pathways are intertwined. The ability of some steroid hormones, such as progestins, to activate multiple steroid receptors, coupled with the complexity of steroid receptor crosstalk, highlights the intricacies of the mechanisms through which hormones used in HT may increase breast cancer risk and promote breast cancer pathogenesis. In order to elucidate the involvement of steroid receptor crosstalk in the mechanism behind HT and increased breast cancer risk, additional comparative studies of hormones used in HT are needed at the cellular level. The aim of this review is to highlight differences between conventional HT and custom-compounded bHT and to discuss known mechanisms behind conventional HT-induced increased breast cancer risk with an emphasis on the role of steroid receptor crosstalk.

Menopause and hormone therapy

Menopausal transition typically occurs in women between the ages of 40 and 60 years and is characterized

by the natural age-related loss of ovarian follicular function leading to decreasing endogenous estrogen, P₄ and testosterone levels (Table 1) (Johnson 1998, Greendale *et al.* 1999). There are three main endogenous human estrogens, namely E₂, estriol (E₃) and estrone (E₁), the latter being the most abundant circulating estrogen in postmenopausal women (Table 1). However, E₁ is not present in sufficient levels to prevent symptoms of menopause, such as amenorrhea, hot flushes, night sweats, vaginal atrophy and mood fluctuations (Johnson 1998, Greendale *et al.* 1999, Bosarge & Freeman 2009). HT was first administered in the 1930s not only to alleviate these menopausal symptoms (Cuzick 2008), but also to prevent the medical implications of decreased endogenous estrogen levels including osteoporosis, Alzheimer's disease, arthritis, coronary heart disease and cataract formation (Johnson 1998, Greendale *et al.* 1999). Today, a large variety of HT regimens are commercially available and can be broadly divided into conventional HT and custom-compounded bHT.

Conventional hormone therapy

The term conventional HT can be interpreted in many ways due to the fast-evolving nature of drug discovery, however, for the purposes of this review, conventional HT will refer to all FDA-approved HT regimens available in the United States of America (USA). Conventional HT regimens are marketed under different brand names and contain either natural, synthetic or bioidentical hormones, which are available in standardized doses and various routes of administration. Depending on the HT, it can be administered orally, subcutaneously, transdermally, intravaginally or by intramuscular injection (Johnson 1998, Marsden 2003).

HT preparations are composed of various hormones that can be ascribed to a specific class. Class A steroids include hormones that are naturally occurring and administered without chemical modification. For example, conjugated equine estrogens (CEEs) containing estrogens such as equilin are extracted from pregnant mare's urine (Bhavnani & Stanczyk 2012, 2014). Although these steroids are naturally occurring, they are not endogenous to the human body (Bhavnani & Stanczyk 2012, 2014). Class B steroids are often referred to as natural or bioidentical; however, these hormones are chemically synthesized from a natural steroidal precursor using numerous chemical reactions (Hudson 1996) and are thus semi-synthetic (Taylor 2005, Cirigliano 2007, Chervenak 2009, Bhavnani & Stanczyk 2012).

Table 1 Serum estradiol (E₂), estriol (E₃), estrone (E₁), progesterone (P₄) and testosterone levels in pre- and post-menopausal women.^a

	E ₂	E ₃	E ₁	P ₄	Testosterone
Pre-menopausal (pg/mL)	7–400	8–2408	12–144	566–15,700	217–2200
Postmenopausal (pg/mL)	1–20	<10	7–44	39–700	461–1050

^aMayo Medical Laboratories, Dunn *et al.* (1981), Cummings *et al.* (1998), Markopoulos *et al.* (1988), Pujol *et al.* (1998), Burry *et al.* (1999), Wright *et al.* (1999), Elmlinger *et al.* (2002), Lewis *et al.* (2002), Chatterton *et al.* (2003), Labrie *et al.* (2003), Wren *et al.* (2003), McTiernan *et al.* (2004), Vashisht *et al.* (2005), Cirigliano (2007), Rothman *et al.* (2011), Falk *et al.* (2012), Du *et al.* (2013), Depypere *et al.* (2015).

Class C steroids differ from class B steroids in that they are synthesized from non-steroidal, rather than steroidal, precursors in a process called total synthesis (Bhavnani & Stanczyk 2012). The shortcoming of class B and C steroids is that various isomers are produced during the synthesis process, with only one of these isomers structurally identical to the endogenous human hormone (Bhavnani & Stanczyk 2012, Gass *et al.* 2015). For example, during total E₁ synthesis, eight racemates (differentiated by the left- and right-handed enantiomers of a chiral molecule) are produced, resulting in 16 isomers of which only one is structurally and biochemically identical to endogenous human E₁ (Bhavnani & Stanczyk 2012). The remaining isomers have different structures and varying degrees of estrogenicity, while some are even completely inactive (Bhavnani & Stanczyk 2012). Lastly, class D steroids are manmade steroidal compounds synthesized either from the same steroidal plant precursors as class B hormones by semi-synthesis or from non-steroidal starting material by total synthesis (Bhavnani & Stanczyk 2012). Examples of class D steroids include estrogens such as estropipate, esterified estrogens and ethinylestradiol (EE), as well as progestins such as MPA, NET-A, LNG and norgestimate (NGM).

Premarin is an example of a HT containing natural CEEs and has been effective in relieving menopausal symptoms from as early as 1942 (Cuzick 2008). Various other estrogens have subsequently become available for use in conventional HT and include synthetic, rather than natural CEEs, bE₂, as well as the less commonly used esterified estrogens, estropipate and synthetic E₂ derivatives including EE, E₂ valerate, E₂ cypionate and E₂ acetate (Table 2). Although estrogen-only HT effectively relieves menopausal symptoms, studies in the 1960s reported increased incidence of endometrial cancer in Premarin users (Smith *et al.* 1975, Weiss *et al.* 1976, Cuzick 2008). This necessitated the addition of a progestin to CEE regimens for women with a uterus, to prevent estrogen-induced endometrial hyperplasia (Cuzick 2008). Progestins are used to mimic the activity of P₄, as they have a longer half-life and a higher bioavailability (Bhavnani

& Stanczyk 2012, Stanczyk *et al.* 2013). Products such as Provera (MPA only) and Prempro (CEE-MPA) (Table 3) thus became commercially available as early as 1965 (Nachtigall *et al.* 1979). Although Provera is produced as a progestin-only HT, it is administered in combination with an estrogen-only HT (Stefanick 2005). Various generations of progestins have subsequently been developed, derived from P₄, T or the MR antagonist spironolactone, where each new generation is designed to have a greater affinity for the PR and elicit biological effects more similar to P₄ than progestins from the earlier generations (Sitruk-Ware 2004, Sitruk-Ware & Nath 2010, Africander *et al.* 2011). Note that P₄ derivatives can be either 17-hydroxy-P₄ derivatives or 19-Nor-P₄ derivatives (Fig. 1).

Progestins currently used in FDA-approved HT include the first-generation progestins MPA and NET-A, second-generation progestin LNG, third-generation progestin NGM and the fourth-generation progestin, DRSP (Fig. 1). Interestingly, not all progestins are used clinically in the USA. For example, although NoMAC is used in HT in Europe (Fournier *et al.* 2005), it is not used in the USA. However, NoMAC and other progestins such as NES and GES (Fig. 1) are currently being investigated in clinical trials for use in contraception in the USA (Sitruk-Ware & Nath 2010, Bahamondes & Bahamondes 2014). The continual evolution of HT is thought to be aimed at designing estrogens and progestogens that effectively manage menopausal symptoms without eliciting unwanted side effects. More recent advances in HT evolution saw the introduction of bioidentical hormones. FDA-approved bE₂-only products (Files *et al.* 2011, The North American Menopause Society (NAMS) 2012 Hormone Therapy Position Statement Advisory Panel 2012) are available in standardized doses in products such as Alora, Vivelle-Dot, Divigel, Elestrin, Estrogel and Estrace (Table 2), or in combination with progestins such as NET-A (Activella, Mimvey, Combipatch), DRSP (Angeliq), NGM (Prefest) or LNG (Climara Pro) (Table 3). Interestingly, although FDA-approved bP₄ is available as Prometrium in the form of a cream or as a pill that is administered together with an estrogen-only HT, there are no standardized

Table 2 FDA-approved estrogen-only HT products.^a

Composition	Products	Route of administration	Administered doses (min–max) (mg)	Serum E ₂ (pg/mL)	Serum E ₁ (pg/mL)
Natural CEE	Premarin	Pill	0.3–1.25	NA	87–4500
		Vaginal cream	0.625*	NA	42–600
Synthetic CEE	Cenestin, Enjuvia	Pill	0.3–1.25	NA	20–85
bE ₂	Alora, Climara, Esclim, Estraderm, Estradot, Menostar, Minivelle, Vivelle-Dot	Patch	0.025–0.1	6–174	9–65
	Divigel, Elestrin, Estrogel	Topical gel	0.025–2.0*	9–67	33–66
	Estrace, Gynodiol	Pill	0.5–2.0	203–355	ND
	Estrace	Vaginal cream	0.1*	ND	ND
	Estrasorb	Topical cream	2.5*	59–70	ND
	Estring	Vaginal insert	2.0	8–63	44–66
	Evamist	Topical spray	1.53 [#]	11–57	NA
	Vagifem	Vaginal tablet	0.01–0.025	6–21	17–28
E ₂ derivatives					
E ₂ valerate	Delestrogen	IM injection	10–40	ND	ND
E ₂ cypionate	Depo-Estradiol	IM injection	1.0–5.0	ND	ND
E ₂ acetate	Femring	Vaginal ring	0.05–0.10	41–76	36–46
	Femtrace	Pill	0.45–1.8	57–177	155–680
Esterified estrogen	Menest	Pill	0.3–2.5	ND	ND
	Estragyn	Vaginal cream	1.0*	ND	ND
Estropipate	Ogen	Pill	0.75–3.0	ND	ND
		Vaginal cream	1.5*	ND	ND

*mg hormone per g cream; [#]mg hormone per single HT spray; ^aBowen *et al.* (1998); Premarin tablets package insert, Wyeth Pharmaceuticals Inc.; Premarin vaginal cream package insert, Wyeth Pharmaceuticals Inc.; Cenestin package insert, Teva Pharmaceuticals USA, Inc.; Enjuvia package insert, Barr Pharmaceuticals, Inc.; Alora package insert, Watson Pharmaceuticals, Inc.; Climara package insert, Bayer HealthCare Pharmaceuticals Inc.; Esclim package insert, Serono Laboratories, Inc.; Estraderm, Novartis Pharmaceuticals Corporation; Estradot package insert, Novartis Pharmaceuticals Australia Pty Ltd; Menostar package insert, Bayer HealthCare Pharmaceuticals Inc.; Minivelle package insert, Novartis Pharmaceuticals Corporation; Vivelle-dot package insert, Novartis Pharmaceuticals Corporation; Divigel package insert, Upsher-Smith Laboratories Inc.; Elestrin package insert, Bradley Pharmaceuticals Inc.; Estrogel package insert, ASCEND Therapeutics Inc.; Estrace tablets package insert, Warner Chilcott Inc.; Gynodiol package insert, Barr Pharmaceuticals Inc.; Estrace vaginal cream package insert, Allergan USA, Inc.; Estrasorb package insert, Novavax, Inc.; Estring package insert, Pfizer Inc.; Evamist package insert, Ther-Rx Corporation; Vagifem package insert, Novo Nordisk Inc.; Delestrogen package insert, Monarch Pharmaceuticals Inc.; Depo-Estradiol package insert, Pfizer Inc.; Femring package insert, Warner Chilcott Inc.; Femtrace package insert, Warner Chilcott Inc.; Menest package insert, Monarch Pharmaceuticals Inc.; Estragyn package insert, Searchlight Pharma Inc.; Ogen tablet package insert, Pfizer Inc.; Ogen vaginal cream package insert, Pfizer Inc.).

bE₂, bioidentical estradiol; CEE, conjugated equine estrogens; E₁, estrone; E₂, estradiol; IM, intramuscular injection; NA, not applicable as these HT products do not contain the relevant hormone; ND, serum concentrations have not been reported.

FDA-approved bE₂-bP₄ combination formulations available. Notably, bP₄ is administered in a micronized form, referring to the fact that the particle size has been decreased to generate finer powders that are more readily absorbed, and thus, have an increased bioavailability to compensate for the short half-life of the natural hormone (Chaumeil 1998, Boothby *et al.* 2004). However, both oral and transdermal micronized bP₄ formulations are dissolved in peanut oil and consequently cannot be used by women with nut allergies (Sood *et al.* 2013, Xie *et al.* 2014, Mirkin *et al.* 2015, Stuenkel *et al.* 2015, Santoro *et al.* 2016). Another alternative HT regimen involves the use of selective estrogen receptor modulators (SERMs). SERMs elicit tissue-selective estrogenic activity by acting as ER agonists in bone tissue, increasing bone mineral density and bone strength, but as ER antagonists in the breast and

endometrium to prevent breast and endometrial cancer (Komm & Chines 2012). FDA approval was recently granted to Duavee, a CEE-SERM (bazedoxifene) combination to be used for the relief of menopausal symptoms as well as to prevent postmenopausal osteoporosis (Kharode *et al.* 2008, Pinkerton *et al.* 2009, Bachmann *et al.* 2010, Kagan *et al.* 2010, Komm & Chines 2012).

Estrogen only and progestogen-containing FDA-approved HT products are outlined in Tables 2 and 3, respectively. These tables indicate the routes of administration and dose range of the various HTs and, where available, the resulting serum concentrations of E₂, E₁, EE, the progestogens or bazedoxifene. Although these conventional HT regimens are still widely used and have proved efficient at relieving menopausal symptoms (The North American Menopause Society (NAMS)

Table 3 FDA-approved progestogen or SERM-containing HT products.^a

Composition	Products	Route of administration	Administered doses (mg)	Serum E ₂ (pg/mL)	Serum E ₁ (pg/mL)	Serum progestogen (pg/mL)
Estrogen Progestin Progestrone only	Prometrium	Pill (100–300 mg)		NA	NA	310–60,600
Micronized bP ₄ [#]	Provera	Pill (2.5–10 mg)		NA	NA	710–1010
Estrogen–progestin combinations						
CEE+MPA	Prempro	Pill	0.3–0.625	NA	79–175	1200–4800
EE+NET-A	FemHRT	Pill	0.0025–0.01	34–38 (EE)	ND	4000–10,700
E ₂ +NET-A	Activella, Mimvey	Pill	0.5–1.0	26–28	196–200	2375–5250
	Combipatch	Patch	0.05	27–71	49–78	386–1060
E ₂ +DRSP	Angeliq	Pill	0.5–1.0	30–64	166–362	2000–85,000
E ₂ +NGM	Prefest	Pill	1.0	39–50	285–325	515–643
E ₂ +LNG	Climara Pro	Patch	0.045	27–54	33–82	110–194
Estrogen–SERM combinations						
CEE+bazedoxifene	Duavee	Pill	0.45+20	NA	≤2600	≤6900 (SERM)

^aProgestogen-only products that are generally prescribed in combination with an estrogen-only product; [#]Stanczyk (2002, 2003); Prometrium package insert, Solvay Pharmaceuticals, Inc.; Provera package insert, Pfizer Inc.; Prempro package insert, Wyeth Pharmaceuticals Inc.; FemHRT package insert, Warner Chilcott Inc.; Activella package insert, Novo Nordisk Inc.; Mimvey package insert, Teva Pharmaceuticals Inc.; Combipatch package insert, Novartis Pharmaceuticals Corporation; Angeliq package insert, Bayer HealthCare Pharmaceuticals Inc.; Prefest package insert, Teva Pharmaceuticals Inc.; Climara Pro package insert, Berlex; Duavee package insert, Pfizer Inc. bP₄, bioidentical progesterone; CEE, conjugated equine estrogens; DRSP, drospirenone; E₁, estrone; E₂, estradiol; EE, ethinylestradiol; LNG, levonorgestrel; MPA, medroxyprogesterone acetate; NA, not applicable as these HT products do not contain the relevant hormone; ND, serum concentrations have not been reported; NET-A, norethisterone acetate; NGM, norgestimate; SERM, selective estrogen receptor modulator.

2012 Hormone Therapy Position Statement Advisory Panel 2017), the reported side effects associated with conventional HT have caused so much alarm that many women have sought alternate menopausal relief in the form of custom-compounded bHT (Bhavnani & Stanczyk 2012).

Custom-compounded bioidentical hormone therapy

Custom-compounded bHT refers to the personalized bHT regimens containing class B steroids that are prepared by compounding pharmacies and can include any number of bioidentical hormones including bioidentical estrone (bE₁), estriol (bE₃), testosterone (bT), dehydroepiandrosterone (bDHEA), bE₂ and/or bP₄ (Chervenak 2009, Bhavnani & Stanczyk 2012). Unlike FDA-approved HT, which is available in standardized doses, a customized dose of bHT is prescribed based on a saliva test that estimates serum hormone levels (Boothby et al. 2004, Santoro et al. 2016). However, this method contradicts a global consensus that the lowest possible dose of HT that effectively relieves menopausal symptoms should be prescribed (Bosarge & Freeman 2009, The North American Menopause Society (NAMS) 2012 Hormone Therapy Position Statement Advisory Panel 2012, de Villiers et al. 2013). Moreover, numerous studies have shown a poor correlation between hormone levels found in saliva and serum, due to saliva hormone levels fluctuating based on time of day, diet and other variables (Boothby et al. 2004, Cirigliano 2007, Fugh-berman & Bythrow 2007, Bosarge & Freeman 2009, Chervenak 2009, Santoro et al. 2016).

The safety and efficacy of custom-compounded bHT is controversial and proponents of bioidentical hormones claim that these hormones are natural and identical in structure to endogenous human hormones; hence, they are safer than conventional HT products (Chervenak 2009). This is despite the fact that the proposed ‘natural’ hormones used in custom-compounded bHT are in fact semi-synthetic and synthesized in a similar manner to the bioidentical hormones used in FDA-approved HT (Cirigliano 2007, Fugh-berman & Bythrow 2007, Bosarge & Freeman 2009, Chervenak 2009). However, because of this ‘natural’ classification, custom-compounding pharmacies may legally dispense products containing bioidentical hormones without obtaining FDA approval for each product (Fugh-berman & Bythrow 2007, Chervenak 2009). This means that personalized hormone preparations are dispensed without the rigorous quality control checks that FDA-approved drugs are subjected to. Furthermore, unlike

Figure 1

Structures of various first (1st)-, second (2nd)-, third (3rd)-, and fourth (4th)-generation progestins. Progestins structurally related to (A) progesterone (P₄) include the first-generation, 17-hydroxy-P₄ derivative, medroxyprogesterone acetate (MPA) and the fourth-generation, 19-Nor-P₄ derivatives, norgestrol acetate (NoMAC) and nesterone (NES), while progestins structurally related to (B) testosterone (T) include the first-generation progestin, norethisterone acetate (NET-A), the second-generation progestin, levonorgestrel (LNG), and the third-generation progestins, gestodene (GES) and norgestimate (NGM). (C) The fourth-generation progestin drospirenone (DRSP) is currently the only progestin structurally related to spironolactone, a MR antagonist. Structures were obtained from Pubchem (<https://pubchem.ncbi.nlm.nih.gov>).

conventional HT products, custom-compounded bHT products lack black-box warnings of the potential adverse effects of HT (Fugh-berman & Bythrow 2007, Bhavnani & Stanczyk 2012, Pinkerton 2014). A major concern raised by randomized FDA checks is the fact that custom-compounded preparations frequently result in accidental under- or overdosing, possibly due to variations in purity and/or human error associated with personalized combination constitution (Cirigliano 2007, Eden *et al.* 2007, Fugh-berman & Bythrow 2007, Bhavnani & Stanczyk 2012, Sood *et al.* 2013, Pinkerton 2014). Moreover, compounded bHT patches have been shown to yield lower serum estrogen levels than bioequivalent standard-dose E₂ patches, emphasizing that the pharmacodynamics of compounded bHT requires further research (Sood *et al.* 2013).

Clinical trials investigating the safety and efficacy of bP₄ creams over a 12-week period have revealed that custom-compounded micronized bP₄ creams do not relieve vasomotor symptoms, inhibit the proliferative effects of E₂ on the endometrium or improve mood swings and libido (Wren *et al.* 1999, 2003, Drisko 2000, Wren 2003, Vashisht *et al.* 2005, Elshafie & Ewies 2007).

It has been suggested that this may be due to insufficient bP₄ absorption and thus low bioavailability (Cooper *et al.* 1998, Burry *et al.* 1999, Wren *et al.* 1999, Carey *et al.* 2000, O'leary *et al.* 2000, Lewis *et al.* 2002). However, the Postmenopausal Estrogen and Progestin Intervention (PEPI) trial revealed that oral micronized bP₄ effectively relieves vasomotor symptoms (The Writing Group for the PEPI Trial 1995, Barrett-Connor *et al.* 1997, Greendale 1998), suggesting that oral micronized bP₄ may be more effective at relieving vasomotor symptoms than localized micronized bP₄ creams.

Custom-compounded bHT often contains bE₂ in combination with bE₃ and/or bE₁ (Taylor 2005, Bosarge & Freeman 2009, Chervenak 2009, Pinkerton 2014). Biest or triest combination regimens can be obtained from compounding pharmacies, where a biest is composed of bE₂ and bE₃ in a 20:80 ratio and a triest is composed of bE₂, bE₃ and bE₁ in a 10:80:10 ratio (Boothby *et al.* 2004, Curcio *et al.* 2006, Fugh-berman & Bythrow 2007, Sites 2008). Proponents of bHT claim that bE₃ and bE₁ are weaker, safer estrogens than bE₂ (Sites 2008, Chervenak 2009, Holtorf 2009) and that bE₃ antagonizes the potent estrogenic activity of bE₂ (Melamed *et al.* 1997, Boothby *et al.* 2004). However, we have recently shown that both bE₃ and bE₁ are not necessarily weaker estrogens than bE₂ in terms of transactivation and transrepression of gene expression, or their effects on breast cancer cell proliferation and anchorage-independent growth (Perkins *et al.* 2017). Moreover, we showed that E₃ did not antagonize E₂-induced gene expression, proliferation or anchorage-independent growth of the MCF-7 BUS breast cancer cell line (Perkins *et al.* 2017). To the best of our knowledge, however, no other detailed comparisons of the agonist and antagonist properties of these hormones for transactivation and transrepression via the ER subtypes have been reported. In fact, the incorporation of E₃ into bHT products appears to be based on murine work conducted by Lemon, more than 30 years ago (Lemon 1975, 1987), showing that E₃ was more protective against carcinogen-induced neoplasms than E₂ or E₁ (reviewed in (Taylor 2005)). However, these claims have not been validated in human models (Melamed *et al.* 1997) and large-scale, double-blinded, placebo-controlled clinical trials evaluating the safety and efficacy of E₃ or bE₃ are lacking. However, some preliminary small-scale trials have suggested that E₃ sometimes relieves vasomotor symptoms but does not protect against bone loss (Yang *et al.* 1995, Takahashi *et al.* 2000), while others have provided evidence that E₃ can protect against bone loss (Cheng *et al.* 1993, Minaguchi *et al.* 1996, Itoi *et al.* 1997,

Kamenov *et al.* 2000, Hayashi *et al.* 2002), highlighting the uncertainties regarding E_3 use. Interestingly, although there are no FDA-approved E_3 -containing HT products (Boothby *et al.* 2004), bE_3 is used in regulated HT products in parts of Europe and Asia (Cirigliano 2007, Lommen & Mead 2013), where it is, usually referred to as E_3 rather than bE_3 (Wright 2005, Conaway 2011).

Androgens such as bT and bDHEA, are also often used in personalized bHT formulations in combination with estrogens and/or progestogens to relieve the symptoms of menopause (Reed-Kane 2001, Boothby *et al.* 2004, Eden *et al.* 2007, Sites 2008). However, observational studies have reported adverse effects of androgen-containing HT such as endometrial cancer, hair loss, acne, hirsutism and deepening of the voice (Greendale *et al.* 1999, Eden *et al.* 2007). In fact, cases of endometrial cancer have been reported in users of oral bHT products containing combinations of bE_2 , bP_4 , bT and bDHEA or bE_1 , bE_2 , bE_3 , bP_4 , bT and bDHEA (Eden *et al.* 2007). Interestingly, various androgens, including the testosterone precursor and methyltestosterone, are approved for HT use in Europe (Davis 2015), while there is no FDA-approved androgen-containing female HT (Pinkerton 2014, Gass *et al.* 2015). Moreover, although there is a lack of clinical trials examining the effectiveness and possible side effects of androgen use in HT, bT and bDHEA are distributed by compounding pharmacies in both the USA (Boothby *et al.* 2004, Cirigliano 2007, Guidozi *et al.* 2014) and South Africa (Golding 2009). In terms of breast cancer risk, the inclusion of bT is especially concerning as testosterone can be aromatized to E_2 within breast tissue (Hickey *et al.* 2012) and endogenous testosterone levels are only marginally decreased after menopause (Table 1). This suggests that the incorporation of bT into an estrogen containing bHT may thus result in greater estrogen exposure than intended, which may increase risk of breast cancer development.

Overall, the lack of large-scale clinical trials investigating the safety and efficacy of custom-compounded bHT such as biest and triest regimens (Cirigliano 2007, Bosarge & Freeman 2009), together with the absence of black-box warnings, lack of thorough regulatory bodies and uncertainties regarding salivary testing, has resulted in a consensus between several organizations including the North American Menopause Society (NAMS), The International Menopause Society (IMS), The Endocrine Society and The European Menopause and Andropause Society (EMAS), recommending against the use of custom-compounded bHT (de Villiers *et al.* 2013).

Hormone therapy and breast cancer risk

Numerous clinical trials and observational studies have associated conventional HT with multiple side effects such as elevated risk of developing breast, ovarian and endometrial cancers, as well as cardiovascular disease and stroke (Nachtigall *et al.* 1979, Obel *et al.* 1993, The Writing Group for the PEPI Trial 1995, Hulley *et al.* 1998, 2002, Johnson 1998, Greendale *et al.* 1999, Notelovitz *et al.* 2002, Waters *et al.* 2002, Writing Group for the Women's Health Initiative Investigators 2002, Million Women Study Collaborators 2003, Barakat *et al.* 2006, Veerus *et al.* 2006, Yaffe *et al.* 2006, Fournier *et al.* 2008b, Tierney *et al.* 2009, Schierbeck *et al.* 2012, Manson *et al.* 2013, Sood *et al.* 2014, Clavel-Chapelon 2015, Hodis *et al.* 2016, Marjoribanks *et al.* 2017). Considering that breast cancer is the most common cancer in women worldwide and the leading cause of cancer-related deaths in women in developed countries (Ferlay *et al.* 2015, Torre *et al.* 2015), the association between HT and breast cancer risk is alarming. Although several studies reported adverse effects associated with HT prior to 2002 (Nachtigall *et al.* 1979, Obel *et al.* 1993, The Writing Group for the PEPI Trial 1995, Hulley *et al.* 1998), it was the findings of the highly publicized Women's Health Initiative (WHI) (Writing Group for the Women's Health Initiative Investigators 2002) that caused alarm and confusion about the safety of HT. The WHI study was a large-scale randomized, controlled clinical trial that evaluated the benefits and risks of CEE alone in hysterectomized postmenopausal women or CEE in combination with MPA in postmenopausal women with a uterus (Writing Group for the Women's Health Initiative Investigators 2002). The results of the trial suggested that CEE-MPA combinations, but not CEE alone, were associated with increased invasive breast cancer risk (Writing Group for the Women's Health Initiative Investigators 2002). In contrast, the Million Women Study (MWS), a cohort study comprising over one million postmenopausal women from across the United Kingdom, found that the use of estrogen alone or estrogen-progestin combinations were both associated with increased invasive breast cancer risk (Million Women Study Collaborators 2003). Interestingly, this study found increased breast cancer risk with all HT preparations investigated, and no difference in risk between specific estrogens (CEE and EE) or progestins (MPA, NET and LNG) (Million Women Study Collaborators 2003).

Many additional studies investigating breast cancer risk associated with HT use have been conducted (Nachtigall *et al.* 1979, Obel *et al.* 1993, Herrington

et al. 2000, Cherry *et al.* 2002, Waters *et al.* 2002, Hulley *et al.* 2002, Notelovitz *et al.* 2002, Greenspan *et al.* 2005, Stefanick *et al.* 2006, Veerus *et al.* 2006, Vickers *et al.* 2007, Fournier *et al.* 2008a, Tierney *et al.* 2009, Schierbeck *et al.* 2012, Clavel-Chapelon 2015, Marjoribanks *et al.* 2017), often with contradictory results. For example, while the Women's international study of long-duration estrogen after menopause (WISDOM) clinical trial also found increased breast cancer risk associated with CEE-MPA use (Vickers *et al.* 2007), the Heart and Estrogen/Progestin Replacement Study (HERS) I, HERS II (Hulley *et al.* 2002) and the Estonian postmenopausal hormone therapy clinical trial (Veerus *et al.* 2006) among others (Nachtigall *et al.* 1979, Herrington *et al.* 2000, Waters *et al.* 2002, Greenspan *et al.* 2005), found no increased breast cancer risk associated with CEE or CEE-MPA. It is thus evident that results from the above-mentioned studies are often contradictory and have resulted in much confusion regarding the safety of these HT regimens. However, it is noteworthy that the studies showing no increased breast cancer risk had significantly less participants (Nachtigall *et al.* 1979, Herrington *et al.* 2000, Hulley *et al.* 2002, Waters *et al.* 2002, Greenspan *et al.* 2005, Veerus *et al.* 2006) than the large-scale WHI, MWS and WISDOM studies (Writing Group for the Women's Health Initiative Investigators 2002, Million Women Study Collaborators 2003, Vickers *et al.* 2007).

Notably, although most clinical and observational studies investigating the association between HT and increased breast cancer risk examined the effects of CEE and MPA, a few studies have in fact investigated other estrogens and progestogens. For example, three clinical trials have reported no increased breast cancer risk associated with the use of oral E_2 alone or in combination with NET-A (Obel *et al.* 1993, Tierney *et al.* 2009, Schierbeck *et al.* 2012), the latter of which was previously shown to increase breast cancer risk when used in combination with CEE in the MWS (Million Women Study Collaborators 2003). Similarly, at least one other study reported no increased risk with the use of an E_2 only patch (Notelovitz *et al.* 2002), while the Estrogen in the Prevention of Reinfarction Trial found no increased risk with the use of E_2 valerate alone (Cherry *et al.* 2002). However, the Kronos Early Estrogen Prevention Study found increased breast cancer risk associated with the use of E_2 patches in combination with oral micronized P_4 , while the Early Versus Late Intervention Trial (ELITE) also found increased risk with oral E_2 used in combination with a P_4 vaginal gel (reviewed in (Marjoribanks *et al.* 2017)). In contrast to the above-mentioned studies

showing increased breast cancer risk with the inclusion of P_4 , the PEPI Trial reported no increased breast cancer risk in women administered CEE plus oral micronized P_4 or CEE plus MPA (The Writing Group for the PEPI Trial 1995). Similarly, the French E3N cohort study found that estrogen (CEE or bE_2) alone or in combination with P_4 or dydrogesterone (a progestin not used clinically in the USA) was not associated with increased breast cancer risk. Interestingly, results from the same study showed that other estrogen-progestin combinations containing the progestins MPA, NET-A, medrogestone, chlormadinone acetate (CMA), cyproterone acetate (CPA), R5020 or NoMAC, were associated with increased breast cancer risk (Fournier *et al.* 2008a, Clavel-Chapelon 2015). This French cohort study also suggested that administration of oral versus transdermal E_2 does not influence the degree of breast cancer risk (Fournier *et al.* 2008a, Clavel-Chapelon 2015). Interestingly, a recent Cochrane review examining the adverse side effects of HT compiled the results of 22 clinical studies, including most of the above-mentioned studies, and suggested that estrogen-progestin HT combinations increased breast cancer risk, while use of estrogen-only HT did not (Marjoribanks *et al.* 2017). It is clear from the above that more clinical and molecular studies investigating the association between different hormones used in HT and breast cancer are needed.

Taken together, the evidence in the literature investigating an association between specific hormones used in HT and increased breast cancer risk is contradictory. However, there are many other hormones used in FDA-approved HT products such as esterified estrogens, E_2 acetate, trimegestone and DRSP, or custom-compounded bHT products such as bE_3 , bE_1 and bT, that have not been investigated in large-scale clinical trials or cohort studies, and thus, it is not known whether these steroid hormones are linked to increased breast cancer risk. An added conundrum is whether the increased risk is due to the initiation of new tumors or the promotion of small, pre-existing tumors. Although still a matter for debate, it is most likely due to tumor promotion (Dietel 2010) as women in the WHI and MWS trials developed tumors within the first year of combined HT use, while breast tumors need an average of 7–10 years to grow to a detectable size (Chlebowski *et al.* 2003). At the molecular level, steroid hormones predominantly elicit their effects by binding to steroid receptors, which are ligand-activated transcription factors belonging to the nuclear receptor superfamily (Griekspoor *et al.* 2007, Aagaard *et al.* 2011).

Steroid receptors as mediators of hormone activity and carcinogenesis

When steroid hormones enter the bloodstream, they bind to various serum binding proteins such as sex-hormone-binding globulin (SHBG), corticosteroid-binding globulin (CBG) and/or albumin (Table 4). SHBG predominantly binds estrogen and testosterone, while CBG binds cortisol and P_4 (Yen *et al.* 2015). The hormones bound to SHBG or CBG are considered unavailable to tissues, while the free, unbound hormones and those bound to albumin are considered biologically available to enter cells of target tissues and elicit a response by binding to a steroid receptor (Pardridge 1981, Kuhn *et al.* 1990, Kuhl 2005). As indicated in Table 4, some estrogens and progestogens bind to SHBG and/or CBG, while others do not, resulting in large differences in the availability of estrogens and progestogens used in HT. For example, while approximately 37% of serum E_2 can bind to SHBG, 16% of E_1 binds, only 1% of E_3 binds and EE does not bind at all. As a result, E_3 , E_1 and EE are mostly available to enter cells of target tissues (Table 4) suggesting that these estrogens may be more abundant than E_2 in target tissues, and therefore, may compete with E_2 for binding to the ER. In the same way, progestins also differentially bind to SHBG as shown by 35.5% of NET and between 47.5 and 73.6% of LNG binding, while MPA and DRSP do not bind at all (Table 4). Considering that the data in Table 4 indicates that progestogens are mostly available, it is plausible that they may be more abundant in target tissues than endogenous steroid hormones, even when administered at low concentrations, and thus, may compete with these hormones for binding to their cognate steroid receptors.

Steroid hormones can permeate the cell membrane and elicit their effects by binding to steroid receptors, such as the GR and MR, as well as the sex-steroid hormone receptors, the ER, PR and AR (Griekspoor *et al.* 2007). A high degree of homology exists between the steroid receptor family, and the receptors are organized into four evolutionary-conserved domains (Fig. 2), namely the N-terminal domain containing the ligand-independent activation function 1 (AF-1) region, the highly conserved DNA-binding domain (DBD), a hinge region and a relatively conserved ligand-binding domain (LBD) containing an additional ligand-dependent activation function (AF-2) region (Giguère *et al.* 1986, Kumar *et al.* 1987, Mangelsdorf *et al.* 1995, Faus & Haendler 2006, Griekspoor *et al.* 2007, Aagaard *et al.* 2011). Generally, unliganded AR, GR and MR are found in the cytoplasm, the ER and PR-A predominantly in the nucleus (Li *et al.*

2005, Yen *et al.* 2015), while PR-B is distributed between the cytoplasm and the nucleus (Li *et al.* 2005, Griekspoor *et al.* 2007). Unliganded steroid receptors are associated with chaperone proteins such as heat shock protein (Hsp)90 and Hsp70 (Pratt & Toft 1997), but dissociate from the chaperone proteins upon ligand binding, as the steroid receptors undergo a conformational change (Griekspoor *et al.* 2007). The ligand-bound cytoplasmic steroid receptor can then enter the nucleus (Griekspoor *et al.* 2007), where it generally binds as a dimer to semi-palindromic DNA sequences known as hormone response elements (HREs) to activate target gene expression (transactivation) (Faus & Haendler 2006, Aagaard *et al.* 2011) or as a monomer to negative HREs (nHREs) or other DNA-bound transcription factors such as nuclear factor kappa B (NF κ B), to repress target gene expression (transrepression) (reviewed in (Faus & Haendler 2006)). Steroid hormones can also elicit non-genomic effects either by binding to membrane-bound receptors to activate signaling cascades, which ultimately result in the downstream regulation of gene expression or by interacting with membrane kinases to activate rapid signaling pathways (Faus & Haendler 2006, Hammes & Levin 2007, Bennett *et al.* 2010, Krug *et al.* 2011, Yang *et al.* 2011, Vernocchi *et al.* 2013, Diep *et al.* 2015, Schwartz *et al.* 2016).

The ER, PR, AR and GR are expressed in most breast cancers, and it is therefore not surprising that they all play functional roles in breast cancer cell biology (reviewed in (Sikora 2016)). Moreover, emerging evidence suggests that their signaling pathways are not always distinct but are in fact extensively intertwined. This too is not surprising considering the high degree of homology between the steroid hormone receptors and their cognate DNA-binding sites (Giguère *et al.* 1986, Kumar *et al.* 1987, Mangelsdorf *et al.* 1995, Faus & Haendler 2006, Griekspoor *et al.* 2007, Aagaard *et al.* 2011, Need *et al.* 2012). In the following sections, we summarize the role of steroid receptors and their interplay in breast cancer, with a focus on the known effects of the estrogens and progestogens via the ER and PR, respectively.

Estrogens and the ER

The first association between estrogen signaling and breast cancer can be traced back to 1896 (Beatson 1896) and since then copious *in vivo* and *in vitro* studies have shown that estrogens promote breast cancer development and progression (Lippman *et al.* 1977, Soto & Sonnenschein 1985, Reddel & Sutherland 1987, Clemons & Goss 2001, Gutendorf & Westendorf 2001, Frasor *et al.* 2003, Lippert

Table 4 Binding of hormones to transport proteins.^a

	SHBG (%)	CBG (%)	Albumin (%)	Free (%)	Available (%)
Estrogens					
E ₂	37	0	61	2	63
E ₃	1	0	91	8	99
E ₁	16	0	80	4	84
EE	0	0	99	1	100
Progestogens					
P ₄	0.6	17.7–36	79.3–90.3	0–2.4	81.7–92.7
MPA	0	0	88	12	100
NET	35.5	0	60.8	3.7	64.5
LNG	47.5–73.6	0	25.5–50	0–2.5	26.4–52.5
DRSP	0	0	95–97	3–5	100

^aDunn *et al.* (1981), Hammond *et al.* (1982), Kuhl (1990), Kuhn *et al.* (1990, 1992, 1994), Schindler *et al.* (2003), Stanczyk *et al.* (2013).CBG, corticosteroid-binding globulin; DRSP, drospirenone; E₁, estrone; E₂, estradiol; E₃, estriol; EE, ethinylestradiol; LNG, levonorgestrel; MPA, medroxyprogesterone acetate; NET, norethisterone; P₄, progesterone; SHBG, sex-hormone-binding globulin.

et al. 2003, Mueck *et al.* 2003, Yager & Davidson 2006, Watson *et al.* 2008, Fernandez & Russo 2010, Liu *et al.* 2015, de Almeida Chuffa *et al.* 2017). Although the precise mechanisms whereby estrogens promote breast cancer is still an area of ongoing research, it is well established that ER-A is crucial for E₂-induced breast cancer cell growth (Couse & Korach 1999, Briskin *et al.* 2010). This critical role for ER-A was highlighted by a study showing that E₂ exposure did not cause breast cancer tumor formation in ER-A-knockout mice (reviewed in (Couse & Korach 1999, Briskin *et al.* 2010)). Considering that the ER is expressed in approximately 75% of breast cancers, current therapies target ER activity or the synthesis of endogenous estrogen (Lanari *et al.* 2012, Lim *et al.* 2016, Doan *et al.* 2017). For example, tamoxifen or fulvestrant are used to antagonize ER-A signaling by blocking or degrading the ER respectively (Santen *et al.* 2009, Lanari *et al.* 2012, Lim *et al.* 2016, McNamara *et al.* 2016, Doan *et al.* 2017), while aromatase inhibitors are used to decrease the production of endogenous estrogens by inhibiting the metabolism of testosterone and androstenedione to E₂ and E₁, respectively.

Estrogens and ER signaling lead to the development and progression of breast cancer largely through the regulation of gene expression (Preston-Martin *et al.* 1990, Dickson & Lippman 1995, Clemons & Goss 2001, Frasor *et al.* 2003, Yue *et al.* 2013, Deroo & Korach 2014, Luo *et al.* 2016). For example, E₂ treatment of the MCF-7 breast cancer cell line results in the upregulation of genes encoding growth factors such as *insulin-like growth factor* (IGF)-binding proteins and *vascular endothelial growth factor* (VEGF) (Dickson & Lippman 1995, Ruohola *et al.* 1999, Mueller *et al.* 2000, Frasor *et al.* 2003, Garvin *et al.* 2006, Walker *et al.* 2007). Furthermore, genes regulating the cell cycle such as *CCND1*, *CCNA2* and

cyclin-dependent kinase 1 (*CDK1*) are also upregulated by E₂ in MCF-7 cells, as are genes promoting proliferation such as *Ki67* (Altucci *et al.* 1996, Frasor *et al.* 2003, Welboren *et al.* 2009). In contrast, E₂ has previously been shown to downregulate the expression of genes inhibiting proliferation such as transforming growth factor beta 3 (*TGFB3*) (Frasor *et al.* 2003, Welboren *et al.* 2009), and genes promoting apoptosis such as *CASP9* (Frasor *et al.* 2003). ChIP-seq analysis mapped ER-binding sites to the promoter regions of the *CCND1*, *CCNA2*, *CDK1*, *Ki67* and *TGFB3* genes in response to E₂ treatment (Welboren

Figure 2

A simplified representation of the structure of steroid hormone receptors. These receptors contain a variable N-terminal domain (A/B) containing the ligand-independent activation function 1 (AF-1) region, a highly-conserved DNA-binding domain (DBD), a hinge region (H) enabling flexibility, and a relatively conserved ligand-binding domain (LBD) containing the ligand-dependent activation function (AF-2) region. ER contains an additional C-terminal domain (C) of which the function is not known. The numbers indicated on the right represent the number of amino acids constituting each steroid receptor. Figure adapted from (Griekspoor *et al.* 2007). A full color version of this figure is available at <https://doi.org/10.1530/JME-18-0094>.

et al. 2009), highlighting the role of the ER in mediating the tumor-promoting effects of E_2 . However, the ER subtypes, ER-A and ER-B, are known to play different roles in breast cancer (Kuiper *et al.* 1996, Barkhem *et al.* 1998, Lazennec *et al.* 2001, Pettersson & Gustafsson 2001, Platet *et al.* 2004, Strom *et al.* 2004, Chang *et al.* 2006, Boothby & Doering 2008, Treeck *et al.* 2010, Lattrich *et al.* 2013, Leygue & Murphy 2013). For example, ER-A has been shown to promote breast cancer pathogenesis by upregulating the expression of *cyclin D1*, while ER-B inhibited its expression (Liu *et al.* 2002). Interestingly, the role of ER-B is dependent on whether ER-A is expressed or not. In the presence of ER-A, ER-B can inhibit ER-A-driven proliferation, while in the absence of ER-A, ER-B promotes proliferation (Kuiper *et al.* 1996, Barkhem *et al.* 1998, Lazennec *et al.* 2001, Pettersson & Gustafsson 2001, Platet *et al.* 2004, Strom *et al.* 2004, Chang *et al.* 2006, Boothby & Doering 2008, Treeck *et al.* 2010, Lattrich *et al.* 2013, Leygue & Murphy 2013). The differential action of the ER subtypes may in part be due to the regulation of subtype-specific target genes (Kian Tee *et al.* 2003, Monroe *et al.* 2003, Platet *et al.* 2004, Stossi *et al.* 2004, Chang *et al.* 2006, Zhao *et al.* 2008, Paruthiyil *et al.* 2009), possibly due to differences in the N- and C-terminals of ER-A and ER-B (Arnal *et al.* 2017). In addition, ER-B has been shown to downregulate the transcriptional activity of ER-A by modulating the recruitment of transcription factors required by the ER-A transcription complex and by increasing ER-A degradation (Matthews *et al.* 2006). While ER-B is expressed in lobular breast cancers and a subgroup of triple-negative breast cancers (TNBC), its expression is lost early in ductal breast cancer (reviewed in (Warner *et al.* 2017)). Although the precise role of ER-B in TNBC is not known, it is thought to be a potential target for treating this breast cancer subtype. For example, it has been shown that postmenopausal women with TNBC respond to tamoxifen treatment due to high expression levels of ER-B (Honma *et al.* 2008). Furthermore, tamoxifen decreased cell growth and increased apoptosis in the ER-A-negative SK-BR-3 breast cell line transfected with ER-B (Treeck *et al.* 2008). Moreover, the use of ER-B selective agonists has been suggested as treatment for the vascular symptoms experienced by menopausal women (Warner *et al.* 2017). The reason for this suggestion is two-fold: (i) the hypothesis that vascular effects in menopause are due to increased activin and follicle-stimulating hormone (FSH) levels because of decreased endogenous E_2 levels and (ii) the observation that ER-B selective agonists increase ER-B expression. ER-B regulates inhibin, which directly inhibits activin and subsequently FSH, suggesting

that ER-B selective agonists could effectively decrease the vascular symptoms of menopause (reviewed in (Warner *et al.* 2017)).

In addition to the full-length ER subtypes, several ER splice variants have been identified in various cell lines; however, it is not clear whether all of these variants are also expressed in tissue and whether they are functional proteins (reviewed in (Heldring *et al.* 2007)). An ER-A 46 splice variant which lacks part of the N-terminal domain has in fact been detected in breast tumor tissue, but its function is still unknown (reviewed in (Arnal *et al.* 2017)). In contrast, several ER-B splice variants are expressed in breast tissue and have been shown to differentially regulate estrogen signaling (Flouriou 2000, Matthews & Gustafsson 2003, Ramsey *et al.* 2004, Wang *et al.* 2005, Leung *et al.* 2006). For example, the ER-B cx splice variant contains a unique sequence in its LBD and although it cannot bind ligand, it forms heterodimers with ER-A, preventing ER-A from activating gene expression (Ogawa *et al.* 1998). In contrast, a second ER-B splice variant which also cannot bind ligand, forms dimers with either ER-A or ER-B, blocking their activity (Maruyama *et al.* 1998).

From the above, it is clear that the role of E_2 and the ER subtypes in breast cancer is complex. An added complexity is the fact that many different estrogens are used in HT, and it is not clear whether these estrogens will elicit similar effects to E_2 on breast cancer. Studies directly comparing the effects of different estrogens on hallmarks of breast cancer such as cell proliferation, migration, invasion and apoptosis are scarce. The limited studies that are available suggest that while both CEE and E_2 increase proliferation, E_2 increased proliferation to a greater extent (Mueck *et al.* 2003, Wood *et al.* 2008). Furthermore, at least three studies have directly compared the proliferative effects of E_1 , E_2 and E_3 and although all studies showed that these estrogens increase breast cancer cell proliferation, differences were observed (Lippman *et al.* 1977, Gutendorf & Westendorf 2001, Lippert *et al.* 2003). For example, our recent study (Perkins *et al.* 2017) and that of Gutendorf and coworkers (Gutendorf & Westendorf 2001) reported potencies in the picomolar range and showed that E_2 was more potent than E_3 , while E_1 was the least potent. In contrast, Lippman and coworkers (Lippman *et al.* 1977) reported potencies in the nanomolar range and showed that E_2 and E_1 were equipotent, and more potent than E_3 . From these studies, it is evident that E_2 is the most potent estrogen; yet, it is not clear how the proliferative effects of E_1 and E_3 compare to E_2 . A study by Lippert and coworkers (Lippert *et al.* 2003) comparatively investigated the effects of E_2 , E_3 and E_1 on proliferation, and although they did

not determine potencies, showed no significant difference in the proliferative effects of 10 nM or 100 nM E_1 , E_2 and E_3 and showed that while E_3 also stimulated proliferation at 1 μ M and 10 μ M, E_2 inhibited proliferation and E_1 had no effect. Interestingly, results investigating the proliferative effects of the synthetic estrogen, EE, relative to E_2 , are contradictory. While we recently showed that EE was less potent than E_2 , one other study of almost 20 years ago showed that EE was as potent as E_2 (Gutendorf & Westendorf 2001). It is thus evident that further comparative studies are required to clarify this ambiguity. To the best of our knowledge, studies investigating the effects of estrogens on migration, invasion and apoptosis only focused on E_2 and while most studies showed increased migration (Zheng *et al.* 2011, Shang *et al.* 2015) and invasion (Albini *et al.* 1986, Thompson *et al.* 1988, van den Brle *et al.* 1992, Zheng *et al.* 2011, Jiang *et al.* 2013, Tchafa *et al.* 2013, Shang *et al.* 2015), as well as decreased apoptosis (Wang & Phang 1995, Song *et al.* 2001, Fernando & Wimalasena 2004, Kampa *et al.* 2005, Tchafa *et al.* 2013) in the ER-A-positive MCF-7 and T47D breast cancer cell lines, a recent study showed that E_2 repressed invasion in the ER-A-positive T47D and BT474 cell lines (McFall *et al.* 2018). In terms of metastasis, we have shown that E_2 , E_3 , E_1 and EE promote the anchorage-independent growth of the MCF-7 BUS cell line to the same extent, suggesting that there is no difference in the metastatic potential of these estrogens.

In terms of gene expression, we and two other studies have directly compared EC_{50} values of E_2 , E_3 , E_1 and EE for both ER-A- and ER-B-mediated transactivation (Bovee *et al.* 2004, Escande *et al.* 2006, Perkins *et al.* 2017); however, the EC_{50} values between the studies differed by up to 750-fold for some estrogens. Although these studies found that E_2 and EE were the most potent estrogens, E_3 was the least potent estrogen in one study (Bovee *et al.* 2004), while we and one other study found that E_1 was the least potent (Escande *et al.* 2006, Perkins *et al.* 2017). These discrepancies may be due to the different model systems or promoter-reporter constructs used, as the first study used yeast cells and a p406-CYC1 yeast expression vector containing two estrogen response elements (EREs) (Bovee *et al.* 2004), one study used a cell line-derived from HeLa cells and stably expressing a plasmid containing one ERE (HELN cells) (Escande *et al.* 2006), while we used the MCF-7 BUS cell line (Perkins *et al.* 2017). In addition to transactivation of gene expression, the estrogen-bound ER can also transrepress gene expression; however, there is a paucity of studies characterizing this mechanism of action. At least two studies have investigated the efficacy

and potency of E_2 and/or EE for transrepression of gene expression via ER-A (Cerillo *et al.* 1998, Harnish *et al.* 2000). However, little was known for other estrogens used in HT or for the transrepressive activities via ER-B until our recent study revealed that E_2 , E_3 , E_1 and EE have similar efficacies for transrepression via ER-A and ER-B (Perkins *et al.* 2017). While the potencies of these estrogens were mostly similar, E_1 was more potent via ER-A, EE more potent via ER-B and E_3 less potent in a model endogenously expressing both ER-A and ER-B (Perkins *et al.* 2017). Considering the scarcity of comparative studies and the fact that our recent study was the first to compare the effects of bioidentical hormones to the endogenous human hormones or synthetic estrogens such as EE, it is imperative that more molecular studies are conducted. This is critical considering that some of the estrogens used in HT and bHT have not been tested in large-scale, double-blinded clinical trials and their safety and efficacy is unknown.

Progestogens and the PR

The role of progestogens including natural P_4 and progestins in breast cancer is not straightforward. Some progestins have been associated with increased breast cancer risk (Writing Group for the Women's Health Initiative Investigators 2002, Million Women Study Collaborators 2003, Fournier *et al.* 2008a, Marjoribanks *et al.* 2017), while others and P_4 have not (Nachtigall *et al.* 1979, Obel *et al.* 1993, Herrington *et al.* 2000, Hulley *et al.* 2002, Waters *et al.* 2002, Greenspan *et al.* 2005, Veerus *et al.* 2006, Fournier *et al.* 2008a, Tierney *et al.* 2009, Schierbeck *et al.* 2012). In addition, results from *in vitro* studies investigating the effects of progestogens on breast cancer cell proliferation are also contradictory. For example, while some studies have shown that P_4 (Carvajal *et al.* 2005), R5020 (Hissom & Moore 1987, Moore *et al.* 2000), MPA (Franke & Vermees 2003, Werner *et al.* 2005) and NET-A (Schoonen *et al.* 1995, Franke & Vermees 2003, Werner *et al.* 2005) promote proliferation of the MCF-7, ZR75 or T47D breast cancer cell lines, others have shown that these progestogens are anti-proliferative in the T47D cell line (Horwitz & Freidenberg 1985, Musgrove *et al.* 1991, Botella *et al.* 1994, Groshong *et al.* 1997, Formby & Wiley 1999). Some studies have even suggested that P_4 and the progestin ORG 2058 are proliferative for one cell cycle, after which they exert anti-proliferative and pro-apoptotic effects (Musgrove *et al.* 1991, Groshong *et al.* 1997). Interestingly, the effects of progestogens on proliferation also seem to be dependent on the absence

or presence of estrogen. For example, while progestogens such as P₄, MPA, NET, LNG, GES and R5020, have been shown to promote proliferation of the MCF-7 cell line, they exerted anti-proliferative effects in the presence of E₂ (Schoonen *et al.* 1995). Investigations into the effects of the progestogens on other hallmarks of breast cancer such as apoptosis, migration and invasion are scarce, and results from the limited studies are ambiguous. For example, some studies suggest that P₄ (Franke & Vermes 2003, Werner *et al.* 2005), MPA and NET increase apoptosis (Werner *et al.* 2005), while others suggest that these progestogens inhibit apoptosis (Ory *et al.* 2001, Franke & Vermes 2003, Moore *et al.* 2006). While it has also been shown that P₄, MPA, NES and DRSP can promote migration (Fu *et al.* 2008, 2010, Diaz *et al.* 2012) and invasion (Kato *et al.* 2005, Fu *et al.* 2008, 2010) in the T47D (Fu *et al.* 2008, 2010) and ZR75 (Kato *et al.* 2005, Diaz *et al.* 2012) breast cancer cell lines, MPA was found to promote migration and invasion of the T47D cell line to a greater extent than P₄, NES and DRSP (Fu *et al.* 2008). Interestingly, P₄, NES and DRSP, unlike MPA, reduced E₂-induced invasion but not migration (Fu *et al.* 2008). Furthermore, *in vivo* studies have shown that P₄, MPA, NET and LNG promote the progression of T47D and/or BT-474 human breast cancer cell xenografts in nude mice (Liang *et al.* 2007, 2010), via a mechanism requiring expression of the potent angiogenic growth factor VEGF, a protein implicated in the progression of tumor growth and metastasis (Hyder *et al.* 2001, Liang *et al.* 2007, 2010). Moreover, at least one study has shown that both P₄ and MPA reactivate stem cell-like properties in pre-existing breast cancer stem cells (Horwitz & Sartorius 2008), supporting the idea that these progestogens reactivate dormant breast cancer cells or potentiate the effects of small, previously undetectable cancers (Horwitz & Sartorius 2008, Eden 2011). In further support of progestins promoting the progression of already established tumors, a recent study showed that MPA increased the expression of CD44 and the activity of aldehyde dehydrogenase, known markers of cancer stem cells, in BT-474 and/or T47D breast cancer cells (Goyette *et al.* 2017). In light of the above, it is clear that more molecular studies are needed, particularly studies directly comparing the effects of the progestogens on hallmarks of breast cancer and the physiology of mammary stem cells. It is well known that P₄ elicits its biological effects primarily by binding to the PR (Grimm *et al.* 2016) and that progestins were designed to mimic the actions of P₄ also by binding to the PR (Sitruk-Ware 2004, Sitruk-Ware & Nath 2010). However, it is known that some progestins can also bind to and elicit biological effects

via steroid receptors other than the PR, such as the AR (Africander *et al.* 2014, Louw-du Toit *et al.* 2017) and GR (Koubovec *et al.* 2005). Thus, whether the observed effects of the progestogens on proliferation and other hallmarks of breast cancer are mediated by the PR, or any of the other steroid receptors, is still an area of ongoing research. However, at least one study has provided evidence that PR knockdown using siRNA (Wargon *et al.* 2015) abrogates MPA-induced breast cancer cell proliferation.

Three PR isoforms, PR-A, PR-B and PR-C, transcribed from different promoters of a single gene, have been identified (Kastner *et al.* 1990, Diep *et al.* 2015). However, only PR-A and PR-B are functional as PR-C lacks a DBD, and thus, cannot bind to DNA to activate gene transcription (Condon *et al.* 2006, Diep *et al.* 2015). Interestingly, approximately 65% of P₄-regulated genes are regulated only by PR-B, while approximately 4% are regulated only by PR-A and 25% are regulated by both PR isoforms (Richer *et al.* 2002, Lanari *et al.* 2012). These somewhat unique gene sets result in different biological roles for PR-A and PR-B, where PR-B mediates P₄-induced normal breast cell proliferation (Conneely *et al.* 2003, Mulac-Jericevic *et al.* 2003), while P₄-bound PR-A is implicated in maintaining ovarian and uterine functions (Conneely *et al.* 2003, Mulac-Jericevic *et al.* 2003, Lanari *et al.* 2012). The transcriptional activity of the individual isoforms is also cell specific and is extensively regulated by post-translational modifications including phosphorylation, sumoylation, acetylation and ubiquitination (reviewed in Diep *et al.* 2015). However, PR-A is generally more transcriptionally active than PR-B in the absence of ligand (Jacobsen *et al.* 2002), while PR-B is more transcriptionally active in the presence of agonist (Faivre & Lange 2007, Lanari *et al.* 2012). The latter may be due to the additional AF-3 region in the N-terminal domain of PR-B, which enables the binding of cofactors to PR-B that cannot bind PR-A (Giangrande *et al.* 1997).

The PR is a well-known ER-target gene and is thus expressed in most ER-A-positive breast tumors (Horwitz *et al.* 1978, Kastner *et al.* 1990, Dunbier *et al.* 2010). Although traditionally thought of only as an indicator of active ER signaling pathways in breast cancer tumors, the role of the PR in breast cancer is quite complex and dependent on multiple factors such as the relative ratio of PR-A to PR-B (Diep *et al.* 2015). PR-A and PR-B are generally expressed at equimolar ratios in the normal mammary gland (Mote *et al.* 2002), resulting in the formation of PR-A/B heterodimers that regulate a specific gene set (reviewed in Lanari *et al.* 2012). In contrast, PR-A and/or PR-B expression is often increased in

atypical breast lesions, dysregulating the ratio of the PR isoforms (Graham *et al.* 1995). This dysregulation disrupts normal PR signaling due to the predominance of one PR isoform and the subsequent formation of homodimers that regulate a unique gene set (reviewed in Lanari *et al.* 2012). Interestingly, PR-A is upregulated in most ductal carcinomas *in situ* and invasive breast cancers (Graham *et al.* 1995, Mote *et al.* 1999, 2002) and is thought to be more stable than PR-B (Faivre & Lange 2007). Although the exact mechanism behind this has not been fully elucidated (Faivre & Lange 2007), it may be due to the fact that PR-B contains six more phosphorylation sites in its N-terminal domain than PR-A, and the increased kinase activity in pre- or early-malignant breast tissue drives PR-B phosphorylation resulting in both PR-B hyperactivity and degradation (Dressing & Lange 2009, Dressing *et al.* 2009). Moreover, the activity of PR-B, as well as the ER, AR, GR and MR, can also be repressed by PR-A under normal cellular conditions (Vegeto *et al.* 1993, McDonnell & Goldman 1994, McDonnell *et al.* 1994, Kraus *et al.* 1995, 1997, Lim *et al.* 1999, Conneely & Lydon 2000, Li *et al.* 2005, Griekspoor *et al.* 2007), suggesting crosstalk between steroid receptors. Interestingly, steroid receptor crosstalk mechanisms have been described in the breast cancer context.

Interplay between ER-A and the PR

Recent evidence in the literature suggests that crosstalk between ER-A and the PR plays an important role in breast cancer pathogenesis (Giulianelli *et al.* 2012, Daniel *et al.* 2015, Mohammed *et al.* 2015, Singhal *et al.* 2016). For example, it has been suggested that the potent PR agonist, R5020, promotes breast cancer progression by activating kinase cascades via a mechanism requiring both ER-A and the PR (Migliaccio *et al.* 1998, Ballare *et al.* 2003). Furthermore, Giulianelli *et al.* (2012) provided evidence of an interaction between ER-A and the PR in breast cancer tissue and cell lines and showed that this interaction is required for MPA-induced breast cancer gene expression and cell proliferation in the T47D breast cancer cell line. Subsequent studies have revealed that the PR can modulate the transcriptional activity and chromatin localization of ER-A through the formation of these ER-A-PR complexes (Giulianelli *et al.* 2012, Daniel *et al.* 2015, Mohammed *et al.* 2015, Singhal *et al.* 2016). For example, Daniel and coworkers (Daniel *et al.* 2015) showed that the unliganded PR can act as a molecular scaffold, resulting in PR, ER-A, PELP-1 (proline-, glutamic acid- and leucine-rich protein 1) and IGF1 complexes that

alter ER-A gene regulation leading to a more aggressive proliferative response upon E₂ stimulation of the MCF-7 cell line. A second study by Mohammed *et al.* (2015) showed that P₄- or R5020-bound PR is recruited to the ER-A complex in both MCF-7 and T47D cells and redirects E₂-activated ER-A chromatin binding such that the gene expression profile is similar to that of PR alone. A similar mechanism was shown by Singhal *et al.* (2016) in primary ER-A- and PR-positive human tumors and is reported to lead to decreased proliferation and an improved clinical outcome (Mohammed *et al.* 2015). The above-mentioned studies suggest that an interaction between ER-A and the PR can be associated with either poor or good prognosis in breast cancer and that the outcome is determined by the absence or presence of PR ligands. Whether this is true for all PR ligands is not known. This is particularly important for progestins used in HT as some progestins from the earlier generations have been implicated in increased breast cancer risk, while clinical trials implicating newer-generation progestins that have a greater affinity for the PR and elicit biological effects more similar to P₄ than progestins from the earlier generations (Sitruk-Ware 2004, Sitruk-Ware & Nath 2010, Africander *et al.* 2011), are mostly lacking. Further studies are thus required to elucidate the role of ER-A-PR crosstalk in breast cancer pathogenesis in response to different progestins. An added complexity in delineating the role of ER-PR crosstalk in response to progestins is the fact that although a few studies report that some progestins and/or their metabolites may bind to the ER (Larrea *et al.* 2001, Pasapera *et al.* 2002, Escande *et al.* 2006, Lemus *et al.* 2009, Louw-du Toit *et al.* 2017), conflicting results are often reported, and most of these studies fail to differentiate between the ER subtypes. In a recent study, we have compared the binding of P₄, MPA, NET-A, LNG, GES, NES, DRSP and NoMAC to the individual ER subtypes and showed that only NET-A, LNG and GES, which are all derived from testosterone, bind to ER-A, while none of the progestogens bind to ER-B (Louw-du Toit *et al.* 2017). Interestingly, it has previously been shown that ER-A is required at least for MPA-induced breast cancer cell proliferation (Giulianelli *et al.* 2012); thus, studies investigating the estrogenic activity of progestins used in HT and whether ER-A is required for the effects of all progestins on proliferation are needed. Furthermore, considering that the ER-B subtype is also expressed in breast cancers, investigations are required to determine whether a similar interaction occurs between ER-B and the PR and what the implications of such an interaction would be. Current molecular studies investigating the role of ER-B in breast cancer cell

biology are however limited, likely due to the lack of an effective commercial antibody for this ER subtype (Nelson *et al.* 2017). A better understanding of the role of ER-PR crosstalk, and whether it is involved in the increased breast cancer risk associated with some progestins, may help with the design of a progestin that can be used in HT without breast cancer risk.

Interplay between the ER and AR

A number of studies have suggested that androgens and the AR play a critical role in breast cancer biology (reviewed in Dimitrakakis & Bondy 2009, Hickey *et al.* 2012, McNamara *et al.* 2014, Tarulli *et al.* 2014, Rahim & O'Regan 2017) and considering that the AR is expressed in about 90% of primary breast tumors (reviewed in Hickey *et al.* 2012), it is not surprising that AR-targeted treatment for breast cancer is actively being investigated. However, the precise role of the AR in breast cancer is dependent on whether ER-A is present. While the AR generally plays an anti-proliferative role in ER-A-positive breast tumors by inhibiting the activity of ER-A (Dauvois *et al.* 1991, de Launoit *et al.* 1991, Hackenberg *et al.* 1991, Birrell *et al.* 1995a, Ortmann *et al.* 2002, Greeve *et al.* 2004, Peters *et al.* 2009), the AR can also mimic the role of the ER-A in ER-A-negative breast cancers and promote breast cancer development (reviewed in Rahim & O'Regan 2017). As a result, clinical trials are currently evaluating the use of selective AR modulators in ER-A-positive breast cancer therapies, and anti-androgens for use in ER-A-negative breast cancer therapies (Rahim & O'Regan 2017). One suggested mechanism whereby the AR can attenuate the activity of ER-A is by displacing ER-A from ER-binding sites, either via binding to androgen response elements that are in close proximity to ER-binding sites in estrogen target genes or by competing with ER-A for binding directly to EREs in target genes (Peters *et al.* 2009, Need *et al.* 2012). Another mechanism may be an AR-mediated increase in ER-B expression, which is known to inhibit the activity of ER-A, as increased ER-B expression has previously been shown in the presence of natural dihydrotestosterone (DHT) and the synthetic androgen mibolerone in MCF-7 and ZR75 breast cancer cell lines (Rizza *et al.* 2014).

Interestingly, it has been shown that the first-generation progestin MPA is a potent AR agonist (Africander *et al.* 2014, Louw-du Toit *et al.* 2017), and that like DHT, it inhibited the transcriptional activity of ER-A in MDA-MB-231 breast cancer cells overexpressing ER-A and the AR (Peters *et al.* 2009). However, AR-mediated effects in breast cancer appear to be a double-edged sword.

For example, MPA treatment has effectively been used to treat breast cancer (Birrell *et al.* 1995b) via a mechanism that potentially promotes AR-induced apoptosis. In contrast, MPA used in HT has been associated with increased breast cancer risk (Writing Group for the Women's Health Initiative Investigators 2002, Million Women Study Collaborators 2003, Fournier *et al.* 2008a) by a mechanism possibly involving the disruption of normal AR signaling (Kempainen *et al.* 1999, Carroll *et al.* 2016). As some progestins can bind to the AR and elicit androgenic effects, while others elicit anti-androgenic effects, it is important to determine the progestin agonist and antagonist properties for AR-mediated transactivation and transrepression of target genes in the same model system. We have performed these experiments for P₄, MPA, NET-A, LNG, GES, NES, DRSP and NoMAC, and showed that the first (MPA, NET-A), second (LNG) and third-generation progestins (GES) display potent AR agonist activity, similar to that of DHT, for transactivation and transrepression of gene expression (Africander *et al.* 2014, Louw-du Toit *et al.* 2017). In contrast, like P₄, the fourth-generation progestins elicit anti-androgenic activity, similar to that of the well-known AR antagonist hydroxyflutamide (Louw-du Toit *et al.* 2017). Considering that some progestins that have been reported to increase breast cancer risk can elicit androgenic activity, studies are required to determine if ER-A-AR crosstalk plays a role in the mechanism whereby some progestins increase breast cancer risk.

Interplay between the ER and the GR or MR

Recent studies have also highlighted roles for the GR, MR and their cognate ligands in breast cancer cell biology. In terms of the GR and its ligands (glucocorticoids), both are involved in mammary gland development during puberty and pregnancy (reviewed in Vilasco *et al.* 2011, McNamara *et al.* 2017). Glucocorticoids have been shown to regulate breast cancer cell proliferation (Mattern *et al.* 2007, Vilasco *et al.* 2011, Courtin *et al.* 2012), invasiveness, motility and adhesiveness via GR-mediated upregulation of oncogenes and downregulation of metastasis suppressor genes (reviewed in Moutsatsou & Papavassiliou 2007). Interestingly, although the GR is expressed in approximately 60% of breast cancers (Abduljabbar *et al.* 2015), no definitive correlation has been found between GR expression and prognosis of breast cancers (reviewed in Vilasco *et al.* 2011). Some studies, however, suggest that its role may be context dependent as high GR expression has been associated with a good outcome in ER-A-positive

cancers, while it is associated with a poor outcome in ER-A-negative cancers (Pan *et al.* 2011). For example, in ER-A-negative MDA-MB-231 cells, GR activation by dexamethasone (Dex) has been shown to increase the expression of genes involved in cell survival, such as *serine/threonine protein kinase 1 (SGK1)* and *dual specificity protein phosphatase 1 (DUSP1)* (Pan *et al.* 2011), while in the mouse xenograft model of MDA-MB-231 cells apoptosis induced by the chemopreventative agent, paclitaxel, was inhibited by Dex (Pan *et al.* 2011). In the presence of the ER, however, ER-A-GR complexes were formed when MCF-7 cells were treated with E₂ and Dex (Karmakar *et al.* 2013, West *et al.* 2016) resulting in the reprogramming of ER-A- and GR-binding sites (Voss *et al.* 2011, Miranda *et al.* 2013, West *et al.* 2016), and the subsequent activation of genes associated with a more favorable breast cancer outcome (West *et al.* 2016). The reprogramming involved an assisted loading mechanism which entailed the GR altering the chromatin landscape to expose novel binding sites to which ER-A can bind (Voss *et al.* 2011, Miranda *et al.* 2013). Moreover, Dex has been shown to antagonize the proliferative effects of E₂ (Zhou *et al.* 1989), while also inactivating estrogens by sulfation due to the activation of estrogen sulfotransferase (Gong *et al.* 2008, Vilasco *et al.* 2011). However, the converse is also true as E₂ has been shown to decrease GR expression and dephosphorylate the GR, thus inhibiting glucocorticoid action (Zhang *et al.* 2009, Vilasco *et al.* 2011). Taken together, although it is clear that the interplay between the ER and GR in breast cancer is complex, targeting of the GR as a potential for novel breast cancer therapies should not be excluded.

The MR has been shown to compensate for the absent GR during specific stages of mammary gland development (Kingsley-Kallesen *et al.* 2002), suggesting that the MR may play a similar role to the GR in breast cancer cell biology (Sikora 2016). Although the MR is expressed in most breast cancer tumors (Martin *et al.* 1984, Sasano *et al.* 1997, Yang & Young 2009), little is known about its role in breast cancer pathogenesis. Nevertheless, the MR ligand, aldosterone, has been shown to increase breast cancer cell proliferation and migration via a mechanism requiring the MR and the G-protein estrogen receptor (GPER), also known as the G protein-coupled receptor 30 (GPR30) (Rigiracciolo *et al.* 2016). This suggests that the MR is also involved in crosstalk mechanisms in breast cancer. Interestingly, at least one study has shown that the MR and ER-A can form a complex in HEK293 cells transiently transfected with cDNA expression vectors for the MR and ER-A (Barrett Mueller *et al.* 2014), thus it is likely that a similar complex formation may be seen in

breast cancer cells. Implications of this putative MR and ER-A crosstalk is not clear. Although beyond the scope of this review, both the GR and MR have also been implicated in crosstalk with the PR (Leo *et al.* 2004), emphasizing the importance of future studies investigating the cellular mechanisms of the GR and MR in breast cancer, as well as the extensive interactions between different members of the steroid receptor family.

Conclusion

Despite the efficacy of FDA-approved conventional HT in relieving the symptoms of menopause, its association with increased breast risk is a major concern. Although both estrogen only and estrogen-progestin HT regimens have been associated with increased breast cancer risk, findings from various clinical trials indicate that estrogen-progestin combinations are associated with a higher risk than estrogen-only HT, suggesting that the progestin component is responsible for the increased risk. These studies, however, only compared a few progestins and considering that many different progestins, some known to elicit differential effects (Hapgood *et al.* 2004, Stanczyk *et al.* 2013, Africander *et al.* 2014), are available it cannot be assumed that all progestins would increase breast cancer risk. However, the alarm surrounding the associated breast cancer risk has caused some women to turn to custom-compounded bHT as an alleged safer, natural alternative. Evidence to support the safety and efficacy of these bHT regimens is however lacking and has resulted in many associations including the NAMS, IMS, EMAS and Endocrine Society recommending against the use of custom-compounded bHT (de Villiers *et al.* 2013). Considering the large variety of hormones used in HT and bHT, and the confusion as to whether or not any of these regimens are safe in terms of breast cancer risk, or whether they initiate or promote breast cancer, it is clear that more clinical and molecular studies directly comparing their mechanism of action are needed. Molecular studies should include the determination of binding affinities, relative agonist and antagonist potencies and efficacies for transactivation and transrepression of various estrogens and progestins for individual steroid receptors. In addition, studies are required to elucidate the effects of different progestins in modulating mammary stem cells and on the reactivation of stem cell-like properties in pre-existing breast cancer stem cells. Moreover, since estrogen-progestin combination regimens are associated with a higher risk than estrogen-only regimens and that progestins elicit differential effects via steroid receptors,

it is possible that signaling via multiple steroid receptors may contribute to the observed increased breast cancer risk. Furthermore, recent studies have revealed roles for interplay between many of the steroid receptors in breast cancer cell biology. If the extensively intertwined nature of steroid hormone receptor signaling pathways can be unraveled, it may be possible to elucidate the mechanism behind progestin-induced breast cancer and design novel progestins that do not increase breast cancer risk.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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