Deep Insight Section

Review

Does DNA ploidy and synthesis phase dynamic accentuate the predictive power of oestrogen and progesterone receptors in breast cancer progression and prognosis?

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Abstract

Oestrogen and progesterone receptors (ER and PR) are closely associated with breast cancer progression. In this review we identify and discuss cellular markers that can accentuate or complement the deployment of ER and PR for predicting prognosis. The focus is on aneuploidy and DNA ploidy which appear to be significant independent predictors of overall survival in many forms of cancer. Their importance in cancer development and progression flows from their origin in the inherent genetic instability. Genetic instability of chromosomes is seen as aneuploidy, chromosomal deletions, translocations and sister-chromatid recombination, and at the DNA level as altered DNA repair, gene amplification and deletion and point mutations. Microsatellite loci of repetitive nucleotide sequences are inherently unstable.

Microsatellite instability is characterised by the loss of DNA mismatch repair activity leading to a hypermutable phenotype. Chromosome abnormalities result from the deregulation of cell cycle and immune checkpoint regulators. Failure of the DNA mismatch repair pathway could be one of the reasons for their incidence, although the available evidence is not unequivocal. Some tumours such as the colorectal carcinomas do not show an indisputable relationship between aneuploidy and microsatellite instability or mismatch repair deficiency.

Epithelial mesenchymal transition (EMT) plays a crucial role in cancer biology. EMT is associated with the emergence and maintenance of cancer stem cells (CSC). Polyploidy and aneuploidy appear as a staging post to the formation of CSCs together with parallel activation of EMT. Chromosomal alterations may occur concomitantly with EMT as well as with the reverse process of mesenchymal epithelial transformation.

Genetic profiling has revealed significant information concerning the abnormal growth kinetics of cancer cells. The DNA ploidy pattern is reflected in the polyploid and aneuploid states, aberrant gene amplification and expression and enlarged S-phase fraction. Aneuploidy may be a consequence of cells entering the S-phase of the cell cycle.
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The expression of these cellular markers is quantified here by image cytometry (ICM) and the accrued data have been analysed by using binomial regression algorithm and the fuzzy K-Nearest Neighbour (FK-NN) classifier to see whether these cellular markers aid the prediction of nodal status and survival of breast cancer patients. The FK-NN analyses have revealed high prediction rates for both nodal involvement and 5-year survival. The FK-NN appears much superior in performance than techniques of logistic regression and multilayer feed-forward backpropagation (MLFFBPNN) the artificial neural network tool. A wide spectrum of evidence is presented here which supports the view that DNA ploidy and SPF acting as complementary factors accentuate the predictive power of ER/PR of breast cancer progression and provides credibility that they could deliver a more reliable prognostic model to assist in patient management.

Keywords
Artificial neural network; Breast cancer progression/prognosis; Cell cycle and immune checkpoints; DNA ploidy/aneuploidy; DNA repair; EGFR/HER2 signalling; Fuzzy k-nearest neighbour analyses; Image cytometry; Logistic regression; Microsatellite instability; Multilayer feed-forward backpropagation; Neural networks; Oestrogen/progesterone receptors; S-phase fraction

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Introduction
Oestrogen and progesterone receptors (also named ERα and ERβ) are closely associated with progression. The expression status of ER and PR is significant in determining response to endocrine therapy. Patients expressing both receptors have the best prognosis and are more likely to respond to hormone treatment than patients with ER+/PR- tumours. There is a view that PR+ patients might respond better to hormonal therapy. Since ER can induce the expression of PR, the reduced response to hormones could be a result of lack of normal ER function. Several growth factors and their receptors, e.g. (epidermal growth factor)/(EGF receptor), activate non-receptor Src kinases to regulate many signalling pathways, including the Erk (extracellular signal-regulated kinases) cascade. In breast cancer cells, both oestrogens and progesterone can activate the Src/Erk pathway. This involves the interaction and crosstalk between their receptors (Migliaccio et al. 1998). Ballaré et al. (2003) have attributed this to the two domains of PR which interact with ER. On the other hand, progesterone can negatively regulate other oestrogen regulated signalling pathways leading to inhibition of proliferation (Chen et al. 2005). Poor prognosis linked with the loss of progesterone/PR function could be a consequence of lack of suppression of ESR1 (ERα) (Thomas and Gustafsson, 2015). Furthermore, ERα and ER related receptors have been regarded as downstream targets of (human epidermal growth factor receptor 2, HER2) signalling (Chang et al. 2011). Even with the play of potential crosstalk between EGFR and HER2 signalling, HER2 overexpression can negate anti-oestrogen therapy. It was shown in an experimental setup some while ago that tumours formed in athymic nude mice by the implantation of ER+ MCF7 breast cancer cells transfected with HER2 did not respond to tamoxifen treatment (Benz et al. 1992). Consistent with this, breast cancers overexpressing HER2 are often not responsive to anti-oestrogen therapy. At present we have no means to determine which patients might benefit from combining anti-oestrogen therapy with blockage of HER2 signalling (Rastelli and Crispino, 2008). Notably, luminal A subtype of breast cancers have shown high PR positivity and are HER2 negative.
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Post-menopausal patients with advanced breast cancer appear to benefit significantly from combination of anti-hormone receptor and anti-HER2 therapy (Prat and Baselga, 2008). Indeed, several signalling systems that can target ER and PR via HER2 can be identified. The crosstalk that takes place between ER and HER2 is obvious from the fact that oestrogen can activate HER2 signalling by inducing its phosphorylation and suppression of HER2 counteracts the effects of oestrogen on cell cycle distribution of an approximately 3-fold increase of S-phase cells. This increase was negated by HER2 suppression (Mullen et al. 2007). How the PR isoforms, PR-A, PR-B and PR-C, function in this context is yet unclear. PR-A is a ligand dependent repressor of PR-B. The PR-A and PR-B isoforms regulate different sets of target genes. The PR-B isoform is associated with increase in cell migration but not on cell proliferation or survival. PR-B activates many progesterone response genes by activating Src/MAPK signalling pathways, which can promote or inhibit cell proliferation (see Sherbet, 2017b). As stated in another location, ER binds directly to the ERE half site/Sp1 binding site in the PR-A promoter (Petz and Nardulli, 2000). Thus much advance has been made on some fronts. But a basic precept has not been explored whether one can identify cellular markers that can accentuate or complement the deployment of ER and PR for predicting prognosis.

Genetic instability and cytogenetic aberrations
Genetic instability parallels the generation of cellular diversity, including the generation of variant sub-populations with metastasising ability, within the tumour. Genetic instability occurs both at the DNA and the chromosomal levels. At the DNA level altered DNA repair properties, gene amplification and deletion and point mutations are indicators of genetic instability. Aneuploidy viz. the presence of abnormal number of chromosome, euploidy, chromosomal deletions, translocations, and sister-chromatid recombination are chromosomal manifestations of genomic instability (see Sherbet and Lakshmi, 1997). Some of the early work on the relevance of DNA ploidy to cancer prognosis has been discussed in detail before (Sherbet, 2006). DNA ploidy has remained as a significant independent predictor of overall survival in many forms of cancer. The aneuploid state has correlated with the expression of established prognostic markers, such as the prostate specific antigen in prostate cancer. Correlations have also emerged between DNA ploidy and the expression of factors such as the matrix metalloproteinases and intercellular adhesive proteins which are conducive to the promotion of cancer invasion. Possibly the unfavourable prognosis seemingly associated with aneuploidy is linked with potential for invasion. Aneuploidy is also seen as being able to predict immune evasion by tumours since fewer immune cell infiltrations seem to be associated with aneuploidy (Davoli et al. 2017). But it is intriguing that they find that activating mutations of oncogenic pathways negatively correlated with the aneuploidy state, which basically runs counter to the view the genetic instability is the driving force of carcinogenesis. But then by definition mutability does reflect genetic instability.

Microsatellite instability
Genetic instability can occur at the nucleotide and or microsatellite level. Microsatellite loci are inherently unstable repetitive nucleotide sequences of varying lengths which occur in the human genome within genes and also between genes. The instability is seen in alterations in the repetitive units within the microsatellites altering the length of the loci. Microsatellite instability is characterised by the loss of DNA mismatch repair activity leading to a hypermutable phenotype. The instability can also affect non-repetitive sequences of the genome. Microsatellite repeats may occur outside the coding regions of genes; nonetheless, they can destabilise genetic function.

The importance of microsatellite instability in tumorigenesis was recognised many years ago (Prolla, 1998). It is known that microsatellite instability can affect cell proliferation related genes and possibly also genes involved in invasion and metastasis. This is significant given that it has been encountered in parallel with cancer progression. Indeed, microsatellite instability has been regarded as a significant prognostic factor and it is a virtually invariable feature of tumours such as colorectal and endometrial cancers linked with the Lynch syndrome of hereditary predisposition to tumorigenesis.

Microsatellite instability is often due to mutation of DNA mismatch repair genes such as , , , and the gene coding for epithelial cell adhesion molecule (Modrich and Lahue, 1996; Chao and Lipkin, 2006; Li, 2008). Inactivating mutations of mismatch repair genes and modulation of gene expression by epigenetic silencing leads to the loss of mismatch repair and to microsatellite instability. EPCAM with the last 3’ exons are deleted results in transcription read-through and silences the downstream MSH2 by promoter methylation. These events are aetiologically linked with (Ligtenberg et al. 2009; Kovacs et al. 2009). The abnormal repair proteins can be assessed to quantify DNA instability. In other
words, it is consanguineous with nucleotide instability.

Given the link between microsatellite instability and defective mismatch repair, genetic instability would be expected to and does markedly influence drug resistance. The emergence of drug resistance under conditions of compromised DNA mismatch repair was recognised many years ago (Pink et al. 1998). A consequence of the loss of mismatch repair faculty is that the cell cannot detect the damage and access the apoptotic pathway. In this context, one has to accommodate the possibility that drug metabolism and the efflux of the drug may also be credible factors. Besides, as noted in the following section, the mismatch repair proteins are also involved in activating the cell cycle checkpoints.

Cell cycle and immune checkpoint regulators and the effects of failure of surveillance

Abnormalities in the regulation of the cell division cycle by aberrant expression and activity of regulators of and interference in checkpoint function are known to result in aneuploidy. Besides cell cycle checkpoint regulation immune checkpoint proteins also regulate the cell cycle. An important outcome of aneuploidy is inappropriate expression of genes associated with tumour growth, invasion and metastasis. The modulation of gene expression and the signalling systems that regulate tumour growth has frequently paralleled the incidence aneuploidy.

The cell cycle traverse is regulated at phase transition points by checkpoint proteins. The G1 (G1/S) and G2 (G2/M) are DNA damage checkpoints with different surveillance functions. The G1 monitors DNA damage and blocks progression at G1/S transition. The interference with DNA synthesis or the repair of DNA lesions arrests the cell cycle at G2/M transition so that damaged cells are not replicated. There are intra-S-phase checkpoint pathways that regulate DNA replication and delay or halt S-phase progression. Several cell cycle and immune checkpoint regulator proteins have been identified to-date. Notable among cell cycle regulators are and the proteins which were recognised as cell cycle checkpoint proteins many years ago (Kuerbitz et al. 1992; Sherr, 1994). They regulate the G1/S checkpoint. This checkpoint is invariably deregulated in tumours. Both p53 and Rb1 are viewed as tetraploid checkpoint proteins since they can suppress tetraploid cells from replicating and suppress tumorigenesis (Margolis et al. 2003; Fujiwara et al. 2005).

Interference with the cytoskeletal machinery also arrests the cells at G2/M transition preventing their entry into mitosis. The spindle or mitotic checkpoint monitors the microtubule integrity and prevents progression if that has been compromised. Deregulation of checkpoint control would lead to chromosomal aberrations and aneuploidy and hyperploidy. In mitosis the sister chromatids attach to the mitotic spindle, are aligned and separated during anaphase. Failure of mitotic checkpoint surveillance, essentially of correct attachment to the spindle, leads to abnormal chromosomal segregation and to aneuploidy. Total failure of mitotic checkpoint function can lead to hyperploid states on account of chromosome non-disjunction. Several highly conserved spindle checkpoint proteins have been identified. The spindle checkpoint proteins, (Mitotic Arrest Deficient 1-3), the , , (Budding Uninhibited by Benzamidazo) 1-3, and Mps1 (Monopolar spindle 1) (see Shah and Cleveland, 2000) have been studied extensively. They operate different pathways (see Amon, 1999; Burke, 2000; May and Hardwick, 2006).

The mitotic spindle checkpoint proteins are also involved with the maintenance of genomic stability. Their deregulation results in chromosomal instability. Mutations have been seen in the genes coding for these checkpoint proteins in many cancers. Colorectal cancer cell lines displaying chromosomal instability also had defects in spindle checkpoint. In cell lines displaying chromosomal instability, the Bub1 gene, encoding an important component of the spindle assembly checkpoint protein, was inactivated by mutation (Cahill et al. 1998). Reduction of expression of eukaryotic protein BUB1B (BubR1) by mutations and haploinsufficiency can result in aneuploidy. Its overexpression has a protective effect (Baker et al. 2008, 2009, 2013).

Cytogenetic aberrations and their influence on tumour biology

In the current environment, the correlation of cytogenetic abnormalities of DNA ploidy with aberrations of SPF has been the subject of much debate. There is ample justification for exploring the interrelationship between DNA ploidy and SPF. As discussed before abnormalities of DNA ploidy are closely correlated with the aggressive behaviour of tumours. Both tend to reflect the rapidity of tumour growth, possibly actuated by different mechanisms. But tumours and tumour derived cell populations are likely to display differential sensitivity to drugs. For example, drugs intercalating into the DNA and microtubule stabilisers would target cells with different chromosomal abnormalities. The growth of polyploid tumours and cell lines might be an outcome of the number of gene copies of the relevant determinants whilst SPF would portray the cells in
the DNA synthesis phase and hence the size of actively proliferating or cycling subpopulation.

It has been suggested that aneuploidy may be a consequence of cells entering the S-phase prematurely. Furthermore, DNA ploidy is associated with expression of growth factor and hormone receptors, a topic at the centre of discussion in this review. Major shifts in DNA index may have some bearing on the progression of cancer since many genomic changes are encountered as chromosomal euploidy and aneuploidy and as chromosomal aberrations, translocations and sister chromatid recombinations. These could have arisen by cumulative genetic changes resulting from loss of cell cycle control. An important proviso to note is the heterogeneity of tumours of the expression of biochemical markers. The heterogeneity also encompasses DNA ploidy (Sherbet and Lakshmi, 1997).

Some features such as DNA index and the size of the SPF have been studied extensively with variable results. Whether these might potentiate or counteract the function of ER/PR has not been investigated. Here we describe the potential of these features in conjunction with the expression of ER and PR and evaluate whether they serve as complementary factors to predict prognosis of breast cancer.

**DNA ploidy and epithelial mesenchymal transition**

Several genetic determinants and their downstream signalling cascades lead to the activation of the developmental mechanism of epithelial mesenchymal transition (EMT). EMT plays a crucial role in cancer biology. A molecular concept of cancer progression has evolved over the years, which has provided a solid basis for the cancer stem cell (CSC) hypothesis. The CSC hypothesis postulates a temporal acquisition of stem cell characteristics upon activation of the EMT pathway, since EMT is invariably accompanied by the generation and the perpetuation of CSCs with corresponding tumour growth, invasion, angiogenesis and metastasis. The emergence of the CSCs has also been linked with drug resistance. Several pathways activated by growth factors and other biological response modifiers steer the cell phenotype towards EMT. Notable among them besides the growth factors are Wnt, Notch and the Hippo. Furthermore, Notch ligands can integrate their signalling with Wnt and TGF-β pathways. The appearance of CSCs is often accompanied by the persistent activation of these signalling systems (Sherbet, 2013, 2017a).

The polyploid state has been implicated as a staging post to the formation of CSCs accompanied by the expression of EMT related transcription factors. It has been postulated that chromosomal alterations can be seen with EMT activation. In ovarian tumours the presence of groups of a subpopulation of giant polyploid cells has been described. These gave rise to CSCs with some groups also displaying EMT characteristics (Zhang et al. 2013, 2014). Whether these cells had gone on to produce metastasis is uncertain since no in vivo assays had been performed. Gao et al (2016) have argued that EMT activation can occur spontaneously and the generated variants undergo chromosomal changes causing the deletion or acquisition of genes whose expression might be connected to cancer progression.

The ability of stem cells to self-renew and differentiate into a wide spectrum of cell types implies the gain of phenotypic diversity and heterogeneity of function. CSCs are also heterogeneous. Further, the innate ability to diversify phenotypically is at the root of intratumoral heterogeneity. Both these features may be spawned by differential signalling to external factors resulting in differential display of CSC as well as EMT markers together with drug resistance and significant connotation for therapy. The common stem cell pathways such as growth factor/receptor, Wnt, and Notch signalling and the downstream effectors such as MAPK, Akt signalling could be differentially activated in most stem cells, including CSCs. This would lead to the diversification of CSCs and their self-renewal bringing about intratumoral heterogeneity in their wake. The reverse process of MET (mesenchymal epithelial transformation) would by similar rationale create intratumoral heterogeneity.

One can translate this concept more precisely with reference to tumour progression to the metastatic state. Although CSC generation and EMT might go hand in hand by virtue of the identity of the signalling systems, diverse pathways might be activated within the EMT signalling that might lead to the generation of functionally different CSCs. The expression of CSC features and EMT activation are regulated by certain transcription factors, whose expression is itself subject to subtle regulation. Their stabilisation by protecting them from ubiquitination is essential for the promotion of metastasis (Lin et al. 2017).

The aberrant expression of mitotic checkpoint proteins has been linked with the emergence of the EMT phenotype. The ribonucleic acid export 1 ( ).
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which is involved in mRNA export, is known to serve as a mitotic checkpoint regulator. Haploinsufficient and/or hypomorphic alleles of RAE1 lead to chromosome mis-segregation and aneuploidy (Baker et al. 2006). However contrastingly, overexpression of RAE1 has been correlated with increased in vitro migration of cell lines achieved by the initiation of EMT as characterised by the enhanced expression of mesenchymal and downregulated expression of epithelial markers. Furthermore, RAE1 overexpression negatively correlated with DFS (disease free survival) and metastasis-free survival (Oh et al. 2017). These authors allude to the effects of the overexpression of RAE on chromosomal instability and aneuploidy. However, they did not look at chromosomal aberrations or instability under conditions where RAE1 was overexpressed. This would have established a direct relationship between the expression of the checkpoint regulator and the initiation of EMT. So some reservations may need to be expressed in relation to how the relationship between RAE1, aneuploidy and EMT might pan out in due course.

The protein is a regulator of cell differentiation, proliferation as well as apoptosis. The overexpression of myc is frequently associated with polyploidy. Vazquez-Martin et al. (2016) found that a whole scenario of myc function correlated with the overexpression of myc-interacting proteins and paralleled EMT activation. Not only does myc interact with proteins overexpressed in the polyploid state, but also with two immune checkpoint proteins, and (PD-L1) (Casey et al. 2016). CD47 regulates many signalling systems related to CSC self-renewal, and tumour initiation, growth and invasion. Tumours adopt certain immune checkpoint pathways especially against tumour-specific T cells. This leads to immune resistance. The CD28 family proteins PD1/ and their ligands are prominent in immunotherapy (Sherbet, 2017a). Tumours displaying mismatch repair deficiency and high microsatellite instability are highly sensitive to immune checkpoint inhibitors (Lee et al. 2016). Many tumour types, among them triple negative (ER-/PR-/HER2-) breast cancers and, overexpress PD-1 and its ligands (Gatalica et al. 2014). Gadducci and Guerrieri (2017) reported recently that endometrial cancers with high microsatellite instability overexpress PD-1 and PD-L1 and so are prone to suppression by blockade of the PD-1/PD-L1 pathway. Therefore, one might be justified in envisioning a rational linkage between microsatellite instability and the incidence and prognostic importance of DNA ploidy.

Oestrogen and progesterone receptors and breast cancer progression

Breast cancer is one of the most common cancers that affects one in ten women, and is a most frequent cause of cancer death in women. Breast cancer treatment and patient management is based on the state of progression of the disease. This is indicated by the presence of the tumour in axillary lymph nodes and the number affected nodes. In recent years much effort has been made to identify molecular and cell markers that might have the potential to accurately assess the state of progression and predict prognosis. Oestrogen and progesterone are known to affect the growth of a variety of tissues, including breast tissue. Oestrogens and anti-oestrogens bind to oestrogen receptors ERα and ERβ. The presence of ER indicates the state of tumour differentiation. The absence of ER in breast cancer as an indicator of poor prognosis, since ER- tumours are resistant to anti-oestrogen therapy, continue rapid growth and result in poor outcome for patients. However, this is not an outcome attributable exclusively to the absence of ER.

ER and PR are closely associated with breast cancer progression. ER and PR expression status is significant in determining response to endocrine therapy. Functional PR might be required for growth signalling by ER. Patients expressing both receptors have the best prognosis and are more likely to respond to hormone treatment than patients with ER+/PR- tumours (Osborne, 1998). Since ER can induce the expression of PR, the reduced response to hormones could indicate a non-functional state of ER. In breast cancer cells, both oestrogens and progesterone and their receptors can collaboratively activate the Src/Erk pathway and promote cell proliferation. On the other hand, progesterone can negatively regulate other oestrogen-regulated signalling pathways leading to the inhibition of proliferation (Chen et al. 2005).

The importance of ER/PR is further highlighted by the possibility that ER/PR signalling can interact with the p53 pathway. Also ER has been implicated in the regulation of this p14ARF-mdm2-p53 pathway (Cho et al. 2006). The transcription factor Twist is known to suppress the p14ARF ( ). This in turn leads to the suppression mediated regulation of p53 and to premature G1/S transition and cell proliferation, a bHLH transcription factor, downregulates the expression of (E-cadherin) and activates EMT. This reasoning is on lines similar to the repression of p14ARF by the T-box transcription factor to initiate EMT. One would recall that Tbx2 is overexpressed in cancer and in embryonic systems (Abrahams et al. 2010) and is a potent activator of EMT (Wang et al. 2012). Finally, transfection of ER
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into MCF7 cells had led to the expression of markers associated with EMT activation (Bouris et al. 2015). Thus the involvement of ER in the p14ARF/mdm2/p53 pathway to EMT activation may be deemed to be complete.

Further evidence one can adduce is that (trefoil factor 1; pS2), a downstream target of ERF, is downregulated in some forms of cancer. TFF1 impedes G1/S transition, inhibits cell proliferation and induces apoptosis by activating p53 (Calnan et al. 1999; Bossenmeyer-Pourie et al. 2002; Haupt et al. 2003). ERα binds to p53 and downregulates p53 expression and suppresses the expression of p53 responsive genes that inhibit cell proliferation. This can also occur by the upregulation of TFF1 (Konduri et al. 2010). Furthermore, ER has been linked with signalling by EGFR and HER2. Of considerable interest from the point of view of progression of breast cancer to the metastatic state is that molecular markers of cancer metastasis such as the metastasis promoter and the suppressor nm23 might conceivably contribute to the metastatic spread in association with functional ER/PR (Grey et al. 2003).

The expression status PR, along with that of ER, is routinely measured in breast cancer specimens. PR is an oestrogen responsive gene and so PR positivity indicates not only ER being present but also functional (Horwitz and McGuire, 1975). However, a small proportion of tumours are ER-/PR+ and still respond more favourably to hormonal therapies than ER-/PR- tumours (Osborne, 1998; Osborne et al. 2005). Also, ER+/PR- may be less responsive to hormonal therapy than ER+/PR+ patients (Lapidus et al. 1998). This demonstrates the non-aligned importance of PR in breast cancer development and treatment, and that it is not just as an adjunct of ER function. We recently showed that a clear distinction can be made between PR and ER, with PR displaying greater correlation than ER with disease progression and prognosis (Caronongan III et al. 2016, Sherbet 2017b). The cell proliferation/survival results depend upon the ER/PR signalling axis subject to the provision of which isoform of ER or PR is functional and the recognition that ER does influence PR function. ERα and ERβ are two ER isoforms. PR also may be expressed in three functionally different isoforms (Kariagina et al. 2008). The promoter of the PR-A isoform contains an ERE half site/Sp1 binding site and ER does bind directly to this site (Petz and Nardulli. 2000). ERα is pro-proliferation whilst ERβ is inhibitory of cell proliferation. Besides, ERα binds to and downregulates PRβ (Sherbet 2017b).

The connotation of ER and PR on DNA ploidy, SPF and cell cycle distribution.
Some features such as DNA index and the size of the SPF have been studied extensively over the years for potential impact on breast cancer progression and prognosis, but no substantive outcome has been recorded. Notably not addressed with apparent intensity is whether these might potentiate or counteract the function of ER/PR. One would recall here that intratumoral heterogeneity is a common feature. Significant heterogeneity in the distribution of many markers including PR, ER, p53, and (Ki-67) has been encountered in breast cancer. The heterogeneity of ER and PR expression has been amply demonstrated using, immunohistochemical and radioactively labelled ligand binding assays. The two assays have also revealed a clear functional distinction between PR and ER in respect of clinical outcome (Table 1). This could be a reflection of the fact that LBA provides information about the totality of receptor expression irrespective of intratumoral distribution. In contrast, IHC based designation of receptor positivity provides the proportion and intensity of staining of tumour cells reflecting potential heterogeneity of distribution.

<table>
<thead>
<tr>
<th>Parameter Tested</th>
<th>IHC</th>
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<tbody>
<tr>
<td>ER vs node</td>
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<tr>
<td>PR vs node</td>
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<tr>
<td>PR vs DFS</td>
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Note: This table emphasises the differential correlation of the receptors with nodal positivity and DFS (disease-free survival). ER positively correlated with node positivity by LBA but PR by IHC. PR status correlated with DFS in both assays, but ER status did not by either assay. This could be suggestive of intratumoral heterogeneity of receptor expression. IHC: Immunohistochemistry; LBA: Ligand binding assay. (Modified from Caronongan III et al. (2017))

Measurement of cellular markers
The method most popularly employed in the study of cellular markers, such as DNA index, size of SPF, and cell cycle distribution (CCD), is flow cytometry. Much less in vogue is Image cytometric data (ICM). The ICM method can measure the cellular features of cell aspirates and in stained tissue sections. Nuclear pleomorphism, which possibly reflects the degree of deviation of cancer cells from normal morphological configuration, is an important criterion in tumour grading. However, it could suffer from subjectivity of assessment. This can be overcome by quantifying the degree of pleomorphism using ICM technology (Lakshmi and
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Sherbet, 1990) and has been measured free of bias. The ICM data mostly relate DNA ploidy, size of SPF and CCD data presented as a ratio of number of cells in G₀, G₁ over the number of cells in G₂M (G₀, G₁/ G₂M). The presence of more than 10% of cells in the G₂M peak of cell cycle is often considered to be an indicator of aneuploidy and therefore the G₀, G₁/ G₂M ratio can be used an additional parameter. A normal cell population will present an SPF around 4% and >10% suggests a high rate of proliferation (Lakshmi and Sherbet, 1990; Naguib et al. 1999). The ICM measures are limited unlike flow cytometry which has a wide spectrum of capabilities. These include immunotyping with fluorescence-labelled antibodies, measuring the intensity of antigen expression, cell sorting and high speed analysis. The limitation of the technique is that one requires strictly unicellular suspensions and hence the adhesive faculty of cells comes into the reckoning. Also recognised is that flow cytometry does not provide any information on the spatial distribution in tissues of the cells of interest (Davey and Kell, 1996; Craig and Foon, 2008).

The perception and perspective of applicability of cellular and molecular markers to prognosis

The potential importance of DNA content and SPF in relation to cancer prognosis has lately received much concerted attention. A recent meta-analysis of breast cancer data has highlighted the association of DNA aneuploidy with higher tumour grade, tumour dissemination to the lymph nodes with ER positivity. ER, PR and DNA ploidy are generally endorsed as useful biomarkers for endometrial cancer. Both ploidy and SPF may reflect extension of endometrial carcinoma to the lymph nodes. Lymphovascular invasion and concomitant reduced recurrence-free survival was associated with the aneuploidy and SPF (Song et al. 2012). Lymphovascular invasion i.e. the presence of tumour emboli, located distal from but not close to the tumour, in the peritumoral region was related prognostically to metastasis-free survival (De Mascarel et al. 2009). However, the tumour emboli focused on by Song et al. (2012) seem to be in close proximity of the tumour. De Mascarel et al. (2009) had ruled out any prognostic value to emboli located close to the tumour. This is compatible with the commonly held view concerning metastatic dissemination. Numerous emboli and tumour cells may enter the lymphovascular system but infiltration into the system may not be fully reflected in overt metastatic foci. Song et al. (2012) had not looked at the steroid receptor status in their work. Nevertheless, Song et al. (2011) had reported earlier that lymphovascular invasion was associated with ER expression, but not PR, HER2 or p53. This was in node-positive breast cancer, but corresponding data relating to node-negatives tumours was also described. So at present some uncertainty surrounds the status of ER/PR in relation to ploidy and SPF. But a recent report has stated that aneuploidy is seen also in ER/PR negative endometrial tumours, where aneuploidy independently predicted survival (Mauland et al. 2017). One has to be mindful of considering the expression status of ER and PR and be circumspect about this since they would have opposing biological effects; so do the ER isoforms biologically oppose each other.

Crosstalk between hormone and growth factor signalling and DNA ploidy

We have arrived at a point when it is arguable whether genetic instability bears any relationship with the expression of ER and PR, cancer progression and with any perceived sensitivity of tumour cells to steroid hormones. In the preceding section we have briefly explored also whether ER/PR expression is related to the incidence of DNA ploidy and related features of SPF and CCD as manifestations of genetic instability. Thus we complete the circuitry of the inter-dependence of genetic instability, steroid receptors and sensitivity of tumours to endocrine therapy, but without invoking causality. Nonetheless, given this background it would seem eminently worthwhile to attempt to determine if the prognostic value of ER/PR status can be enhanced by combining the steroid expression status with cell proliferation features such as DNA ploidy, and SPF measurements and CCD.

The basic thesis of a potential link between genetic instability manifested as DNA ploidy and sensitivity to endocrine therapy was postulated some time ago. ER positivity of the breast cancer cell line T47D corresponded with DNA ploidy (Reddel et al. 1988). However, one has to add a rider here that the sensitivity of the breast cancer cell lines to anti-oestrogens is complicated by a number of factors. The cell line T47D is ER+/mutant p53+, MCF-7 is ER+/wild-type p53+ and MDA-MB-231 is ER-/mutant p53+. Furthermore, MDA-MB-231 cells do indeed overexpress HER2 (see Sherbet, 2013). So the sensitivity or insensitivity of these cell lines to growth suppression might be determined by more than a single growth promoter.

It was reported over a couple of decades ago that among the biomarkers which included p53, ER, PR, EGFR and HER2, DNA ploidy was found to be most highly predictive of recurrent endometrial cancers. HER2 was a significant factor, but surprisingly EGFR overexpression was not (Lukes et al. 1994). However, we do not know much about the inter-relationship between them and whether the
expression of a specified marker influences the significance of the rest. The discordance noticed between the importance of HER2 and EGFR is difficult to interpret in terms of their consequence to prognosis. One should recall that EGFR ligands can also interact with HER2 and generate biological effects. More recently, some effort has been made to check if the level of DNA ploidy was associated with the expression of PR and HER2 (Dayal et al. 2013). Low DNA index was associated with PR positive state and wildtype P53, compatible with their proliferation suppressor effects. Compatible also is the finding that high DNA index correlated with HER2 positivity. However, it would have been useful to know the relationship between ploidy and ER and PR isoforms, which subserve opposing functions.

Much archival work can be cited which has revealed a significant correlation between the expression of both EGFR and HER2 and the associated enhanced cell proliferation with aneuploidy breast cancers (Fernandez et al. 2002). The situation obtaining in ovarian cancer is totally at variance with this. Although EGFR expression was more frequently noted in overt cancers as opposed to borderline malignancies, the expression levels showed no correlation with DNA ploidy (Nagai et al. 2001). Recent research tends to be more positively inclined about this topic. Uesugi et al. (2017) stated that there was a significant linkup between the overexpression of HER2 as well as p53 and microsatellite instability. In an opposing view Birkness et al. (2018) found no correlation between chromosome polysomy, i.e. the presence of multiple copies of chromosome, with HER2 amplification. However, polysomy is an indicator of genetic instability. So Birkness et al. (2018) have advocated that microsatellite instability and chromosomal instability may be unrelated and not inter-dependent events. But in sharp contrast to this in colorectal cancer chromosome copy numbers have correlated with microsatellite instability (Jasmine et al. 2012). Therefore any distinction between chromosomal instability and microsatellite instability may yet be regarded as inauthentic. There is further substantiation of this below. In both EGFR and HER2 overexpression has strongly correlated with aneuploidy. Activation of Akt and EGFR/HER2 was related to DNA aneuploidy (Hisamatsu et al. 2016). Yaglom et al. (2014) demonstrated that HER2 was able to induce genetic instability by downregulating the pathways of repair of double strand DNA lesions. The ambiguity in the interrelationship between the overexpression of these growth factor receptors and chromosomal instability gains further crediblity with the revelation in many recent studies that in a -deficient environment genetic instability can occur independently of DNA double strand repair (Gupta et al. 2009). This leads one to speculate that activation of the HER2/PI3K/Akt signalling axis could have led to genetic instability.

Alu (short interspersed element) Sine repeats and Line (long interspersed elements) elements are a recognised source of genetic instability. They are retrotransposons which can produce major genetic changes with the potential to lead to tumorigenesis. There is a general perception that methylation of the transposable elements suppresses their mobility and is conducive to genetic stability. Hypomethylation is encountered in many disease states including cancer; it is associated with aberrant gene activation and is possibly one of the provisions by which genetic instability might drive the neoplastic process. The insertion of the mobile elements can affect the expression of the genes in the proximity of the site of insertion. Since the epigenetic changes are stably inherited, the methylation status of the transposable elements assumes much functional significance (Ikeda and Nishimura, 2015). Genomic instability can lead to the generation of chromosomal abnormalities (Ba et al. 2012; Saito et al. 2010). The postulate here is that hypomethylation of these elements leads to chromosomal instability and to genetic abnormalities such as loss of heterozygosity and chromosomal translocations and that this would have serious implication for disease prognosis. Although loss of heterozygosity has been shown to occur no linkup is seen between hypomethylation and the incidence of genetic abnormalities. It is of some interest to note in the present context that hypomethylation of both Alu and Line elements was associated with a breast cancer subpopulation overexpressing HER2 and p53. Also hypomethylation of a Line element was seen in ER- tumours (Park et al. 2014). These authors omitted to check ER status in this work. As stated in an earlier section PR-A does contain an ERE half site/Sp1 binding site to which oestrogen can bind and so PR-A can respond to oestrogen even in the absence of ER.

Notwithstanding the dissensions concerning the relationship of growth factor and steroid receptors to genomic instability, aneuploidy is considered to be significantly predictive of breast cancer prognosis (Xu et al. 2016). Aneuploid tumours showed high SPF and this correlated with advanced stage disease (Pinto et al. 2013, Bianco et al. 2013). Dayal et al. (2013) reported that in the breast cancer series that they had analysed high SPF was associated with poor survival. Indeed SPF was identified some while ago as a significant factor for the prediction of nodal involvement and survival (Naguib et al. 1999; Seker et al. 2002).
In abnormal DNA ploidy and the loss of PTEN gene that suppresses the anti-apoptosis effect of Akt signalling and the deletion of 6q15 correlated significantly with tumour grade and stage and nodal involvement. These deletions reflect chromosomal instability. The deletion of 6q15 which harbours gene is a frequent event in prostate cancer. The loss of this gene corresponds with Gleason grade. The MAP3K7 (mitogen-activated protein kinase kinase kinase 7) gene suppresses cell proliferation and invasion (Wu et al. 2012). This kinase negatively regulates the cell survival factor NF-kB. Combining DNA ploidy and the deletion status, both arising from genetic instability, were markedly powerful predictors of poor prognosis (Lennartz et al. 2016). So too in colorectal tumours aneuploidy has been linked with advanced Duke stage and found to be predictive of prognosis (Laubert et al. 2015).

Alexiou et al. (2013) investigated the intracranial tumours and Cell cycle analyses and SPF determinations differed markedly between low grade and high grade gliomas. Furthermore, they found most glioblastomas to be aneuploid. However, Carloni et al. (2017) found that DNA ploidy was not related to SPF in . They found hyperdiploid was encountered more frequently in primary carcinomas than in recurrent tumours. The primary tumours were larger in size than the recurrent ones. But one cannot place much weight on the tumour sizes as regards growth potential. Although SPF did not show any relationship to ploidy, higher SPF correlated with reduced patient survival in the hyperdiploid group. SPF has been viewed by many as an independent prognostic factor, but information is scarce regarding the influence of ER/PR or growth factor family receptors on its expression.

The perspective of DNA ploidy, SPF and ER/PR in cancer progression and prognosis

Some years ago, O’Reilly et al. (1990) reported that the median SPF was higher in aneuploid tumours as compared with diploid tumours and that it correlated with high Bloom-Richardson histological grade, but they found no correlation between SPF and DNA ploidy, nodal status or steroid receptor expression. The Bloom-Richardson system is probably not as reproducible as one would like, but cell proliferation index is a better prognostic marker.

Much valuable information about the relevance of cellular markers for the prediction of cancer progression has also been collated using data obtained by ICM technology. We had noted previously that both nodal involvement and 5-year disease-free survival could be predicted a high degree of accuracy when ER/PR status was combined with DNA ploidy, SPF and CCD represented by the G0/G1/G2/M ratios the cell cycle distribution. CCD is indeed a measure of DNA index. It would be reasonable to suggest that our analysis does show a conspicuous link between nodal involvement and ER/PR expression status together with DNA index as revealed by DNA ploidy and CCD.

**Cellular and molecular marker expression analysis by binomial regression algorithm**

Even with this background information there have been no significant efforts to examine the utility of these cellular features in the background of steroid receptor expression to evaluate whether they might serve as complementary factors to predict prognosis of breast cancer. Andronas et al. (2003) analysed the possible influences of ER and PR expression in breast cancer in conjunction with the cellular markers and the influence they brought to bear individually or in combination on the nodal dissemination and 5-year DFS of patients with breast cancer.

The query addressed was whether DNA ploidy, SPF and CCD displayed significant correlation with the presence of metastatic tumour in the regional lymph nodes and patient survival and to project any potential enhancement of the prognostic value of the steroid receptors by combining with cellular markers in patient management. The analyses, carried out using Matlab technical computing environment (Maths Works Inc.), were based on an algorithm designed and implemented to perform polynomial regression analysis of the data. Andronas et al. (2003) concluded that ER and PR exerted differential effects on the cell features examined. Whilst PR influenced both DNA ploidy and SPF, ER had no influence on either. However, there was some interrelationship between these two features since DNA ploidy/SPF taken together were influenced by ER but not by PR (Tables 2).

**Table 2. Influence of ER/PR on DNA ploidy, SPF and cell cycle distribution by polynomial regression analysis**

<table>
<thead>
<tr>
<th>Cellular marker</th>
<th>ER</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA ploidy</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>SPF</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DNA and SPF</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>DNA and G0G1/G2M (CCD)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G0G1/G2M (CCD)</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Based on Andronas et al. (2003).

**Table 3. Prediction of nodal status and survival by polynomial regression analysis**

<table>
<thead>
<tr>
<th>Steroid Receptor Status</th>
<th>Prediction accuracy</th>
<th>Nodal status</th>
<th>5-year DFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER+/PR+</td>
<td>10/16 (81%)</td>
<td>15/16 (94%)</td>
<td></td>
</tr>
<tr>
<td>ER+/PR-</td>
<td>8/14 (55%)</td>
<td>12/14 (86%)</td>
<td></td>
</tr>
</tbody>
</table>
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| ER-/PR- | 11/16 (69%) | 13/16 (88%) |

Based on Andronas et al. (2003). DFS: Disease free survival

It is obvious from the data generated by these analyses that the expression of both ER and PR provides high prediction accuracy for the both nodal status and 5-year DFS. In the ER+/PR- group the accuracy nodal prediction was markedly reduced indicating that ER might not be fully active due to the absence of PR. Even more interesting is that the prediction rates for nodal status as well as survival ER-/PR- group was more or less similar to that in the ER+/PR- group, but far lower that obtained with the ER+/PR+ group (Table 3). Given that ER influenced all the cellular markers but PR influenced only SPF, it would not be unreasonable to suggest from these prediction rates that the cellular markers could be regarded as partly independent prognostic factors.

The multilayer perceptron (MLP) has been shown to be an effective vehicle for exploring the predictive potential of these biomarkers. The MLP-based analysis has provided accurate and reliable prediction for breast cancer given that an appropriate design and validation method was employed (Mojarad et al. 2010, 2011). Nevertheless, we have looked for supportive evidence by analysing the data set of Andronas et al. (2003) aided by more sophisticated analyses, viz. by using the Fuzzy K-Nearest Neighbour (FK-NN) algorithm, of which the methodology is briefly described below.

The **Fuzzy K-Nearest Neighbour (FK-NN) algorithm**

The two techniques widely used to analysis data relating to cancer prognosis are logistic regression as a statistical method, and the artificial neural network tool MLFFBPNN (multilayer feed forward backpropagation neural network). The concept of fuzzy sets and fuzzy logic introduced by Zadeh (1965) has been applied in various disciplines, including medicine, and shown to provide a viable alternative to both artificial neural network based approach and statistical methods (Szczepaniak et al. 2000). The FK-NN algorithm proposed by Keller et al. (1985) is a powerful pattern classifier.

The FK-NN classifier has been successfully used for the prediction of nodal status and survival in series of breast cancer patients. The FK-NN based feature evaluation index is calculated using the class memberships, computed by means of the FK-NN algorithm and actual class memberships, which were previously known. Similar to pattern class memberships, this measurement gives a degree of importance between 0 and 1 for the subsets of the factors indicating how significant the subset is. The subset that yields the highest value of the index is considered as the most important one. The index can be used together with the predictive accuracy, as a secondary measurement, to precisely identify the most and the least important factor(s) and subset(s) of the factors (Seker et al. 2000a, b; 2002). 

**FK-NN outcome of the predictive importance of molecular and cellular markers**

Previously we analysed the utility of cellular markers which included histology type, tumour grade, DNA ploidy, SPF, G1, G2/M, and pleomorphism indices employing logistic regression and MLFFBPNN techniques. The major focus in extending the inquiry was to analyse the outcome of the FK-NN technique and to compare its effectiveness with logistic regression and MLFFBPNN, the latter two techniques having been widely used for cancer prognosis.

The FK-NN method yielded the highest predictive accuracy of 82-88% for both nodal involvement and survival analyses obtained from the two subsets of tumour grade, SPF, nuclear pleomorphism index and tumour histology type, DNA ploidy, SPF, G0/G1/G2/M ratio (Seker et al. 2002). An overview shows that the predictive accuracy for MLFFBPNN in respect of both nodal involvement and survival is higher than the accuracy obtained with logistic regression analyses. But the performance of the FK-NN is much superior to that of logistic regression and MLFFBPNN. Given this outcome, it would appear that the FK-NN technique may be deemed as a more reliable prognostic factor models (Seker et al. 2000a, b; 2002).

Now returning to the question of the effects combining the biomarkers with ER/PR, we recently carried out FK-NN analyses of the expression status of the biomarker set comprising ER/PR, DNA Ploidy, and CCD and the clinical outcome. This has allowed a valuable insight into the effects of ER/PR. Unsurprisingly, the subset which included all the biomarkers seemed to be the best model that yielded the highest accuracy for predicting nodal status and patient survival (Seker and Sherbet, 2018 unpublished work) (Table 4). But the previous study in which ER/PR had not been included found SPF to be the significant predictive factor (Seker et al. 2002). Possibly, in the presence of ER/PR the predictive effect of SPF may have been diluted or diminished. Obviously ER/PR exerts substantial influences over the analytical outcome. This is possibly a result of the pro-proliferation effects that ER can exert, so counteracting the significance of SPF.
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Table 4. Prediction of nodal status and survival by FK-NN analysis

<table>
<thead>
<tr>
<th>Prediction</th>
<th>Model Parameter (K)</th>
<th>Biomarker Set</th>
<th>-Accuracy</th>
<th>-Sensitivity</th>
<th>-Specificity</th>
<th>Standard Deviation for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodal Status</td>
<td>1</td>
<td>[ER/PR; DNA Ploidy; CCD]</td>
<td>0.6522</td>
<td>0.7155</td>
<td>0.5537</td>
<td>0</td>
</tr>
<tr>
<td>Survival</td>
<td>1</td>
<td>All the Biomarkers</td>
<td>0.8040</td>
<td>0.8616</td>
<td>0.5967</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

Note: In order to find the best FK-NN model, number of neighbourhoods (K) between 1 and 8 was assessed. In order to independently assess the predictive performance of all the models and make sure that the prediction is not biased towards particular sub-set of the samples selected, 2-fold cross validation was used, which is repeated x100 as different sub-sets of the data were randomly selected for each run. The mean and standard deviation are presented over 100 runs of the 2-fold cross validation in the following tables. The 2-fold cross validation means that 50% of the samples is used for training and the other half for testing. The results presented in the table above are the ones obtained for the test samples. (Seker and Sherbet, 2018 unpublished data)

Recently, Pradhan et al. (2011) noted that tetraploid stage I and II showed greater recurrence than diploid ones, albeit not as high as aneuploid tumours with high DNA index. The diploid tumours showed far superior 5-year recurrence free survival. This confirms previous findings by Susini et al. (2007) that DNA aneuploidy was associated with higher risk of recurrence of endometrial cancer and also with reduced DFS. The patients with aneuploid tumours also carried a markedly lower 10-year survival risk than patients with diploid tumours. Whether p53, ER, and PR are important prognostic factors is still an open question. The ER/PR status might be associated with low grade and early stage endometrial carcinomas (Kounelis et al. 2000). However, some caution is probably warranted since differentiating between grades on ER/PR status is considered problematic and also the high grade tumours may be heterogeneous in many ways. Our overview of the current status of the affiliation and complementarity between ER/PR and the cellular markers is summarised in Table 5.

Table 5. Comparison of ER/PR and DNA ploidy/SPF states in cancers

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>ER/PR</th>
<th>Cellular marker DNA ploidy/SPF/CCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal</td>
<td>?</td>
<td>+ (?)</td>
</tr>
<tr>
<td>Prostate</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Endometrial</td>
<td>+ (?)</td>
<td>+</td>
</tr>
<tr>
<td>Ovarian</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Breast</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: The + symbol in both columns denotes positive relationship between ER/PR and DNA ploidy/SPF. When the relationship is not unambiguous it is indicated by + (?) symbol. For colorectal and prostate cancers the relationship has not been explored.

There are reasonable grounds which indicate that exposure to steroid hormones may reduce the risk of colorectal cancers in post-menopausal women (Johnson et al. 2009). Besides ER/PR other factors have been previously implicated in the perceived reduced colon cancer risk. Oestrogen therapy is a form of treatment for prostate cancer. But it may not be an ER/PR dependent outcome. Diethylstilbestrol reduces testosterone levels and in this way suppresses the growth of prostate cancer. Furthermore, the precise effect of ER/PR expression in prostate cancers is not satisfactorily resolved (Kowalska and Piastowska-Ciesielska, 2016).

There is extensive evidence, both research and epidemiological, about the role of steroid hormones in the pathogenesis of these cancers. Overall, these thoughts are compatible with the sensitivity of breast and ovarian cancers to the therapeutic suppression of ER/PR. The linkup between the two parameters is unambiguous in breast and ovarian cancers and there are indications that it may subsist also in endometrial cancers. This review highlights that as far as breast cancers are concerned, the inclusion of the biomarkers DNA ploidy, SPF and CCD (G0 G1/ G2:M) with ER/PR status would enhance the predictive utility of steroid receptor expression.

Epilogue
The ER/PR axis has a well-established role in cancer development and progression is widely used in assessing whether a cancer is benign or likely to be aggressive. The question posed here is whether cellular markers of ploidy, aneuploidy, SPF and cell cycle distribution, which also reflect the inherent genetic instability and the deregulated growth of cancers arising from the failure of cell cycle and immune checkpoints, can function in a complementary capacity with ER/PR. The wide...
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spectrum of evidence reviewed here does support the view that these markers accentuate the predictive power of ER/PR in respect of breast cancer progression. It also provides ample credibility to the view that they could assist in patient management.

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