Description and Pre-clinical Validation of Dynamic Molecular Determinants of Sensitivity to Aromatase Inhibitors in Breast Cancer.

PhD Thesis

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September 2007

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Breast Cancer

Cancer originating in the mammary gland is the most common type of cancer in women. The lifetime risk of breast cancer for a woman in developed countries has been calculated at around 1 in 7 to 1 in 10. When it comes to Catalonia, the latest analyses report an accumulated lifetime risk of developing breast cancer of 1 in 11 with a 1 in 33 probability of death due to this disease[2]. This means that around 10% of the female population will be diagnosed with breast cancer at some point of their life. Out of these patients, around 30-40% will eventually die of this disease, mainly due to the development of metastases, an incurable condition in most types of cancer.

This high incidence, the complexity and the economic costs of the treatment for this disease make breast cancer one of the most relevant health problems in our society.

A neat decline in breast cancer specific mortality rate has been observed over the last twenty years[3]. This increasing trend in patient survival rates can be attributed to both early diagnosis and improved treatment efficacy. Widespread population-based screening programmes implemented in Western countries over the last thirty years have allowed the diagnosis of the disease at earlier stages with the consequent higher rates of curability. On the other hand, better staging procedures and improvements in surgery and radiotherapy have led to increased local control rates while minimizing mutilation and the number of untoward effects related to treatment of the breast.

Considered as a systemic disease even from its earliest stages, improved systemic treatments have resulted in an important breakthrough in patient outcome. To date, systemic therapies used as a complement to local treatments in curative settings include chemotherapy and targeted approaches such as hormonal manoeuvres and anti-Her-2/neu treatments. Among these, hormone treatment has undoubtedly the best established role in the adjuvant setting of most patients as well as the most favourable toxicity/efficacy profile.

When cancer becomes incurable, mainly following its metastatic spread, local treatments (surgery and radiotherapy) have a very limited role while systemic

treatments have shown to be highly effective in prolonging life and providing improved symptom control and better quality of life to the patients.

The availability of an increasing number of systemic therapies has urged the development of research strategies to better define the sub-groups of patients most likely to benefit from one therapy or another. This is relevant in both the adjuvant and the metastatic settings. To this effect, tumour characteristics and the patients which they identify as more or less likely to benefit from a certain treatment are known as predictive factors and are of great importance. These factors are taken into account to minimize long term side effects and economic costs in the adjuvant setting, in which most patients may survive cancer. On the other hand, predictive factors are useful to improve the quality of life of patients with incurable disease in whom therapeutic approaches are aimed at following the "*primum non nocere*" rule to a maximum.

This thesis is focused on the utility of novel predictive markers in the use of hormonal treatment in patients diagnosed with breast cancer.

The origin of breast cancer and hormone dependence

Virtually all breast adenocarcinomas (by far the most common type of breast cancer and, herewith, referred to as breast cancer or breast carcinoma when the contrary is not specified) appear to originate in normal breast glandular epithelium coating the lumen of terminal duct lobular units (TDLUs). The sequence from normal TDLU to invasive carcinoma follows a long evolution period in which epithelial cells can typically be found in different stages of progression to cancer. Thus, when normal epithelium has begun its malignant transformation it undergoes a first stage of excessive proliferation known as hyperplasia followed by the appearance of cells showing aberrant characteristics (atypical ductal or lobular hyperplasia). At a later stage, known as carcinoma i*n situ*, these cells acquire a full malignant phenotype,except the ability to invade the surrounding parenchyma through the basal membrane. Nonetheless, in its final phase the carcinoma cells break through the basal membrane and become an

invasive carcinoma. Progression from normal TDLU epithelium to cancer has been characterised according to the similarity at all cell-biology levels of the cancer cells and the surrounding carcinoma *in situ* or hyperplasia in the biopsies of patients with cancer[4-6].

Nevertheless, the biological progression to carcinoma is not as simple as described above. To illustrate the complexity of this process, clinical evidence have shown that, even pre-malignant lesions which are closest in similarity to invasive carcinoma (the high-grade carcinoma *in situ*), may not always evolve to cancer if left to their natural evolution. Although it is virtually impossible to determine the percentage of cases of ductal carcinoma *in situ* which may progress to invasion, it has been calculated to be less than 50-60% [7]. Hence, atypical hyperplasia and carcinoma *in situ* are non-obliged precursors of breast cancer.

Since breast cancer originates in normal breast epithelium it can be induced that stimuli that normally result in breast gland proliferation will also boost the growth of breast cancer cells, intrinsically characterised by their proliferation advantage.

Mammary gland epithelium proliferation presents a marked dependence on hormones. This way, glandular units of the female breast present a cyclic growth and atrophy throughout the menstruation cycle, with the most marked hyperplasia during pregnancy and lactation, and regression following menopause. Although dependent on multiple hormones, the most important regulator of these changes is oestradiol (E_2) through its interaction with the cellular oestrogen receptor (ER). Of the 3 forms of oestrogens, namely oestradiol, oestrone and oestrone-sulphate, oestradiol is the effector.in which androgens are converted by the aromatase enzyme.

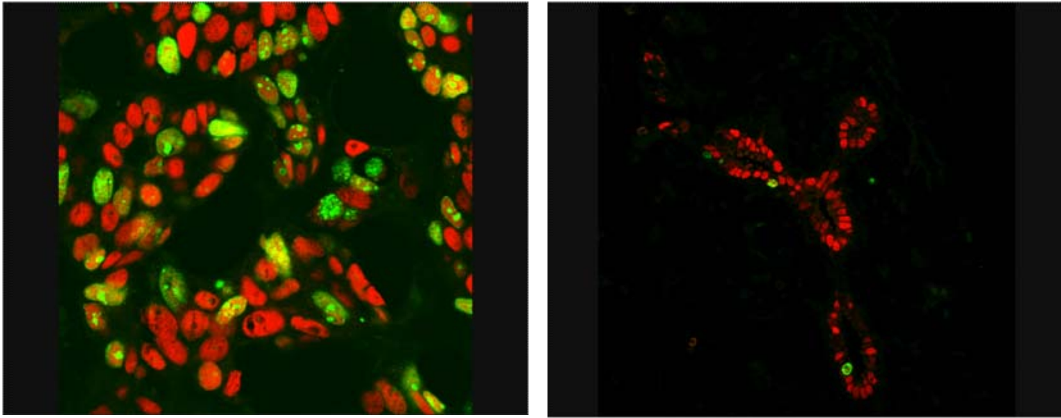
Breast cancer growth depends on E_2. In 1896 Beatson reported the first successful treatment of a pre-menopausal breast cancer patient using the excision of the ovaries [8]. This report described the first evidence of the hormone dependence of breast

cancer and provided evidence of the potential use of hormonal manoeuvres for its treatment.

Ulterior clinical evidence has definitively established the importance of oestrogen in the development, progression and treatment of breast cancer. The risk of breast cancer rises with early onset of menstruation, late menopause and late age of first pregnancy. Likewise, longer duration of hormone replacement treatment after menopause is related to a higher risk of breast cancer development [9, 10].

Since oestrogens seem to be of great importance in breast epithelium growth and E_2 interacts directly with ER to direct cell biology, it can be induced that most mammary epithelial cells should be positive for the presence of ER. However, no more than 30% of breast epithelial cells are ER positive (thereby showing expression of ER using immune staining) at one time point. What is even more paradoxical is that most, if not all, proliferative mammary epithelial cells are ER negative [11], a fact which has not, as yet, been well explained [5].

In contrast with the findings in normal breast, in the malignant conversion through atypical hyperplasia and carcinoma *in situ* towards invasive breast cancer, ER positive and proliferating cell populations tend to coincide [12]. Along with this higher proliferation index of ER positive cells, the percentage of ER positivity is increased, with around two thirds to three quarters of ductal carcinoma *in situ* and invasive breast cancer cases showing ER positivity, defined by positive staining of ER in more than 10% of the cells [13]. Around 25-30% of breast cancers are ER negative (see figure below)



Double immunofluorescence of ER (red) and proliferation marker Ki-67 (green).

Left panel: ER positive breast carcinoma. Several cells show co-expression of ER and Ki-67. *Right panel*: normal breast duct. Most cells are ER positive but do not proliferate. The few proliferative cells do not show ER positivity.

At a molecular level, in a significant minority of cases, ER positivity of breast cancer cells can be explained by oestrogen receptor 1 (ESR1) gene amplification, which is mainly concentrated in tumours with a higher percentage of ER-positive cells[14]. In the remaining cases, ER-positive staining may be explained by epigenetic or post-translational mechanisms of overexpression. In addition to facilitating the understanding of the intriguing nature and origin of breast cancer, the presence or absence of ER in breast cancer cells is of major importance when making treatment decisions, particularly refering to hormonal therapies (see below).

Oestrogen drives breast cancer biology by interaction with ER

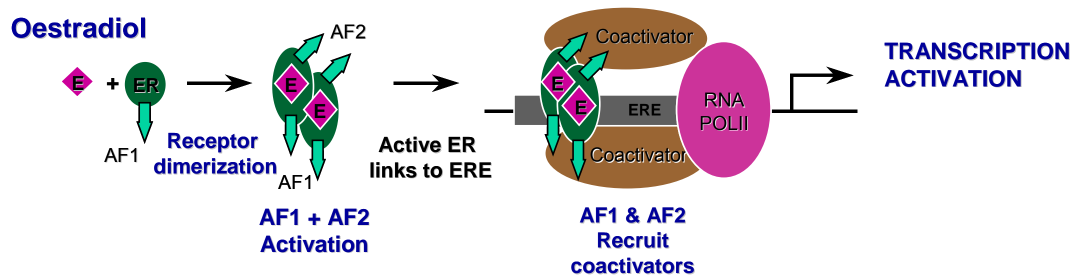
It has been well established that the activity of all oestrogen sensitive cells is mediated by the binding of E_2 to ER[15]. Although the oestrogen receptor has different types, two are the most important, namely ERα and ERβ. The latter has recently been described and, although it seems to play an important role in the modulation of ERα activity and may be of paramount importance to understand the efficacy of hormonal treatments, its

role has not been clearly defined [16]. Herewith and unless otherwise specified, we will refer to the α form when ER is mentioned.

The ER was defined as a cell receptor with exclusive nuclear location and its activity was considered to be solely derived from its direct interaction with sequences of DNA where the complex E_2-ER could bind and initiate gene transcription (the oestrogen responsive elements or EREs)[17]. Nevertheless, this classical genomic mechanism of action has recently been enriched by other alternative modes of action of the complex E_2-ER. The complexity of cell biology results in a permanent interaction and cross-talk of the different pathways initiated by the E_2-ER binding. In order to understand the diverse mechanisms of action of oestrogens, these different pathways can be divided into classical genomic, non-classical genomic and non-genomic.

In the classical genomic mechanism of action (the best understood and probably most important), E_2 arrives to the nucleus through the cell membrane and induces activation of the receptor. In fact, ER dissociates from heat shock proteins, and undergoes conformational changes, dimerization and phosphorylation. The activated ER binds to oestrogen response elements (EREs). Two different domains, activating function-1 (AF-1) and AF-2, mediate positive regulation of gene expression by ER[18]. AF-1 is at the N-terminus of the receptor. Its function is regulated by phosphorylation and does not seem to depend on hormone-regulation, whereas AF-2 is in the ligand-binding domain of the receptor and is hormone-dependent. The two activating domains act synergistically, although some gene promoters are activated independently by AF-1 or AF-2. Co-regulatory molecules that interact with the ER–ligand complex modulate the transcriptional activity of ER. In particular, the transcriptional activity of ER is enhanced by the binding of co-activators such as nuclear-receptor co-activator 1 (NCoA1 or SRC1), NCoA2 (TIF2) and NCoA3 (AIB1, TRAM1, RAC3 or ACTR) to the AF-2 domain [19]. These proteins form large complexes that enhance ER-driven transcription by different mechanisms including recruitment of histone-acetyltransferase (HAT) at the promoter site. In contrast, co-repressor proteins such as nuclear-receptor co-repressor

1 (NCoR1) and NcoR2 influence ER-induced transcription, at least in part, by recruitment of histonedeacetylase complexes.



E=Oestradiol.
ER= Oestrogen receptor.
AF1=Activation function 1.
AF2=Activation function 2.
ERE=Oestrogen eesponsive elements.
RNA POLII=RNA polymerase II.

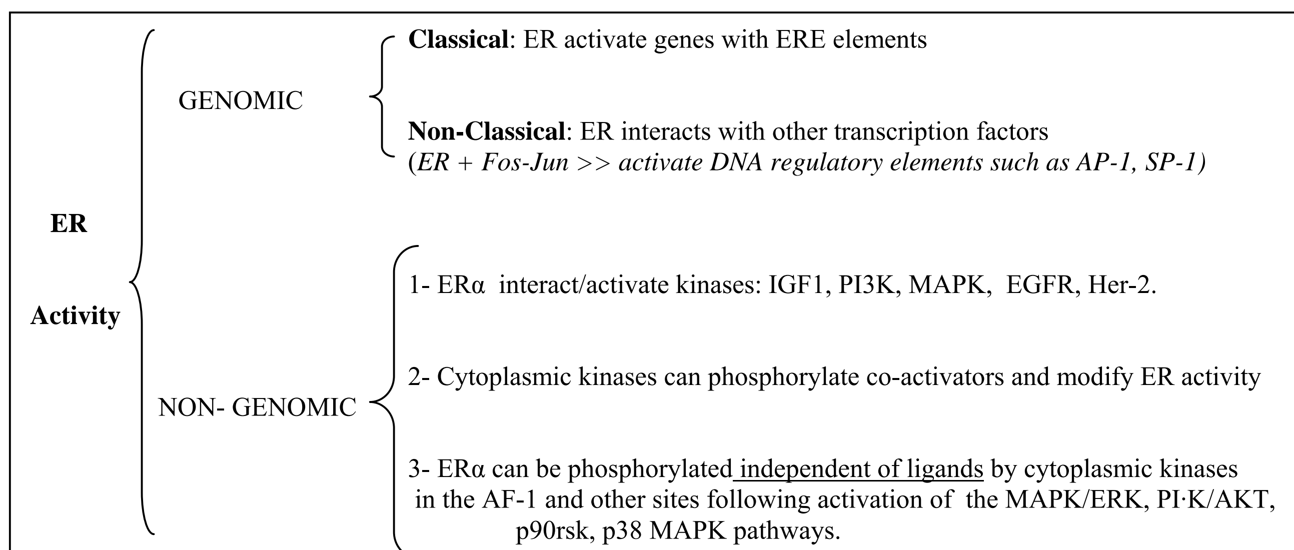
Initially supposed to be mainly located proximal to the promoter sequences of genes, full genome screening for ER binding sites has demonstrated that most EREs are located at *cis*-regulatory regions different from the promoters[20]. Screening has also better defined the existence of ER-DNA binding sites alternative to the EREs. The AP-1 binding sites are DNA sequences where ER bind, and also result in gene activation/repression. This is known as the non-classical genomic mechanism of action. This AP-1 element binding seems to depend on other transcription factors such as JUN and FOS[21]. In the time-dependent gene activation/repression activity of ER, the AP-1 dependent gene population seem to be enriched in genes mainly repressed in a second time.

With respect to the nuclear actions dependent upon the direct interaction of ER with DNA, several reports have shown the ability of ER to interact with cytoplasmic kinases in a cross-talk between cytoplasmic kinase pathways and ER genomic action. In this way, ER can directly interact with the pathways depending on the insulin-like growth factor receptor (IGFR-1), Src, the phospatidylinositol 3-kinase (PI3K), the mitogen activated protein kinase (MAPK), the epidermal growth factor receptor (EGFR) and

ErbB-2[22-25]. Conversely, the cytoplasmic kinases can modulate ER activation via the phosporylation of co-factors that activate or repress ER activity on DNA[26-28].

Finally, in the scenario of the non-genomic interactions of ER, it has been shown that ER can be directly phosporylated by the kinases resulting in its ligand-independent activation. These non-genomic activities of ER generate a complex landscape in which ER plays its cornerstone cell biology role in constant interaction with the cytoplasmic kinases (summarized in Fig 1); the so-called ER-growth factor cross-talk.

This cross-talk has great importance in the modulation of hormonal treatments and, particularly, in the acquisition of resistance to such therapy.



Gene regulation produced by ER activation

Once ER is activated and binds to DNA it results in the promotion of the transcription or repression of the different genes to which it bind. Several reports have comprehensively studied the changes in gene expression providing extensive lists of genes regulated following the binding of E_2 to the ER in *in vitro* models of oestrogen - dependent breast cancer[29-31]. It is beyond the scope of the introduction to this thesis to perform an extensive review of the knowledge on these changes. Nevertheless, some general ideas can be outlined from these screening studies:

- The number of genes regulated by ER activation is very high and covers a full range of cell functions (cell proliferation, cell-matrix interaction, cellular transport, homeostasis, signalling pathways…) thereby demonstrating the importance of oestrogen-driven processes in cell biology.
- The number of genes in which expression is repressed by E_2 is similar or even higher to those up-regulated. This fact is not contradictory to the pro-transcriptional activity of ER. Actually several mechanisms have been suggested to explain this repression such as the inhibition of NF-κB or the sequestering of transcriptional co-activators that may be necessary for gene expression[32, 33]. Most of the down-regulated genes seem to be functionally related to transcription, signal transduction and enzymatic reactions.
- Different groups of genes are regulated in a time-dependent fashion. Thus, some genes are rapidly up or down-regulated while others change their expression pattern at a later stage. A time course examination of the gene changes shows an earlier induction of the up-regulated genes while those which are down-regulated seem to be repressed a second time[30], suggesting that the down regulation of genes produced by ER may be mediated by intermediate factors which up-regulate themselves by ER in a first instance, resulting in a later blockade of other gene expression. It has also been hypothesized that the time dependence of ER activity and the selective up or down regulation may depend on the type of binding established with the DNA, with the genes harbouring EREs in the regulatory sequences being more rapidly up-regulated and those carrying AP-1 sequences being mostly repressed a second time[20].
- The functional categories of genes regulated by ER seem to be enriched in genes related to transcription, signal transduction, cell cycle, enzymatic reactions, cellular transporting and cell communication, adhesion and motility.

- There is a high degree of overlap when these studies of gene regulation are performed in different cell lines.

Although these comprehensive screening studies provide a good portrait of oestrogen-directed biology in cell lines, there is a remarkable lack of parallel studies performed in breast cancer *in vivo*. These studies may be of great importance given the marked differences to be expected between a cell growing in a culture dish, virtually without any interaction with other tissues etc., and a human tumour cell growing in complex tissue surrounded by stroma within a stressing environment exposed to hypoxia, immunity and other vital processes.

Endocrine dependency of breast epithelial cells provides a great opportunity for the treatment of breast cancer.

Since Beatson's findings[8] hormonal dependence of breast cancer has been used to fight the disease. The different strategies implemented have ranged from ovarian ablation in pre-menopausal women (for years the only feasible endocrine treatment) to the new aromatase inhibitors including ER down-regulators, selective oestrogen receptor modulators (SERMs), LHRH analogues, androgens, progestagens and high doses of oestrogens. Herewith we will refer to the current pharmacological approaches routinely used by clinicians, namely SERMs (basically tamoxifen), third generation aromatase inhibitors (anatrozole, letrozole and exemestane) and ER down-regulators (fulvestrant). The remaining drugs have largely been abandoned because of their lack of efficacy or excessive toxic effects.

SERMs

For years selective oestrogen receptor modulators [mostly tamoxifen since raloxifen and toremifen, also approved for treatment, share almost all the biologic properties with tamoxifen but are less well known and of little use in this setting] have been the only pharmacological approach to endocrine treatment of breast cancer. Tamoxifen was

registered in 1969 as a potential contraceptive treatment but later proved to be an effective therapy against metastatic breast cancer[34]. Established as a cornerstone of palliation in advanced breast cancer, a large meta-analysis of early breast cancer trials also found it to be an effective treatment. Tamoxifen has therefore become an important pharmacological agent in the adjuvant setting of the disease 5 years after resection surgery [35]. The addition of tamoxifen to surgery in patients with positive nodes (high risk) achieved an absolute improvement of 11% in the 10-year survival rate. Nevertheless, this benefit was negligible in ER-negative patients. This latter issue was a major finding providing oncology with the first, and to date, most commonly used predictive factor of treatment activity.

Tamoxifen is a dual agonist-antagonist that binds to ER and produces a conformational change in the receptor that impedes the binding of co-activators, thereby arresting AF-2 mediated transcriptional activity[36]. In breast cancer cells, tamoxifen mainly plays an antagonist role by arresting cell proliferation and inducing apoptosis. Nonetheless, its agonist side is not absent in the effect of tamoxifen on breast cancer cells. When compared to a pure antagonist such as fulvestrant, which completely blocks oestrogen-driven gene regulation, several genes are still regulated by E_2 even in the presence of tamoxifen[31]. However, it is of note that these differences do not result in a substantial difference in the clinical activity of tamoxifen compared to fulvestrant[37].

The dual antagonist-agonist effect of tamoxifen is responsible for several side-effects of the drug, some of which are desirable (delay in bone resorption in post-menopausal women) while others are the source of major treatment-attributable toxicity (endometrial hyperplasia and cancer).

Aromatase Inhibitors

For years taxoxifen was the only useful endocrine treatment, and was only overcome in its anticancer activity by the third generation aromatase inhibitors (AIs): anastrozole and letrozole, which share a non-steroidal biochemical structure, and exemestane, with

an steroidal structure. These are few differences among these AIs and they are the most effective endocrine treatment for ER-positive breast cancer in post-menopausal patients[38]. The mechanism of action of AIs differs substantially from tamoxifen. Whereas tamoxifen produces a blockade in the ability of E_2 to induce the activation of ER, AIs simply produce an almost complete deprivation of the substrate of the reaction, E_2.

As previously stated, together with oestrone and oestrone sulphate, oestradiol, are all products of the enzymatic activity of aromatase on androgens. The conversion of androgens into oestrogens in pre-menopausal woman takes place mainly in the ovarian cells surrounding the follicles. The activity of aromatase in this setting is very important and the levels of circulating E_2 are correspondingly high. Aromatase inhibitors cannot significantly decrease ovarian aromatase activity and are of little use in pre-menopausal patients with breast cancer.

Conversely, when the pool of follicles comes to an end and women undergo menopause, the ovary stops oestrogen production. Thereafter, E_2 levels are much lower but are still present. Although E_2 production continues thanks to aromatase activity, this no longer occurs in the ovaries but rather in several tissues incuding fat tissue, adrenal glands, muscles, brain, breast parenchyma and breast cancer itself when present[38]. The activity of aromatase is substantially lower after menopause, such that it may be blocked by aromatase inhibitors, achieving aromatase inhibition rates over 95%[39-42]. Thus. E_2 are reduced to a minimum leaving ER without its natural substrate and stopping ER lead gene transcription.

This different mechanism of action results in different clinical activity and, although nowadays, the differences are slight in most cases, AIs have shown to be more effective than tamoxifen in all treatment settings of ER-positive breast cancer in post-menopausal women.

Initially tested in patients with advanced disease, both anastrozole and letrozole showed a better disease control when compared to tamoxifen. The differences were

not significant in terms of the tumour response rate for anastrozole but were so for letrozole. Taking into account the favourable toxicity profile of the AIs, they rapidly became first line therapy in this setting[38].

Tamoxifen versus AIs in first line metastatic or locally advanced breast cancer

Author	Treat.	n	Response rate	Clinical benefit rate	TTP (months)
Mouridsen	Letrozole	453	30%	49%	9.4
	Tamoxifen	454	20%	38%	6.0
Nabholtz	Anastrozole	171	21%	59%	11.1
	Tamoxifen	182	17%	46%	5.6
Bonneterre	Anastrozole	340	33%	56%	8.2
	Tamoxifen	328	33%	56%	8.3
Eiermann	Letrozole	154	55%	--	--
	Tamoxifen	170	36%	--	--
Ellis	Letrozole	154	60%	--	--
	Tamoxifen	170	41%	--	--

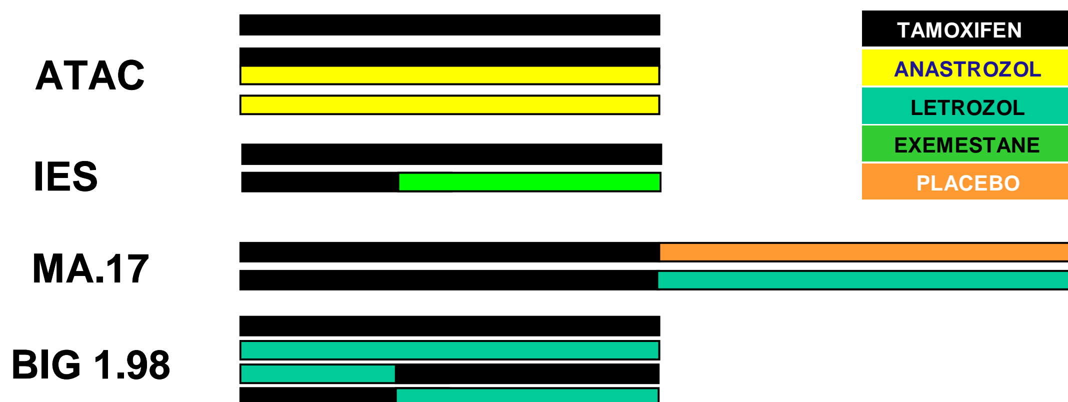
Underlined values show a statistically significant benefit in favour of AIs
Modified from IE Smith & M Dowsett. NEJM 2005

Remarkably, one setting in which AIs, particularly letrozole, have shown a neat superiority over tamoxifen has been the primary hormonal treatment of localized or locally advanced breast cancer. One clinical study demonstrated that letrozole over-performed tamoxifen in response rate in this setting[43]. These clinical results were paralleled by a higher degree of proliferation abolition in the letrozole arm[44]. Interestingly, these differences were of particular magnitude in favour of letrozole in the subset of tumours with overexpressed epidermal growth factor pathways (Her-1 and Her-2). This fact has generated new hypotheses on the potential mechanisms involved in the resistance to endocrine treatments (see below).
Following the success of AIs in the setting of advanced disease, multiple trials were initiated to test whether their efficacy in the adjuvant setting differed from the standard five years of tamoxifen. A thorough discussion of these trials would be lengthy and out of the scope of this review. Summarizing, all three third generation aromatase inhibitors have shown prolonged disease free survival rates compared to 5 years of tamoxifen.

Despite the good prognosis of most of these patients, the complexity of these trials, the need for long follow up and the limited differences between treatments have resulted in a lack of improvement in the overall survival rates to date in most of these trials. Nonetheless, AIs have changed the previous standard[45]. Several strategies can be used to include AIs in post-surgical treatment schedules, ranging from the initial introduction of an IA over 5 years, to the prolongation of adjuvant treatment with AIs after 5 years of tamoxifen. The so-called "switching" strategy includes initial treatment with tamoxifen for 2-3 years followed by an IA until completion of the 5 years. See summary table below.

While the best strategy to use AIs in the adjuvant setting remains to be defined, at present their inclusion in this scenario is state-of-the-art.

Summary of the AI versus Tam 5 pivotal adjuvant trial results in terms of disease free survival (DFS) and overall survival (OS)



	ATAC	IES	MA17	BIG 1.98
Result	Anas > Tam	Tam-Exe > Tam	Tam-Let > Tam	Let > Tam
DFS	HR 0.83; p 0.005	HR 0.75; p <0.001	HR 58%; P<0.001	HR 0.82; p 0.007
OS	ns	ns	node + HR 61; p 0.04	ns

ns: not significant

Nevertheless, resistance to endocrine treatment is almost a universal fact.

Despite all the above considerations on the efficacy of endocrine treatments, resistance to these therapies is almost a universal fact. This can be illustrated with some clinical observations:

- Almost no ER-negative tumours respond to hormonal treatment, the first feature of resistance.
- Depending on the risk groups, even under endocrine treatment after surgery, in the adjuvant setting a number of patients present relapse of ER-positive disease.
- Two main features illustrate resistance in the locally advanced or metastatic settings in which endocrine therapy is used without prior tumour excision and if only ER-positive tumours are considered:
    1. No more than 50-60% of ER-positive tumours respond to the currently most effective hormonal therapy (namely letrozole in post-menopause), demonstrating up-front or *de novo* resistance to endocrine treatment in about half of ER-positive tumours.
    2. Despite initial benefits, virtually all tumours undergoing hormonal therapy eventually relapse if not excised. Thus, practically all tumours under prolonged hormonal therapy acquire resistance.

Based on these clinical observations the scientific community is making an important effort in studying the mechanisms involved in resistance to endocrine treatments. The following list summarizes the potential mechanisms producing resistance:

- Changes in the ER expression or function.
- Modulation by ERβ.
- Pharmacological issues.
- Altered expression of co-regulators.
- Supersensitivity of ER.

- Increased growth factor signalling.

1- Change in ER status

ER positivity is not a non-modifiable feature. Indeed, it has been calculated in around 15-20% of initially ER-positive tumours which, at the time of relapse or re-growth, show lack of ER expression[46]. Some of these cases may be explained to incorrect initial determination but most represent either a real down-regulation of ER or the clonal selection of the ER-negative cells of an initially heterogeneous tumour. Although this highlights the need for re-evaluation of ER at the time of tumour progression, it occurs in a minority of cases and cannot be considered responsible for resistance in most instances. Several mutations[47] and variant messenger RNA[48] of ER have been described; nevertheless its role in endocrine treatment acquisition is unclear.

2- Modulation by ERβ

ERβ, a second oestrogen receptor, seems to be a modulator of ERα and may play a role in the ERα independent growth resulting in resistance[16, 49]. Nonetheless, to date, its importance has not been sufficiently clarified.

3- Pharmacological issues

Tamoxifen, the most studied drug in reference to resistance, is metabolized through the cytochrome p450 complex. Some variants in enzymes of this complex have shown to carry prognostic implications[50]. Of interest, certain relevant drug interactions may diminish tamoxifen efficacy. For example, paroxetin, a selective serotonine reuptake inhibitor commonly used for the treatment of hot-flushes (the most frequent side effect of tamoxifen), reduces the levels of an active metabolite of tamoxifen[51].

4- Altered expression of co-regulators

The tissue specific dual agonist-antagonist activity of tamoxifen has allegedly been determined by the availability of co-activators or co-repressors in the different organs. This modulator effect of the co-regulators may result in resistance. For example, high levels of the co-activator AIB1 have been related to a worse outcome in tamoxifen-treated patients[52]. It should, however, be taken into account that most of these predictive-factor studies are retrospective and therefore provide limited evidence In particular, in reference to AIB1, its response modulator may be confounded by its dependence on Her-2.

5- E_2 Independence acquisition

Based on cellular models in which E_2 dependent cell lines are grown under oestradiol deprivation for months until they acquire the capacity of becoming oestrogen independent, several groups have studied the processes leading to this resistant-stage[53-55]:

- A first feature of these cells is the so call "hypersensitivity" of its ER. In this stage, there is an elevated level of ER with an increased rate of translocation to the cytoplasm. This ER seems to respond to minimal doses of E_2 through a cytoplasmic effect on the MAPK signalling pathway that, in turn, activates proliferation. Cells at this stage are extremely sensitive to all processes derived from exposition to oestradiol. Thus, the apoptosis induction usually takes place when the wild-type cells are exposed to high doses of E_2 (paradoxically), and are triggered by much lower doses of the hormone.
- In another study on this model the "supersensitive" stage was described. Here, the transcriptional activity of ER is maintained via its activation by growth factor pathways. At this stage ER is almost independent from E_2 or responds to residual traces of oestrogen in the medium. The growth rate of these cells slows down when insulin is removed from the medium. This observation allowed the

study of the role of phosporylation of ER at its serine 118 residues that seem to activate the receptor independently of its natural ligand.

- Other observations in this model suggest alternative growth depending on other pathways such as the PI3K/AKT.

6- Cross-talk

The radical importance of the acquisition of resistance of the cross-talk between growth-factor pathways and ER is also confirmed by the clinical observation that Her-2 positive tumours are much more sensitive to letrozole than to tamoxifen. On the basis of the above mentioned clinical trials[43, 44] and on previous *in vitro* findings, it was hypothesised that the activation of the Her-1 and Her-2 driven pathways results in a modulation of the cytoplasmic activity of tamoxifen on the ER. When Her-2 is activated, tamoxifen apparently has mostly an agonist activity on cytoplasmic ER, impeding its anti-tumour activity. Since AIs follow a completely different therapeutic strategy, this modulation is not possible as the ER is not activated due to the simple lack of substrate, thus AIs were expected to have a clear advantage over tamoxifen in these tumours. Although not yet overwhelmingly agreed, to date, Her-2 positivity is the only phenotypic criterion that seems to make tamoxifen relatively contraindicated (as discussed previously).

Summarizing this paragraph on endocrine treatment resistance, the plasticity of cancer cells seems to make them particularly skilful either in finding alternative pro-growth signal pathways and circumventing the lack of oestrogen-driven stimulus when switched off or in re-activating the ER pathway independent of the abundance of its ligand. This ability to by-pass the effect of endocrine treatments may be shown very rapidly resulting in the so called up-front or *de novo* resistance or may take longer to be patent making the tumours initially sensitive and becoming resistant at a later stage.

Given this scenario of the endocrine treatment of breast cancer, which challenges call for translational research from the clinic?

Obviously, a major issue in the search for better results in endocrine treatment in breast cancer is the development of novel and more effective agents. This may be achieved by more in depth knowledge of the pathways involved in resistance to thereby target these new agents to the genes or gene products most implicated in the loss of hormonal treatment efficacy.

Bearing in mind the urgent need for new agents and focusing on the challenges that clinicians face nowadays in the every day clinics two of them could be highlighted:

1. The need for better predictive factors.
2. The need for new early surrogate markers of treatment efficacy which correlate well with long-term patient outcome.

1- The need for better predictive factors.

Regarding the predictive information on the expected efficacy of endocrine agents little has been introduced in the daily routine practice beyond ER positivity. As previously commented, taking the most efficacious endocrine treatment into account, ER positivity accurately predicts response in no more than 60% of the cases (response which does not always correlate with longer survival). This means that the factor of choice has a positive predictive value of no higher than 60%. Conversely, its negative predictive value is higher than 90%.

Many studies have been aimed at the search for new predictive markers as discussed below. Nevertheless, to date, no predictive marker is routinely used (with the exception of Her-2 positivity as indicated beforeforehand). This need is patent both in the early adjuvant and in the advanced disease settings. When making decisions as to the complementary systemic treatment to be implemented after surgery, a more precise prediction of response to endocrine agents would be of great help to avoid chemotherapy in patients with tumours showing extreme dependence on ER or,

alternatively, to stress the need for treatments other than hormonal therapy when the tumours are likely to rapidly develop resistance to ER pathway inactivation.

Regarding the metastatic setting, in which the quality of life is of utmost importance, avoiding long exposure to treatments before they can be deemed ineffective, would probably result in longer progression free survivals and better palliation.

2- *The need for new early surrogate markers of treatment efficacy which correlate well with the long term outcome of patients.*

Nowadays tumour volume reduction, usually referred to as response, is considered the gold standard as a prognosticator of long-term benefit and survival. This paradigm can be challenged in two settings: that of primary hormonal treatment of early operable or locally advanced breast cancer and metastatic disease.

Three examples may illustrate the limitations of tumour reduction as a marker of efficacy:

- In a recent study by our group, we demonstrated the weakness of the correlation between radiological and pathological assessment of response to primary hormonal treatment prior to primary tumour excision. Almost one third of the cases were deemed to be stable disease following hormonal treatment evaluated by radiology before surgery while the pathologist observed extensive changes attributable to treatment. Thus, while these cases were evaluated as obtaining little benefit from endocrine treatment assessed by tumour response criteria, they were clearly sensitive to this therapy[56].
- In the metastatic setting, clinical experience has revealed that, considering endocrine treatments, clinical benefit is not always mediated by tumour shrinkage and a common endpoint is the stabilization of the disease. This has a biological rationale if we consider that response assessment by tumour reduction is a common standard in chemotherapy and has been extrapolated to the remaining anticancer therapies, including endocrine. Nevertheless, the

efficacy of active chemotherapy is mediated through the induction of cell death while hormonal treatment results in the withdrawal of a pro-growth signal which usually, but not always, correlates with apoptosis induction when the principle feature of treatment activity is diminished proliferation[57]. This means that, when endocrine therapies are active they may result in the discontinuation of growth but not necessarily tumour shrinkage. Although hormonal treatments are the first therapies targeted, the introduction of novel targeted drugs in the current oncology arena has re-opened the debate as to the convenience of the use of new endpoints or means of assessment beyond traditional tumour response standards[58].

This prolonged time before treatment efficacy can be assessed exposes patients to inactive and toxic treatment in a significant number of cases.

- Lastly, as the first explanatory example of the need for new markers of clinical benefit beyond response, two recent trials compared anastrozole and tamoxifen in adjuvant and neo-adjuvant settings, respectively. While in the post-surgical trial anastrozole was superior in long-term efficacy[59], these results were not paralleled in the neo-adjuvant setting where no differences were seen in the response rates between anastrozole and tamoxifen[60]. Although this lack of difference may be attributable, in part, to the short duration of treatment (3 months) it is remarkable that the changes in the biomarkers correlated better with the more effective performance of anastrozole in the long-term than tumour shrinkage itself[39, 61].

All these facts highlight the need for new markers of treatment efficacy in the arena of endocrine therapy. This thesis is focused on the search for novel tools in this setting.

What are the most important breakthroughs in the search for new predictors or reporters of endocrine treatment activity?

1. Oestrogen and progesterone receptors

As has previously been mentioned, the first, major advance in the tailoring of endocrine treatment was made when the relation between ER and benefit from tamoxifen was firmly established[35]. Progesterone receptor, a protein firmly linked to ER, is reported to be a marker of ER pathway integrity[62]. Despite the large number of studies which have been focused on the value the progesterone receptor positivity or negativity as a predictor of response to different types of endocrine therapy; no clear conclusions have yet been drawn on this issue[63, 64].

2. New basal predictors of response to hormonal therapies

Many studies have focused on the search for the description of novel molecular or clinical characteristics of breast cancer which, determined at the time of diagnosis, may allow prediction of endocrine treatment efficacy beyond the mere positivity of ER or progesterone receptor. Unfortunately, most of these studies have correlated the initial tumour phenotype at surgical excision with the possible event of relapse under adjuvant endocrine treatment, mostly with tamoxifen. This approach makes it difficult to draw conclusions given that the predictive value of these factors is confounded with the prognostic value. Thus, it is difficult to determine whether a tumour which does not relapse after resection under hormonal treatment is due to the efficacy of the treatment or the low tendency of the tumour itself to relapse.

The neoadjuvant setting of early and locally advanced breast cancer is probably the best to study these potentially predictive factors. In this setting these factors may be correlated with their biological activity comparing the initial tumour features with those at the time of surgery after endocrine treatment followed by the study of their relation with long-term outcome after excision. Nevertheless, in most correlation, this correlation has not been made. Some of the putative individual predictive factors that have been studied include: bcl-2, p53, Her-2, Her-1, Cyclin D1, heat shock proteins, ki-67, and pS2[65-69]. The complexity of tumour cell biology and the important inter-

dependence of most of the individual factors make it difficult for any of these factors to surpass the performance of ER. Indeed, with the exception of Her-2 (see before) these factors are of little use for physicians.

The availability of new technologies which allow comprehensive screening of gene expression in cells has generated a renewed interest in the search for predictive/prognostic markers related to hormonal agents. Of interest, one of these multi-gene platforms has been approved by the regulatory agencies for its use in this setting, albeit this platform was based on large retrospective series with the confounding factor of treatment efficacy[70]. This new technology may improve ER value but clearly requires further validation before its introduction in the state-of-the-art armamentarium.

One of the major breakthroughs in breast cancer research in the last years has been the definition of breast cancer subtypes harbouring prognostic value based on microarray technology,[71, 72]. One of these sub-types, the so-called luminal A, is characterized by its over-expression of ER-related genes, its high ER scoring and its good prognosis. This is allegedly the tumour subset most likely to benefit from endocrine treatments However, apart from ER positivity, the predictive value of this platform has yet to be determined.

3. Dynamic biomarkers of response

Since the initial tumour phenotype has limitations in predicting tumour behaviour when exposed to endocrine treatment, one possible approach is *in vivo* testing of efficacy. Tumour phenotyping may be a useful tool if assessed early before tumour response to thereby not expose patients to prolonged, inactive and potentially toxic treatment. Some non-invasive methods for tumour metabolism assessment are being explored in this setting with encouraging results[73, 74]. The conclusion of these studies may be that the early decrease in metabolism following initiation of treatment may be a good predictor of later benefit.

Nevertheless, the most extensively studied dynamic factor has been the Ki-67 protein. Ki-67 is a non-histone nucleolar protein tightly linked to cell-cycle. Its expression is universal in cells going through the cell cycle while it is absent in cells in G_0. The feasibility of its determination in frozen and paraffin-embedded tissues using immunohistochemistry techniques and its high efficacy in reporting proliferation has prompted its extensive use in cancer research. The prognostic and predictive values of Ki-67 have been studied In translational research. A recent review by this author and colleagues summarizes its nature and value in early breast cancer[1].

Ki-67 has prognostic value for long-term outcome after excision when determined up-front. Nevertheless its correlation with other features such as histological grade or ER positivity makes its prognostic value secondary to other features in the decision making of oncologists.

The availability of minimally invasive tumour sampling techniques in early breast cancer (i.e. fine needle aspiration, core-cut biopsy) has prompted investigators to study whether changes in Ki-67 score occur after a variable treatment period and may provide a better predictive or prognostic value than base-line measurements. If so, these early changes might provide a valuable intermediate marker of treatment benefit, particularly in relation to drug development.

Studies with MCF-7 xenograft tumours in nude mice with oestrogen deprivation[75] or anti-oestrogen hormonal treatments[57]provide encouraging proof of principle. Each of these treatments results in a rapid decrease of Ki-67 within 1 week of treatment. The study by Johnston et al demonstrated a complex relation between Ki-67 decrease, changes in apoptosis and tumour regression following oestrogen withdrawal compared to tamoxifen (Fig 1). Oestrogen withdrawal induced a profound decrease in Ki-67 (circa 5-fold) accompanied by an increase in apoptosis (circa 4-fold) and this resulted in tumour regression. With tamoxifen, lesser reductions in Ki-67 (circa 2-fold) and increases in apoptosis (circa 3-fold) were seen and these were associated with no more than stabilisation of tumour size. These findings suggest that although smaller

changes in Ki-67 and apoptosis could lead to slowing of tumour growth they could still be associated with progressive disease. The implications of these findings for clinical studies are discussed below.

Before assuming that a change in any biomarker is due to intervention, the intrinsic variability of the marker without the mediating intervention should be assessed. Few reports have addressed this issue. Our studies on the reproducibility of Ki-67 measurements in core-cut biopsies suggest that a change in Ki-67 score of at least 32-50% between two determinations is required to consider the difference as attributable to treatment effect *for an individual patient* [76, 77]. These individual requirements do not apply in the same way to populations but are important to take into consideration in the statistical powering of these studies. Further data on the variability in the measurements of Ki-67 are provided by the placebo arms of several short-term pre-surgical studies[78-81] which largely support our estimates of variability. However, different biopsy sites, staining and counting procedures may affect this variability.

(Ki-67 / AI) **GI**	A	B	C
+ E_2	(51.0 / 0.86) **59.3**	(42.7 / 0.77) **55.4**	(30.8 / 0.48) **64.1**
+ E_2 + tam		(24.7 / 2.48) **9.9**	(30.8 / 0.69) **44.6**
- E_2		(9.0 / 3.68) **2.4**	(11.8 / 3.85) **3.0**

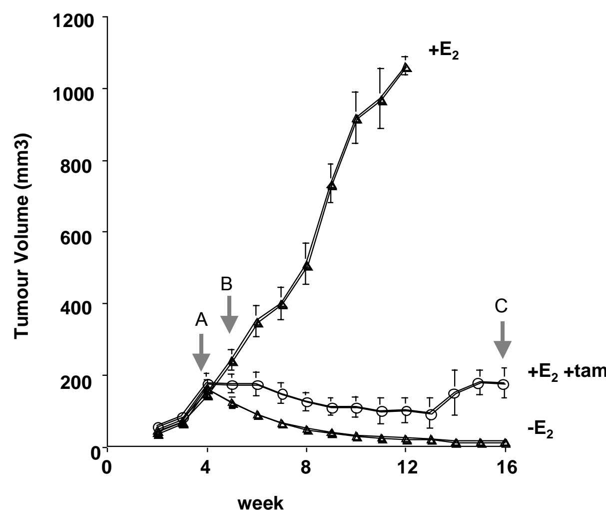


Fig 1. Taken from Urruticoechea et al[1]
Effect of tamoxifen (tam) on the antagonism of oestradiol (E_2) supported growth of MCF-7 xenografts in nude mice. Control groups consisted of either E_2 treatment alone plus placebo capsule (+E_2) or withdrawal of E_2 support plus placebo capsule (-E_2). All tumours were initially established over 4 weeks with E_2 and measured weekly. Table above: determination of growing index (GI) at three different time points. Data points: means; Bars: standard error; AI: apoptotic index

In the clinical setting, effective hormonal treatments suppress Ki-67 levels in both short-term (less than 6 weeks)[78-87] and long-term (eg 12 weeks) studies[44, 88-92]. This has also been shown with withdrawal of hormone replacement therapy[93]. As would be expected, these changes are only seen in hormone receptor positive tumours[79-81]. Reduced Ki-67 levels with hormonal therapy are illustrated in figure 2 which shows the substantial falls in Ki-67 at two weeks and 12 weeks in 56 patients treated with anastrozole prior to surgery[94]. At 2 weeks only 4 patients did not show a reduction in Ki-67 levels. The geometric means of reduction were 76 and 82% at 2 and 12 weeks respectively. Some patients showed increases in Ki67 between 2 and 12 weeks which may be an early indicator of resistance to therapy. It is, however, difficult to distinguish these relatively modest increases in individual patients from the between-sample variability.

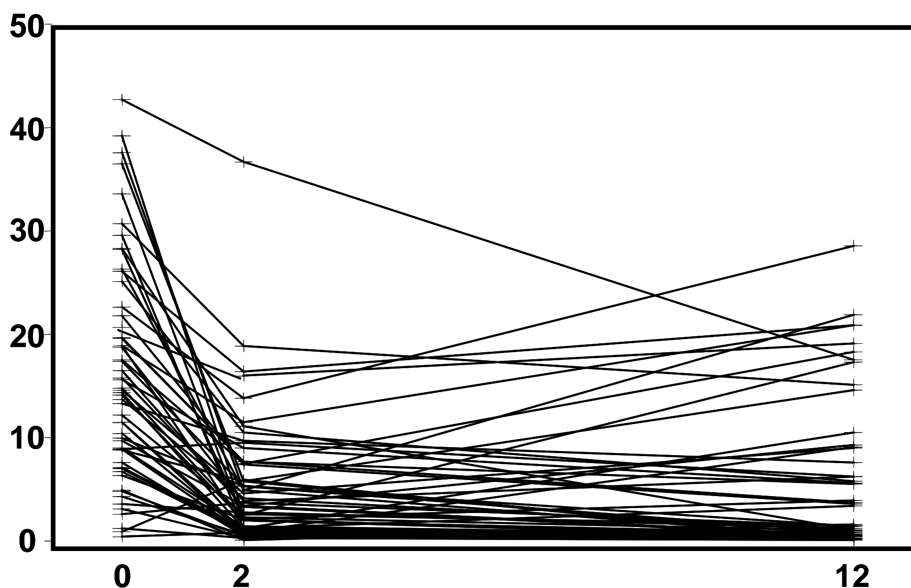


Fig 2. Taken from Urruticoechea et al[1]
Individual Ki-67 changes at 2 weeks and 12 weeks in 56 hormone receptor positive patients treated with anastrozole

The reduction in Ki-67 with endocrine therapy probably reflects the well-described cytostatic effect of the drugs[95]. Changes in Ki-67 occur early and well before

significant tumour regression is seen suggesting that the role of treatment selection of a less proliferative cell population has probably little to do with these changes.

Decrease in Ki-67 also occur with chemotherapy and mixed chemo-hormonal treatments[76, 77, 89, 96-102]. To our knowledge only one study has failed to show this[103] and our own study suggests this may be related to the timing of the second biopsy[103, 104]. The reduced proliferation with chemotherapy may be, at least partly, due to increased apoptosis in actively proliferating cells[105] such that the residual population would be enriched by Ki-67-negative cells. The relationship of the decrease in Ki-67 during cytotoxic chemotherapy over 2-3 months with long-term outcome has been addressed in two studies[96, 97] which, on multivariate analysis, found that a decrease in Ki-67 of more than 25% and a residual score <10 % each predicted a longer disease free survival.

Early changes in Ki-67 have also been found to correlate positively with clinical and/or pathological response in early breast cancer with both hormone therapy and chemotherapy[42, 44, 76, 89, 90, 92, 96-99]. In these studies the timing of the 2nd biopsy varied. The relationship with clinical and/or pathological response within 2 weeks is particularly useful since these changes preceded clinical response while the changes at 12-16 weeks are usually concordant, rather than predictive, with clinical response.

This issue has recently been supported by a report on the results of IMPACT trial[60, 106, 107]. This trial compared 12 weeks of neo-adjuvant treatment with anastrozole or tamoxifen and the combination of both drugs in post-menopausal women with ER-positive early breast cancer, followed after surgery by the same regimen as adjuvant treatment. A decrease in Ki-67 was determined in a biopsy taken 2 weeks after initiation of treatment and predicted the better long-term outcome seen in the parallel adjuvant trial with anastrozole[108].

The possibility that changes in Ki-67 might predict long-term efficacy (after 10-14 days of treatment) has opened a new scenario for clinical research in early breast cancer.

This so-called "short-term pre-operative" setting refers to the time between diagnosis of primary operable early breast cancer and the surgery itself, an interval when treatment is not usually administered. Thus, drugs with established safety and low toxicity may be tested and compared against placebo using a decrease in Ki-67 as the primary end-point. This model has been exploited in the assessment of several treatments which have mainly been hormonal[78-82, 86, 87] but have more recently included inhibitors of signal transduction pathways[109, 110]. Most of these studies showed a significant reduction in Ki-67 in the treatment arms compared with placebo or no treatment.

Different treatments have also been compared in this short-term setting to obtain rapid information on potential differences in efficacy[79, 80, 82, 85]. Comparative drug studies require that the different drugs have similar pharmacokinetics or the use of schedules to compensate for different pharmacokinetics. The drugs compared should also have a mechanism of action that affects proliferation in a similar way (or another end-point such as apoptosis). These approaches could also determine the differential effectiveness of drugs on subgroups of patients to be assessed and thereby lead to the identification of targets to select optimal therapy. For example, a recent report by Ellis et al found differences in Ki-67 response to letrozole and tamoxifen depending on whether the tumours over-expressed Her1 and/or Her2[44] and these results paralleled differential clinical response in the same subgroups[111]. These differences had been previously suggested in a pooled analyses of several smaller studies in the short-term preoperative setting[112]. The effect of Her2-targeted therapy combined with endocrine therapy on Ki-67 would seem to be a rational approach to evaluate efficacy.

While the above rationale and examples of the use of early changes in Ki-67 suggest an increasingly growing interest in the use of this protein as an intermediate marker of treatment benefit, challenges in the handling of the data obtained must be met to allow its widespread use in clinical practice. One complication is the non-normal distribution of the data which logarithmic transformation may not normalise[44]. Neither is it clear whether the proportional reduction in Ki-67 is the most relevant measure for predicting

outcome. The above arguments suggest that proportional reduction may be an appropriate parameter for predicting benefit in the adjuvant setting but that the residual (on-treatment) level of Ki-67 may be a better predictor of response and/or absolute long-term outcome since this is more likely to relate to the growth rate of the persistent disease. Currently available and future data sets should examine these possibilities.

In summary, endocrine treatment is one of the most relevant treatments used today in breast cancer. However, the need for better tools for the prediction of its efficacy and to monitor its activity is currently one of the greatest challenges of this therapy.

With the background provided in this Introduction, the aim of this thesis is to describe new dynamic markers which are more effective than Ki-67 in the prediction of response to aromatase inhibitors.

Objectives of the first study

1. To study the regulation in gene expression induced by 2 weeks of treatment with either anastrozole or letrozole in ER-positive breast cancer tissue in post-menopausal patients using high throughput technology.
2. To describe the genes with the most significant regulation by treatment with anastrozole and letrozole in this setting.
3. To compare the relation of this gene regulation process with previous knowledge obtained from *in vitro* models.
4. To compare the magnitude of the changes in gene expression between the two treatments.
5. To correlate the changes in gene expression with the changes in Ki-67 measured by immunohistochemistry.
6. To integrate the changes in gene regulation in a global index of dependence on oestrogen (GIDE).
7. To study the potential predictive value of this GIDE testing its correlation with changes in Ki-67 measured by immunhistochemistry and with other well known predictive factors of aromatase inhibitor activity.

8. Breast Cancer RESEARCH

Breast Cancer Research

Molecular response to aromatase inhibitor treatment in primary breast cancer

Breast Cancer Research 2007, **9**:R37 doi:10.1186/bcr1732

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ISSN	1465-5411
Article type	Research article
Submission date	31 January 2007
Acceptance date	7 June 2007
Publication date	7 June 2007

Molecular response to aromatase inhibitor treatment in primary breast cancer

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Abstract

Background: Aromatase inhibitors such as anastrozole and letrozole are highly effective suppressants of estrogen synthesis in postmenopausal women and the most effective endocrine treatment for hormone receptor positive breast cancer in such women. Little is known of the molecular effects of these agents on human breast carcinomas *in vivo*. Methods: We randomized primary estrogen receptor positive breast cancer patients to treatment with anastrozole or letrozole for 2 weeks prior to surgery. Expression profiling using cDNA arrays was conducted on pretreatment and post-treatment biopsies. Sample pairs from 34 patients provided sufficient RNA for analysis. Results: Profound changes in gene expression were seen with both aromatase inhinitors including many classical estrogen-dependent genes such as *TFF1*, *CCND1*, *PDZK1* and *AGR2* but also many other genes that are likely to represent secondary responses: decrease in the expression of proliferation-related genes were particularly prominent. Many up-regulated genes are involved in extracellular matrix remodelling including collagens and members of the small leucine-rich proteoglycan (SLRP) family (*LUM*, *DCN*, *ASPN*). No significant differences were seen between letrozole and anastrozole in their molecular effects. The gene changes were integrated into a global index of dependence on estrogen (GIDE) which enumerates the genes changing by at least 2-fold with therapy. The GIDE varied markedly between tumours and related significantly to pre-treatment levels of HER2 and changes in immunohistochemically detected Ki67. Conclusions: Our results identify the transcriptional signatures associated with aromatase inhibitor treatment of primary breast tumours. Larger datasets using this approach should enable the identification of estrogen-dependent molecular changes, which are the determinants of benefit or resistance to endocrine therapy.

Introduction

Approaching 80% of human breast carcinomas express estrogen receptor (ER) alpha protein at clinically significant levels and are considered ER-positive (ER+). Estrogen deprivation or antagonism, are effective treatments for many but not all patients with such tumours. The selective ER modifier, tamoxifen has been the predominant treatment for the last two decades and improves survival in ER+ patients receiving this as adjuvant therapy post-surgery [1]. However, in postmenopausal women aromatase inhibition with the non-steroidal inhibitors anastrozole and letrozole has now been shown to be more effective than tamoxifen as adjuvant therapy [2]. Letrozole and anastrozole are highly specific for the aromatase enzyme and inhibit whole-body aromatisation by 99% and 97%, respectively [3]. Aromatase inhibitors (AIs) therefore provide a highly selective and essentially complete withdrawal of estrogen in postmenopausal patients. Proliferation of malignant cells as measured by expression of the nuclear antigen Ki67 is reduced in >90% of ER+ primary breast carcinomas by treatment with AIs [4, 5]. This suggests that almost all ER+ tumours derive some proliferative stimulus from estrogen and may be considered hormone responsive; in some patients, however, this effect may be only modest. We have recently found that the difference in the change in Ki67 after 2 weeks' treatment with anastrozole, tamoxifen or the two drugs in combination was predictive of relative recurrence free survival (RFS) in a parallel adjuvant trial of the same treatments [6]. Additionally, Ki67 levels after 2 weeks treatment significantly correlated with RFS of the same patients in the presurgical study [7]. Both of these findings support the validity of short-term changes in Ki67 as an intermediate marker of the clinical effectiveness of endocrine therapy. It seems likely, however, that Ki67 is an imperfect marker of proliferation and that changes in gene expression other than those related to proliferation may be involved in determining the clinical effectiveness of estrogen deprivation.

Transcriptional profiling of estrogen responses in ER+ human breast cancer cell lines

and model systems *in vitro* leads to changes in the transcription of large numbers of genes [8, 9], however, very little is known of these effects *in vivo* or how these effects vary between tumours and whether these molecular changes fully encompass the determinants of clinical response. Biopsy of tumours before and during pre-surgical treatment with an aromatase inhibitor allows the study of estrogen-dependent effects across a range of ER+ breast carcinomas in situ. We therefore evaluated the effects of estrogen deprivation with letrozole or anastrozole on Ki67 expression and transcriptional profiles in ER+ breast carcinomas *in vivo*. Such an approach might provide insights into the mechanisms of clinical benefit and allow the development of a predictor of that benefit. Specific aims were (i) to determine whether there was a significant difference between letrozole and anastrozole on (a) change in Ki67 (reported elsewhere) and (b) changes in gene transcription, (ii) to identify the genes which change with aromatase inhibition and to integrate these as a global index of dependence on estrogen (GIDE) (iii) to assess how the most prominent gene changes relate to those reported *in vitro* with estrogen stimulation and (iv) to determine the relationship between the GIDE and previously described putative determinants of benefit from endocrine therapy such as HER2 and Ki67 expression.

Materials and Methods

Patient samples. Postmenopausal patients with primary ER-positive (Allred scores 2-8; NB scores of 2 are conventionally regarded as ER-negative, [10]) breast cancer were randomized to presurgical treatment for 2 weeks with letrozole (2.5mg/d po) or anastrozole (1mg/d po). Multiple core-cut biopsies were taken with a 14-guage needle before treatment and at surgery from 54 patients and were either immediately frozen in liquid nitrogen for RNA analysis or fixed in neutral buffered formalin for immunohistochemistry. RNA from each frozen biopsy was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Microarray hybridizations. Total RNA integrity was confirmed on an Agilent 2100 BioAnalyser prior to linear T7

amplification using a RiboAmp kit (Arcturus). 4µg of amplified aRNA was labeled with either cy3 or cy5 and hybridized to Breakthrough 17K cDNA microarrays in replicate dye swap hybridizations as previously described [11]. The Breakthrough 17K microarray platform and all primary microarray data have been submitted to Array Express (ArrayExpress submission number E-TABM-180). Annotation of the Breakthrough 17K cDNA microarray based on build 189 of Unigene is provided as supplementary information - BT17K_array_annotation. Analysis of microarray data. Expression values from spots with hybridisation artefacts or extremely low intensities were flagged in Genepix 5.1 (Axon Instruments) then converted to missing values and removed from the analysis. The raw intensity values were then converted to log2 ratios of sample to reference (M values) and log2 average spot intensity (A values) for all subsequent pre-processing and analysis. The loess local regression function was used to remove biases due to the combined effect of spot intensity and the row-group to which the spot belonged, and then to remove the more global bias across the slide. A quantile filter was used to remove data which had average intensity or A values below the 25th percentile in 60% or more of the hybridisations. The M values for each hybridisation were rescaled so as to remove the relationship between increasing dispersion of M values with increasing dispersion of A values across the hybridisations. This latter transformation did not involve extensive rescaling of the data and although it clarified the relationships found in this study, these were all apparent without this step. The replicate dyeswap hybridisations were then averaged. This left 14024 genes that were used for paired (pre/post) differential gene expression analysis using SAM version 2.21 [12]. In order to focus on the more extensive gene fluctuations between samples, further reductions in the number of genes used for some analyses were based on filtering out genes with low inter-sample variation. We used the inter-quartile range (IQR) as a robust estimate of gene variation and used a stringent threshold at IQR = 0.75 (2418 genes remaining). In order to map the gross phenotypic changes

across the samples the following supervised analysis was chosen. A core set of genes were selected using the weighted Kolmogorov-Smirnov statistic due to its robustness and flexibility. The maximum number of genes (421) that gave minimum leave-one-out cross validation in separating pre- from post-treatment samples using the k-nearest-neighbor algorithm (k = 7), were retained. Agglomerative clustering (see below) was used to separate the 421 selected genes into ten clusters of co-regulated genes. Each cluster was then represented by the average M value of it's genes for each sample (metagene) following centering and rescaling across samples. All clustering used the flexible beta agglomerative clustering algorithm with the correlation distance measure was used to cluster both genes and arrays. Clustering heat maps were produced with Java Treeview 1.0.12 software. Correlations were performed with Spearman rank or Pearson correlations. Immunohistochemistry. Conventional immunohistochemistry was performed on each biopsy using antibodies for ER-alpha clone 6F11, (Novocastra), PR clone PgR 636, (DAKO) and KI67 clone MIB-1, (DAKO) according to the manufacturers instructions.

ER and PgR immunohistochemistry was quantitated according to the Allred score [10]. Ki67 immunohistochemistry is reported as the number of positive cells amongst 1000 malignant cells counted and is expressed as a percentage. Real-Time PCR. Quantitative real time PCR was conducted on 5 genes of interest (*CCND1*, *PDZK1*, *FAS*, *TFF1*, *MAN1A1*). Total RNA from the same RNA preparations as used for microarray analysis was reverse transcribed using random primers and Superscript III (Invitrogen) according to manufacturer's instructions. A reverse transcription negative control was included to account for any genomic DNA contamination. cDNA samples were subjected to quantitative PCR using Taqman_ (Applied Biosystems) on an ABI Prism 7900HT with primers designed by Primer Express, or Quantitect SYBR green (Qiagen) on an Opticon Monitor 2 with primers designed by Primer 3 in two different laboratories. Primer sequences are shown in Supplementary Table S1. Pretreatment/posttreatment changes were estimated after normalization using the

geometric mean of the 2 reference genes that had been shown to be unchanged in the expression during treatment with AIs (*TBP*, *KIAA0674*).

Results

RNA of sufficient quality and quantity was obtained from 34 pretreatment/post-treatment pairs of samples: the following results refer solely to those samples. Patient clinical information is summarized in Supplementary Table S2. The samples were clustered to determine whether pre- and post- biopsies aggregated together as nearest neighbors in clustering dendrograms. Half of pre/post biopsy pairs were found to co-aggregate whether based on all 14034 measured genes (17/34), or 2418 genes (18/34) which were filtered to retain the most variable genes. Similar proportions of co-aggregating pairs were also found using other algorithms (e.g. complete linkage and group average linkage, data not shown). Separation of paired biopsies in this analysis contrasts with other studies in which the differences in gene expression amongst breast tumours is far greater than that observed as a result of treatment with chemotherapeutic agents [11, 13].

A heatmap diagram from the clustering of the 2418 most variable genes amongst the 68 biopsies is shown in Figure 1. Clusters of genes containing some of the most important known markers of breast tumour phenotypes are shown in greater detail: *ESR1* (A), *MKI67* (B), *ERBB2* (C) and *TFF1* (D). *ESR1* and *ERBB2* gene expression are inversely correlated in these samples (r=-0.57, p=0.0005 Pearson correlation) as has been shown in many other studies of breast tumours. The samples with the lowest *ESR1* and/or high *ERBB2* invariably have pre-/post- biopsy pairs that co-aggregate as nearest neighbours and account for more than half (11/17) of the co-aggregating pairs. The *ERBB2* cluster contained several genes present in the *ERBB2* amplicon on chromosome 17q12-21 including *GRB7*, *THRAP4* and *STARD3*, highly overexpressed in the 4 HER2 amplified cases. Data files for Java Treeview are provided as

supplementary information.

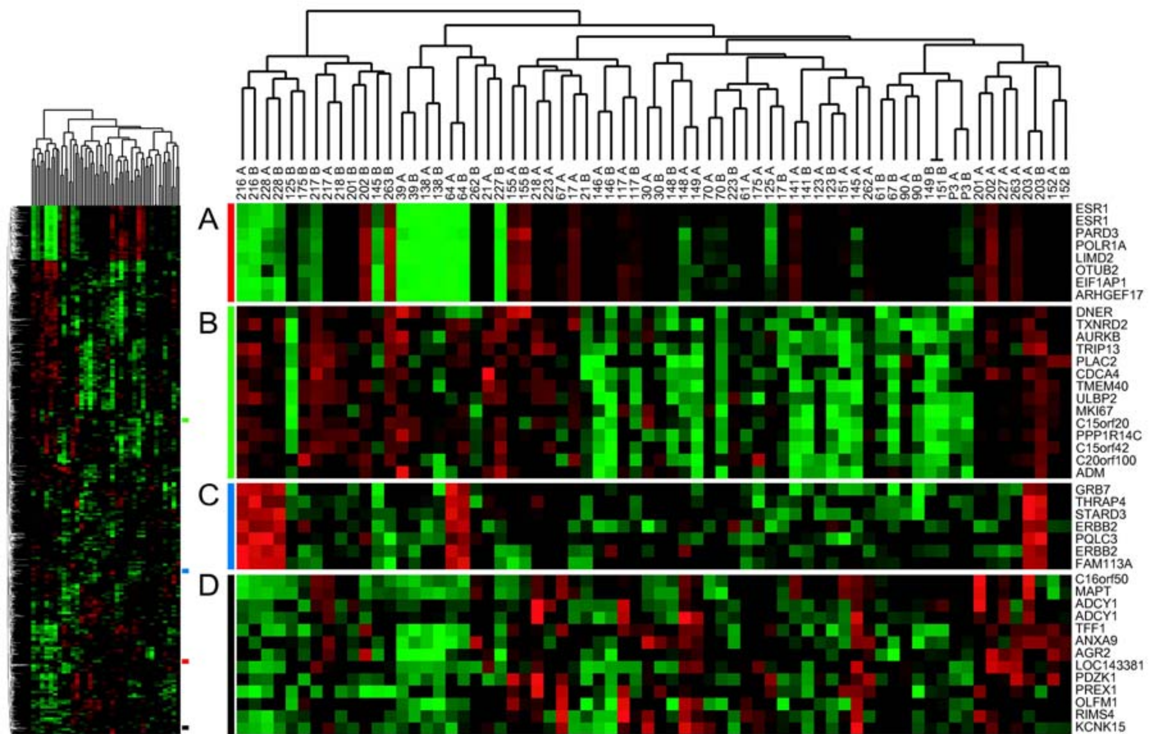

Figure 1

Heatmap of unsupervised clustering of pretreatment and posttreatment biopsies. A heatmap of the unsupervised clustering of the 34 pre-treatment and post-treatment samples (labeled A and B respectively) using 2418 of the most variable genes is shown. The entire heatmap is shown in miniature on the left. Clusters containing the genes ESR1 (A), MKI67 (B) ERBB2 (C) and TFF1 (D) are shown in detail. 18 out of 34 pairs of biopsies co-aggregated at the first or second level in the sample dendrogram.

To summarize the effects of estrogen deprivation on gene expression we have derived a global index of dependence on estrogen (GIDE). This index was defined as the number of genes changing by at least 2-fold between each pair of biopsies irrespective of the direction of change. This index positively correlated with change in the proliferation marker Ki67 (Spearman rank rho=0.533, p=0.0022, Figure 2A) and

negatively with the expression of ERBB2 (Spearman rank rho=-0.381, p=0.0282, Figure 2B). Although no patients with a high GIDE were amongst the lowest in terms of *ESR1* expression, overall there was not a significant correlation between the two. A complete summary of GIDE data is provided in Supplementary Table S3.

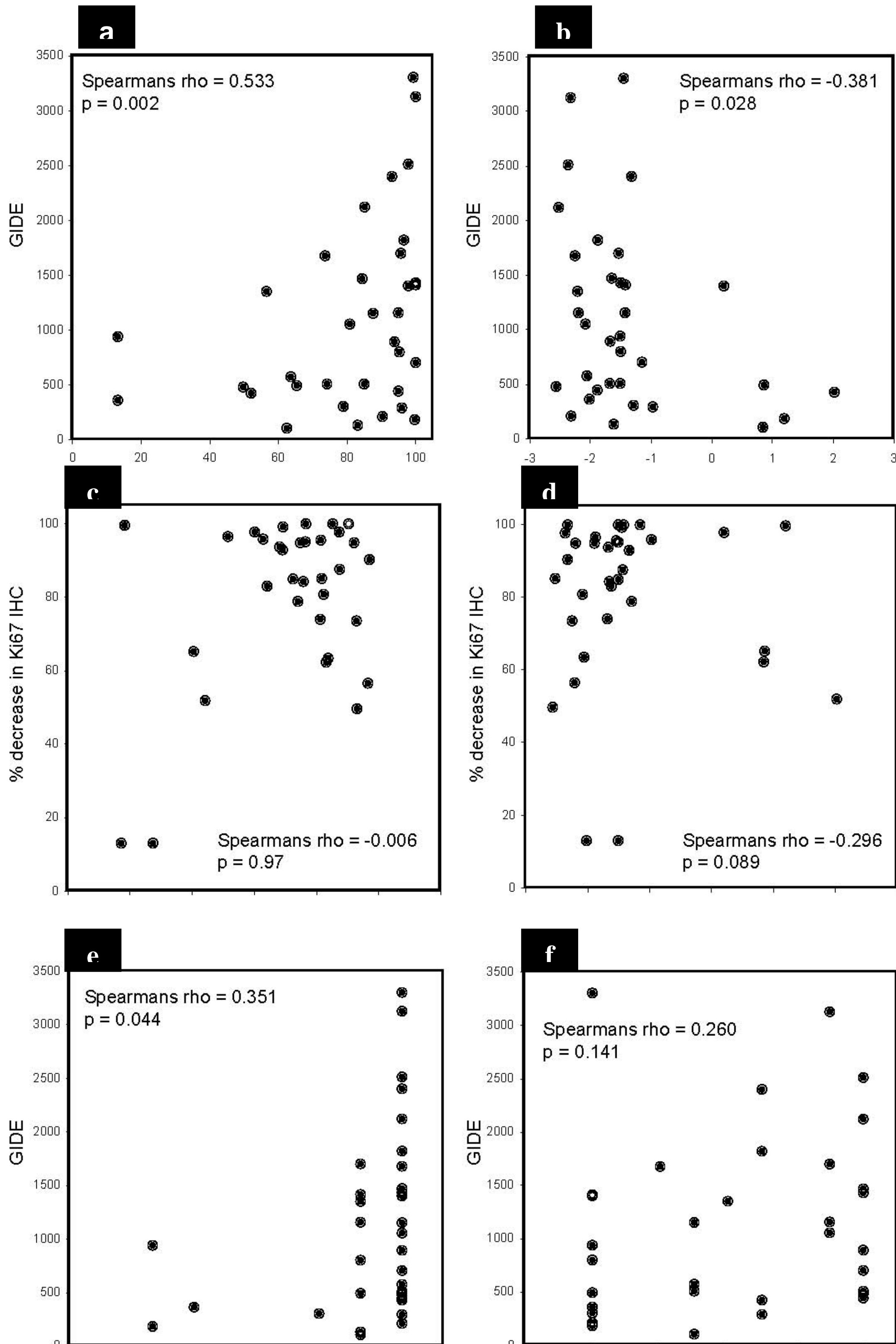

Figure 2

Comparison of GIDE score and change in Ki67 immunohistochemistry with ESR1 and ERBB2 expression. (a) Significant positive correlation of the GIDE scores for each pair of biopsies is shown to the % decrease in Ki67 immunohistochemistry. (b) Significant negative correlation of the GIDE score is shown to the pre-treatment expression of ERBB2 as derived from microarray profiling. The comparison of the change in Ki67 immunohistochemistry (% decrease) is shown to pre-treatment ESR1 expression (c) and ERBB2 expression (d). Comparisons of GIDE scores with pre-treatment immunohistochemical measurements (Allred scores) are shown for ER (e) and PgR (f).

The primary endpoint of the study was the reduction in tumour proliferation measured by the change in the biomarker Ki67 by conventional immunohistochemistry. The relationship between change in Ki67 immunohistochemistry and microarray expression of *ESR1* and *ERBB2* is shown in Figure 2C and 2D. Tumours expressing low levels of ER or high levels of *ERBB2* showed less reduction in Ki67 staining following AI treatment.

Correlations of the GIDE with immunohistochemical measurements of ER and PgR (Allred score) are shown in figures 2E and 2F. In these samples there was a significant correlation of the GIDE with pre-treatment ER staining but not with that of PgR. There was no significant difference between letrozole and anastrozole in their effects on the GIDE or on Ki67 confirming the result for the whole patient set [14].

A paired SAM statistical analysis identified 1395 genes up-regulated and 1264 genes down-regulated by AI treatment using a local false discovery rate threshold of 1%. Significantly changing genes were then ranked according to their average fold change and the top 40 down regulated genes are listed in Table 1 and the top 40 up regulated genes are listed in table 2 (the complete list is shown in Supplementary Table S4). The most consistently down-regulated genes included *TFF1*, *PDZK1*, *AGR2*, *TFF3*, STC2 and *CCND1*. The most consistently up-regulated genes included *LUM*, *CALD1*, *ASPN*,

DCN, *PDGFRA*, *VIM*, *SPARC*, *MAN1A1* and *FAS*. Q-RT-PCR confirmed significant up-regulation of MAN1A1 and FAS ($p<0.05$ for each) and down-regulation of *TFF1*, *PDZK1*, CCND1 ($p<0.01$, <0.001 and <0.001, respectively (data not shown). The complete list of up and down regulated genes was subjected to gene ontology analysis using Onto-Express and Pathway Express [15].

The change in expression in some of these key index genes in individual patients is shown in Figure 3**;** the change in Ki67 immunohistochemistry is also shown for comparison. The majority of tumours show large changes in the expression of these genes. However, changes in the expression of individual estrogen responsive genes did not clearly identify tumours with a poor anti-proliferative response. Different subsets of tumours showed the largest or smallest responses in expression changes for each different gene.

Table 1 *Genes down regulated by AI treatment. The table shows the first 40 down-regulated genes from a paired SAM analysis, which identified 1264 genes down-regulated by AI treatment below a local false discovery rate of 1% (lfdr). The genes are ranked according to their fold change.*

ID	SYMBOL	DESCRIPTION	UNIGENE	Fold	lfdr
HSI182A05	TFF1	Trefoil factor 1 (breast cancer, estrogen-inducible)	Hs.162807	0.26	0.09
HSI054D07	HBB	Hemoglobin, beta	Hs.523443	0.31	0.00
HSI047B01	PDZK1	PDZ domain containing 1	Hs.444751	0.35	0.00
HSI035H02	CYP2B6	Cytochrome P450, family 2B6	Hs.1360	0.41	0.04
HSI075H09	AGR2	Anterior gradient 2 homolog (Xenopus laevis)	Hs.530009	0.41	0.08
HSI031E07	STARD10	START domain containing 10	Hs.188606	0.42	0.00
HSI183G10	TFF3	Trefoil factor 3 (intestinal)	Hs.82961	0.43	0.00
HSI147F09	ZBTB20	Zinc finger and BTB domain containing 20	Hs.570657	0.45	0.00
HSI182A08	STC2	Stanniocalcin 2	Hs.233160	0.47	0.00
HSI147F08	KTN1	Kinectin 1 (kinesin receptor)	Hs.509414	0.47	0.00
HSI059H10	LOC143381	Hypothetical protein LOC143381	Hs.388347	0.47	0.00
HSI147F10	MSI2	Musashi homolog 2 (Drosophila)	Hs.134470	0.49	0.00
HSI177G07	EST	Transcribed locus	Hs.443277	0.50	0.10
HSI053H02	UBE2C	Ubiquitin-conjugating enzyme E2C	Hs.93002	0.52	0.10
HSI096C06	MAPT	Microtubule-associated protein tau	Hs.101174	0.52	0.00
HSI049A02	ERGIC1	ER-golgi intermediate compartment 1	Hs.509163	0.53	0.09
HSI040C08	AZGP1	Alpha-2-glycoprotein 1, zinc	Hs.546239	0.55	0.00
HSI133F06	EST	Transcribed locus	Hs.159264	0.55	0.00
HSI182E08	PLAT	Plasminogen activator, tissue	Hs.491582	0.55	0.00
HSI033B05	LY6E	Lymphocyte antigen 6 complex, locus E	Hs.521903	0.56	0.00
HSI048F12	CCND1	Cyclin D1	Hs.523852	0.56	0.10
HSI085G12	KCNK15	Potassium channel, subfamily K, member 15	Hs.528664	0.57	0.00
HSI177H07	PCBP3	Poly(rC) binding protein 3	Hs.474049	0.57	0.10
HSI032D02	ABCA3	ATP-binding cassette, sub-family A3 (ABC1)	Hs.26630	0.57	0.00
HSI182A02	TFF3	Trefoil factor 3 (intestinal)	Hs.82961	0.58	0.04
HSI025A03	FGD3	FYVE, RhoGEF and PH domain containing 3	Hs.411081	0.58	0.00
HSI070B06	AP1S1	Adaptor-related protein complex 1, sigma 1 subunit	Hs.489365	0.58	0.08
HSI057H12	GNB2	Guanine nucleotide binding protein beta 2	Hs.185172	0.58	0.00
HSI080H11	SEMA3F	Semaphorin 3F	Hs.32981	0.59	0.00
HSI054G06	NUSAP1	Nucleolar and spindle associated protein 1	Hs.511093	0.59	0.00
HSI124D07	RIMS4	Regulating synaptic membrane exocytosis 4	Hs.517065	0.59	0.00
HSI065C09	CNNM2	Cyclin M2	Hs.500903	0.59	0.00
HSI080F02	PREX1	PIP3-dependent RAC exchanger 1	Hs.153310	0.59	0.00
HSI095H09	C6orf97	Chromosome 6 open reading frame 97	Hs.130239	0.59	0.00
HSI161G02	EST	Transcribed locus	Hs.570637	0.60	0.00
HSI045G02	UBE2T	Ubiquitin-conjugating enzyme E2T (putative)	Hs.5199	0.60	0.00
HSI046F10	TOP2A	Topoisomerase (DNA) II alpha 170kDa	Hs.156346	0.60	0.00
HSI183D04	AR	Androgen receptor	Hs.496240	0.61	0.01
HSI183A08	SLC9A3R1	Solute carrier family 9 , member 3 regulator 1	Hs.396783	0.61	0.00
HSI025G02	SHARPIN	SHANK-associated RH domain interactor	Hs.529755	0.61	0.08

Table 2

Genes down regulated by AI treatment. The table shows the first 40 up-regulated genes from a paired SAM analysis, which identified 1365 genes up-regulated by AI treatment below a local false discovery rate of 1% (lfdr). The genes are ranked according to their fold change.

ID	SYMBOL	DESCRIPTION	UNIGENE	Fold	lfdr
HSI022G08	LUM	Lumican	Hs.406475	2.87	0.11
HSI101E05	ODF2L	Outer dense fiber of sperm tails 2-like	Hs.149360	2.80	0.07
HSI027H04	IGJ	Immunoglobulin J polypeptide	Hs.381568	2.73	0.07
HSI082D05	RNH1	Ribonuclease/angiogenin inhibitor 1	Hs.530687	2.51	0.00
HSI056B04	COL3A1	Collagen, type III, alpha 1	Hs.443625	2.50	0.00
HSI182D05	MRC1L1	Mannose receptor, C type 1	Hs.461247	2.45	0.06
HSI067F08	C21orf70	Chromosome 21 open reading frame 70	Hs.410830	2.44	0.00
HSI127E04	CALD1	Caldesmon 1	Hs.490203	2.37	0.11
HSI030C06	PTPRC	Protein tyrosine phosphatase, receptor type, C	Hs.192039	2.36	0.11
HSI067H02	ASPN	Asporin (LRR class 1)	Hs.435655	2.34	0.00
HSI066B08	COL14A1	Collagen, type XIV, alpha 1 (undulin)	Hs.409662	2.28	0.11
HSI049B12	COL1A2	Collagen, type I, alpha 2	Hs.489142	2.28	0.00
HSI049G07	DCN	Decorin	Hs.156316	2.28	0.00
HSI067E05	MRC1L1	Mannose receptor, C type 1	Hs.461247	2.22	0.05
HSI101D05	IFT122	Intraflagellar transport 122 homolog (Chlamydomonas)	Hs.477537	2.17	0.03
HSI183E05	PDGFRA	Platelet-derived growth factor receptor, alpha	Hs.74615	2.14	0.09
HSI031A12	FSTL1	Follistatin-like 1	Hs.269512	2.12	0.00
HSI183H01	COL5A2	Collagen, type V, alpha 2	Hs.445827	2.11	0.00
HSI055A11	ECM2	Extracellular matrix protein 2	Hs.117060	2.08	0.09
HSI018G02	SPON1	Spondin 1, extracellular matrix protein	Hs.445818	2.06	0.00
HSI183B01	PDGFRA	Platelet-derived growth factor receptor, alpha	Hs.74615	2.04	0.01
HSI037C02	CPVL	Carboxypeptidase, vitellogenic-like	Hs.233389	2.03	0.06
HSI062B08	SAS10	Disrupter of silencing 10	Hs.322901	2.01	0.00
HSI129E10	ADAM12	ADAM metallopeptidase domain 12 (meltrin alpha)	Hs.386283	2.00	0.00
HSI183G08	RGS1	Regulator of G-protein signalling 1	Hs.75256	1.98	0.18
HSI054F01	VIM	Vimentin	Hs.533317	1.97	0.00
HSI048C08	CTGF	Connective tissue growth factor	Hs.410037	1.97	0.00
HSI183G05	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	Hs.111779	1.96	0.00
HSI139G09	ADAMTS2	ADAM metallopeptidase with thrombospondin motif 2	Hs.23871	1.96	0.00
HSI018D05	FBLN1	Fibulin 1	Hs.24601	1.95	0.00
HSI040E08	DUSP1	Dual specificity phosphatase 1	Hs.171695	1.95	0.00
HSI082C05	MAN1A1	Mannosidase, alpha, class 1A, member 1	Hs.102788	1.94	0.01
HSI098G12	RARRES1	Retinoic acid receptor responder 1	Hs.131269	1.94	0.00
HSI044C09	SAT	Spermidine/spermine N1-acetyltransferase	Hs.28491	1.92	0.09
HSI030F05	HTRA1	HtrA serine peptidase 1	Hs.501280	1.92	0.00
HSI088D11	CILP	Cartilage intermediate layer protein	Hs.442180	1.91	0.00
HSI040E09	MME	Membrane metallo-endopeptidase (CALLA, CD10)	Hs.307734	1.91	0.00
HSI028G12	PDGFRA	Platelet-derived growth factor receptor, alpha	Hs.74615	1.89	0.00
HSI060C05	FN1	Fibronectin 1	Hs.203717	1.89	0.00
HSI045G12	CXCL12	Chemokine ligand 12 (stromal cell-derived factor 1)	Hs.522891	1.88	0.00

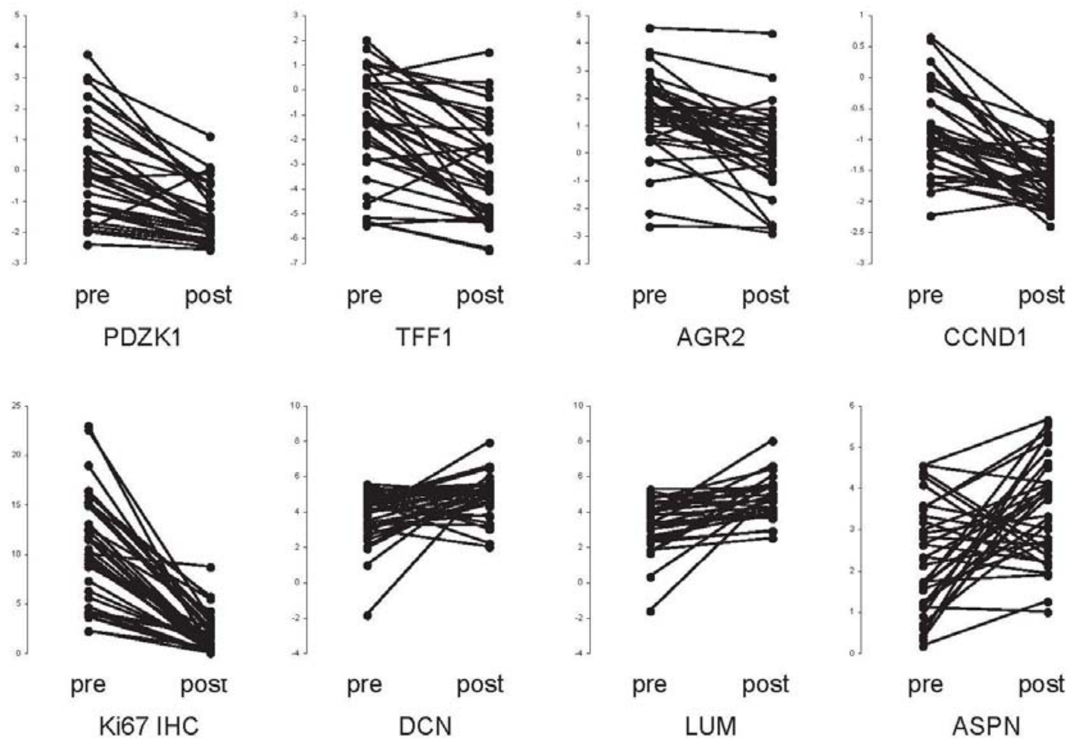

Figure 3

Expression changes in key index genes in response to AI treatment. Individual log ratio measurements are plotted and joined with a line in each of the paired biopsies. Individual results are shown for the down regulated genes PDZK1, TFF1, AGR2 and CCND1 and the up-regulated genes DCN, LUM, and ASPN. The percentage decrease in Ki67 immunohistochemistry (Ki67 IHC) is shown in the bottom left panel for comparison.

To map the gross phenotypic changes of the tumours in response to AI treatment relative to their initial states we selected a core set of 421 genes that distinguished pre from post treatment biopies (see methods). These were used to produce the heatmap shown in Figure 4 and separated the biopsies into predominantly pre- and post-treatment arms. Three of the 4 HER2 amplified cases had pre-treatment profiles that segregated in the post-treatment arm (216, 228, 64). The fourth (203) was the only case that expressed high levels of both ESR1 and *ERBB2*. Seven of the 8 pre-treatment biopsies that were incorrectly grouped included 7 of the 10 biopsies with the lowest pre-treatment expression of *ESR1* (217, 216, 228, 138, 39, 64, P3). Data files for Java Treeview are provided as supplementary information (421.cdt, 421.atr, 421.gtr, 421_annotations).

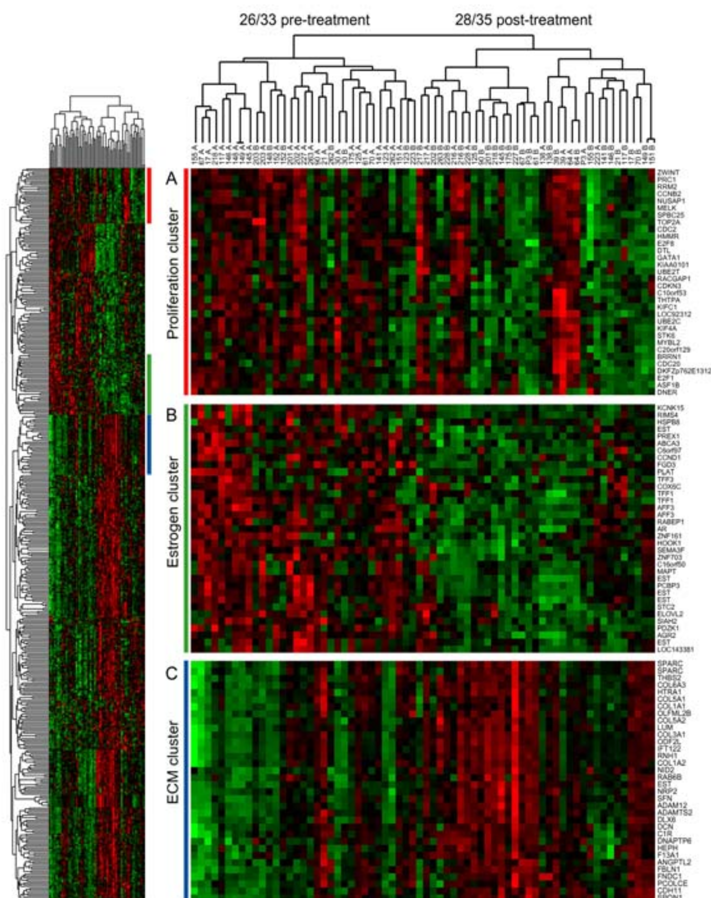

Figure 4 Supervised clustering of pre and post treatment biopsies. The 421 genes which best distinguished pre- and post-treatment biopsies were used to cluster the samples in the heatmap shown on the left. Three clusters of genes are shown in greater detail on the right. (A) a proliferation cluster representing genes associated with proliferation and cell cycle progression (B) an estrogen cluster of known highly estrogen responsive genes (C) and an ECM cluster of genes known to be involved in extracellular matrix remodeling.

Three clusters in this supervised analysis clearly represented distinct pathway related

phenotypes based upon the ontology of the genes they contain (Figure 4). Genes in the “proliferation cluster” showed a highly significant overlap with a previously characterized breast cancer proliferation signature [16]. We labeled a cluster containing many genes known to be classically estrogen responsive in breast cancer as an “estrogen cluster” and one including collagens and other genes involved with extra cellular matrix deposition as an “ECM cluster”. Figure 5A shows the combined effect of treatment on the estrogen and proliferation metagenes (mean of each cluster’s M values) as a vector diagram in which the pre- and posttreatment samples are joined by an arrow. Tumours with extremely low baseline levels of estrogen-dependent gene expression and HER2 amplified tumours show very little change in either cluster (e.g. 39, 138 *red arrows* and 218, 216, 64, *green dots, respectively*). Perhaps most importantly, this analysis identified a number of cases that had major reductions in expression of the estrogen metagene with minimal impact on the proliferation metagene (e.g. 145, 262, 263, *blue arrows*). Figure 5B shows the interaction of the estrogen metagene and the ECM metagene. The ECM metagene is clearly up-regulated in the majority of biopsies irrespective of pre treatment levels of *ESR1* and estrogen metagene values (*red arrows*). The proliferation metagene showed the highest positive correlation (r=0.51, p=0.000029) to the change in Ki67 immunohistochemistry of any of the 9 metagenes (eg. estrogen metagene: r=0.31, p=0.102).

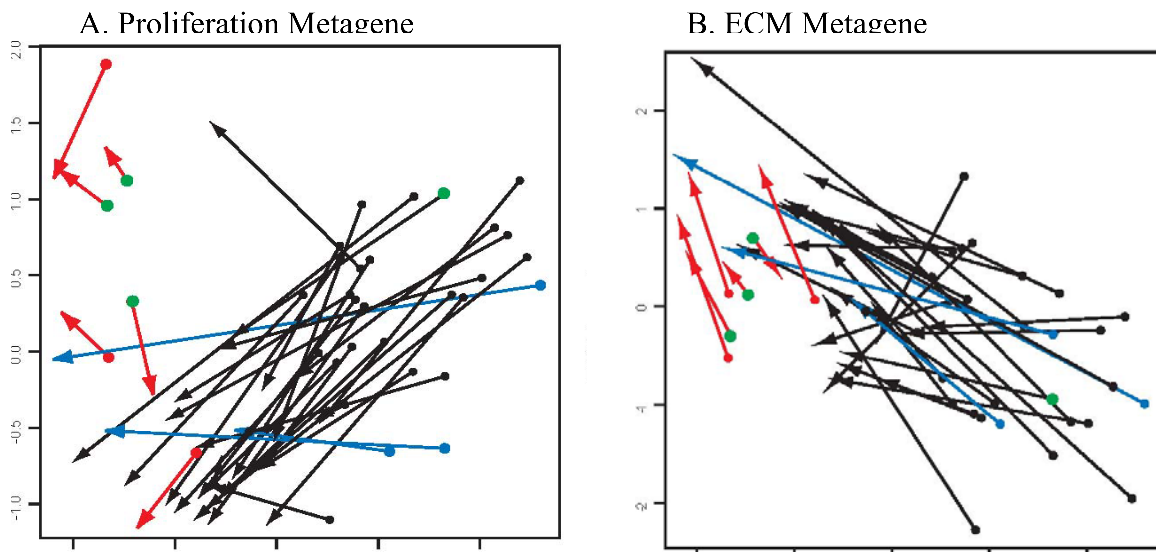

Figure 5

Vector diagrams of metagenes representing estrogen response, proliferation and extracellular matrix remodelling (ECM). Metagene values derived from the mean values of all the genes in each of the clusters in Figure 4 are plotted and connected with a line from dots (pre-treatment values) to arrowheads (post-treatment values). Estrogen metagene values are compared with the proliferation metagene (A) and the ECM metagene (B). The 6 biopsies with the lowest pre-treatment estrogen metagene are coloured in red. The 4 biopsies with HER2 amplification and high ERBB2 expression are shown with green dots and samples with the lowest responses in the proliferation metagene are highlighted in blue.

Array profiling also identified sets of genes both positively and negatively correlated with ER in these biopsies. Intersection of genes associated with ER and those identified as estrogen responsive showed that only 10% of the genes most highly correlated with high *ESR1* expression were down-regulated by estrogen deprivation *in vivo***.** A complete list of genes whose expression correlates with *ESR1* with a Pearson correlation of greater or less than 0.5 is given in Supplementary Table S5.

Discussion

Anastrozole and letrozole are highly specific and efficient inhibitors of the aromatase

enzyme leading to profound estrogen deprivation in postmenopausal women [17]. These agents are also the most effective treatment for breast cancer in postmenopausal patients and have become the standard of care over recent years [2]. Here we have used gene expression profiling by microarray to identify the longitudinal differences in gene expression between matched pre- and post-treatment biopsies of tumours from patients treated with AIs. The data generated in this study are biologically relevant in terms of identifying genes that respond to estrogen withdrawal in primary breast tumours in vivo and clinically relevant in identifying genes or groups of genes that may be used to understand and predict the response of patients to AI treatment. While many reports have examined estrogen-regulated gene expression in breast cancer cells and model systems generating a comprehensive genome-wide catalogue of estrogen responsive genes [18] there are as yet few reports using an aromatase inhibitor as a biological probe of estrogen-dependent expression profiles in human breast carcinomas *in vivo* [19, 20]. The number of patients included in our study was too small for confidence in matters of detail but important broad messages may be developed. There have been several reports over the last few years utilizing expression profiling of breast tumours and demonstrating that the expression of ER by breast carcinomas is a consistently dominant feature in their transcriptional profile [13, 21, 22]. Although these studies have identified many hundreds of genes that are significantly associated with ER expression, it is has not been clear which of these genes are directly responsible for estrogen responses in tumour cells. The current study indicates that only a small proportion of the genes correlating with ER status are estrogen-responsive *in vivo*. In this study we included only ER positive tumours (plus 3 tumours with Allred scores of 2, conventionally considered ER negative) [10]. Correlations between gene expression and ER in the current dataset were therefore made in relation to degree of ER expression rather than due to ER positivity or negativity. Nonetheless we observed strong correlations between ER and many genes which have previously been shown to be strongly associated with ER positivity including

GATA3, *FOXA1*, *AGR2*, *AR* and *STC2* in microarray profiling studies of mixed ER+ and ER- tumours [21-23]. The current study indicates that only a small proportion of the genes correlating with ER status are estrogen-responsive in vivo. The GIDE may be a useful approach to characterizing the overall biological reactivity of a tumour to and dependence on estrogen. The data indicate that there is a continuum of such dependence with one tumour showing 3304 genes changing more than 2-fold over the 2-week treatment period while another showed only 105. These data recapitulate the continuum of change shown by Ki67 immunohistochemistry, which indicates that almost all ER+ tumours show an antiproliferative response to estrogen deprivation although this is highly variable between patients. The data from the GIDE similarly suggest that few ER+ tumours are completely non-responsive to estrogen deprivation. There was only a modestly significant relationship between the GIDE and the pretreatment IHC level of ER: the current data suggest that high ER expression may be necessary for a tumour to be highly responsive (high GIDE) but that some tumours with a high ER have only a moderate or poor biological response. The GIDE may be a useful endpoint for investigation of the mechanisms of resistance to hormonal therapy. One putative mechanism is through overexpression of growth factor receptors such as HER2. Although HER2 was associated with a low GIDE, in all but one case these tumours also had low ER as has been previously observed [24]. PgR positivity has generally been regarded as indicative of an intact ER mechanism. An association with a higher GIDE might have been anticipated and while there was a trend to a positive association with higher PgR expression this was not significant, possibly because of the limited numbers of samples. While the GIDE would benefit from a proven association with clinical outcome we have recently shown that 2-week change in Ki67 was predictive of long-term outcome after treatment with endocrine agents in the adjuvant setting [6, 25, 26] and in this study the GIDE is significantly associated with change in Ki67. The profound changes in transcriptional profiles found in some but not all tumours in this study suggest that it is possible that predicting clinical response to an

aromatase inhibitor by transcriptional profiling may, as with Ki67, be more precise when conducted on tumours shortly after starting treatment. There have been many reports of the transcriptional profiling of estrogen responses in breast cancer cell lines in *vitro*, including those in MCF-7 [8, 9], T47D [27] and ZR75.1 [28] breast cancer cell lines and their derivatives [29, 30] as well as those using model systems in experimental animals [31, 32]. These studies identified many hundreds of genes up- and down regulated by estrogen treatments. Computational and experimental attempts have also been made to integrate these data and catalogue all the estrogen responsive genes and estrogen response elements in the genome [18, 33]. Many of the genes up-regulated by estrogen *in* vitro were down-regulated by AI treatment in our study including the majority of classically estrogen responsive genes (*TFF1*, *TFF3*, *CYP2B6*, *PDZK1*, *AGR2*). *TFF1* (pS2) is one of the best characterized estrogen responsive genes in breast cancer [8, 34-36]. CYP2B6 is dramatically up-regulated by estrogen in ZR75.1 cells although it is not expressed in MCF-7 cells [37]. *PDZK1* has been consistently identified as one of the genes most highly up-regulated by estradiol in MCF-7 cells [8, 9]. *AGR2* is another classically estrogen responsive gene expressed in both cell lines and ER+ breast tumours [38] which has been been associated with a poor response to hormonal therapy [39]. One of the genes that we found to be significantly down-regulated by AIs was aromatase itself (*CYP19A1*). The current observation supports earlier evidence of a positive autocrine feedback loop [40].

In contrast to the genes down-regulated by AI treatment, the up-regulated genes are not represented by those which are directly down-regulated by estrogen in cell lines in *vitro*. Gene ontology analysis of the up-regulated genes identified pathways associated with the regulation of the actin cytoskeleton, cytokine-receptor interactions and focal adhesion more commonly associated with the functions of stromal components than epithelial cells (*VIM*, *CTGF*, *FN1*, *SPARC*). The genes most highly up-regulated by AI treatment include several members of the of the small leucine-rich proteoglycan (SLRP) family (*LUM*, *ASPN*, *DCN*) which regulate matrix remodeling. Lumican is not

expressed in cancer cells in breast cancers but in fibroblasts and is associated with a high tumour grade, low estrogen receptor levels and young age [41]. Decorin is preferentially expressed in stromal areas in proliferating endometrium, directly up-regulated by estrogens in stromal endometrial cells *in vitro* [42] and also up-regulated in mouse uterus by estrogen treatment [43]. Asporin is closely related to biglycan which has been shown to be down-regulated by estrogen in the stroma of normal human breast tissue in a mouse xenograft model [32]. The effect of this stromal signature on patient survivial is unclear but reduced SLRP family expression has been observed in poor prognosis ER negative breast cancer [44]. There are several possible mechanisms for this up-regulation of a stromal signature clearing response to AI treatment. It was notable that the genes representing this stromal signature were up-regulated independent of high level ER alpha expression in tumour cells. It is possible that up-regulation may result from an interaction with stromally expressed ER beta [45]. For example, CD36 has been shown to be directly up-regulated by estrogen via ER beta [46] and both lumican and *PDGFRA* were induced by the SERM raloxifene in U2OS cells transfected with ER beta [30]. Among the other genes up-regulated by AI treatment in our study are genes representative of the normal profiles of luminal and myoepithelial phenotypes [47, 48] which are not driven by high level ER overexpression including *RARRES1*, *MME*, *TCF4*, SFN and *CAV1*. This represents a joint up-regulation in post-treatment biopsies of a basal/stromal phenotype which has also been shown in estrogen treatments of normal human breast tissue in xenograft studies [32]. Taken together these findings highlight the fact that studies identifying estrogen responsive genes in cell lines do not take into account the diversity of responsiveness, composition and genetic backgrounds seen in primary ER+ breast tumours. While many of the gene changes are likely to be directly transcriptionally regulated by estrogen it is also likely that the majority are a secondary consequence of estrogen deprivation and the resulting inhibition of breast tumour proliferation by AI treatment. Recently, Oh et al. [38] have attempted to integrate data on estrogen

responsiveness of MCF-7 cells in vitro with gene expression and clinical outcome data from 65 ER+ and/or PR+ breast cancer patients to predict outcome for hormone responsive breast cancer. The study used only the 383 genes up-regulated by estrogen treatment in this single cell line but, a very high dose (1μM) of estradiol was used and dosage differences have been suggested to compromise comparisons of transcriptional signatures [49]. The identification of a comprehensive profile of estrogen responsive genes in tumours deprived of estrogen in vivo may be expected to provide a much better basis upon which to classify the estrogen response of breast tumours than *in vitro* studies. Robust gene selection methods were used to identify genes that together best separated pre from post treatment samples. Cluster analysis using these genes identified groups associated with proliferation and estrogen responses. The 32 genes that constitute the "proliferation cluster" contain 17 of those reported by Dai *et al.* [16] as a proliferation signature containing critical genes predicting the long-term clinical outcome of patients with ER+ breast tumours. To summarize both the "estrogen cluster" and the "proliferation cluster" we used metagene values to depict the relative changes in tumour phenotype in response to AI treatment. In most tumours there was a co-ordinated decrease in both of these clusters, however we observed that in some tumours these facets of phenotype change were uncoupled. A better understanding of the mechanisms that lead to a poor anti-proliferative response in the presence of a good response in the “estrogen cluster” of genes is likely to provide a guide to additional treatments for ER+ breast cancer and may be possible with an extension of this study to larger numbers of tumours.

Conclusions

In summary, short-term estrogen deprivation with aromatase inhibitors leads to profound changes in transcriptional profiles. Although many of the genes have been previously described in cell culture studies as responsive to estrogen stimulation, many additional estrogen responsive genes were identified that responded to estrogen deprivation *in vivo*, particularly those which are repressed by estrogen. The study

revealed complex changes in estrogen responsive pathways, proliferation and matrix remodeling which cannot be simply summarized by the ER status of the tumours or completely recapitulated in cell line studies. The global changes in gene expression can be integrated into a GIDE that we found to be associated with previously established correlates with clinical outcome. Studies of this type, which link with clinical outcome, should enable the key genes that underpin clinical response/benefit to be established and may be expected reveal the molecular features of tumours responsible for sensitivity and resistance to estrogen deprivation.

List of abbreviations

ER – Estrogen receptor

PgR – Progesterone receptor

AI – Aromatase inhibitor

ECM – extracellular matrix

SLRP - small leucine-rich proteoglycan

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Additional files available on-line:

Additional file 1: mackay_supplementary_table__s1.xls, 11K
http://breast-cancer-research.com/imedia/1351855289130638/supp1.xls
Additional file 2: mackay_supplementary_table_s2.xls, 39K
http://breast-cancer-research.com/imedia/1378876157130638/supp2.xls
Additional file 3: mackay_supplementary_table_s3.xls, 15K
http://breast-cancer-research.com/imedia/2017778721410861/supp3.xls
Additional file 4: mackay_supplementary_table_s4.xls, 1963K
http://breast-cancer-research.com/imedia/1874438445141086/supp4.xls
Additional file 5: mackay_supplementary_table_s5.xls, 133K
http://breast-cancer-research.com/imedia/1835363230130638/supp5.xls
Additional file 6: mackay_bt17k_array_annotation.xls, 2913K
http://breast-cancer-research.com/imedia/4134672611306382/supp6.xls
Additional file 7: mackay_2418.atr, 2K
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Additional file 8: mackay_2418.gtr, 91K
http://breast-cancer-research.com/imedia/1764398523145998/supp8.gtr
Additional file 9: mackay_421.cdt, 212K
http://breast-cancer-research.com/imedia/5819839481459988/supp9.cdt
Additional file 10: mackay_421.atr, 2K
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Additional file 11: mackay_421.gtr, 14K
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Additional file 12: mackay_2418_annotation.xls, 447K
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Additional file 13: mackay_421_annotation.xls, 96K
http://breast-cancer-research.com/imedia/7286013411459989/supp13.xls

Objectives of the second work

1. To test the feasibility of obtaining adequate samples for gene expression study through minimally invasive techniques in an animal model of post-menopausal oestrogen-dependent breast cancer.
2. To test the feasibility of the study on the regulation of gene expression in serial samples.
3. To study the changes in a limited number of candidate genes, selected from the previous study, following short-term treatment with letrozole in this animal model of aromatase inhibitor-sensitive human breast cancer.
4. To compare the short-term regulation in expression among these genes in order to select those with the most profound regulation.
5. To compare the regulation of these genes with that undergone by Ki-67, the best gene studied in this setting.
6. To choose the best candidates among these genes for future human studies on dynamic molecular reporters of AIs efficacy.

Breast Cancer Res Treat
DOI 10.1007/s10549-007-9676-7

PRECLINICAL STUDY

Pre-clinical validation of early molecular markers of sensitivity to aromatase inhibitors in a mouse model of post-menopausal hormone-sensitive breast cancer

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Received: 21 June 2007 / Accepted: 26 June 2007
Springer Science+Business Media B.V. 2007

“Early Molecular Markers of Response to Aromatase Inhibitors”

Pre-clinical Study

Pre-Clinical Validation of Early Molecular Markers of Sensitivity to Aromatase Inhibitors in a Mouse Model of Post-Menopausal Hormone-Sensitive Breast Cancer.

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ABSTRACT

Introduction. Changes in breast cancer cell biology following hormonal treatment have been claimed as promising predictor markers of clinical benefit even outperforming clinical response. From previous work we selected 10 genes showing both a well known regulation by oestrogen and a high level of early transcriptional regulation following therapy with aromatase inhibitors. Here we use an animal breast cancer model to explore the feasibility of the determination of their expression in minimally invasive samples and to further assess the magnitude of their regulation by letrozole.

Animal and Methods. Aromatase inhibitor sensitive breast cancer tumours were grown in athymic mice under supplement with androstenedione. Following initial tumour growth animals were assigned to a control group or to receive letrozole at two different dosages. Fine needle aspirates were obtained at the moment of treatment assignation and one week later. Expression of the following genes at both time points was determined: Ki-67, Cyclin D1, pS2, Trefoil Factor 3, PDZ domain containing 1, Ubiquitin-conjugating enzyme E2C, Stanniocalcin 2, Topoisomerase 2 alfa, MAN1A1 and FAS.

Results. Fine needles aspirates were found to be a feasible and reproducible technique for RNA extraction. Trefoil Factor 3, pS2, CyClin D1 and Stanniocalcin 2 were significantly downregulated by letrozole. Among them pS2 appears to be most sensitive to aromatase inhibitor treatment even differentiating sub-optimal from optimal letrozole dosage.

Discussion. We present pre-clinical evidence to justify the exploration in clinical trials of pS2, Trefoil factor 3, Cyclin D1 and Stanniocalcin as dynamic markers of oestrogen-driven pathway activation.

INTRODUCTION

Breast glandular epithelium grows and differentiates under the stimulus of oestradiol. Once breast cancer develops from epithelial progenitors oestradiol deprivation can result in tumour regression. The nuclear oestrogen receptor α (ER) is the most important predictor of benefit derived from hormonal treatments [1] and despite multiple reports on novel determinants of hormone-sensitivity no other marker has been introduced into routine practice. Yet 40-50% of ER positive tumours do not respond to the best hormonal treatment strategy (i.e. aromatase inhibitors in the postmenopausal woman) [2].

In an attempt to improve the value of the existing pre-treatment predictors of response to hormonal treatments, several groups have studied pharmacodynamic biomarkers potentially related to treatment efficacy [3-12]. Such an approach has the advantage of an in-vivo assessment of the sensitivity to therapy. Provided that these changes happen early enough they may become biomarkers that identify, from the very beginning, hormone-sensitive tumours beyond ER.

Changes induced by hormonal agents in the percentage of tumour-cells positive for Ki-67 (a universal marker of proliferation) have been shown to harbour valuable information to classify ER positive breast tumours as sensitive or resistant to aromatase inhibitors and tamoxifen [13-18]. Nevertheless and despite being promising, the use of Ki-67 is this setting presents important limitations.

Some groups have extensively described changes in gene regulation driven by oestrogen or oestrogen antagonists in breast cancer cell models but, to date, no extension of these data into clinical studies has been reported [19, 16].

In a previous study we described, using cDNA microarray technology, those genes showing early regulation of expression by aromatase inhibitors[20]. Briefly, core biopsies were taken from postmenopausal patients diagnosed with localized breast cancer before and after two weeks of treatment with either anastrozole or letrozole. Messenger RNA was obtained and hybridized onto cDNA microarrays to screen for

those genes significantly regulated by these drugs that produce a highly effective abolition of circulating and intratumoral oestradiol in the postmenopausal woman [21].

Out of those genes that are most consistently regulated through all samples some have a well known biological link to oestrogen driven processes. We selected a set of those genes on the basis of the previous knowledge of their oestrogen dependence and of the magnitude of the changes observed.

In the present study we attempt, in a preclinical setting, to confirm whether these markers are truly regulated by letrozole, the most effective inhibitor of aromatase [17] . To do so we have used a well established animal model for aromatase inhibitor sensitive breast cancer [22, 21]. We also explored whether determination of these genes is feasible using minimally invasive techniques (fine needle aspirates). Those genes harbouring the most promising regulatory profile will be candidates to be further validated for their predictive value in the clinical trial setting.

ANIMALS AND METHODS

Animal model

Animals were maintained in the facility of the Institut de Recerca Oncològic-IDIBELL (Barcelona), AAALAC unit 1155, and all procedures were carried out in accordance with international guidelines. Forty female athymic mice, 6 weeks of age, were purchased from Harlan Italy (Milan). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water *ad libitum*. Mice were bilaterally ovariectomized and allowed to recover for 2 weeks prior to experimental use. Pellets containing 60-day release of 15 mg androstenedione (Innovative Research of America®) were inserted subcutaneously at the same time but in a distant site from tumour cells implant. MCF-7 cells transfected with the aromatase gene (MCF-7 Arom1 cells) [21] were grown to 80% confluence in DMEM culture medium containing 10% FCS, 1% penicillin and streptomicin, 2mM

glutamine, 1mM sodium pyruvate, 10 μg/ml insulin and 600 μg/ml G418 to maintain selection for neomycin–resistant cells. Cells were scraped and resuspended in matrigel. Each mouse received a subcutaneous injection at the right flank with 0.1 ml of cell suspension containing 10^7cells.

Tumours were allowed to grow under androstenedione supplementation with twice weekly monitoring of their volume assessed manually with a caliper (volume= 4/3 r_1^2 x $r_{2;}$ were r_1 is the smallest radius and r_2 the largest).

Once the tumour volume was over 0.5 cm^3 mice had a new hormone pellet placed and were randomized into one of the three following treatment groups: Control: daily oral gavage with vehicle; “Suboptimal dose”: Letrozole 2mg/kg/day given by oral gavage and “Optimal dose”: Letrozole 20 mg/kg/day given by oral gavage. Letrozole (kindly donated by Dean B. Evans, Novartis pharma AG, Basel, Switzerland) was dissolved in a 0.5% solution of carboxymetilcellulose and administered in a total volume of 0.2 ml once every two days.

Mice were sacrificed by cervical dislocation 4 weeks after randomisation before tumour volume of control group reached 2 cm^3.

RNA extraction and quantification

Samples for RNA were obtained from each mouse by fine needle aspirates (FNA) using a 20-gauge needle at the time of treatment group randomisation (T_0) and one week later (T_1). FNA technique had been previously tested in an independent tumour set and proved to yield samples containing over 80% of human breast cancer epithelial cells. Aspirates were immediately taken into TriZol reagent (Life Technologies®) by multiple passages of the fluid through the needle and syringe. Total RNA was extracted according to TriZol manufacturer’s recommendations. The concentration of RNA was determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer (Nanodrop®). The integrity, DNA contamination and size distribution of total RNA

purified was checked by agarose gel electrophoresis and using an Agilent 2100 bioanalyzer.

A two step real time reverse transcription polymerase chain reaction technique (RT-PCR) was used to determine relative expression levels of eleven different mRNAs using the ABI Perkin Elmer Prism 7700 Sequence Detection System (Applied Biosystems®). One microgram of RNA from each sample was reverse transcribed using hexamers with the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems®).

Twenty nanograms of each sample were analyzed in triplicate by RT-PCR for ten different test genes and one housekeeping gene [Ki-67, Cyclin D1 (CCND1), Trefoil Factor 1 (TFF1 or pS2), Trefoil Factor 3, PDZ domain containing 1 (PDZK1), Ubiquitin-conjugating enzyme E2C (UBE2C), Stanniocalcin 2 (STC-2), Topoisomerase 2 alfa (TOP2A), Mannosidase alpha class 1A member 1 (MAN1A1), Fas TNF receptor superfamily member 6 (FAS) and Beta glucuronidase (GUSβ) as housekeeping gene] using gene specific primers designed to include intron-exon boundaries and as human specific sequences.

For the quantification of gene expression the comparative threshold cycle number ($\Delta\Delta Ct$) was used. The ΔCt value for each gene was determined by subtracting the average housekeeping gene Ct value from the average target Ct value. The calculation of $\Delta\Delta Ct$ was done by subtraction of the ΔCt of each gene at T_1 from the value at T_0. Final results were presented as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Mann-Whitney test for one to one comparison (Control-2 mg/kg, Control-20 mg/kg, 2 mg/kg-20 mg/kg) and Anova test for global comparison between groups were used with threshold p values for significance adjusted following Bonferroni correction for multiple observations. An initial p value of 0.05 was adjusted to 0.001 for one to one

comparisons and to 0.002 for Anova global comparison test. These stringent conditions allow only for detection of very significant changes in gene expression.

RESULTS

Tumour growth

Twenty-six mice out of the initial 40 showed an adequate tumour growth 8 weeks after cell implantation to allow randomisation into the three treatment groups (64% success rate). Six mice were assigned to the control group and ten to each Letrozole dosage group. Mean tumour volume in the three groups did not show statistical difference at T_0 and was 0.50 cm^3 in the control group, 0.51cm^3 in Suboptimal Dose group and 0.47cm^3 in the Optimal Dose group.

Tumours maintained an ascending tumour growth curve in the control group reaching a 200% of the initial tumour volume 4 weeks after randomisation. Mice in the Letrozole treatment groups showed a dose-dependent tumour volume reduction mimicking a clinical response (Figure 1). The two different dosages were selected in order to compare gene expression between an optimal and a sub-optimal dose (20 mg/kg/day and 2 mg/kg/day respectively). As expected both treatments resulted in a marked reduction of tumour volume with differences between both. One week after T_0 tumour volume was 93 % of baseline in 2 mg/kg and 91% in 20 mg/kg, 71 and 57% at 2 weeks, 46 and 30% at 3 weeks and 28 and 14% at 4 weeks, respectively. These changes were not statistically different between the treatments. Due to the short-term scope of this experiment no differences in time to re-growth were observed.

At T_1, one week after treatment assignation, when the second FNA was performed, differences in tumour volume among the three groups were very subtle (118%, 93% and 91% from volume at T_0 for control, Suboptimal and Optimal Dose groups respectively). These minor differences allow the exclusion of differences in gene expression being attributable to heterogeneity of the samples due to a potential smaller volume in the Letrozole treatment groups.

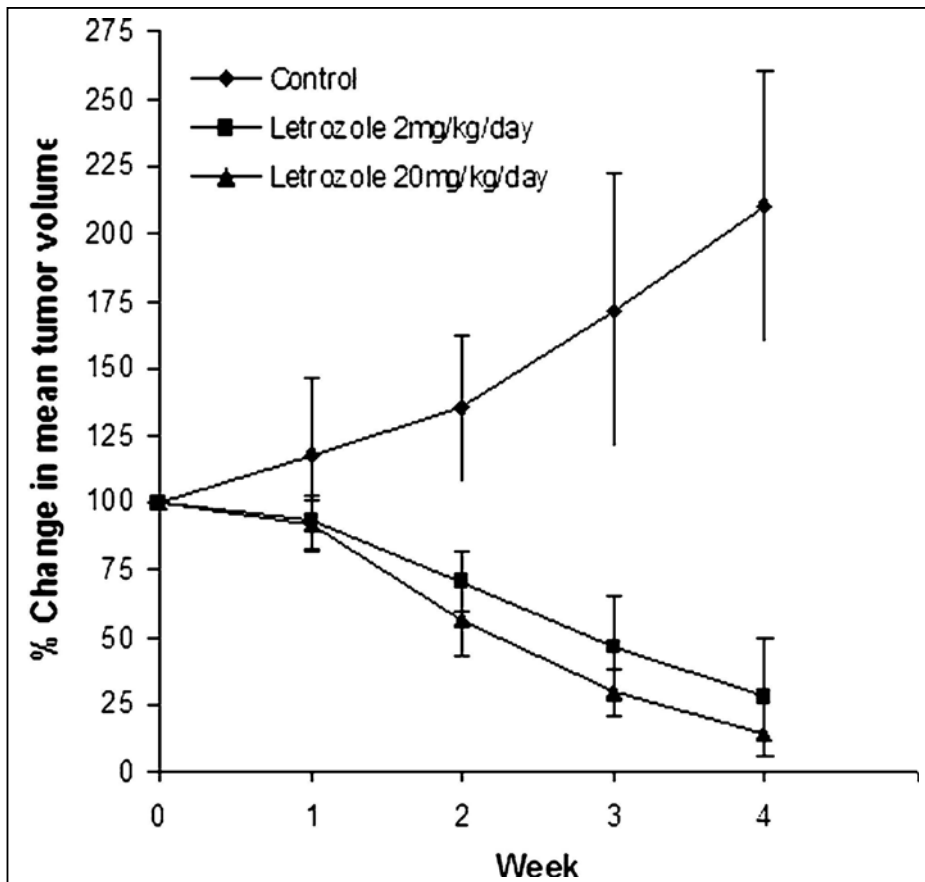

Figure 1: Curves of tumour growth starting at T_0 and expressed as percentage of tumour volume at T_0 at each time point.

Sampling

One FNA was performed at each tumour and at each time point. All of the procedures yielded high quality samples. RNA amount from each FNA ranged from 1 to 15 micrograms with a median amount of 6 µg. The spectrophotometer assessment showed all samples to have 260 / 280 ratios in the range of 1.8 to 2.

Regulation of gene expression

Details on the mean relative expression of each of the tested genes in each of the treatment groups are provided in table 1A.

All genes showed differences in expression among groups with a tendency to the down-regulation. This regulation resulted in minor changes in three genes: FAS, MAN1A1 and TOP2A. The two former had been previously characterized to be up-regulated in breast cancer following oestradiol deprivation.

Ki-67, Cyclin D1, pS2, TFF3, PDZK1, UBE2C and Stanniocalcin 2 all showed clear downregulation in the treatment groups when compared to control and even between sub-optimal and optimal dosage groups (fig. 2). After Bonferroni correction these changes among groups reached global statistical significance in the Anova test for Cyclin D1, TFF3, pS2 and Stanniocalcin 2 (table 1A).

When statistical differences between mean values for each gene were evaluated, TFF1 (pS2) showed highly significant regulation comparing control with 20 mg/kg dosage and 2 with 20 mg/kg dosage (table 1B).

Ki-67 is the most previously studied gene in relation to its down-regulation with hormonal treatments and, hence, it has become a comparator for other genes. Ki-67 showed a marked down-regulation following exposure to letrozole, nevertheless this regulation did not reach statistical significance when Bonferroni adjustment was applied. The magnitude of its regulation (0.9377 in the control versus 0.9376 in Suboptimal Dose and 0.4223 in Optimal Dose) was markedly inferior to that for those genes with statistically significant regulation. Finally, it was of little value to discriminate control from sub-optimal treatment.

Table I. Expression of the tested genes in response to Letrozole in the athymic mice xenograft breast cancer model.

A

Treatment	Control	TR1	TR2	p Value
TFF1	0,64 ± 0.19	0,30 ± 0.1	0,05 ± 0.03	**1 x 10^{-6} ***
TFF3	1,35 ± 0.3	0,98 ± 0.2	0,63 ± 0.18	**9.6 x 10^{-4} ***
STC2	1,53 ± 0.4	1,51 ± 0.4	0,51 ± 0.2	**9 x 10^{-4} ***
CCND1	0,94 ± 0.2	0,94 ± 0.16	0,42 ± 0.1	**8.5 x 10^{-4} ***
PDZK1	0,54 ± 0.27	0,31 ± 0.15	0,06 ± 0.08	0.002
UBE2C	0,46 ± 0.13	0,31 ± 0.09	0,20 ± 0.07	0.006
MAN1A1	0,82 ± 0.14	0,98 ± 0.09	0,68 ± 0.16	0.010
FAS	0,70 ± 0.22	0,61 ± 0.16	0,40 ± 0.1	0.029
Ki-67	0,34 ± 0.2	0,32 ± 0.09	0,17 ± 0.08	0.066
TOP2A	0,46 ± 0.15	0,47 ± 0.27	0,36 ± 0.17	0.711

B

Gene	C vs TR1	C vs TR2	TR1 vs TR2
pS2 (TFF1)	0.019	**6.6 x 10^{-4} ***	**4.3 x 10^{-5} ***
TFF3	0.055	0.004	0.018
CCND1	0.953	0.019	0.002
STC2	0.859	0.011	0.001

A- Mean (± standard deviation) gene expression presented as $2^{-\Delta\Delta Ct}$ [**C**: control; **TR1**: Letrozole 2mg/kg/d; **TR2**: Letrozole 20 mg/kg/d]; and Anova test for global comparison.

B- P values of the statistical analyses of differences in mean gene expression between groups. One to one comparison by Mann Whitney test of those genes showing significant regulation in the global comparison.

* In **bold** those differences with statistical significance after Bonferroni correction.

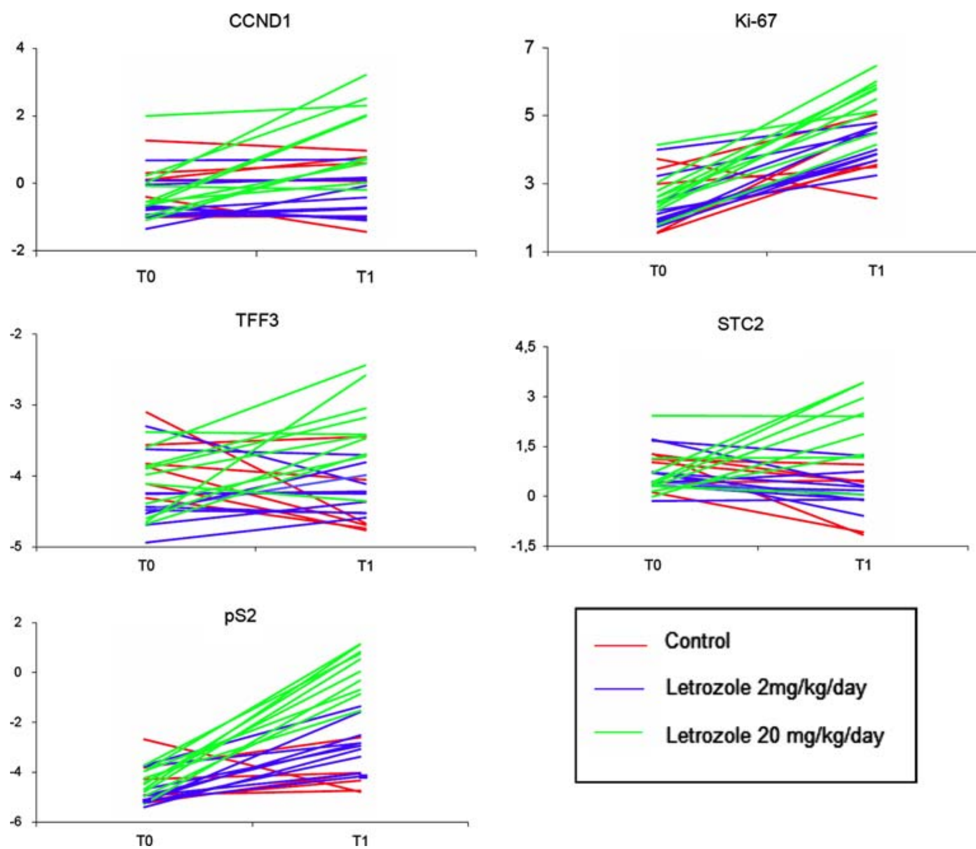

Figure 2: Changes in expression (expressed as ΔCt) for each tumour, at T_0 (basal) and T_1 (one week after treatment), for the five most significant genes. An increasing number of PCR cycles to get the signal threshold (Ct) reflects a decreased expression.

DISCUSSION

The efficacy of endocrine treatments faces two major challenges. A moderate efficacy with response rates hardly ever over 60% even when selecting ER positive tumours reveals a major problem in the identification of up-front ("de-novo") resistant tumours. Secondly, virtually all initially responding tumours develop resistance with time ("acquired resistance"). These tumours restart their growth either due to the loss of efficacy of the treatments to abolish ER dependant signalling or to the development of alternative pro-growth signalling pathways leading to tumour independence from oestrogen [19, 23].

As an added challenge to the clinical management of hormonal treatments the accuracy of tumour reduction as a surrogate marker of long term clinical benefit is currently debated. Many patients with metastatic disease who do not present with tumour regression, nonetheless benefit from long progression free periods with symptomatic improvement. On the other hand recent well powered trials have failed to extrapolate results from the comparison between two endocrine treatments in terms of tumour response in the pre-operative setting with the performance of the same drugs in terms of long term benefit in the post-surgical adjuvant scenario [24]. In either case (advanced and early disease) prolonged periods of treatment may be necessary to classify tumours as sensitive or resistant on the basis of tumour reduction [25]. This fact exposes patients to the risk of either a long ineffective treatment or to the early stop of an effective but slow working tolerable treatment because of the absence of tumour volume reduction in the first months of therapy.

As an alternative to ER, dynamic molecular markers of tumour response present the advantage of an in-vivo assessment of the ER driven growth and, if they happen early enough, may represent a good mean of characterizing de-novo resistance in ER positive tumours. To this extent the most widely explored marker, at a protein level, is

the change in proliferation-marker Ki-67 following endocrine therapy [20]. Several reports have correlated the decrease in the percentage of cells staining for Ki-67 happening up to 2 weeks after initiation of hormonal treatment with later tumour volume reduction. More importantly, there is some evidence supporting the superiority of these early changes to anticipate long term benefit when compared to tumour reduction after 3 months of treatment [24]. This putative better predictive value of changes in proliferation compared with tumour volume reduction may be explained by the existence of tumours that, even arresting cell division rate following an effective therapy, take a long time to reduce tumour volume and are so considered resistant on clinical judgement.

Despite these encouraging results of Ki-67 as a dynamic predictive marker there are some conflicting results on its performance and some limitations in its use. One of these limitations is the need for a biopsy for Ki-67 assessment that limits the serial determination of the marker to 2 or 3 time points at the maximum. This restricts the exploration of the value of Ki-67 as early predictor of tumour re-growth following the development of resistance. The difficulties for the normalisation of Ki-67 scoring among pathologists and the low correlation between protein staining and the level of RNA have also contributed to the limited use of the marker.

We present pre-clinical results on novel candidates for clinical development as markers of ER dependent pathway activation and endocrine treatment efficacy. We have performed this study using an animal model that has been shown to predict the behaviour of human ER positive breast cancer when exposed to aromatase inhibitors [22, 21]. Extrapolation of our results to human tumours demands great caution given the expected differences between both settings. Particularly with regard to the lack of a human microenviroment in the animal model. The fact that samples obtained by FNA in humans are usually highly enriched in tumour epithelial cells is encouraging regarding the value of the present markers comparing to other sampling techniques (core-biopsy)

in which high representation of stromal cells may confound the gene expression findings.

The use of FNAs for the sample extraction has been previously reported in mouse xenograft tumours although not extensively [26]. One of the major goals of this study was to determine whether this method, that allows sampling without the removal of highly invasive biopsy of the tumour, is feasible and provides high quality RNA.

Out of the total number of 14,000 genes studied in our previous work[20] a paired SAM statistical analysis identified 1,395 genes up-regulated and 1,264 genes down-regulated by aromatase inhibitor treatment using a local false discovery rate threshold of 1%. In order to select the 10 most up-regulated we selected genes with fold change > 4 and < 0.63 for down-regulated ones. These thresholds are arbitrary and resulted in the identification of highly regulated genes that could be candidates for a low throughput analyses with RT-PCR as the present. Out of these twenty genes we selected those ten with the best proved biological link to ER in order to increase the likelihood of being significant in the animal platform. Hence the clinical relevance of our findings.

The selection of one week after baseline for the second sampling is based upon the need to avoid that differences in tumour volume between groups result in bias during sample acquisition. In contrast to changes at the protein level, more frequently determined after two weeks of treatment in the clinical setting, changes in gene mRNA expression are expected to happen earlier in particular in this model where tumour growth curves vary much more rapidly.

Following the present feasibility study we have been able to select a small number of genes that show a more sensitive regulation by aromatase inhibitors than Ki-67 at the transcription level and that can be determined in minimally invasive samples allowing multiple sampling serial determinations. These two facts encourage the further clinical exploration of these markers either as early predictors of treatment efficacy or as

markers of acquired resistance to endocrine treatments when determined in a repetitive fashion during treatment.

Four of the ten explored genes, namely pS2 (trefoil factor 1; TFF1), TFF3, Cyclin D1 and STC2 seem to meet criteria for further development in the clinical setting. All of them are known to be regulated by oestrogen either directly through ER responsive elements [25] present in their gene sequence or indirectly by associating with AP-1 and Sp1 transcription factor complexes and their respective binding sites. Although cyclin D1 gene transcription is directly inducible by oestrogen, there is no ERE-related sequence in the promoter region [27]. Instead, the cyclin D1 promoter contains multiple regulatory elements, including binding sites for AP-1, STAT5, NF-kB, E2F and Sp1 [28].

Normal functions attributed to trefoil factor (TFF) genes include protection against mucosal injury, stabilization of the mucous layer and acceleration of repair of mucosal damage in the adult gastro-intestinal tract. In addition they have a role in tumour biology. Trefoil factors tend to be overexpressed in tumours in which normal tissue counterparts do not express them or do it at a very low level (e.g., breast) and are usually absent or reduced in tumours in which normal counterparts express them at high level (e.g., stomach). TFF1 is a breast cancer specific gene [29] tightly linked to ER activation. Initially described in MCF-7 cells it has been also found in breast cancer biopsies [30, 31]. In oestrogen-treated MCF-7 cells, TFF1 expression is directly controlled at the transcriptional level via the ERE in its promoter [32]. TFF3 is only expressed in oestrogen-responsive breast cancer cells and its expression is regulated by oestrogen in these cells [33].

Stanniocalcin 1 and 2 are genes initially found to participate in the calcium homeostasis of the bony fish [34]. STC2 has been identified as an oestrogen-regulated gene that coexpressed with ER mRNA in breast carcinomas [35] with potential ER/Sp1-binding elements in its gene sequence [36]. Its biological role in human has not been well

characterized so far but it has been claimed to represent a molecular marker of ER activation.

TFF1 presented the most pronounced regulation in our model even showing differences between a sub-optimal and an optimal dose of letrozole. This fact encourages its development as a comparator between different hormonal treatments. However, the small sample size in our experiment does not allow a formal comparison of the differential power of these four genes to discriminate ER driven proliferation and, although pS2 seems to be a finer marker, no definitive conclusions should be drawn to this respect on the basis of these results.

The reason for a non-significant level or regulation of the rest of tested genes may lie on an insufficient statistical power of our experiment. Alternatively the differences in the cell-population components of samples between the previous study on which gene selection is based (core-biopsies) and the present (FNA) may account for this result.

In conclusion, our findings in this pre-clinical model support the clinical investigation of the regulation of pS2, TFF3, CCND1 and STC2 as dynamic markers of ER activation and ER dependent growth in serial, minimally invasive, breast cancer samples.

If these results are paralleled by clinical findings a first application could be the oestrogen dependency determination of tumours through short courses of endocrine treatment even before surgery.

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Integrated Discussion

Summary of individual discussions

In the first clinical setting, short-term oestrogen deprivation with aromatase inhibitors was shown to result in profound changes in the gene expression profile of ER-positive breast cancer. Although many of genes have previously been described to be responsive to oestrogen stimulation in cell culture studies, many additional oestrogen responsive genes were identified to respond to oestrogen deprivation *in vivo*, particularly those which are repressed by oestrogen. This study revealed complex changes in oestrogen-responsive pathways, proliferation and matrix remodelling, which cannot be simply summarized by the ER status of the tumours or completely recapitulated in cell line studies. An index of the magnitude of the effect of oestradiol deprivation was derived and expressed as the number of genes showing significant regulation, the so-called GIDE. This index was found to be associated with previously established correlates with clinical outcome.

In the second experimental setting, four candidate genes previously known to depend on oestradiol, pS2, TFF3, Cyclin D1 and STC2 were selected as the best candidates for further validation as reporters of ER pathway status. Assuming the major differences between the human and mouse settings, the stringent statistical conditions and the known value of the model provided good proof of principle on the validity of these biomarkers. As a major added value of the experiment, the feasibility of the serial determination of these markers in minimally invasive samples was determined.

The novelty of this work lies mainly in the biologically meaningful approach to the description of new biomarkers.

The lack of previous evidence on the changes happening in gene regulation in human breast cancer samples following oestrogen deprivation has been highlighted in the Introduction. Several studies have provided in depth insight on the biological processes which take place in cell cultures but this setting presents serious limitations when it is

the basis for clinically relevant tests. The biological heterogeneity of all the diverse entities simplistically grouped under the denomination of "breast cancer" makes complex approaches mandatory when basing any clinically applicable conclusion on basic research. This heterogeneity has recently been made even more patent through the studies of cancer taxonomy based on high throughput technology. Although, to some extent, the classification of tumours with microarray expression studies is similar to that of 3 classical immunohistochemistry markers, the number of exceptions to this rule is high and the differences in the biology of tumours that share the ER/PgR/Her-2 phenotype cannot be disregarded. This diversity should be taken into account in any research project targeting the discovery of new biomarkers with either predictive or prognostic value and means that the first steps of this process must be performed over clinical samples covering a full range of tumour characteristics. Moreover, the complexity of the interaction between cancer cells of epithelial origin and the surrounding stroma and the well known importance of this interaction directing tumour biology requires that both cell populations be considered in the clinical biomarkers development process.

Our study initiates the particular gene selection process with a screening approach performed on core biopsies (hence, including tumour and stroma) with no intermediating cell selection processes. As opposed to more "purist" approaches in which tumour epithelial cells are selected through cell sorting processes (laser microdissection, scratching processes, etc), this "global profiling" policy presents the advantage of producing more comprehensive portraits of the tumours. It is obvious that the results are more complex to reproduce given the expected noise introduced by a heterogeneous sample with a variable percentage of tumour cells in the samples. To this effect the microarray data processing method used by our group allowed selection in highly stringent statistical conditions providing a maximum guarantee that the selected genes undergo a significant regulation in their expression.

Two steps are of particular interest in the methodology of microarray processing in order to select only those genes with a biologically meaningful regulation. Following local regression for removal of technically biased data, M values were adjusted to eliminate increasing dispersion correlated to increasing A value variation. This is a conflictive step that, in the opinion of some experts, may artificially introduce an observer driven *a priorism* which is that this increasing variation is not biology meaningful but rather a technical bias. From our point of view, despite the risk of discarding some important information, this adjustment results in safer selection. In any case, and as stated above this adjustment did not substantially change the results.

Using the interquartile range as an estimate of gene variation, the threshold of 0.75 resulted in a relatively low (although still very high, as discussed) number of genes considered to be truly regulated. Again, at the risk of losing important information, we decided to play it safe and only consider genes in which the regulation was highly significant.

Although in the biomarker discovery processes investigators often rush from initial screening studies to retrospective (less frequently prospective) clinical series to further determine the informative value of the candidate markers, it would be better to include additional studies of the markers and their determination methods prior to large scale studies in patients. This is of particular interest when the value of the marker lies in its change through time and an intervention. This way, each particular marker, if intended to be used individually, requires studies of the changes which occur without therapeutic intervention in order to avoid attributing changes, which may simply reflect the inherent variability of the marker (gene expression in this case) over time, to treatment activity. These data are also imperative for the statistical design of future clinical studies with these markers.

The issues highlighted above lead to the second part of this thesis and are of particular relevance for future characterisation of the candidate markers with different sampling

methods and gene expression determination technology; hence, the need for further preclinical development of the markers before progressing to the clinical setting.

The third major difference between the first and second study is the setting which confers a more comprehensive character to this thesis and avoids bias in the biomarker selection process which may explain a future potential lack of value in the clinical setting.

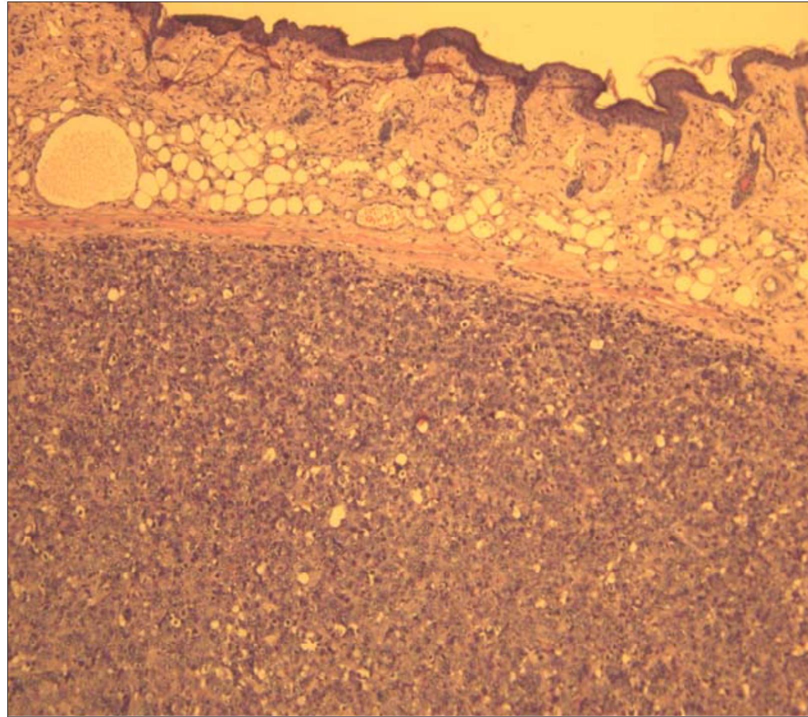
<u>There are three major differences between the study settings of the two studies.</u>

1. The setting. Compared with the study of small, operable, original breast tumours performed in the first study, the preclinical validation is based on a cell line implanted in mice. The MCF-7 cells, originally obtained from the pleural fluid of a patient with lobular breast cancer with pleural metastasis[113], represent the most frequently used platform for the preclinical study of breast cancer in general and oestrogen-dependent breast cancer in particular. Despite their origin, these cells have undoubtedly undergone many interventions and on selection in culture they may present important differences compared with original breast cancer cell biology. In our case, in particular, stable transfection of the aromatase gene was performed.

   In addition to the differences in the epithelial cancer cells themselves, the tumour model used in the second paper implies human cancer cells growing in a mouse stroma environment. These tumours tend to grow as solid spheroids with no stromal cells inside, surrounded by mice inflammatory and fatty tissue coating layer (see figure below). This differs greatly from the human setting in which tumours grow as a heterogeneous mixture of tumour epithelium and breast connective tissue.

   These differences may be reflected in gene expression regulation and unquestionably represent a challenge for the validation of the original results

in such a different setting. On the other hand, agreement in the results would confirm their robustness.



MCF 2A cell tumour growing subcutaneously in a mouse. No significant stromal population is seen among the epithelial cells. The mouse connective tissue forms a capsule surrounding the tumour.

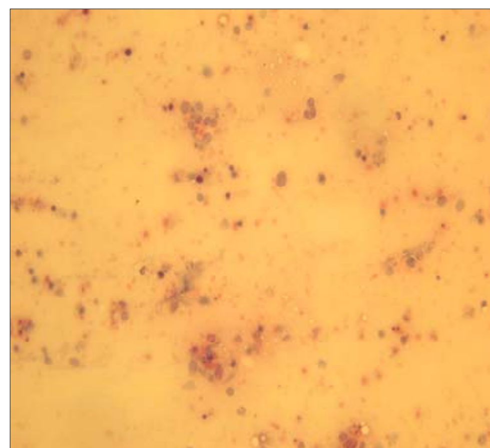
2. The sampling method. In the first study samples were obtained using a 14 gauges needle (core biopsies). This means that the samples obtained were cylinders of tissue (approximately 1mm wide and 1 cm long). These biopsies included tumour epithelial cells and their surrounding parenchyma in addition to fibrous tissue, fat, vessels and inflammation mediating cells. The cancer cell content of these biopsies usually ranges between 15 and 80% but the median is slightly above 30%. This is particularly patent in this study in which the tumours studied were small in size. Thus, the expression profile observed in these samples will probably include non-tumour cells and will thereby demonstrate the possible changes in gene expression regulation in the surrounding stroma of patients receiving AI treatment.

On the other hand, fine needle aspiration was the sampling method implemented in the second study. With this method, the extract contains isolated cells or small groups thereof due to the disruption produced by the fine needle in tissue cohesion. If this method is performed adequately the samples include large numbers of epithelial cells, especially when the tumour sampled in homogeneous, dense and the cell population is mainly of epithelial origin. The resulting samples are usually composed of more than 80 % of cancer cells (as reported herein). This significant difference in the sampling method (illustrated in the figure below) may probably explain the differences in the results between the two studies as discussed below.

Figure Below.

Left panel: Core biopsy. Including full tissue structure and stroma.

Right panel: FNA. Scattered cells or small cell clusters are seen. The sample mainly contains epithelial cells.



3. Gene expression determination technology. The principles on which the cDNA microarray technology and the real time-polymerase chain reaction (RT-PCR) are based are the same and the nature of the results should be similar. Nevertheless, there are major differences in the sample preparation itself and in the method of reporting gene expression between the two methods which may account for differences in the quantification.

   In the sample preparation process for later hybridisation on the microarray, one single round of T7 linear amplification is performed and a total of 4 μg of amplified aRNA is hybridised to the 17000 spot microarray. The results are normalised against both a reference and the average spot intensity. Conversely, when using RT-PCR, following retro-transcription of total RNA into cDNA, 20 ng of cDNA are amplified by sequence-specific primers in subsequent cycles before the threshold signal of the quenched probe is detected. This detection usually happens after 19 to 30 cycles. Normalisation is made against a house-keeping gene expression.

   All these marked differences usually result in a higher sensitivity for gene expression changes with RT-PCR compared with the microarray. This is used as a mean of confirming the nature of the expression observed with the microarray (as was done in our paper) but calls for caution and intermediate validation when microarray findings are to be extrapolated for further determination with RT-PCR.

<u>The selection of the candidate genes for individuals is one of the most conflictive steps of this thesis study.</u>

As outlined in the first study in Results, the main initial finding of the screening phase was the large number of genes that undergo significant regulation after such a short period of oestradiol deprivation. It should be taken into account that both drugs (anastrozole and letrozole) require from 2 to 4 days to achieve the maximum abolition

of oestradiol levels in blood, which means that the effective treatment time is even reduced to less than 2 weeks. This short time of E_2 deprivation results, nevertheless, is a major change in most cell processes. This is particularly notorious if we compare these extensive changes with those which occur after chemotherapy (see Results in 1st study).

Of this large number of genes showing significant regulation, more than 2,400, albeit a very limited number, had to be selected for individual study in the animal platform.

In order to be extremely stringent in the selection process and to avoid selecting any gene that may not undergo real changes in expression, we only considered the top 40 up or down regulated genes as eligible. Although selection from this list of 80 genes implied a high risk of discarding genes which may have been extremely relevant for the purposes of biomarker selection, we assumed this risk. Out of these genes with a highly significant regulation we selected 9 with well established direct regulation by ER. This selection process is biased by previous knowledge and very probably omits genes that may be of high relevance for the purpose of the study. Nevertheless, the planned number of genes selected for further study was no larger than ten (including Ki-67). It is known that the 9 genes selected are directly regulated by ER in breast cancer cells thereby guaranteeing their potential value as reporters of ER-driven pathway activation status and ruling out, to a maximum, their possible regulation in non-epithelial cells. Although of biological interest, this possibility was not considered appropriated for a study performed in FNA samples containing RNA from almost exclusively epithelial cancer cells.

In 2 of the 10 candidate markers the regulation was not paralleled in the two experimental settings

FAS and MAN1A1, up-regulated by oestradiol deprivation in the microarray study, did not show a significant change (non-significant trend to down regulation) in the animal model setting.

The FAS gene encodes a protein member of the TNF receptor superfamily. It plays a pivotal role in programmed cell death and its activity mirrors apoptosis. At the time of the design of the animal study, an initial list of the 40 most significantly up-regulated genes obtained from the microarray studied included FAS. Unfortunately, the definitive analyses performed when the animal study was almost finished reflected a less prominent regulation of the gene, falling to the 179th position. This may be the first reason why the results of the two studies do not coincide. Moreover, it should be taken into account that some recent reports question the direction in which FAS expression is regulated by oestradiol and have reported its involvement in the phenomenon of E_2-enhanced apoptosis[114, 115]. It may, therefore, be argued that E_2 deprivation may even reduce FAS expression thereby making it a sub-optimal candidate.

As previously mentioned, larger quantities of both FAS and MAN1A1 (a Golgi apparatus membrane protein), were found to be present in the mRNA profile of biopsies taken following treatment with the aromatase inhibitors. The fact that the biopsies contain a significant stromal cell population requires particular caution when up-regulated genes are considered. This is because the presence of higher levels of some genes after treatment may reflect sustained expression in non-cancer cells while epithelial cells have stopped the expression of cancer process-related up-regulated genes following growth signal deprivation. Nonetheless, this effect will clearly not be present in samples in which only epithelial cells are profiled.

Why Ki-67 was introduced as a comparator.

As has been extensively commented in the Introduction to this thesis, Ki-67, determined at a protein level using immunohistochemistry (IHC) techniques, is the best dynamic marker of AIs activity to date. One of the objectives of the first study was to study the correlation between gene expression changes and Ki-67 determined by IHC. As a result of this correlation study it was shown that the GIDE index correlated

positively with a decrease in Ki-67 by IHC. This fact raises high interest in the study of the GIDE in future studies as a novel and better dynamic predictive tool.

Nevertheless, measured at an mRNA level on the microarray, Ki-67 showed a clear down-regulation and ranked 132nd and GIDE did not correlate as well with Ki-67 mRNA as it does with Ki-67 IHC (data not shown). This is not particularly surprising since it is well known that the correlation between Ki-67 mRNA and protein is far from perfect.

In any case we deliberately introduced Ki-67 in the list of 10 genes to be studied in the animal model as a reference for the direction of its regulation (the up-regulation of Ki-67 through treatment would have been very difficult to explain). At the same time it was of great importance for us to test which of the candidate genes over-performed Ki-67 at the mRNA level in order to encourage us in their future development.

The utility of the markers herein described

- Up-front resistance identification.

  As previously outlined, dynamic markers of response were initially developed in an attempt to characterise resistance to hormonal treatments in ER-positive tumours.

  The almost universal decrease in Ki-67 expression following hormonal treatment extensively discussed before led us to consider that more than being complete refractory to hormonal treatment up-front resistant tumours undergo a reduction in growth but tend to very rapidly circumvent the inactivation of the ER signal. This is in contrast to sensitive tumours that take longer to overcome this blockade. To this effect, a panel of markers that non-specifically reports not only proliferation but both proliferation and ER pathway activation status, are used to provide more accurate information on treatment efficacy.

  If human clinical trials with a long follow-up show these markers to be useful to unveil ER-positive resistant tumours, they could be used in the short pre-operative setting. As an example, on diagnosis of ER-positive early, initially operable, breast cancers, treatment with an AI may be administered prior to excision and the

changes in these markers examined. This would be of great value to assist post-operative decisions on adjuvant treatment and would be particularly helpful in certain subsets of patients (such as patients over 60 years old) in whom the benefits of chemotherapy are dubious and determination of hormonal treatment-sensitive patients is of key importance.

- Aromatase inhibition efficacy monitoring during prolonged treatment.
    - In the primary hormonal treatment of early or locally advanced breast cancer.

      Although for years primary hormonal treatment was confined to use in patients with locally advanced (non-resectable) disease unfit for chemotherapy or in elderly or frail patients in whom the risk of surgery was too high, its indications have been recently broadened. Thus, an increasing number of patients receive on or off-trial hormonal treatment, mainly with AIs in post-menopause patients, to induce tumour reduction prior to surgery despite initial intervention adequacy.

      One of the greatest challenges in this primary hormonal treatment is to define the most adequate time of surgery. Several studies have determined that a treatment period of less than 4 months substantially reduces the percentage of patients achieving response, with some tumours starting to respond up to one year after treatment initiation. Thus, the optimal time of treatment in this setting before surgery is indicated somewhere between 4 and 12 months. Nevertheless, this prolonged period before surgery, needed to rule out resistance, may expose a substantial number of patients (30 to 50%) to non-effective treatment. Moreover, complete responses are a very infrequent event in this setting and most patients achieve a plateau after initial response, hence continuation of treatment is of little benefit if tumour reduction is the goal.

On the other hand, a significant number of patients under primary hormonal treatment are frail and surgery is only considered in cases of hormonal treatment failure.

All these patients would derive a great benefit from the development of markers such as those described here since

a. they are determined in a sample obtained using a virtually painless method.
b. They may thereby be serially determined,
c. and accurately determine either the lack of treatment efficacy or the re-start of ER-dependent or independent growth, therefore indicating the need for surgery.

- In the metastatic setting.

  As summarized in the Introduction, the lack of good markers of treatment efficacy in patients under palliative hormonal treatment leads to almost half of ER-positive patients receiving non-effective treatments for long periods of time. Stable disease status is a blind box in which the treatment efficacy to stop progression and the difficulty to identify progressive disease are mixed.

  In the clinical practice it is not unusual for patients showing stable disease assessed by the usual radiological means to experience a lack of symptom control or an increase in serum markers making the decision as to the need for a change in treatment truly difficult.

  In this setting markers that report ER pathway status and the proliferation activity of the tumour cells, initially and throughout treatment, would greatly aid in decision making.

- In the clinical research of new treatments.

Years of clinical, basic and translational research by several groups on the mechanism of resistance to hormonal treatments have provided convincing evidence on the need to approach this resistance through the combination of hormonal agents with other selective growth signal blockers. At present, several pathways have been described which, once activated, lead to re-growth of tumours under endocrine therapy (see Introduction). On the other hand, increasingly selective blockers of one or several of these pathways are progressively available for clinical investigators.

On the combination of several drugs, one of the greatest challenges in clinical research is to distinguish the efficacy of the combination from that of each drug. One of the most frequent approaches, nowadays, is to start treatment with one drug only, and, after a short period in which the effect of this drug is assessed by pharmacodynamic markers, the combination treatment is started and the markers are re-checked to evaluate the effect of the addition of the second drug (usually the drug under investigation).

Given the limitations of pharmacodynamic markers and the relatively little value of tumour reduction to assess this kind of treatment activity, novel markers such as the those studied in this thesis would be extremely useful to evaluate the efficacy of the new treatments abrogating the re-activation of the ER pathway that usually characterises resistance to AIs and other endocrine therapies.

Another investigational setting in which new markers can assist research is the early Phase II pharmacodynamic trials which are now frequently performed in the short pre-operative setting. The aim of these trials is to characterise the potential activity of a drug in an early breast cancer setting. The new drugs is administered over a short treatment time (similar to that used in the first of the two papers of this thesis) and its effect on the cell targets is evaluated. If the drug is biologically active, it may be worth

investing in its development in this setting. Thus, the markers herein described may aid in better profiling of the activity of the new drugs, especially if the target is in the ER pathway.

Summary

- Short term treatment with aromatase inhibitors produces profound changes in ER-positive breast cancer leading to a high number of genes undergoing rapid regulation of its expression.
- A full profile of genes regulated by aromatase inhibitor treatment is provided.
- The complexity of the changes shown in several pathways, including ER, proliferation and extra-cellular matrix-related pathways overwhelms the capacity of cell culture studies to characterise them.
- A Global Index of Dependence on Oestrogen (GIDE) has shown a promising correlation with known negative and positive predictive factors of response to aromatase inhibitors and warrants future development in this setting.
- Trefoil factor 1 (pS2), Trefoil factor-3, Stanniocalcin 2 and Cyclin D1 are down-regulated early by aromatase inhibitors.
- The 4 markers can be serially determined using RT-PCR technology in samples obtained by fine needle aspiration.
- Changes described in the clinical setting in the regulation of the 4 markers by AI treatment have been confirmed in an animal model of AI-sensitive breast cancer.

Conclusions

- The comprehensive study of transcriptional changes following aromatase inhibitor treatment provides better insight into the mechanisms underpinning the response to this therapy.
- TFF-1, TFF-3, STC2 and CCND1 are potential dynamic markers of response to aromatase inhibitors and their study in the clinical setting is warranted in order to evaluate:
    - The predictive value of their early change
    - The long-term prognostic value of their down-regulation
    - Their value as reporters of ER pathway and cell-growth activation.

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