

Review Article

Endocrine Resistance in Breast Cancer

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Around 70% of all breast cancers are estrogen receptor alpha positive and hence their development is highly dependent on estradiol. While the invention of endocrine therapies has revolutionised the treatment of the disease, resistance to therapy eventually occurs in a large number of patients. This paper seeks to illustrate and discuss the complexity and heterogeneity of the mechanisms which underlie resistance and the approaches proposed to combat them. It will also focus on the use and development of methods for predicting which patients are likely to develop resistance.

1. Introduction

Approximately 70% of breast cancers are considered estrogen receptor alpha (ER α) and/or progesterone receptor (PR) positive, and the hormone estrogen (17 β -estradiol, E₂) represents the primary stimulant in the growth and development of these tumours [1]. Thus deprivation of estrogen signalling through endocrine-targeted therapy has become the mainstay of treatment in ER α -positive disease. Despite the benefits, endocrine therapy resistance eventually occurs in a large number of patients and represents a significant issue for optimal clinical management [2]. In recent years a large body of work has focused on trying to understand the underlying mechanisms leading to resistance and approaches for their circumvention, as well as developing methods to predict which patients are likely to develop resistance and are therefore in need of additional or alternative therapies. While numerous mechanisms have been proposed in addition to those discussed in this review, a major lesson from the laboratory has been the realisation that resistance is both highly complex and heterogeneous and it is clear that more work is needed to identify and improve clinical outcome of patients with ER-positive breast cancers.

2. Estrogen Production

In both pre- and postmenopausal woman estrogen production occurs locally in the normal tissues of subcutaneous

fat, the breast, muscle tissue and bone, where it is produced by the enzymatic conversion of androgens (androstenedione and testosterone) by aromatase [1, 3–8] (Figure 1). Within breast cancer tissue, expression of aromatase occurs mainly in fibroblasts [9]. Residual levels of estrogen are also commonly found circulating in the blood and are around 20-fold higher in postmenopausal women compared with premenopausal women, despite the loss of ovarian estrogen production [4, 5, 10]. In postmenopausal women there is a noticeable correlation between risk of breast cancer and levels of circulating estrogen in the blood plasma [11]. Aromatase transcription and protein levels have been shown to differ between the quadrants of the breast in women with breast cancer, being considerably increased in the quadrant containing the tumour [12, 13]. Evidence suggests that this is primarily due to tumour cell-released factors such as prostaglandins and inflammatory cell released cytokines such as IL6, IL11 and TNF α , which enhance the activity of aromatase in fibroblasts through intracellular cAMP signalling and regulation of the aromatase gene (CYP19) via an alternative non-standard promoter [1, 14, 15].

3. Estrogen Signalling

In hormone-dependent cancers, estrogen taken up from the blood plasma or from local production diffuses into the cancer cell and binds ER, thus causing the dissociation of

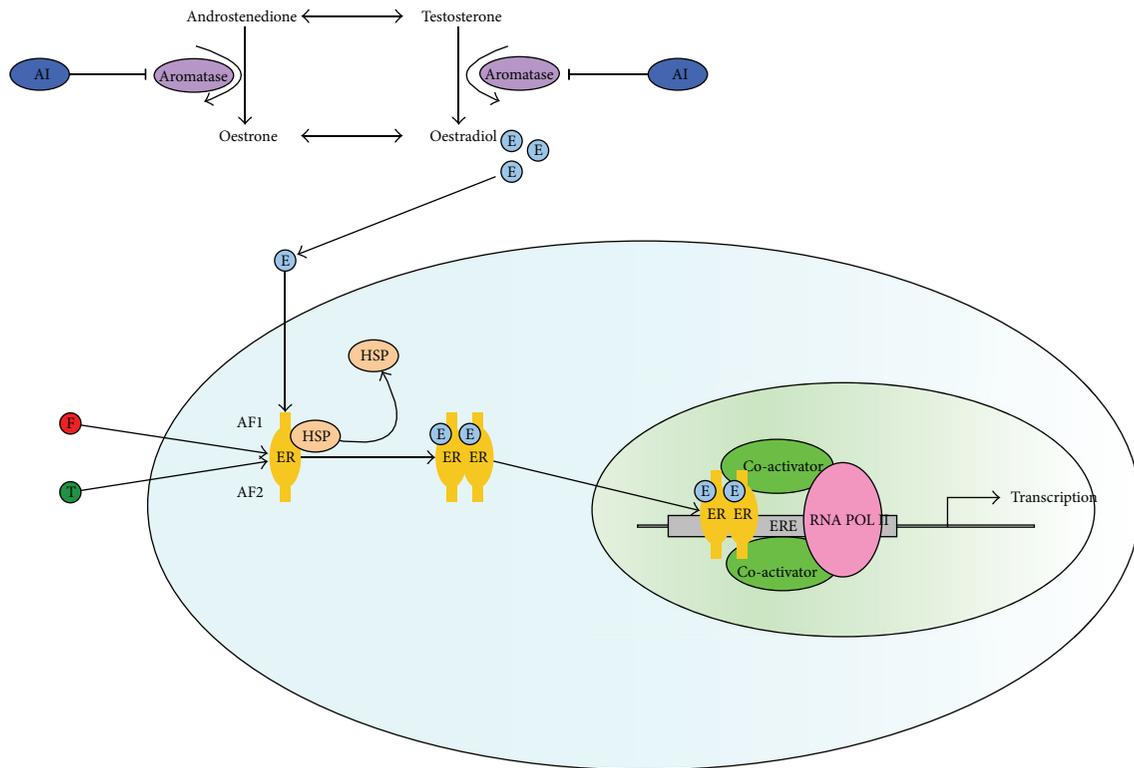


FIGURE 1: Diagram showing local estrogen production via the enzymatic conversion of androgens to estrogen by aromatase and estrogen receptor signalling in breast cancer. Estrogen (E), estrogen receptor alpha (ER), estrogen response element (ERE) and heat-shock proteins (HSP). The targets of commonly used endocrine therapies in the pathway are shown: aromatase inhibitors (AI), tamoxifen (T) and fulvestrant (F).

heat shock proteins from the ER molecule. The ligand-bound molecule dimerises, associates with other coactivator or corepressor proteins and subsequently binds to conserved estrogen response element (ERE) sequences within the promoter regions of genes over which it exerts transcriptional control [1, 16] (Figure 1). ER is a nuclear receptor encoded by the *ESR1* gene; it comprises two distinct transactivation domains: activation function (AF)-1 in the amino-terminal region and AF2 in the carboxy-terminal region [17–19]. AF1 is regulated by growth factors acting through the MAPK pathway whereas AF2, located in the ligand binding region of ER is activated by estrogen [17, 20]. Full agonist activity requires both AF1 and AF2 to be active [21]. Studies have shown that ERE bound ER is ubiquitinated and targeted for proteosomal degradation, suggesting that each ER molecule is destined for only one cycle of estrogen signalling [22]. EREs were first discovered flanking the regions of estrogen-regulated vitellogenin genes in *Xenopus laevis* and have since been found in the promoter regions of several hundred human genes, with a minimum consensus sequence consisting of a 13 base-pair palindromic inverted repeat: 5'-GGTCANNNTGACC-3' (N: any nucleotide) [23–25]. Some EREs have been identified which have imperfect palindromic sequences, differing from the consensus sequence by one or more nucleotides and are often less responsive to ligand-bound ER than consensus sequence EREs [26]. The association of ligand-bound ER with EREs is thought to be achieved via either of two mechanisms:

(i) “direct binding” in which the molecule binds to the EREs and associates directly with coactivator/corepressor molecules and the RNA polymerase II transcription initiation complex, or (ii) “tethering” whereby the ligand-bound ER does not bind DNA but rather interacts with another DNA-bound transcription factor either stabilising that factor, or recruiting additional cofactors to the complex [27, 28]. The latter is thought to be the mechanism by which ligand-bound ER associates with transcription factor SP1 [27]. Via these transcriptional associations estrogen can induce proliferation of cancer cells which overexpress ER. It should be noted that non-ligand-bound ER complexes can also bind to EREs, without the ability to regulate gene expression it was thought [17, 29–31] however, recent chromatin immunoprecipitation sequencing data (ChIP-seq) from MCF7 breast cancer cell lines suggests that endogenous ER α can bind DNA in the absence of the ligand and that transcripts are produced at those sites [32].

Although the exact mechanisms by which estrogen drives proliferation are yet to be fully elucidated, a number of potential models have been proposed [33]. In normal human breast tissue, cells which do not express ER proliferate via paracrine signalling, whereas in tumours an autocrine action occurs in which ER-positive cells proliferate [34]. It has been reported that estrogen promotes transition through the G1/S phase of cell cycle via a number of pathways involving the induction of cyclin-D1 expression by ligand-bound ER,

mediated by one or more transcription factors including: c-Jun, c-Fos, and ATF-2 at the API promoter site, or via an SP1 transcription factor dependent pathway [35–41]. Furthermore, ligand-bound ER has also been shown to bind cyclin-D1 and, as a complex, regulate the expression of that gene and other downstream genes [39, 42]. Cyclin-D1 subsequently binds and activates CDK4 and CDK6, which regulate G1/S phase transition through the phosphorylation of RB1. The latter can no longer inhibit E2F/DP1 complexes, thus allowing them to activate the transcription of S-phase entry genes such as those encoding cyclin-E1 and cyclin-A1 [35, 36, 43, 44]. Other studies suggest that ligand-bound ER may promote G1/S phase transition by induction of c-MYC which leads to activation of CDC25A and CDK4 gene transcription [45–47]. Active CDC25A dephosphorylates CDK2 leading to the inhibition of RB1 and p130 and transcriptional activation of E2F/DP1 complexes which in turn upregulates S-phase entry genes [36, 48, 49]. Alternatively, it has been proposed that ER could activate G1/S phase transition via redistribution and downregulation of p21 and P27KIP1 thus removing their inhibitory control over key cell cycle progression proteins such as CDK2 [49, 50]. It is thought this might be achieved by ubiquitin targeting for proteosomal degradation or by nuclear export via membrane-bound ER associated with ERK2 and CRM1 [51, 52].

4. Endocrine Therapy

In 1896 George Beatson, a Glasgow surgeon, showed that patients with advanced breast cancer had regression of metastatic disease following oophorectomy, providing the first contemporaneous published evidence of a link between hormones and breast cancer. By 1937 Dodds and Robertson had synthesised diethylstilboestrol and its anti-tumour activity was demonstrated although its use was limited by severe side effects (Figure 2) [53]. In 1973, the synthetic estrogen blocker tamoxifen was licensed for use in the treatment of hormone-positive breast cancer and became the mainstay of endocrine therapy for the next 30 years [54]. Today, endocrine therapy constitutes a major treatment modality in ER-positive breast cancer and can be used alone or in addition to chemotherapy, which has more associated toxicity [55–58]. Indeed, studies have shown it to provide more benefit in the adjuvant setting in postmenopausal women with ER-positive breast cancer than doxorubicin or taxane-containing chemotherapy [59, 60]. Endocrine therapies work by manipulating endocrine signalling by the exogenous administration of hormone antagonists designed to inhibit the biosynthesis and/or activity of estrogen. Endocrine therapies are considered to be cytostatic rather than cytotoxic, leading to reduced proliferation and reduction of growth rate [61]. At the simplest molecular level, they achieve this through the arrest of cell cycle in G1/S phase [62]. Today, several types of endocrine therapies exist and are used commonly in the treatment of ER-positive breast cancer in postmenopausal women.

First synthesised in the 1960s, tamoxifen is a selective estrogen receptor modulator (SERM) (Figures 1 and 2).

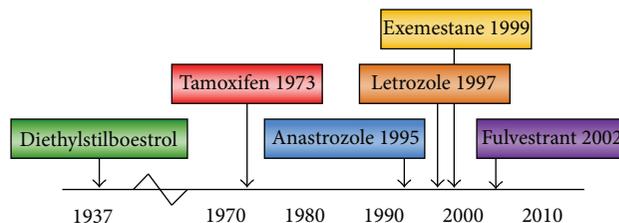


FIGURE 2: Timeline of approval for endocrine therapy agents.

It functions by disrupting the estrogen signalling pathway by competitive intranuclear binding to ER, causing a conformational change to the subsequently formed ER dimer involving the shift of helix 12 into an adjacent coactivator site (AF2), thus blocking the binding of the coactivator, which significantly reduces the level of estrogen-regulated gene transcription [21, 63, 64]. However, this complex has been shown to exhibit partial estrogen-agonist properties due to the remaining activity of AF1 [1, 21]. A newer yet similar class of endocrine therapies also exist which are known as selective estrogen receptor downregulators (SERDs) and these include fulvestrant, which was first approved for use in 2002. Fulvestrant functions to downregulate ER by competitive binding to ER dimers and by causing immobilisation of ER in the nuclear matrix which is accompanied by degradation via the ubiquitin-proteasome pathway [65] (Figures 1 and 2). It has been shown to be more potent than tamoxifen *in vitro* and does not exhibit the partial estrogen-agonist properties associated with tamoxifen in murine models. This is due to the fact that both AF1 and AF2 activities are suppressed, blocking cofactor recruitment at the ERE site of estrogen responsive genes [21, 66, 67].

Another major group of endocrine therapies in routine clinical use are third-generation aromatase inhibitors (AIs), which comprise two drug types. Firstly, the irreversible steroidal inhibitors (type 1), including exemestane which are androstenedione analogues and secondly, the non-steroidal inhibitors (type 2), which include letrozole and anastrozole [1, 68–73] (Figures 1 and 2). AIs seek to disrupt estrogen signalling by either: irreversible and inactivating binding (type 1), or reversible and competitive binding (type 2) to the aromatase enzyme; thus significantly reducing local estrogen biosynthesis and hence intratumoural levels of estrogen [1, 74–76]. Indeed, in the adjuvant setting, letrozole, anastrozole and exemestane have been shown to be more effective than tamoxifen with a significant reduction in the rate of relapse [73, 77–79]. Endocrine therapies can also be used in the treatment of ER-positive breast cancer in premenopausal women where their use is usually combined with drugs such as goserelin (zoladex) to suppress ovarian estrogen production [80].

In postmenopausal women, adjuvant treatment represents the major clinical setting for endocrine therapy, where long-term adjuvant systemic treatment is targeted against micro-metastatic disease [2]. Indeed, several studies have reported overwhelming evidence of a high correlation between the adjuvant use of endocrine therapy and reduction

in the risk of recurrence [81]. Endocrine therapy can also have an important role in the neoadjuvant setting where systemic treatment may be indicated for 3 to 4 months prior to surgery in postmenopausal women with large and/or technically inoperable tumours. This treatment is intended to shrink the tumour so that, in locally advanced disease surgery becomes possible and in large operable breast cancers, breast conserving surgery (BCS) can be performed [2, 56]. The Immediate Preoperative Anastrozole or Combined with Tamoxifen (IMPACT) study was a phase 3 clinical trial which showed that 46% of 124 ER-positive postmenopausal women initially recommended for mastectomy became eligible for BCS following 3 months of neoadjuvant anastrozole [82]. Similar results were found in a study of exemestane in which 85% of 40 patients deemed unfit for BCS at diagnosis became eligible following 6 months of neoadjuvant therapy [83]. Indeed by measuring proliferation levels of malignant cells using the expression of nuclear antigen Ki67, studies have shown a reduction in proliferation in approximately 90% of ER-positive primary breast tumours treated with and responsive to AIs, confirming that these tumours derive significant proliferative stimulus from estrogen and that this can be potently suppressed by endocrine therapy [84, 85]. Endocrine therapy can also be used in the treatment of advanced or metastatic disease to prolong survival, either as a monotherapy or as part of a sequenced treatment regimen with chemotherapy, palliative surgery or radiotherapy [86].

5. Resistance

Despite the benefits of endocrine therapy in the treatment of ER-positive breast cancer, resistance to treatment eventually occurs in a large number of patients [2]. Clinically, resistance can manifest as relapse or cancer recurrence during or after completion of adjuvant therapy, following surgery or in rare cases after complete pathological response (elimination of all cancer tissue) following a period of drug therapy. Alternatively, in the neoadjuvant setting, resistance can be observed as clinical progression of primary disease, usually constituting an increase in primary tumour size or disease spread to regional nodes or beyond to more distant metastatic sites. Pathological changes such as increased tumour grade or increased proliferation are indicators of potential resistance to therapy. In the neoadjuvant setting, resistance occurs as either a primary lack of response (no change or an increase in tumour size and no evidence of pathological response) early in treatment, implying innate resistance, or later following a period of response, suggesting acquired resistance [87]. Studies have shown that recurrence on adjuvant endocrine therapy occurs in approximately 10–15% of patients with early stage ER-positive breast cancer within 5 years [88] and recurrence rates are as high as 30% by 15 years [77]. Others have suggested that as many as 40–50% of all ER-positive patients treated with adjuvant endocrine therapy will eventually relapse [89]. In the neoadjuvant setting, an observable clinical response occurs in 50–70% of patients [74, 83, 90]. The majority of patients with advanced or metastatic

ER-positive disease acquire resistance within 2-3 years of commencing endocrine therapy [91–93].

6. Studying Endocrine Therapy Resistance

Resistance to endocrine therapy has been investigated using a number of different approaches which extend from fundamental cell line studies in culture or as xenografts in immunosuppressed animals, to clinical adjuvant and neoadjuvant treatment studies [2].

6.1. Cell Line Studies. A number of cell line based studies, both as *in vitro* cultures or as *in vivo* xenografts, have been used to investigate endocrine therapy resistance and have involved endocrine therapy-treated breast cancer cells transfected with the aromatase gene (CYP19) [78, 94, 95]. Such studies have elucidated important findings and have advantages including the ability to assess dynamic changes with numerous time-points which is not practical in studies involving patients [96]. However, several limitations exist which include an inability to accurately model the heterogeneity known to be present between and within individual primary breast tumours due to their clonality. They are also unrepresentative of the local tumour microenvironment found in patients; in particular, they lack stromal and immune components [2]. Cell line models do not represent a realistic model to evaluate endocrine resistance as seen in clinical practice, as many of the therapies such as aromatase inhibitors, which in patients target the peripheral tissue sites of aromatase activity, cannot be reliably studied. Furthermore, many of the mechanisms identified in cell lines have been found to have no or limited clinical utility.

6.2. Adjuvant Setting. Investigations involving adjuvant treatment are limited because the primary tumour has been surgically removed and is thus not available for on-treatment molecular analysis. Furthermore, measuring the response to treatment relies on monitoring survival and disease recurrence. These require long-term follow up with carefully documented study outcomes which are difficult to monitor successfully, as recurrence may be the result of inherent cancer aggressiveness rather than acquired resistance to therapy. Additionally, for studies to produce meaningful results the patient sample size must be sufficiently large to be statistically valid and data collection on relevant outcomes will take considerable time and effort to acquire [2].

6.3. Neoadjuvant Setting. The neoadjuvant setting presents a number of advantages for investigating the characteristics of response and resistance. The primary tumour remains in place during treatment and clinical response can be determined by considering changes in tumour volume as measured by 3D ultrasound or mammography. In addition, tumours can be biopsied, often at multiple and sequential times, allowing for assessment of dynamic changes in gene expression or protein levels as treatment continues. These data can be related to clinical response allowing for a dynamic

comparison of clinical and molecular response in both responsive and resistant tumours [2, 74, 97, 98].

7. Mechanisms of Resistance

Several different resistance mechanisms have been described which set the basis for continued research aimed at improving the outcome of endocrine treatment. Response to endocrine therapy essentially manifests at a molecular level as a G1/S phase arrest in cell cycle, a feature which is lacking in resistance cancer cells [62] (Figure 3). The resistant phenotype is characterised by maintained/high expression of the cell cycle machinery genes including molecules such as: cyclin-D1 and cyclin-E1, essentially driving proliferation [99] often in tandem with pro-survival signals including high expression of anti-apoptotic proteins such as BCL2 and low levels of proapoptotic proteins such as BAK, BIK and caspase 9 [100, 101].

High expression of cyclin-D1 is associated with activation of CDK4 and CDK6 and progression to the S-phase of cell cycle. Studies have linked high expression of cyclin-D1 to tamoxifen resistance and high expression of cyclin-E1 to letrozole resistance [102, 103]. Another study identified a correlation between high expression of cyclin-E2 and resistance to tamoxifen [104]. Several investigations have also demonstrated the aberrant expression of other key players associated with the regulation of cell cycle including: C-MYC, RB1, p21 and P27KIP1, which have all been implicated with endocrine therapy resistance [49, 105–107]. While activity of the cell cycle machinery is central to the maintenance of proliferation in endocrine therapy-resistant cells, the underlying mechanisms which regulate and control the machinery, and hence resistance to therapy, are complex, highly heterogeneous, vary with regard to estrogen-dependence and remain poorly understood. Work to-date in this field has been geared towards predicting which patients are likely to have or acquire resistance to therapy and to stratify subgroups of high-risk patients, in a move towards personalised therapy, based on their underlying molecular characteristics of resistance so as to develop tailored, novel or combinatorial treatment protocols to circumvent resistance and improve outcome in the population as a whole.

7.1. ER Expression in Tumours with Innate Resistance. Studies have suggested that innate resistance may be linked to lower levels of ER, which might suggest that the drive to proliferation of these cancers is not as dependent on estrogen as those expressing higher levels of ER. The current use of the Allred score for assessing ER levels by IHC staining categorises all tumours with greater than 1% of positively stained cells as potentially ER-positive despite the enormous variation within that group and does not cope well with intratumoural heterogeneity, with parts of the tumour less ER-positive than others [108, 109]. However, rather than a mechanism explaining resistance this suggests that endocrine therapy alone may be not be an appropriate treatment option for these patients.

7.2. Progesterone Receptor. Clinically, the decision to treat with endocrine therapy is primarily based on ER status; however, IHC levels of progesterone receptor (PR) are also determined at diagnosis. The steroid hormone progesterone is the ligand for the PR and its binding causes restructuring with dimerization and dissociation of the complex to the nucleus where it binds DNA and modulates transcription. Estrogen signalling via the ER has been shown to upregulate the expression of the PR and thus the majority of ER-positive patients are also PR-positive. However, a subset is PR-negative, and some studies have linked this genotype with innate resistance. Indeed, tumours which are ER-positive and PR-negative display a poorer response to endocrine therapy and a more aggressive phenotype than ER-positive/PR-positive tumours, and some reports have shown that the ER-positive/PR-positive population has a significantly better prognosis compared with the ER-positive/PR-negative group [110]. Studies looking at differential chromosomal loss and gain between ER-positive/PR-negative and ER-positive/PR-positive tumours have demonstrated loss of regions containing genes associated with tumour suppression and apoptosis in the PR-negative genotype. Furthermore, gains have been identified in regions of PR-negative tumours which encode genes including: MAP3K3, RPS6KB1 and ZNF217. Amplification of these genes could lead to activation of the PI3K-AKT-mTOR pathway, which has been implicated with endocrine therapy resistance [111]. However, despite the better prognosis associated with the ER-positive/PR-positive genotype, some patients still fail to respond to endocrine therapy. A recent study suggested that this may be due to the progesterone mediated assembly and activation of a transcriptional enhanceosome complex involving AP1, STAT3, PR and HER2 at the cyclin-D1 promoter which drives breast cancer growth and development [112].

7.3. Estrogen Receptor

7.3.1. ER Posttranslational Modifications. A number of post-translational modifications of ER have been reported, including phosphorylation, methylation and sumoylation which influence its interaction with other members of the ER signalling pathway. It has been suggested that aberrations in the posttranslational modification of ER could be linked to endocrine therapy resistance [113, 114]. ER can be phosphorylated at a number of different sites including serine-118, serine-167 and threonine-311 within the AF1 binding domain as well as in other domains. Phosphorylation and activation of ER at key positions can result from a number of pathways including: the MAPK/ERK pathway in response to growth factors such as epidermal growth factor (EGF), the PI3K-AKT pathway in response to insulin-like growth factors and the p38-MAPK pathway in response to stress or various cytokines [115, 116]. As tamoxifen can still bind partially activated ER, overexpression and cross-talk between these pathways regulating ER activation might explain the partial agonist capabilities of the drug.

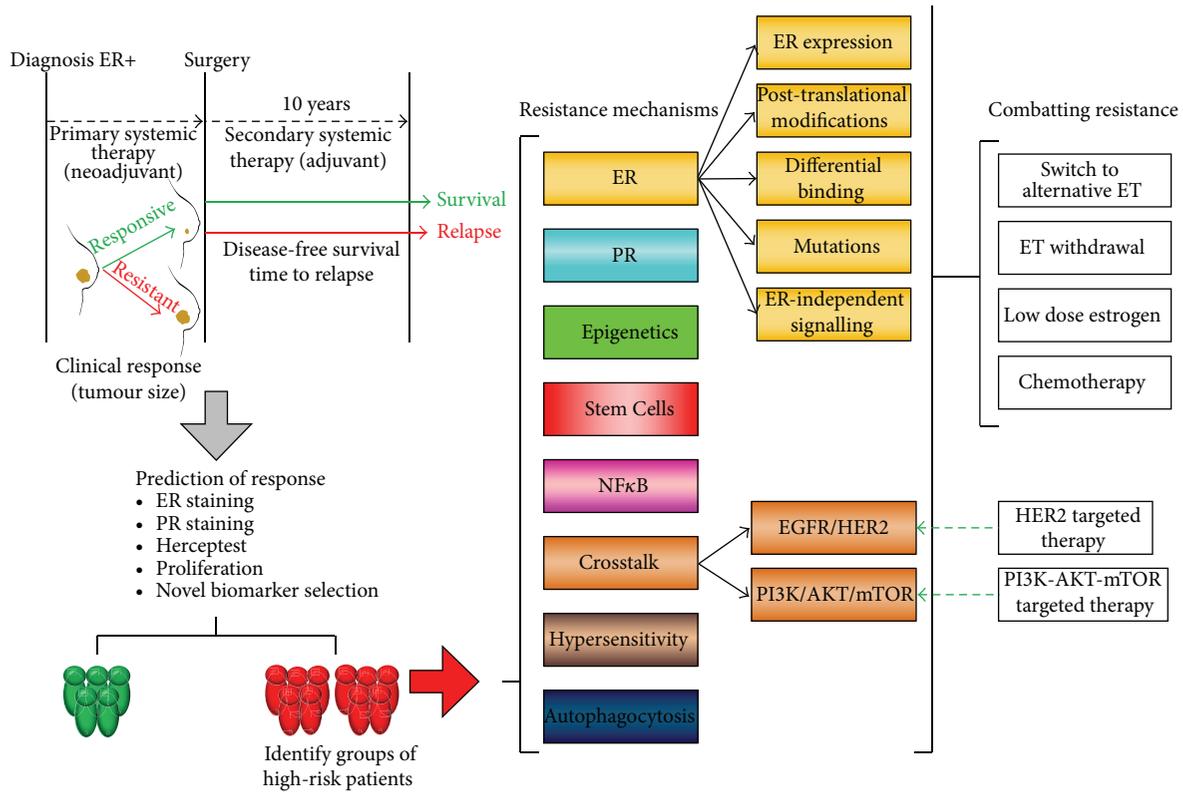


FIGURE 3: Summary of resistance in breast cancer showing the clinical manifestations of resistance in the neoadjuvant and adjuvant settings, the clinical need to accurately identify high risk patients, an overview of some of the best described resistance mechanisms and potential treatments and therapeutic strategies currently under investigation to combat resistance.

7.3.2. Differential ER Binding. A recent study looked at genome-wide ER binding events in primary breast tumours of patients sensitive and resistant to tamoxifen and revealed that in tamoxifen-resistant cancers ER is still recruited to the chromatin and binds regulatory regions in a pattern that is unique to resistant tumours [117]. The resistant phenotype may be due to selection and expansion of a resistant subpopulation of cells, or alternatively could involve the rapid reprogramming of ER binding by FOXA1, which has a known role in ER-chromatin interactions in response to growth stimuli [117–120]. Forkhead motifs and EREs were found to be enriched within the DNA regions which showed increased ER binding in tamoxifen-resistant cell lines and in primary tumour specimens of patients with a poor clinical outcome, providing further evidence for the FOXA1-mediated reprogramming model of ER binding [117]. These findings suggest that ER may have an important role to play in tamoxifen resistance by binding to a distinct set of regulatory elements giving rise to a unique gene expression profile which promotes tumour progression and confers resistance to therapy.

7.3.3. Activating Mutations in ER. A recent clinical sequencing study in patients with advanced ER-positive breast cancer identified a D538G mutation within ER in endocrine therapy resistant patients causing a change from aspartic acid to

glycine at position 538 within the ligand binding domain. Importantly, the mutation was found in distant metastatic sites but not in the primary tumour. The D538G mutant ER was found to confer constitutive ligand-independent transcriptional activity which mimicked that of estrogen-bound wild-type ER with reduced tamoxifen binding affinity. Overexpression of mutant ER was found to enhance proliferation and confer resistance to tamoxifen [121]. Similar studies have also identified additional ER mutations in the ligand-binding domain which also result in constitutive activity and may represent potential mechanisms for acquired endocrine therapy resistance [122, 123].

7.3.4. ER-Independent Signalling. Other reports suggest that innate resistance may be a feature of ER-positive tumours in which proliferation is regulated by an ER-independent signalling mechanism. Some studies have shown that acquired resistance can occur in tumours with low levels of ER resulting from loss of expression or mutations inactivating its encoding gene (ESR1), suggesting an ER-independent driving mechanism for proliferation [124, 125]. However, loss of ER is only seen in approximately 15–20% of resistant breast cancers and the incidence of inactivating ER mutations is even lower, with less than 1% of resistant cases reported having this genotype [126–129]. Alternative mechanisms involve expression of truncated isoforms of ER such as ER α 36 or other

estrogen-related receptors such as estrogen-related receptor gamma (ERR γ), both of which have been associated with reduced response to tamoxifen [130, 131]. Tamoxifen works by inactivating estrogen binding to wild-type ER, resistance in the case of ER α 36 overexpression could be a feature of lower binding affinity to the truncated isoform of the molecule. Studies have shown that expression of ER α 36 can be induced by BMP2, a member of the bone morphogenetic protein family of proteins which are known to have roles in regulation of cell fate and cancer development, suggesting a potential role for this molecule in endocrine therapy resistance [132]. Resistance related to ERR γ overexpression might suggest an important role for this molecule in an alternative estrogen signalling pathway [133, 134]. Furthermore, it should be noted that the estrogen receptor exists as two distinct isoforms: ER (ER α) and ER β . The exact role of ER β is not clear, however studies have shown that tamoxifen can bind ER β and that tamoxifen-bound ER β can activate API regulated genes, possibly by altering the balance of associated coactivators and corepressors at the promoter site [135, 136]. Indeed, increased ER β expression has been reported in tamoxifen resistant breast cancers and data from a recent study suggested that the ratio of ER α to ER β may be important in predicting response to tamoxifen and anastrozole in the neoadjuvant setting [137, 138].

7.4. Crosstalk with Growth Factor Signalling Pathways

7.4.1. EGFR and HER2 Signalling. Resistance to endocrine therapy is common in ER-positive breast cancers that overexpress HER2 [139]. Many studies have reported cross-talk between ER and receptor tyrosine kinases (RTKs) such as HER1 and HER2, which are receptors for epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF1, somatomedin 1) [140, 141]. Overexpression of these receptors suggests that tyrosine kinase signalling is driving proliferation and evasion of apoptosis in these cancers, representing either a primary mechanism in the case of innate resistant tumours or a switch in driving mechanisms to evade the action of endocrine therapy in tumours with acquired resistance [142, 143]. Studies have also shown that EGF signalling can lead to an EGF-induced ER with regulatory control, dependent on API, over a set of genes commonly overexpressed in HER2-positive breast cancer, which are distinct from those regulated by estrogen-induced ER [139]. These data suggest that proliferation may be driven by a distinct EGF-dependent mechanism which is independent of estrogen signalling. One recent study suggested that long-term endocrine therapy facilitates the translocation of GPR30 to the cell surface, promoting activity of the EGFR pathway [144]. One group has suggested that EGF-induced ER might arise from EGF signalling in response to soluble stromal factors including fibronectin and matrix metalloproteases, secreted into the tumour microenvironment by fibroblasts, which associate with tumour cell membrane-bound β 1 integrin, thus activating the PI3K/AKT pathway and MAPK/ERK pathway leading to ER phosphorylation and activation [145]. Alternatively, another study demonstrated

that dysfunctional TP53 can lead to activation of the EGFR pathway, thus decreasing estrogen dependence for proliferation [146]. Furthermore, this indicates that patients with ER-positive/HER2-positive breast cancer could benefit from endocrine therapy combined with immune therapy targeted at EGF receptors. This mechanism fits well with studies which have suggested that ligand-bound ER is associated with repression of EGFR and HER2 [147]. Depletion of estrogen by endocrine therapy could lead to expression of these molecules via loss of activated ER repression. Studies have shown that expression of a transcription factor known as PAX2 is associated with reduced incidence of endocrine therapy resistance due to its role in the ER-mediated repression of HER2 [148]. One study reported that the response of cells to tamoxifen is regulated by competition between the ER coactivator AIB1 and PAX2 binding to the *cis*-regulatory elements in intron 4 of HER2. Indeed, they showed that a decrease in expression of PAX2 in tamoxifen resistant cells correlated with an increase in HER2 expression. Furthermore immunohistochemical staining of tamoxifen treated-ER-positive breast cancer tumours showed that PAX2 expression in the absence of AIB1 correlated with recurrence-free survival (RFS) and a low rate of HER2 expression. Conversely, patients with higher expression levels of PAX2 and AIB1 had a higher rate of RFS [148, 149]. It should also be noted that the mechanisms behind overexpression of HER2 are complex and have been shown to involve genetic and epigenetic modification, as well as alterations in upstream regulators such as FOXP3 and transcription factor GATA4 [150, 151].

7.4.2. PI3K-AKT-mTOR Pathway and Somatic Mutations. PI3K is activated by growth factor receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). PI3K phosphorylates PIP2 to produce PIP3 which recruits several molecules such as PDK1 and AKT to the plasma membrane which, on activation, drive cell cycle progression and survival [152–154]. The pathway is negatively regulated by PTEN and INPP4B which dephosphorylate PIP3 and PIP2 respectively [155, 156]. AKT activates mTORC1 which regulates protein synthesis. The PI3K-AKT-mTOR pathway interacts with ER both directly and indirectly. AKT can phosphorylate ER, which increases estrogen-induced, tamoxifen-induced and ligand-independent ER transcriptional activity [157, 158]. In addition, PI3K promotes c-Jun phosphorylation, which complexes with c-Fos to form the AP-1 complex, known to be involved with ER transcription [159–161]. Studies have also shown that the PI3K pathway can be activated by HER2, the overexpression of which has been linked to a weaker response to endocrine therapy and poor prognosis following adjuvant therapy. In this model, activation of the PI3K pathway confers resistance to tamoxifen, fulvestrant and deprivation of estrogen [143, 157, 162]. Somatic mutations, which represent the most common in ER-positive breast cancer, have been described in key members of the PI3K-AKT-mTOR pathway including PI3KCA, PIK3CB, AKT1, AKT2, PTEN, and INPP4B, which have been implicated with aberrant activation and potential dependence on the pathway [163]. Interestingly,

a recent study showed increased expression of key PI3K-AKT-mTOR pathway members including phosphorylated mTOR, 4EBP1, and P70S6 in metastatic sites compared with primary sites in patients who received adjuvant endocrine therapy, as opposed to no difference in expression in an untreated cohort, suggesting the compensatory activation of the PI3K-AKT-mTOR pathway as a possible mechanism leading to acquired resistance [164].

7.5. NF κ B Signalling and Inflammation. NF κ B plays an important role in processes such as cell survival and proliferation [165]. It can promote proliferation through regulation of key cell cycle genes including cyclins and CDKs and can mediate growth and survival signals via the PI3K-AKT-mTOR pathway [166, 167]. Additionally, NF κ B has been shown to have a role in blocking apoptosis through cross-talk with ER and regulation of the BIRC3 gene [168]. NF κ B has been reported as overexpressed in some endocrine therapy resistant breast tumours and many groups have alluded to its role in endocrine therapy resistance [169–175]. Some investigations have reported cross-talk between ER and NF κ B signalling in which cooperative binding to transcriptional response elements can lead to specific gene expression [176]. Conversely, ER has also been shown to inhibit NF κ B via a mechanism involving displacement of NF κ B coregulators such as CBP at NF κ B response element sites [177]. One study suggested the involvement of TGF β -activated TAB2 in tamoxifen resistance in which a phosphorylated, active form of TAB2 exports the corepressor protein NCoR from the nucleus, thus translocating it from EREs, resulting in loss of response to tamoxifen [178]. TAB2 has also been implicated in the activation of NF κ B via the IKK complex in response to IL1 stimulation [179].

Alterations in the NF κ B cascade have also been identified in endocrine therapy resistant cells. In tamoxifen resistant cells, expression of the NF κ B subunit p50 was increased. Nevertheless, it remained unchanged in fulvestrant resistant cells compared with the sensitive cells. Conversely, expression of the p65 subunit was found to be increased in fulvestrant resistant cells but remained unchanged in tamoxifen resistant compared with sensitive cells. The most abundant form of NF κ B is the p50-p65 heterodimer and these findings suggest that resistant cells may utilise different strategies for upregulating the activity of this molecule [165]. Furthermore, the phosphorylation levels of p65 at its serine-536 site were found to be increased in endocrine therapy resistant cells. Phosphorylation at this site is necessary for optimal activity of the molecule and was shown to enhance its transactivation potential [180].

Some risk factors identified for breast cancer, including increased age and menopause, are associated with increases in indicators of systemic inflammation such as higher levels of circulating proinflammatory cytokines [181, 182]. Other risk factors such as pregnancy and obesity have been linked to the promotion and maintenance of a local inflammatory microenvironment in the breast [183]. Tumour associated macrophages (TAMs) have been found to comprise up to 50% of a breast tumour mass in some patients. Indeed, increased

macrophage infiltration in breast tumours has been positively correlated with increased angiogenesis as well as reduced relapse-free and overall survival [184, 185]. One study in which TAMs were cocultured with ER-positive breast cancer cells revealed an increase in cancer cell invasiveness compared with cultures with no TAMs present. This was reported to involve a mechanism in which an inflammatory cytokine known as TNF α , produced by macrophages, led to activation of NF κ B and JNK pathways [186]. Indeed, high expression of the classic macrophage marker CD68 in breast cancer is associated with poor prognosis and lack of response to endocrine therapy [187]. Furthermore, increased circulating levels of TNF α have been associated with advanced tumour stage, lymph node metastasis and local invasion [188, 189]. TNF α has been shown to stimulate proliferation in some ER-positive cell lines through increased expression of cyclin-D1 by a mechanism dependent on NF κ B [190, 191]. Upon TNF α stimulation the p65 NF κ B subunit is phosphorylated at serine-536 via the IKK complex. Following IL1 stimulation the PI3K-AKT pathway mediates phosphorylation of p65 at serine-536 via an unknown mechanism, although TBK, IKK, and p38 have all been implicated with IL1-induced p65 phosphorylation at serine-536 [192, 193]. TNF α -induced transcriptional activity of NF κ B has been found to be significantly increased in endocrine therapy resistant cells compared to sensitive cells. This is thought to be due to the increased expression of NF κ B subunits and increased levels of p65 phosphorylation. Interestingly, PR also has a known anti-inflammatory role in breast cancer cells and the loss of its expression in a subset of endocrine therapy resistant ER-positive cell lines which overexpress NF κ B has been reported [194, 195].

7.6. Breast Cancer Stem Cells. There is now a large body of evidence suggesting an important role for stem cells in the development of breast tissue and that cancer stem cells (CSCs) can be found in breast cancers [196]. In breast cancer these undifferentiated, clonogenic cells are linked to increased invasive and metastatic phenotype. However, their frequency is dependent on tumour grade, disease, stage and molecular subtype [197–201]. Normal breast stem cells are thought to be basal-like and mainly ER-negative. Consequently, it is thought that CSC development is not influenced greatly by hormones such as estrogen and that these cells may be resistant to endocrine therapy as a result of low ER expression, with any partial response attributed to paracrine signalling from nearby differentiated ER-positive tumour cells. Normal breast stem cells are regulated by EGF receptor and other growth factor receptor signalling. Some groups have suggested that the observed increase in EGF receptor expression in endocrine therapy resistant tumours may reflect a greater proportion of CSCs selected by endocrine therapy [196]. Furthermore, one study showed that letrozole-treated tumours appeared to have an expression signature that was more like “claudin-low” subgroup of tumours originally described by Perou et al. (2000) and that posttreatment tumours were enriched for stem cells compared to pretreatment samples [202, 203]. A recent

study reported a mechanism for tamoxifen resistance which involved the SOX2-dependent activation of Wnt signalling in CSCs. Silencing of the SOX2 gene reduced the CSC population and restored sensitivity to tamoxifen as did inhibition of the Wnt signalling pathway [204].

7.7. Hypersensitivity to Residual Estrogen. Hypersensitivity to estrogen has also been suggested as a potential mechanism of endocrine therapy resistance [1]. Aromatase inhibitors function to dramatically reduce estrogen biosynthesis, although residual amounts of estrogen remain in tissues [205]. Cell line studies have shown that after prolonged deprivation of estrogen some cells develop hypersensitivity to residual estrogen. Reports have suggested that this phenomenon is associated with an increase in expression of HER2 and subsequent overactivity of the MAPK pathway resulting in changes in the phosphorylation of ER conferring its hypersensitivity to residual estrogen [206, 207]. Other researches have indicated that mutations in ESR1 may give rise to mutated ER which has increased interactions with the protooncogene tyrosine-protein kinase (SRC) family of coactivators and changes in promoter binding dynamics linked to hypersensitivity to estrogen [208].

7.8. Epithelial-Mesenchymal Transition. Epithelial-mesenchymal transition (EMT) is a morphological change which has been demonstrated in some epithelial tumours [209, 210]. EMT is associated with a loss of differentiation and loss of intracellular adhesion, a feature of an invasive phenotype characterising advanced metastatic disease [211–213]. Intracellular adhesion is an important feature of tissue architecture maintenance and can limit cell movement and proliferation. It is primarily mediated through the adherens junction (AJ) which are complexes of calcium-dependent transmembrane cadherin receptors, the cytoplasmic domains of which link to the actin cytoskeleton via α -catenin and β -catenin [214]. One study reported that development of tamoxifen resistance in cell lines is associated with an enhanced motile and invasive phenotype characterised by loss of intracellular adhesion and partial EMT. This phenomenon is thought to be brought about by the EGF-signalling mediated activation of β -catenin and the subsequent increased expression of β -catenin regulated genes known to be involved in tumour progression. Inhibition of EGF signalling in the same cells reduced β -catenin activity and promoted intracellular adhesion [215]. This suggests a possible role for EGF signalling (involving β -catenin) in the manifestation of an aggressive phenotype of endocrine therapy resistant tumours. In another study, overexpression of the transcription factor zinc finger protein SNAI1 (Snail) resulted in EMT. In this case ER expression was also lost as a result of Snail binding to the promoter region of ESR1 and causing deacetylation of histone H3K9 [216]. This represents a potential EMT-associated mechanism by which endocrine resistance could develop and might suggest the involvement of epigenetic mechanisms.

7.9. Epigenetics. Epigenetic mechanisms including DNA methylation have been shown to be responsible for determining and maintaining cell fate and for the stable differentiation of cells [217]. An increasing body of evidence is building to suggest an important role for DNA methylation in cancer including the silencing of tumour suppressing genes, activation of oncogenes, and promotion of metastasis [218–220]. More recently, changes in DNA methylation have been linked to endocrine therapy resistance [221, 222]. One study described a correlation between silencing of the promoter region of ER by methylation and reduction in ER expression as a potential mechanism leading to resistance [223]. Studies comparing endocrine therapy (tamoxifen or fulvestrant) resistant and sensitive cell lines have identified a pattern of methylation characterised by promoter hypomethylation in the resistant cell line compared with the sensitive [221]. This mechanism of hypomethylation was further outlined in a report showing the development of tamoxifen resistance in sensitive cell lines treated with a DNA methylation inhibitor (5-azacytidine) [222]. One study also suggested that the hypermethylation of the ER β gene is associated with tamoxifen resistance [224]. Additionally, it has been shown that ER itself participates in epigenetic control. When ER binds EREs within the genome it recruits cofactors involved in epigenetic control including: NCOR1, NCOR2, SRC1, and AIB1 [225, 226]. Tamoxifen resistance has been linked with dysregulation of these cofactors suggesting a possible role for epigenetic mechanisms in endocrine resistance. Indeed, a recent study suggested that a novel concept that prolonged tamoxifen exposure could induce epigenetic silencing of a set of estrogen responsive genes with functions linked to the negative control of proliferation [227].

7.10. Autophagocytosis. Autophagocytosis is a cellular catabolic degradation process involving the lysosomal machinery whereby aggregated proteins, unfolded or misfolded proteins, or damaged subcellular organelles are degraded in response to stress or nutrient deprivation in an attempt to restore metabolic homeostasis. While the role of autophagocytosis in endocrine therapy resistance remains poorly understood, the process is known to be both pro-survival and pro-death with the final cell fate dependent on its extent and duration [228, 229]. Some studies have shown that inhibition of autophagocytosis can resensitise resistant cells to tamoxifen suggesting that it might have a role in resistance in some cancers [228]. One study identified a protein known as HSBP8 as having a role in regulating autophagocytosis in endocrine therapy resistant cells [230].

8. Predicting Response to Endocrine Therapy

There is an urgent need to identify early on treatment those patients who are unlikely to gain any benefit from endocrine therapy, thus sparing them from prolonged periods of ineffectual and redundant therapy and possibly exposing them to high risk side-effects. This is prudent in neoadjuvant treatment, where the aim is to down stage large or locally advanced tumours in order that they become operable or less

extensive surgery becomes possible; and in the adjuvant setting, to identify patients who would benefit from additional or alternative therapies following relapsed disease (Figure 3).

8.1. Pathological and Clinical Response. Following three to four months of neoadjuvant endocrine therapy, 60–80% of tumours demonstrate signs of pathological response. This usually includes both a decrease in overall tumour volume, and at a pathological level, a decrease in tumour cellularity with an increase in fibrosis or formation of fibrous connective tissue, and in some cases, a decrease in histological grade [231, 232]. Complete pathological response (CPR) to endocrine therapy is rare, but the incidence of CPR increases with increasing length of treatment [233]. It should be noted that quantitative measurements of partial pathological response rely on subjective assessment without robust formal criteria, and agreement between pathologists is variable; reported to be in the range of 50–86% [234, 235]. Indeed, whilst the clinical significance of tumour grade is well recognised, almost half of all tumours are histologically classified as grade 2, and interobserver reproducibility of grade is lacking, although there has been great effort to improve reproducibility [89]. A number of pathologically responding tumours decrease in size and this constitutes the clinically used definition “clinically responsive” [2]. Indeed, clinical and pathological response correlates significantly in the majority of tumours [233]. However, approximately 20% of tumours are discordant in this respect, showing either a clinical response (decrease in overall tumour volume) without evidence of pathological response or demonstrating pathological evidence of response whilst not reducing in volume [235, 236]. These clinical and pathological determinants of response require repeated clinical or radiographical measurements and biopsy histological assessment. Whilst they offer an effective end-point for categorising overall response, they often do not manifest soon enough to be suitable for predicting, early-on-treatment, which tumours are likely to respond or otherwise to endocrine therapy [2].

8.2. Proliferative Response. Significant decreases in proliferation are seen in approximately 80% of ER-positive tumours after 3 months of endocrine therapy and can be seen as early as 10–14 days of treatment in some tumours. Levels of proliferation are routinely established by measuring changes in expression of the protein Ki67. However, some tumours display variable patterns of Ki67 expression such as an initial reduction followed by recovery to pretreatment levels, a delayed change, and in some cases little difference over a treatment period [236]. Ki67, discovered in 1989, is a nuclear nonhistone protein which was reported to be universally expressed in proliferating tissues and to be absent in quiescent cells, establishing it as a marker of proliferation. More recently however we have learned that expression of Ki67 varies throughout the cell cycle which could influence the identification of proliferating cells [237]. Indeed some studies have demonstrated Ki67 expression in the G1 phase of cell cycle to be minimal [238, 239]. That being said, Ki67 expression has been shown to correlate positively with alternative markers

of proliferation such as proliferating nuclear antigen and minichromosome maintenance protein 2 (MCM2) [240, 241]. Whilst the association between Ki67 and response to neoadjuvant chemotherapy has been demonstrated [242, 243], no significant correlation has been reported with neoadjuvant endocrine therapy [244–246]. Indeed, whilst an early reduction in proliferation is positively and significantly correlated with both clinical and pathological response (sensitivity) to endocrine therapy, there is discordance in a number of cases, and so this alone lacks specificity in determining which cancers are resistant to endocrine therapy [236, 247].

8.3. Molecular Response Markers. The most commonly used predictive molecular marker for endocrine therapy is ER. It has a strong negative predictive value with ER-negative tumours hardly, if ever, responding to endocrine therapy and around 50–70% of ER-positive tumours responding well. However, still around 30–50% of ER-positive patients will exhibit innate or acquired resistance to endocrine therapy [87]. Another commonly used molecular marker is PR. This molecule is regarded as a classical marker of estrogenic activity and is reduced in 70–80% of cases treated with endocrine therapy [233, 236]. However, loss of PR expression can occur independently of clinical and pathological response [236, 248]. Indeed, studies have shown increased response rates in PR-positive tumours compared with PR-negative tumours; however response to endocrine therapy can occur in both [248–250]. A recent study which sought to determine the prognostic significance of PR reported that, in multivariate models including ER and other standard clinicopathological features, PR did not contribute significant prognostic information [251]. Similar results were found with other classical markers of estrogenic activity including pS2 [248]. It has been shown that ER-positive tumours overexpressing HER2 are less likely to respond to endocrine therapy, although the number of such cases is small and they do not account for all resistant cases [252, 253]. The use of single molecular markers to predict response is far from adequate given their poor correlation with clinical and pathological response [254, 255]. Even expression of ER, clinically the most widely used molecular marker, has only around 50–70% accuracy in endocrine therapy response prediction in ER-positive cases.

8.4. Multigene Signatures

8.4.1. Gene Expression Profiling. The introduction of high-throughput gene expression profiling technologies, such as gene expression microarrays, applied to translational research has revolutionised the way breast cancer is understood, highlighting the importance of heterogeneity and the fact that distinct molecular subtypes of the disease exist which can affect the same anatomical site [256]. Indeed, much research has focused on the development of multigene signatures using high-throughput gene expression microarray technology, which have been used for molecular subtype classification, confirming the importance of key disease drivers such as ER and HER2 signalling, and for prognosis [256–258]. A number of studies have focused on identifying

subsets of patients with a favourable prognosis, in whom the absolute benefit of systemic adjuvant chemotherapy is small compared with associated toxicity, in order that they could forgo this treatment [259]. Mammaprint and Oncotype DX are two such prognostic signatures available for clinical use, both developed to estimate recurrence risk in node-negative early breast cancer [187, 260, 261].

8.4.2. Commercial Profiling: Prognostic Assays. The 70-gene Mammaprint test uses fresh tissue and microarray technology to evaluate expression of genes associated with proliferation, invasion, metastasis, tumour stroma, and angiogenesis in both ER-positive and ER-negative cancers [261]. It was given Food and Drug Administration (FDA) approval in 2007 to be used as a prognostic predictor (indicating risk of relapse within 5 years) for breast cancer patients aged under 61 years of age, with lymph node negative stage 1 or 2 tumours up to 5 cm in size. Those patients with poor prognosis and ER+ tumours are recommended to receive adjuvant chemotherapy in combination with endocrine therapy, whereas those with favourable prognosis and ER-positive tumours are recommended to receive only adjuvant endocrine therapy [256]. It should be noted that Mammaprint is of limited clinical use in ER-negative breast cancer with only 0–4% of such patients predicted to have a good prognosis [262–266]. An independent validation of the Mammaprint signature demonstrated that prognostic accuracy is highly time-dependent and may be more suitable as a predictor of early relapse [261, 265]. Furthermore, no clinical validation studies of the Mammaprint signature have been performed in randomised trial populations [267–269].

The 21-gene Oncotype DX test uses “formalin fixed paraffin embedded” (FFPE) tissue with quantitative real-time PCR (qRT-PCR) to measure expression of 16 genes associated with proliferation, estrogen regulation, HER2, and invasion in hormone receptor positive cancers, as well as 5 reference genes [187, 260, 270]. The test outcome is presented as a recurrence score (RS) ranging from 0 to 100 to predict the risk of 10 year distant recurrence. In clinical use the RS is subdivided into three groups: low (<18), intermediate (18–31), and high (>31), and several publications have shown that ER-positive breast cancer patients with low RS have a low risk of recurrence and derive little benefit from chemotherapy, whereas the reverse is true for those with high RS [270–274]. According to the National Comprehensive Cancer Network (NCCN) guidelines for breast cancer treatment, patients with a low RS should receive endocrine therapy alone, whilst those with a high RS should also receive additional chemotherapy. However, the ideal clinical management strategy for those with an intermediate RS remains at present unclear; emphasising a considerable limitation with the use of Oncotype DX [256].

More recently, a microarray diagnostic test known as MapQuant DX was launched to accurately measure tumour grade and risk of metastasis, predict response to chemotherapy, and indicate proliferation. It measures expression of a published 97-gene signature to determine a “genomic grade index” (GGI) [275–278]. When applied to an independent

validation cohort the GGI had strong association with histological grade, although 9% of grade 1 tumours were classified as having high GGI and 14% of grade 3 tumours were classified as having low GGI. However, GGI was found to be more strongly associated with relapse free survival than histological assessment of grade. Furthermore, GGI was able to stratify histological grade 2 tumours into prognostically significant high or low grade groups. In independent validation studies GGI has also been shown to be independently prognostic of outcome (risk of recurrence) in tamoxifen treated patients. Tumours with high GGI profiles were also reported to respond well to neoadjuvant chemotherapy despite having a significantly worse outcome than low GGI tumours [275]. The MapQuant DX test was recently converted to an 8 gene assay (PCR-GGI) based on quantitative real-time PCR (qRT-PCR) measurements [279]. While the evidence for genomic grading is compelling, independent validation of the MapQuant DX and PCR-GGI systems has not yet been documented. Furthermore, their ability to discriminate between high and low grade in ER-negative tumours is limited [280, 281].

Theros is a qRT-PCR based assay designed for lymph node negative ER-positive breast cancer which has been treated with surgery alone. The test is reported to assess risk of recurrence and benefit from endocrine therapy. The test measures the ratio of expression of homeobox gene HOXB13 to interleukin IL17BR [256]. It has been shown to identify ER-positive tamoxifen-treated patients with a high risk of recurrence and predict outcome in ER-positive patients, either adjuvant systemic therapy naïve or tamoxifen treated. However, its ability to predict benefit from neoadjuvant hormone therapy or from chemotherapy remains to be seen [282, 283]. Furthermore, its use has limitations in lymph node positive disease [284]. It should also be noted that neither the MapQuant DX/PCR-GGI nor Theros tests have been included in the NCCN guidelines for breast cancer treatment [256].

8.4.3. Prognostic Signature Research. Several other gene signatures, which are not as yet commercially available, have also been reported in an attempt to refine tumour classification and improve prognostication.

The “sensitivity to endocrine therapy” (SET) index comprises a signature of 165 genes coexpressed with ER in 437 patients (unrelated to treatment or outcome). The association of the signature with distant relapse risk was evaluated in 5 independent validation cohorts: 2 cohorts ($n = 225$ and 298) of patients who received adjuvant endocrine therapy, a cohort who received adjuvant chemotherapy followed by endocrine therapy ($n = 122$), and two cohorts who received neither adjuvant endocrine nor chemotherapy ($n = 208$ and 133). The SET index was found to be significantly associated with distant relapse and risk of death in endocrine therapy and chemotherapy plus endocrine therapy treated cohorts, but not in systemic therapy naïve cohorts. Whilst this signature has been extensively validated in independent cohorts and been shown to predict survival benefit, there is as yet no evidence suggesting accurate prediction of response to therapy or inherent prognosis [285].

More recently a further PCR based assay—EndoPredict (EP), has been validated to predict the likelihood of distant recurrence in patients with ER-positive, HER2-negative breast cancer treated with adjuvant endocrine therapy. The test uses RNA levels of a panel of 8 genes (plus 3 reference genes), as determined from RT-PCR of FFPE tissue, which are used to calculate an EP score. A combination of EP score, nodal status, and HER2 status is used to calculate a comprehensive risk score: EPclin. Using this, 58%–68% of women from two large phase III trials, who were classified as having high/intermediate risk of recurrence according to clinical guidelines, were predicted to have a low recurrence risk based on their EPclin score. The rate of occurrence of distant metastases in this group was found to be only 5%. Further validation work has shown reproducible performance of the assay and negligible interlaboratory variation. The authors suggest that the EPclin score may be used to identify ER-positive postmenopausal women with a limited number of clinical risk factors who may not benefit from adjuvant chemotherapy [286–289]. EPclin does show promise in identifying subgroups of patients with a low risk of recurrence which may out-perform currently utilised clinical guidelines; however there is once again no evidence, as yet, to suggest that it can predict response to therapy or inherent prognosis. Furthermore, as with other tests such as Oncotype DX, patients' risk is defined with a score on a continuous scale and the optimal clinical management approach for those with intermediate scores remains unclear.

8.5. Lessons from Prognostic Signature Research. Numerous prognostic signatures have been proposed beyond those already discussed including signatures assessing: amplification of cyclin-D1, inactivation of p53, activation of PI3K pathway, MAPK cascade, and prediction of invasion to name a few [290–295]. Most prognostic signatures have significant agreement in their prediction of outcome and identification of similar groups of patients despite the fact that the overlap of individual gene lists is negligible [280, 281, 296–302]. Meta-analysis of different prognostic signatures, looking at genes, pathways, and networks revealed that classification of patients into favourable or poor prognostic groups relies heavily on the expression of proliferation-associated genes. Indeed, some signatures were found to perform better when only proliferation genes were used to predict prognosis [281]. Furthermore, this analysis confirmed that the prognostic power of most signatures, particularly the four commercially available assays (Mammprint, Oncotype DX, MapQuant DX, and Theros) was limited to the ER-positive/HER2-negative subgroup of breast cancers, providing more evidence for proliferation as the key determinant of prognosis in this subgroup [280, 281]. The study and use of prognostic signatures has disclosed important heterogeneity features of breast cancer, including the fact that within all ER-positive disease arises a spectrum of tumours ranging from low proliferative favourable outcome tumours to highly proliferative poor outcome disease. ER-negative tumours conversely represent distinct entities, driven by distinct molecular aberrations, whose prognosis may not be determinable using proliferation

gene based signatures [273, 303, 304]. Indeed, recent work has suggested the importance of immune response genes in the prediction of outcome in ER-negative breast cancer [305–307].

8.6. Predicting Response to Endocrine Therapy. Unlike prognostic signatures, relating gene expression to risk of relapse; predictive signatures determine features associated with response, or lack of it, to a particular therapy [256]. Such research is aimed at determining the ideal systemic therapy and the magnitude of benefit derived from it for individual patients, in a move towards personalised therapy [256, 296].

A number of predictive multigene signatures have been reported which directly relate gene expression to the clinical and pathological response to endocrine therapy in ER-positive patients, the majority of which have not yet been validated for clinical use. One of the first signatures developed for endocrine therapy came from a study to predict clinical response to tamoxifen in patients with recurrent disease (local/regional relapse or distant metastasis). Gene expression profiles of primary tumour samples from 112 patients who later recurred and received tamoxifen were derived. Of the 112 patients, 52 responded with an objective decrease in size of recurrent mass and 60 had progressive disease. A 44-gene signature was derived, which included genes associated with estrogenic regulation, apoptosis, extracellular matrix remodelling, and immune response. The signature was reported to predict endocrine therapy outcome and time to progression in ER-positive patients with recurrent disease. In independent validation the gene signature was shown to predict response to tamoxifen in 77% of patients, outperforming the commonly used ER marker which predicts response in 50–60% of metastatic patients [308]. However, it should be noted that this signature has not yet been shown to be of significant predictive value in patients with early stage disease.

One of the first gene expression studies focused on response to aromatase inhibitors was published in 2007 by Mackay et al. [309] and reported response to letrozole and anastrozole during a short preoperative (14 days) treatment period using sequential biopsies from 34 patients. The study revealed that short term estrogen deprivation by aromatase inhibition led to profound changes in transcriptomic profile, including genes associated with proliferation and estrogen signalling. While many of the changed genes reported had been previously identified in cell line studies, many additional estrogen responsive genes were identified in this study. Importantly, the study revealed complex changes in matrix remodelling and stromal interactions which cannot be easily studied in cell lines. The reported response gene changes were integrated into a global index of dependence on estrogen (GIDE), a measure of the number of genes with at least a 2-fold change on treatment. The GIDE was found to be significantly associated with on-treatment changes in Ki67 and with pretreatment levels of HER2 [309]. While time-course studies in cell lines have revealed much about the response to estrogen deprivation, cell lines are not representative of the *in vivo* tumour microenvironment and may

not be an ideal model for investigating endocrine therapy resistance. This pilot study was one of the first to use multiple biopsies from the same patient, allowing for an assessment of changes in gene expression rather than static expression levels at a given time point. By comparing on-treatment with pretreatment in the same patient, this valuable approach has allowed for identification of key genes which are consistently changed over a number of patients as a direct result of a given therapy. While the design of this study is novel and may have potential for determining key predictive genes associated with sensitivity or resistance to endocrine therapy, several limitations are apparent. Firstly, the study is underpowered with only 34 patients, and no independent validation was performed to confirm the findings. Furthermore, given that the study was based on only 14 days of treatment prior to surgery, clinical response could not be directly evaluated, as changes in tumour size take longer than 14 days to manifest. Instead, changes in immunohistochemical Ki67 were used as a surrogate for clinical response, which despite correlation with both clinical and pathological response, has been shown to be a suboptimal predictor of response due to discordancy in some patients. As a result, there is currently no conclusive evidence demonstrating the predictive capacity of this signature.

In 2007, Miller et al. [97] published a similar study designed to investigate changes in gene expression associated with short-term neoadjuvant letrozole therapy also using pretreatment and on-treatment (14 days) biopsies from the same patient. From gene expression profiling of 58 patients, 143 genes were identified which were consistently changed between pretreatment and 14 days biopsies. Using the most significantly and consistently changed genes, patients were stratified into 4 distinct molecular groups by hierarchical clustering, reinforcing the concept of heterogeneity of response [97]. However, the clinical significance of the molecular subgroups remains to be seen. The molecular changes observed in both Mackay et al. [309] and Miller et al. [97] studies were largely consistent, with aromatase inhibitor treatment leading to suppressed expression of genes associated with proliferation and estrogenic signalling and increased expression of genes involved with stromal remodelling, cell adhesion, and immune response [2]. Importantly, these changes were identifiable within two weeks of treatment, long before clinical changes and pathological changes in morphology could be seen, raising the potential for determining early-on-treatment which patients are likely to respond to and adapting therapeutic protocols in resistant patients.

In 2008, Harvell et al. [310, 311] published one of the first gene expression signatures reported to discriminate between tumours clinically responsive and non-responsive to endocrine therapy, which used clinical response after extended therapy (4 months) as the end-point criteria. They published a 50 gene signature which was later refined to a 25 gene signature based on pretreatment gene expression levels. However, the signature was based on expression profiles from only 6 patients, (3 responsive and 3 non-responsive), rendering the study significantly underpowered. In addition, no independent validation of their findings was carried out.

In 2009, Miller et al. [74] published the largest study of its type at the time in which they presented a gene expression signature reported to discriminate between tumours clinically responsive and nonresponsive to aromatase inhibitors. Findings were based on the same microarray gene expression dataset used in their 2007 publication [97]. Neoadjuvant clinical response to letrozole in 58 patients was evaluated by changes in 3D ultrasound measurements taken over a 3 month treatment period (immediately prior to surgery), giving rise to 37 responding tumours and 15 showing lack of response. Gene expression analysis revealed 205 covariables consistently differentially expressed between clinically responding and resistant tumours, which distinguished between the two response groups. Of the 205 genes, 69 were differentially expressed in pretreatment samples, 45 were differentially expressed in 14-day samples, and 91 were significantly different when considering expression changes between pretreatment and 14-day samples. Hierarchical clustering based on 205 genes separated clinically responding and resistant tumours into two distinct groups. The most predictive genes were found to be associated with protein biosynthesis, in particular, ribosomal proteins. Interestingly, changes in proliferation associated genes and estrogenic signalling genes were found to occur in both clinically responding and nonresponding cases [74]. The major limitation of this study is the lack of validation of predictive capacity in an independent cohort of patients which as yet has not been reported. In addition, the assessment of clinical response (at least 50% reduction in tumour volume by 3 months) was an arbitrary threshold and does not allow for a satisfactory and clear differentiation between clinically responsive and resistant tumours; for example, a tumour with a reduction of 51% would be classed as clinically responding whereas a tumour with a 50% reduction would be resistant.

8.7. Considerations When Developing Predictive Gene Signatures

8.7.1. Sample Size and Patient Heterogeneity. A number of multigene signatures have been reported to predict subgroups of ER-positive patients unlikely to respond to endocrine therapy. However, many of these studies are underpowered, with findings based on relatively small numbers of patients in the training set and as a result may not be representative of the population [312]. When considering gene signatures derived from a single time point, such as before treatment, this problem may be confounded by patient heterogeneity (variables such as age, BMI, tumour size, tumour grade, tumour histological subtype, lymph node involvement, metastasis, additional medical conditions or drug regimens, and inherent genetic differences). By considering consistent changes in gene expression between sequential biopsies (before and after/during treatment) from the same patient in a pairwise fashion, the issues of patient heterogeneity can be somewhat minimised and significantly changed genes are more likely to be directly related to treatment response. Nevertheless, for statistical validity it is desirable to recruit the largest number and most representative patients as possible.

8.7.2. Microarray Bias. The predictive capacity of many reported signatures is often not reproducible in external datasets [258]. This can be in part due to the small unrepresentative sample size in the original training set, although, it can also be related to issues of microarray bias. Several studies have alluded to microarray bias as a major contributory factor affecting the reproducibility of microarray data derived results [258, 312, 313]. Bias can occur at all stages of microarray experiments from patient selection and sample processing to choice of microarray platform. Bias of this nature can lead to results becoming dataset-specific. Some studies have reported methods which can lead to a reduction in microarray bias, which have been shown to significantly improve reproducibility [312, 313].

8.7.3. Independent Validation. A major limitation of published predictive gene signatures is a lack of independent validation. This is essential to confirm that the reported findings are real, strongly associated with clinical outcome and that they out-perform or improve upon currently clinically used predictors of response. Successful validation is an essential first step if any new predictive signatures are to be endorsed in the clinic to improve patient care [257]. Furthermore, most predictive signatures are derived from microarray data, which should be considered a surrogate for gene expression, implying a further need for validation of results in external independent datasets and by alternative methods for measuring gene expression such as qRT-PCR. Independent validation is often carried out using publically available external datasets which may or may not be similar in design to the pilot study. Important factors for consideration when selecting an appropriate validation study for endocrine therapy response might include: the patient cohort (age range, BMI range, and menopausal status), disease features of the cohort (tumour size, spread to nodes, metastasis, and ER/PR/HER2 status), treatment (drug, length of treatment window), and assessment criteria used for response. With this in mind, it should be noted that, while novel experimental designs, such as gene expression profiling of multiple biopsies taken from the same patients over a period of treatment, are promising and may yield important information, a lack of similar external experimental datasets with which to validate findings is likely.

8.7.4. Response Criteria. Predictive multigene signatures for neoadjuvant response to therapy are designed by comparing differences in gene expression, or differences in changed gene expression, between tumours defined as responsive or non-responsive. The predictive capacity of such signatures is highly dependent on the criteria used for response assessment. Such criteria include clinical, pathological, and molecular assessment of response and, while these show reasonable concordance, they do not agree for every case. Therefore, if the aim of a neoadjuvant predictive signature is based primarily on the clinical need to reduce tumour size sufficiently to allow either surgery if the cancer was inoperable or breast conservation if it was operable only by mastectomy, then it would seem logical to use clinical response assessment (as

determined by changes in periodic 3D ultrasound over the treatment period) as the primary end point [2]. However, pathological and proliferative responses do represent useful secondary end points. Nevertheless, it should be noted that using proliferation as an end-point might lead only to identification of proliferation associated genes, which have been indicated as being poor predictors of clinical response to aromatase inhibitors [74]. If clinical response (changes in tumour size or volume) is to be used as the main end-point, then attention must be given to the cut-offs applied for each response group. Rather than applying a single arbitrary cut-off where all tumours above a certain value are classed as resistant and all below this value are responsive, it may be beneficial to design a predictive signature based on 2 well characterised groups of tumours that respond well or not at all. For example, tumours which reduce by a least 70% by 3 months would be classed as responsive, while tumours which increase or decrease by no more than 50% by 3 months would be classed as nonresponsive; all tumours in-between (intermediate clinical response) could be excluded from the development of a predictive signature design. This approach may lead to the identification of a predictive signature that has greater power to differentiate between responsive and nonresponsive tumours. It may also be able to subsequently stratify the intermediate clinical response group into clinically relevant responsive or nonresponsive subgroups, ultimately identifying which individual patients are likely to benefit from alternative or combination therapy.

8.7.5. Heterogeneity in the Resistant Patients. Previous studies have alluded to heterogeneity within the clinically resistant group despite a similar clinical response to treatment [314]. However, predictive multigene signatures for endocrine therapy in ER-positive patients to-date have failed to take this heterogeneity into account, instead considering all clinically resistant cases as belonging to the same molecular group. For this reason, predictive capability is likely to be dataset-specific, with reproducibility highly dependent on the frequency and distribution of distinct molecular subtypes within validation datasets compared with the training set. The exploration and characterisation of distinct molecular subgroups within the clinically resistant patients may be an important consideration in the development of signatures with greater predictive accuracy and reproducibility. Indeed, it should be noted that the accurate elucidation of distinct molecular subtypes will doubtless require a large sample number, once again reinforcing the importance of this factor in the experimental design.

8.7.6. Clinical Application of a Predictive Signature. The application of gene expression technology in the field of breast cancer research has yielded much important information and has vastly improved understanding of the disease at a molecular level [258]. However, there are a number of important considerations to address before it can be readily applied in a clinical setting to aid diagnostic and treatment decisions [257]. Foremost, a predictive signature must demonstrate clear clinical benefit for patients. Furthermore, it must be

shown, ideally with prospective independent validation, to out-perform or improve upon currently used parameters for guiding clinical decisions. In addition, thought must be given to the specific technology chosen for the assay and to its implementation. Technologies range from expensive high-throughput commercial or custom microarray platforms to low cost lower-throughput qRT-PCR assays. While most predictive signatures are developed from microarray data, there may be potential to convert resultant signatures to lower cost technologies such as qRT-PCR as was the case with Oncotype DX and MapQuant DX [187, 260, 270, 273]. Another benefit of qRT-PCR is that the assay can be effectively performed on formalin-fixed paraffin embedded (FFPE) tissue with greater ease than with microarray systems. Importantly, FFPE tissue is far easier to collect, store, and work with than fresh frozen tissue. A number of factors can affect the choice of technology: firstly, costs can be prohibitive and studies must be conducted to assess the cost-to-benefit ratio [257]; secondly, the number of genes included in the assay is a point for consideration, as large numbers of genes may preclude the application of lower-throughput technologies such as qRT-PCR. There is also a known issue with reproducibility; microarray bias can significantly impact the reproducibility of results and can manifest from differing technical variables (platforms, RNA extraction, processing, and hybridisation techniques) used across different sites [312]. It may be possible to minimise bias by instigating a strict common protocol for implementation of a predictive assay at different sites. However, commercial enterprises including Oncotype DX and Mammaprint address the issue by only offering their assay at one controlled site using the same validated technology and delivered by the same technicians.

9. Combating Resistance

A number of treatment strategies have been used clinically and assessed in trials to counteract endocrine therapy resistance in breast cancer, including alternating or combining agents [315–317] (Figure 3). While combining tamoxifen and aromatase inhibitors simultaneously does not appear of benefit, patients resistant to tamoxifen have been shown to respond when treatment was switched to an aromatase inhibitor [1]. A number of studies have also assessed the combination of endocrine therapy with the selective estrogen receptor downregulator, fulvestrant. One study combining fulvestrant with anastrozole reported an improved overall survival of 6 months [318]. However, this study included a primary endocrine therapy-naïve cohort of patients treated with this combination as a first line therapy and the results suggested that the benefit was limited to this group alone. The FACT study was a randomised trial of anastrozole with or without fulvestrant for patients pretreated with endocrine therapy and they reported no benefit from the combined therapy [319]. However, it should be noted that the results of the CONFIRM trial which set out to compare 500 mg fulvestrant with 250 mg in women who progressed after previous endocrine therapy suggested that the higher dose was associated with statistically significant increases in

progression-free survival (PFS) and no increase in toxicity, a finding which brings into question the conclusions of the previous trials in which not enough drug was being administered [320].

Another approach has involved augmenting standard endocrine therapy with agents designed to resensitise resistant tumours to endocrine therapy by targeting pathways and molecules recognised as drivers of resistance. One such approach has been the combination of endocrine therapies with HER2-targeted therapies such as trastuzumab and lapatinib, which have shown some promise in endocrine therapy resistant cancers which overexpress HER2 [321, 322]. A number of studies have also shown that the use of PI3K-AKT-mTOR pathway targeted therapies such as the mTOR inhibitor everolimus and EGFR inhibitor gefitinib can reverse the PI3K-AKT-mTOR mediated resistance to endocrine therapy when used in combination with endocrine therapy [321, 323–327]. Indeed, everolimus is now in wide use in combination with exemestane (a steroidal aromatase inhibitor) after being shown to improve PFS in second or third line treatment of patients with ER-positive metastatic breast cancer [163, 328, 329]. A recent study also reported benefit from everolimus plus fulvestrant in tamoxifen resistant ER-positive metastatic breast cancer [330].

Lab-based studies have also yielded some promising results. One cell line study revealed that cells resistant to tamoxifen can be resensitised to its growth-inhibitory effects by targeting and blocking the action of the NF κ B pathway [165]. Another group suggested that by inhibiting O-6-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein they identified as being overexpressed in some tamoxifen resistant cell lines, sensitivity to tamoxifen could be restored [331]. Another reported that a CDK2 inhibitor could reverse endocrine therapy resistance in tumours over-expressing cyclins-E1 and E2 [104].

A novel approach involved the withdrawal of endocrine therapy. Cell line studies have provided evidence for the growth-inhibitory effects of withdrawal from endocrine therapy, although there is little clinical evidence to support this. One group reported a clinical trial which suggested that resistance to endocrine therapy could be minimised by intercalating therapy with periods of withdrawal [332]. A further treatment option was suggested in which low-dose estrogen is intercalated with aromatase inhibitors [333]. Indeed, before the development of drugs such as tamoxifen, high-dose estrogen represented a major therapy for the treatment of hormone-dependent breast cancer. It was thought to work by inducing apoptosis via extrinsic Fas/Fas ligand and intrinsic mitochondrial pathways [334]. Another study reported that the apoptosis inducing action of estrogen involves endoplasmic reticulum stress response and inflammatory response genes [335]. One group demonstrated in hormone-dependent xenograft models that loss of response to letrozole was accompanied by upregulation of HER2 and MAPK pathways and downregulation of ER and aromatase activity, which was reversed by replacing aromatase inhibitors with low dose estrogen treatment for a short period of time, thus resensitising the cells to estrogen and hence aromatase inhibition [333]. The major problem with these lab based cell

line studies is that they take no account of the wide range of cancers seen in clinical practice. Their value at best is to provide leads for pathways or mechanisms of resistance that might be targeted.

10. Conclusions

A wide range of distinct mechanisms have been described that have been implicated in endocrine therapy resistance in breast cancer including lower levels and heterogeneity of ER expression, posttranslational modifications and differential binding of ER, ER-independent signalling including EGF, HER2, and PI3K-AKT-mTOR pathways, and NF κ B signalling, as well as phenomena such as stem cells, EMT, epigenetics, estrogen hypersensitivity, and autophagocytosis. Together, these findings suggest that resistance to endocrine therapy is complex and heterogeneous and may differ from patient to patient, between primary and acquired resistance and even between endocrine therapy types. Improved understanding of the underlying mechanisms will significantly aid the development of new therapeutic strategies including novel drug targets with which to combat resistance. The majority of trials thus far have focused on combining or alternating endocrine therapy agents, intercalating treatment with PI3K-AKT-mTOR, EGF, or HER2 pathway targeted therapy or the addition of chemotherapy. Some trials have shown promise; however, it seems clear that given the heterogeneity of resistance mechanisms and the toxicity and side effects associated with some alternative treatments, biomarker selection to stratify patients into clinically meaningful high and low risk groups in a move toward personalised therapy will be a crucial part of successfully combatting resistance to endocrine therapy in the ER-positive population.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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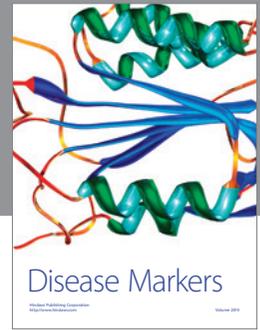
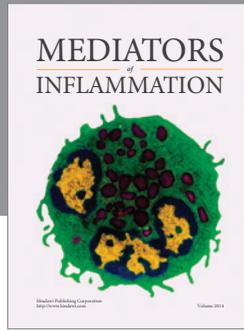
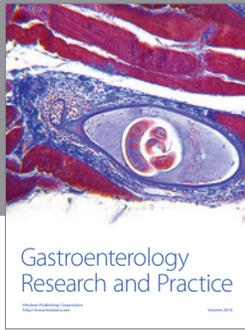
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