Cross-talk between the Oestrogen Receptor and the Human Epidermal Growth Factor Receptor (HER) family: Role in resistance to Tamoxifen treatment in breast cancer.

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1. Tovey,S.M., Dunne,B., Forsyth,A., Witton,C.J., Cooke,T.G., and Bartlett,J.M. (2005). Can molecular markers predict when to implement treatment with aromatase inhibitors in invasive breast cancer? Clinical Cancer Research. *11*, 4835-4842.

2. Tovey,S.M., Witton,C.J., Bartlett,J.M., Stanton,P.D., Reeves,J.R., and Cooke,T.G. (2004). Outcome and human epidermal growth factor receptor (HER) 1-4 status in invasive breast carcinomas with proliferation indices evaluated by bromodeoxyuridine labelling. Breast Cancer Research. *6*, R246-R251.

List of Abbreviations

AF-1, AF-2	transcription-activating function 1,2
ATAC	Arimidex, Tamoxifen, alone or in combination Trial
BH3-	BCL-2 homology 3 domain
DBD	DNA binding domain
ERα	Oestrogen Receptorα
ERE	oestrogen response elements
FISH	Fluoresence in situ hybridyzation
H&E	hematoxylin and eosin
HER	Human epidermal growth Factor Receptor
HSP	heat shock proteins
4ICD	HER4 intracellular domain
IES	Intergroup Exemestane Study
IHC	Immunohistochemistry
LBD	ligand-binding domain
MAPK	Mitogen-activated protein kinase pathway
NEAT	National Epirubicin Adjuvant Trial
NTD	N-terminal domain
pERαSer118	Phosphorylated ERα at Serine 118
pERαSer167	Phosphorylated ERα at Serine 167
pHER2	Phospho specific HER2
PI-3K	phospatidylinositol-3 kinase
PR	Progesterone Receptor
TACE	TNFα-converting enzyme

TEAM	Tamoxifen Exemestane Adjuvant Multinational Trial
TMA	Tissue microarray

Summary

Tamoxifen is a potent anticancer agent and has long been the standard for adjuvant endocrine therapy in breast cancer in the United Kingdom. Despite this success, resistance to Tamoxifen is a significant clinical problem; almost all patients with metastatic disease experience progression and up to 20% of early breast cancer patients relapse whilst on adjuvant treatment. The primary mechanism of action of Tamoxifen is via competitive inhibition of oestrogen binding to oestrogen receptors. The subsequent reduction in expression of oestrogen related genes such as growth factors and angiogenic factors results in reduced cell proliferation, enhanced apoptosis and reduced cell growth. However as the complexity of oestrogen receptor function is increasingly revealed, particularly in terms of its mechanism of action and interactions with other signalling pathways, our understanding of Tamoxifen activity and the development of resistance against it has also expanded.

Tamoxifen resistance may be either "*de novo*" (present prior to Tamoxifen treatment) or "acquired" during the course of treatment. Identifying the biological mechanisms behind Tamoxifen resistance is important particularly as increasing clinical trials evidence implies superiority of aromatase inhibitors over Tamoxifen. It is not currently clear whether this superiority is the case for all ER positive patients, or whether there is a particular group of patients resistant to Tamoxifen whose tumours may be sensitive to aromatase inhibitors.

The HER family of tyrosine kinase receptors (HER1-4) initiate a complex signal transduction cascade modulating cell proliferation, survival, adhesion, migration and differentiation. We have postulated that cross talk between the HER family and the ER may underlie development of Tamoxifen resistance in breast cancer. We have

used tissue microarray technology and immunohistochemistry in analysis of a retrospective database of 402 ER positive, Tamoxifen treated patients to test the hypothesis that overexpression of HER family members 1-3 is associated with phosphorylation of the ER and with clinical relapse on Tamoxifen ('Tamoxifen resistance').

We have demonstrated that HER1-3 (but not HER4) and PR status can identify time-dependent *de novo* Tamoxifen resistance, with risk declining markedly after 3 years of tamoxifen treatment. We also demonstrate for the first time *in vivo* expression of phosphorylated ERα at the cell membrane of breast cancer cells and have demonstrated a correlation between HER1-3 status and *membranous* phosphorylated ER. This supports recent reports of an interaction between membrane-bound ERα and the HER family and its pathways and provides further evidence for the non-classical actions of the ERα at the membrane. We confirm previous reports that phosphorylated ERα in the nucleus is associated with higher ERα expression and a more differentiated phenotype suggesting that it acts as a marker of an intact, functional, ERα signalling pathway.

Data presented here demonstrates the significant role played by HER1-3 in promoting Tamoxifen resistance in hormone responsive breast cancers. It also emphasises the different role of HER4 in this context. This work is particularly clinically relevant with recent trials suggesting that the apparent superiority of aromatase inhibitors over Tamoxifen may be linked to the expression of specific tumour markers. In fact our results parallel those from the ATAC and IES trials which suggest that, whilst PR negative patients derive greater benefit from initial aromatase inhibitor treatment, PR status has no impact on response when given as delayed treatment to those who were disease free on Tamoxifen after 3 years.

Taken together, these data strongly supports the conclusion that the predictive value of PR, and possibly HER1-3, expression is time dependent and identifies patients at high risk of *de novo* Tamoxifen resistance. The ability to select, at diagnosis, patients at high risk of early relapse on Tamoxifen could provide the opportunity to tailor their adjuvant therapy on an individual basis, either in terms of an aromatase inhibitor or by supplying them with a HER family inhibitor such as Iressa or Herceptin to be used in conjunction with their endocrine treatment.

There is some evidence that, in contrast to genomic activity, non-genomic ERα activity can be stimulated by SERMS such as Tamoxifen. Therefore Tamoxifen may be incapable of breaking (or even may stimulate) any cycle linking non-genomic and genomic ERα with the growth factor pathways. From our results, we speculate that membrane bound ERα, in conjunction with the HER family, may be responsible for initiating tumour cell proliferation even in the presence of Tamoxifen resulting in *de novo* Tamoxifen resistance. These results have implications for establishing ERα status, particularly in the clinical diagnostic setting, as more detailed analysis in terms of location and function of the receptor may become the norm for diagnostic testing in the future.

Further research is required to determine the mechanisms relating to Tamoxifen resistance particularly in regard to the nature of the interactions at the membrane between the ER and HER family. Testing these hypotheses in the context of the redesigned TEAM trial will provide a valuable insight into the most appropriate future therapeutic options for differing sets of breast cancer patients.

Chapter 1: Introduction

1.1 Breast cancer

Breast cancer is the most common female malignancy with more than 40,000 cases diagnosed in the UK in 2000. The lifetime risk of developing breast cancer is 1 in 9 and its incidence is on the increase (www.show.scot.nhs.uk/isd/). Survival rates however are improving year on year from a 5 year survival rate of 52% in the 1970s to 80% during the years 1998-2001 (www.statistics.gov.uk/).

Breast cancer rarely occurs in women under the age of 25 years. Thereafter, the incidence increases steadily until the menopause, where the rate of increase slows. 5-10% of cases have a known familial predisposition but for the majority aetiology is not always clear. Exposure to increased endogenous (early menarche and late menopause) or exogenous hormones has been implicated as have environmental factors such as, diet/obesity, alcohol and physical activity (Muti, 2004).

1.2 Endocrine Therapy

Endocrine systemic therapy is an effective and minimally toxic method of treating hormone responsive breast cancers. Indeed endocrine manipulation has been used in varying forms since the discovery of the beneficial effects of oophorectomy on young women with locally advanced breast cancer (Beatson AT, 1896). Now cancers expressing oestrogen receptors are treated with some form of endocrine therapy to abolish the proliferative effect of oestrogen on these tumours. This may be achieved by depleting the environment of oestrogen with either aromatase inhibitors or the lutenising-hormone-releasing agonist goserelin. Alternatively, treatment that involves

competition with oestrogens for binding to the ERα can be used, such as the 'selective oestrogen receptor modulator' Tamoxifen.

1.2.1 Tamoxifen

Tamoxifen was originally developed as an oral contraceptive, but its potential anti-oestrogenic properties were subsequently recognised (Harper and Walpole, 1966) and was introduced as a endocrine agent for the treatment of breast cancer. Initially hormone treatment was given to all patients but trial evidence confirmed responsiveness in the oestrogen receptor positive patients only (Early Breast Cancer Trialists' Collaborative Group, 1998; Stewart, 1992). Tamoxifen subsequently became the first line agent in endocrine responsive breast cancer.

The primary mechanism of action of Tamoxifen is via competitive inhibition of oestrogen binding to oestrogen receptors. The subsequent reduction in expression of oestrogen related genes such as growth factors and angiogenic factors results in reduced cell proliferation, enhanced apoptosis and reduced cell growth (Osborne, 1998). However it is not a pure antagonist, and in fact exerts both agonist and antagonist effects. Generally it is an agonist in bone and endometrium (Kedar et al., 1994; Love et al., 1992), whereas it is used for its antagonist effect on genes important for cell proliferation or survival in the breast.

Tamoxifen is a potent anticancer agent and is well established in clinical practice for use in ERα positive breast cancer. The Scottish Cancer Trials Breast Group results demonstrated tamoxifen, taken for a median duration of 60 months, significantly prolonged disease-free survival overall (P = .0001), in both premenopausal (n=214) - and postmenopausal (n=533) node negative patients. Analysis of a large number of randomised trials (Early Breast Cancer Trialists' Collaborative Group, 1998; Early

Breast Cancer Trialists' Collaborative Group, 2005) demonstrated a 50% proportional reduction in the recurrence rate and a 28% proportional mortality reduction for ERα positive patients taking adjuvant Tamoxifen for 5 years. These overviews demonstrated a long term and increasing benefit of Tamoxifen with survival benefits maximised at 5 years of treatment.

As well as remaining the standard adjuvant therapy for women with ERα positive tumours following surgery, Tamoxifen can also achieve tumour regression in many patients with locally advanced or metastatic disease. It is generally well tolerated and responses are longer compared to cytotoxic chemotherapy (Dowsett, 2001).

Side effects of Tamoxifen include menopausal symptoms such as hot flushes and it carries an increased risk of thromboembolic disease. Its partial agonist effects include uterine proliferation (with an associated increased risk of uterine malignancy) as well protective oestrogen like effects on bone metabolism resulting in an increase and stabilisation of bone density. Despite its proven benefits however, resistance often develops to Tamoxifen, with almost all patients with metastatic disease, and some on adjuvant treatment eventually relapsing (Osborne, 1998). This is despite the fact that most cases retain the nuclear steroid receptors (Clarke et al., 2001b). Tamoxifen resistance may be either "*de novo*" (present prior to Tamoxifen treatment) or "acquired" during the course of treatment. *De novo* resistance may partially be explained by Tamoxifen being used to treat ERα negative tumours or by inadequate or inaccurate measurement of the ERα status. However despite this it appears that a proportion of ERα positive tumours are intrinsically hormone-independent (McGuire, 1980).

Despite many years of use, there is still no adequate explanation as to why some potentially sensitive tumours become resistant to Tamoxifen. However as the

complexity of oestrogen receptor function is increasingly revealed, particularly in terms of its mechanism of action and interactions with other signalling pathways, our understanding of Tamoxifen activity and the development of resistance has also expanded. Identifying the biological mechanisms behind Tamoxifen resistance is important, particularly as increasing clinical trials evidence implies superiority of aromatase inhibitors over Tamoxifen (Dowsett, 2003).

1.2.2 Aromatase Inhibitors

Aromatase is a cytochrome P450-dependent enzyme, which acts at the last step in the synthesis of oestrogen by catalyzing the conversion of androgens to estrogens. Aromatase inhibitors work by preventing conversion of androstenedione to oestrone and oestrodiol in peripheral tissues (fat, muscle etc) and locally within established breast tumours (Reviewed in (Carpenter and Miller, 2005). By inhibiting aromatase, the levels of plasma oestrogens are reduced by over 97% in postmenopausal women (Brodie and Njar, 1996).

Earlier aromatase inhibitors also affected adrenal corticosteroid function but current third generational potent selective inhibitors do not have these potentially toxic side effects. They may be steroidal (Exemestane) or non-steroidal (Arimidex, Letrozole). Steroidal AIs are analogues of androstenedione and are noncompetative, irreversible inhibitors of the aromatase enzyme. The non-steroidal inhibitors act by binding reversibly to the enzyme complex. Both classes have been shown to reduce circulating oestrogen to 1% to 10% of pretreatment levels (Buzdar et al., 1997). Initial evidence with regard to the efficacy of these newest aromatase inhibitors has come from the neoadjuvant (Ellis et al., 2001) and metastatic settings (Mouridsen et al., 2001; Nabholtz et al., 2003) and these drugs are commonly used as the first line

treatment in these cases. In the adjuvant setting whilst emerging data on the efficacy of aromatase inhibitors does not yet identify a group of patients for whom additional benefit is derived in terms of overall survival, the ATAC (Arimidex, Tamoxifen, alone or in combination), IES (Intergroup Exemestane Study) and BIG-98 studies all demonstrate a clear benefit in terms of disease free survival (Coombes et al., 2004b; Howell and on behalf of ATAC trialists' Group, 2004; Coates et al., 2007). It is not currently clear whether this superiority is the case for all ERα positive patients, or whether, as is widely predicted, there is a particular group of patients resistant to Tamoxifen whose tumours may be sensitive to aromatase inhibitors. There is however, growing evidence that specific tumour markers may be used to identify tumours which exhibit resistance to Tamoxifen and/or demonstrate enhanced responsiveness to aromatase inhibitors (Schiff et al., 2004; Ross and Fletcher, 1998; Dowsett, 2003). These markers include both conventional markers of endocrine responsiveness (ERα and PR) and receptor tyrosine kinases such as the human epithelial growth factor (HER) family of receptors.

It has been suggested that cross-talk between the oestrogen receptor and HER family may underlie Tamoxifen resistance (Schiff et al., 2004) and thus may also go some way towards explaining the superior clinical results demonstrated with aromatase inhibitors.

1.3 Oestrogen receptor

In order to explore the potential mechanisms underlying Tamoxifen resistance, it is necessary to first attempt to understand the complex behaviour of the oestrogen receptor. Oestrogen action is mediated through the oestrogen receptors ERα and ERβ; both members of the nuclear hormone receptor superfamily. Although more information about ERβ is now available, most of our knowledge about the role oestrogen plays in breast cancer is with regard to its' actions via ERα.

ERα is a 65kDa nuclear transcription factor receptor expressed in 46-77% of breast cancers (Robertson, 1996). The conventional pathway for ERα action is well described (Figure 1). The ERα exists as a monomer bound by heat shock proteins. Subsequent ligand binding causes dissociation of the heat shock proteins and alteration of receptor conformation. In this classical pathway the activated receptors then homodimerize, and in a complex with coregulatory molecules bind to oestrogen responsive elements (ERE) in the promoter region of target genes, to alter transcription (Klein-Hitpass et al., 1988; Kumar and Chambon, 1988). The targets of proliferative oestrogen/ERα action include genes such as *fos*, *jun-B* and *myc* (Weisz and Bresciani, 1993), cell cycle-controlling gene products such as cyclin D1 (Musgrove et al., 1993), cyclin-dependent kinase inhibitors (Planas-Silva and Weinberg, 1997) and growth factors and their pathways (Migliaccio et al., 1996; Dickson and Lippman, 1995).

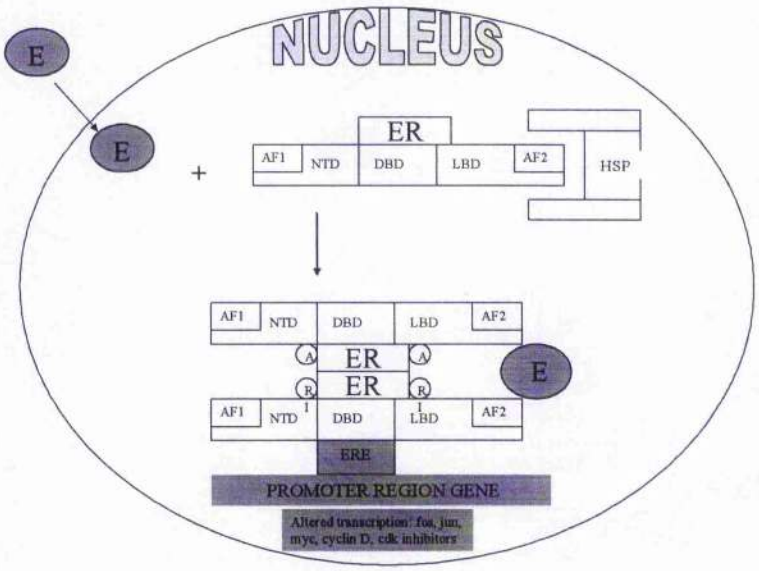

Figure 1

The ER is composed of an N-terminal domain (NTD), a central DNA binding domain (DBD) and a C-terminal ligand-binding domain (LBD). Oestrogen binding to the ER causes dissociation of the heat shock proteins (HSP). The activated receptor subsequently homodimerize and form a complex with co-activators (A) and co-repressors (R), which binds to oestrogen response elements (ERE) in the promoter region of target genes.

Like other nuclear steroid receptors, the ERα is comprised of an N-terminal domain (NTD), a central DNA binding domain (DBD) and a C-terminal ligand-binding domain (LBD). The DBD mediates ERE recognition. The LBD in region E contains an oestrogen–inducible transcription-activating function called AF-2 (Kumar et al., 1987; Tzukerman et al., 1994). In addition there is a constitutively active

transcription-activating function AF-1 which is located in the NTD region of the receptor (Tora et al., 1989). AF1 and AF2 can function independently or synergistically to activate transcription and promote signalling depending on the cell and promotor context (Kumar et al., 1987; Shang and Brown, 2002).

ER activation and its' subsequent cellular actions are in fact more complex than originally thought and deviate from the original concept of the classical nuclear receptor at more than one level. A background to this will now be outlined, and discussed further when considering the implications of this in the development of Tamoxifen resistance.

1.3.1 Receptor phosphorylation

Modification of proteins through phosphorylation is a key mechanism by which the activity of transcription factors can be regulated (Shao and Lazar, 1999). The ER has the potential to be regulated via phosphorylation at all of its domains which results in post-translational modifications. Phosphorylation at serines 104 /106, serines 118 and 167, serine 236 and on tyrosine 537 has been demonstrated using deletional or point mutation analysis (Ali et al., 1993; Castoria et al., 1993; Le Goff et al., 1994; Chen et al., 1999).

The AF-1 region is phosphorylated by components of growth factor pathways (Figure 2) such as MAPK kinases (Kato et al., 1995) and Akt/Protein Kinase B (Campbell et al., 2001) or by cyclin dependent kinases (Chen et al., 2000; Rogatsky et al., 1999) to promote co-activator recruitment and transactivation. The DBD can be phosphorylated by PKA (Chen et al., 1999) to inhibit transcriptional activity via decreasing DNA binding and regulation of dimerisation. In addition the AF2 region

can be phosphorylated by Src kinases on tyrosine 537 (Arnold et al., 1997) to enhance dimerization, ligand binding and co-regulator recruitment.

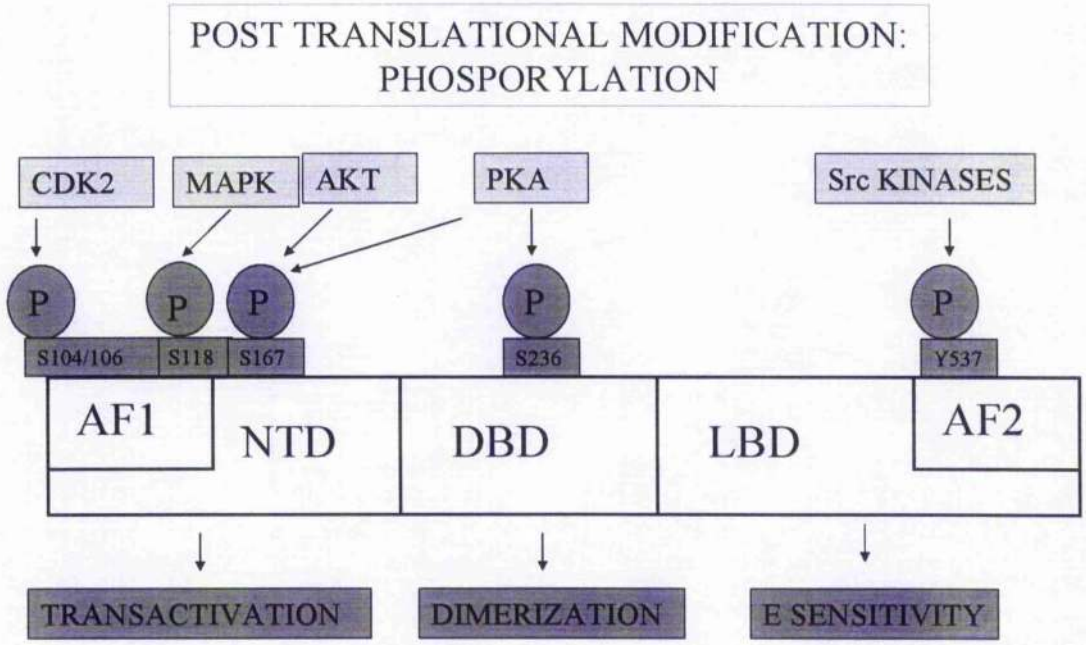

Figure 2

The AF1 region can be phosphorylated by MAPK kinases, Akt or cyclin dependent kinases. The DBD has been shown to be phosphorylated by PKA and the AF2 region by Src kinases.

1.3.2 Ligand independent action

The function of the AF-1 region, which controls ligand-independent activation, seems to depend on various factors. AF-1 action appears to be more apparent in various cell types (Berry et al., 1990; Webb et al., 2000), in the presence of high levels of AF-1 coactivators (Webb et al., 1998), or following activation of components of growth

factor pathways such as MAPK, JNK kinase (Font and Brown, 2000; Kato et al., 1995) (Feng et al., 2001) or AKT (Campbell et al., 2001) activation. In addition reports have suggested that cells with high levels of ER expression are able to self-generate ligand independent AF-1 transactivation and growth (Fowler et al., 2004).

1.3.3 Non-genomic action

There is increasing evidence for the non-genomic actions of ER, particularly as an explanation for the more rapid (seconds to minutes) signalling that can be produced by oestrogen stimulation (Simoncini et al., 2000; Kousteni et al., 2001; Castoria et al., 1999). Under these circumstances the ER is able mediate its rapid actions via direct association with signal transduction components without any transcriptional activity taking place (Losel et al., 2003). Some of these non-genomic actions appear to be taking place outside the nucleus, either in the cytoplasm or at the membrane.

Oestrogens appear to be able to mediate some actions through a putative membrane associated oestrogen receptor (Watson and Gametchu, 1999; Simoncini et al., 2000; Pappas et al., 1995). In the late 1970s Pietras & Szego (Pietras and Szego, 1977) reported the presence of high affinity binding sites for oestrogen associated with the plasma membranes of the MCF7 human breast cell line. This observation was largely ignored until recently, when evidence for non-genomic actions of ERα increased. Pappas et al co-incubated rat pituitary cells with an antibody specific to intracellular ER and a fluorescent ER-BSA conjugate. They demonstrated that these labels colocalize on cells suggesting that mERα may be structurally similar to intracellular ERα. In addition using confocal microscopic methods, translocation of full length ERα into the membrane has been demonstrated in response to oestrogen (Song et al., 2002) and transfection of ERα negative cells with ERα resulted in 5% of ER locating

to the membrane with the remainder mostly in the nucleus (Razandi et al., 1999). Recent work has suggested that these membrane receptors may be particularly important in the role oestrogen plays in 'survival' or anti-apoptotic mechanisms (Razandi et al., 2000). Oestrogen stimulation of membrane ERα has been shown to result in G protein activation, which results in rapid stimulation of protein kinase C, protein kinase A, MAPK and PI3K (Kelly and Levin, 2001; Marquez and Pietras, 2001). There is also evidence that this membrane receptor may directly signal via activation/cross talk with the membrane growth factor EGFR (Razandi et al., 2000). Note that this also provides a mechanism for a positive feedback loop as downstream growth factor pathway members such as MAPK and Akt may then phosphorylate the ERα as discussed previously.

1.3.4 Co-regulators

ER-mediated gene transcription is regulated at yet another level depending on co-regulator availability and function. The receptor interacts with corepressors or coactivators that inhibit or enhance its activity on target genes respectively. In the inactivated state, the ERα is bound to corepressor complexes. On oestrogen binding, the conformational change in AF2 facilitates an interaction with co-activators (Ali and Coombes, 2002). Three related co-activators, known collectively as the p160 co-activators, stimulate ERα activity following ligand stimulation, via interaction with AF-2 (reviewed (Leo and Chen, 2000; McKenna et al., 1999)). These 3 proteins are known as nuclear receptor co-activators NCOA1 (also known as SRC1), NCOA2 (also known as TIF2 or GRIP1) and NCOA3 (also known as P/CIP, ACTR, AIB-1, RAC3 or TRAM1).

1.3.5 Non-classical response elements

As well as regulating transcription through classical EREs in the promotor regions of target genes, the ER also interacts with a growing number of non classical response sites (Saville et al., 2000; Weatherman and Scanlan, 2001). These sites do not necessarily require DNA-protein interactions between the receptor and promotor element, but instead regulate transcription through protein-protein interactions between the receptor and other transcription factors such as the fos jun complex (AP-1) and Sp-1.

1.4 Mechanism of action of Tamoxifen and potential mechanisms for resistance.

Tamoxifen is not a pure antagonist, and in fact exerts both agonist and antagonist effects. Generally it is an agonist in bone and endometrium (Kedar et al., 1994; Love et al., 1992), whereas it is used for its antagonist effect on genes important for cell proliferation or survival in the breast.

The antagonist effect of Tamoxifen arises from inhibition of the AF-2 transcription-activating function (despite allowing release from heat shock proteins and ERE binding) (Figure 3) (Parker, 1996). However unlike steroidal anti-oestrogens such as ICI 182,780, (Wakeling et al., 1991) it does not appear to antagonise the AF-1 function (McDonnell et al., 1995). Tamoxifen therefore allows weak AF-1 activity although in most cases this is insufficient to increase gene transcription (Berry et al., 1990). However, in circumstances where the AF-1 region can become activated, this activity becomes more significant. This has been thought to explain the mechanism of action behind Tamoxifens' tissue specific partial agonist effects. The ERα activity in breast epithelium is due mainly to AF-2 therefore Tamoxifen acts largely as an antagonist, whereas in the uterus, the role of AF-1 is more significant, explaining its agonist effects there.

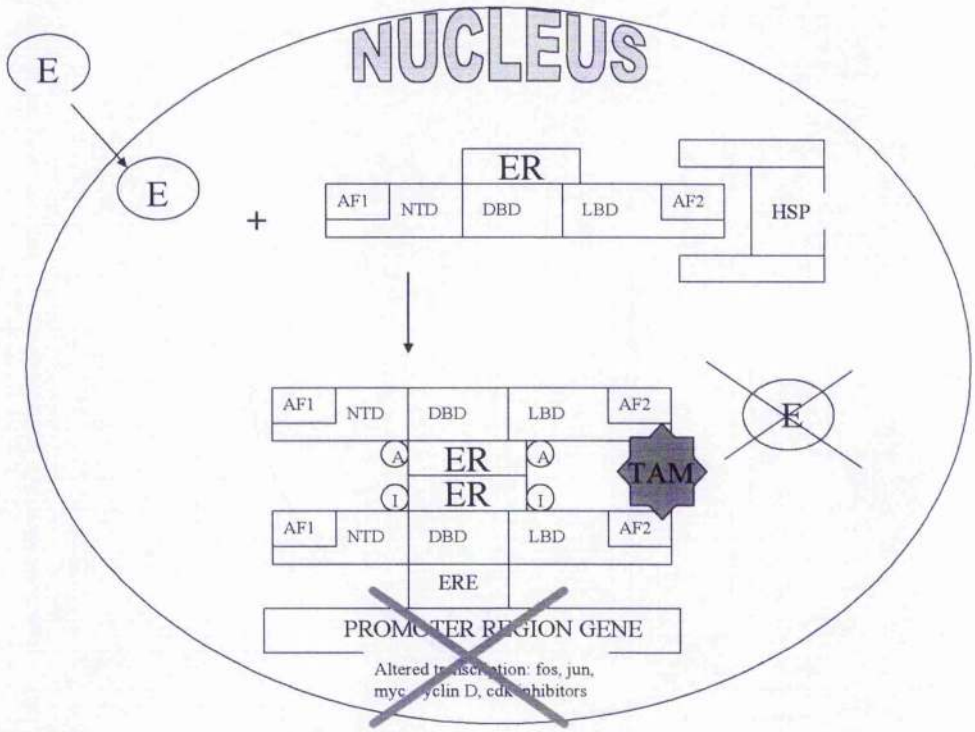

Figure 3

Tamoxifen (TAM) antagonises AF2 transcription activating function but not AF-1 function.

Several mechanisms have been proposed to explain Tamoxifen resistance (Osborne, 1998):

1.4.1 Loss of ERα

Loss of ERα expression, particularly in metastasis, has been proposed as a mechanism for the development of resistance to hormonal therapy. Most ERα positive breast cancers will also contain populations of ERα negative cells. Under the selective pressure of anti-oestrogen treatment, the ERα negative cells may come to predominate so accounting for an apparent switch in their tumour receptor status (Graham et al., 1992). However there is also convincing evidence that ERα expression is a stable

phenotype (Robertson, 1996). We know that at least 2/3rds of tumours that become resistant to tamoxifen continue to express ERα (Encarnacion et al., 1993). Tamoxifen resistant cell lines frequently demonstrate sustained ERα content and remain responsive to pure anti-oestrogen therapy (Brunner et al., 1993). We also know that tumours that have become resistant to Tamoxifen as an initial endocrine therapy will often go on to respond to a 2nd line anti-hormonal treatment such as megace, an aromatase inhibitor or oophorectomy (Murray and Pitt, 1982; Robertson et al., 1989; Sawka et al., 1986), indicating the continued presence of a functional ERα.

1.4.2 Variant/mutant ERα

Mutation of the ER gene could affect function or response to Tamoxifen. There is a naturally occurring ER mutant that recognises Tamoxifen as an agonist only (Fuqua, 1994). However the effect that these mutants have on clinical resistance *in vivo* is yet to be determined (Hopp and Fuqua, 1998).

1.4.3 Presence/altered expression of ERβ

In 1996 a second ER was identified (Mosselman et al., 1996) with subsequent identification of various isoforms such as ERβ1(wildtype), ERβ2 (identical to βcx), ERβ3, ERβ4, ERβ5, and ERΔ5), (Lewandowski et al., 2002; Ogawa et al., 1998; Tong et al., 2002). ERβ is highly homologous to the ERα and has been shown to bind estrogens with an affinity similar to that of ERα, and activates expression of reporter genes containing oestrogen response elements in an oestrogen-dependent manner. When a gene does not require activation of the ER's AF-1 function, ERα and ERβ appear equivalent at regulation of transcription (Cowley and Parker, 1999). However, in addition to acting as a homodimer, ERß also heterodimerizes with ERα (Pace et al.,

1997). Evidence suggests that the expression of functional ERα/β heterodimers, may result in unique patterns of gene regulation, many of which are distinct from the genes regulated by the ER homodimers (Monroe et al., 2005).

It has been suggested that an altered ratio of ERα: ERβ may occur during carcinogenesis (Leygue et al., 1998) with the ERα proportion progressively increasing as the cells acquire a more aggressive phenotype. This alteration in ERα: ERβ ratio may also play a role in Tamoxifen resistance (Speirs et al., 1999; Salvatori et al., 2003). Recent in vitro evidence has confirmed that high levels of ER-beta predicted for improved disease-free and overall survival in patients treated with adjuvant tamoxifen therapy (Hopp et al., 2004b). However more studies are required to examine the role of various isoforms with a report from small numbers of patients suggesting ERβcx in primary lesions correlated with a poor response to tamoxifen (Saji et al., 2002).

1.4.4 Tamoxifen agonist stimulation of growth

In some patients it appears that Tamoxifen therapy can stimulate tumour growth. This is illustrated by the clinical response sometimes shown after Tamoxifen is stopped because of progression, and explains a lack of response to oophorectomy in premenopausal women who remain on Tamoxifen (Wiebe et al., 1993). In addition clinical trials have suggested that longer treatment with adjuvant Tamoxifen is no better, and may possibly be worse, than the standard 5 year treatment (Fisher et al., 2001; Early Breast Cancer Trialists' Collaborative Group, 1998).

MCF-7 breast cancer cell line engrafts can be selected *in vivo* to become stimulated by tamoxifen (Osborne et al., 1987). More recently an explanation for this has been proposed involving a disturbance between ERα and its' coactivators and corepressors

(Smith et al., 1997; Osborne et al., 2003) (Figure 4). It is suggested (Takimoto et al., 1999) that Tamoxifen antagonism may not be a passive competitive process but may involve active recruitment of corepressor or coactivator proteins to produce mixed responses. These studies suggest that an increasing ratio of antagonist specific coactivators to corepressors may lead to inappropriate agonist activity so producing the hormonal resistant state.

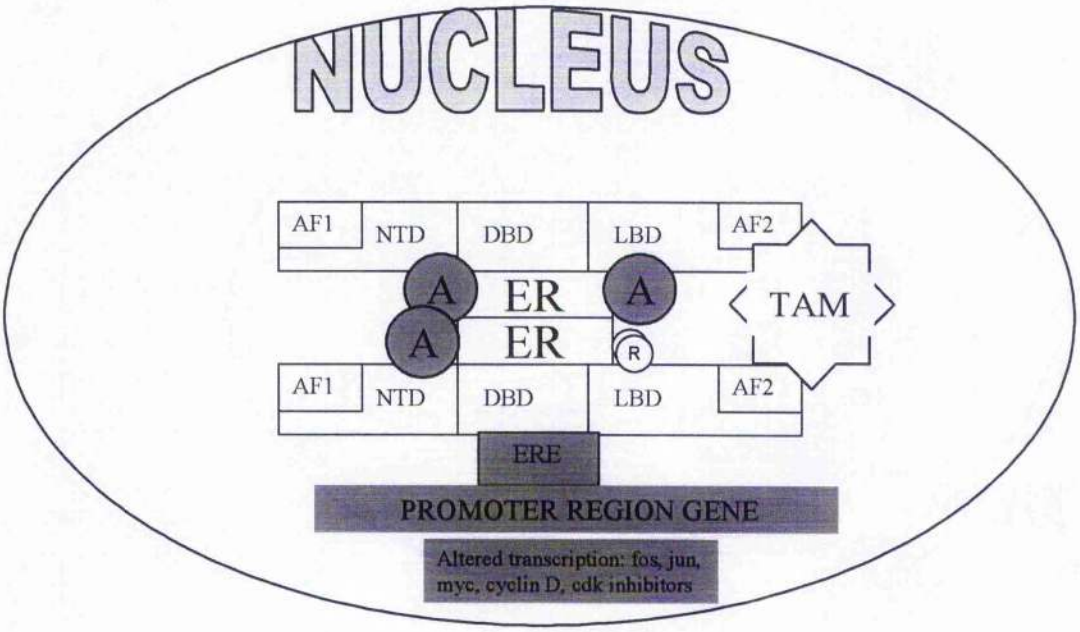

Figure 4

An increasing ratio of coactivators (A) to corepressors (R) may lead to inappropriate Tamoxifen agonist activity.

One particular coactivator AIB1, which may be activated by growth receptor pathways (Font and Brown, 2000), has been linked to poor prognosis in Tamoxifen treated ERα positive patients (Osborne et al., 2003). In addition levels of the co-

repressor N-CoR was reduced in an animal model of tamoxifen resistance (Lavinsky et al., 1998).

Other oestrogen-like effects of Tamoxifen may stem from ERα action at genes with alternative non-classical response elements such as AP-1 or Sp-1 sites (Saville et al., 2000). For example ERα has been shown to enhance AP-1 activity in the presence of tamoxifen (Weatherman and Scanlan, 2001; Webb et al., 1999).

1.4.5 Development of ligand independent ER mediated transcription

The concept of ligand independent activation of ERα, particularly in regard to activation of the AF-1, has already been discussed. There is considerable evidence that ligand independent activation can follow the activation of components of growth factor pathways such as MAPK, JNK kinase (Font and Brown, 2000; Kato et al., 1995) (Feng et al., 2001) or AKT (Campbell et al., 2001). Activation of the normally quiescent AF1 region may promote gene transcription even in the presence of tamoxifen (Figure 5).

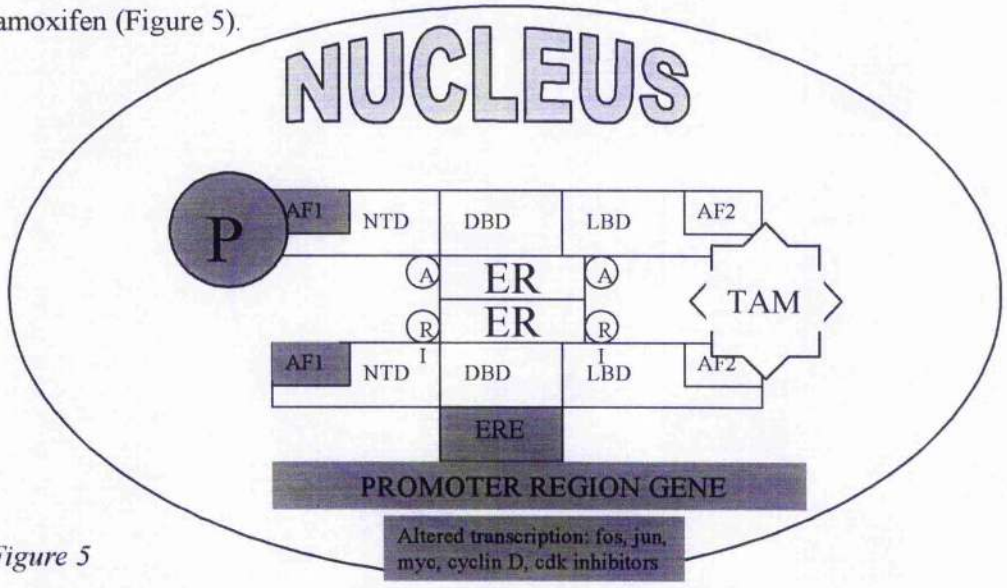

Figure 5

Phosphorylation of AF-1 region by components of growth factor pathways may lead to ligand independent activation despite presence of Tamoxifen.

Evidence for the role of the HER family in this process and in development of subsequent Tamoxifen resistance will be discussed in the following chapters. However cell lines acquiring oestrogen independence often retain their responsiveness to anti-oestrogens (Clarke et al., 1989) thus it may be that oestrogen independence and anti-oestrogen resistance involves overlapping but separate mechanisms.

1.4.6 Role of Growth Factors.

Growth factors have been shown to produce oestrogen-like effects in ER positive breast cancers even in the absence of oestrogenic stimuli (Bunone et al., 1996; El Tanani and Green, 1997). Thus growth factor signalling may play a critical role in regulating the response of breast cancer cells to both oestrogens and anti-oestrogens.

Whilst tamoxifen blocks the oestrogenic induction of growth factors such as TGF-α, IGF-1 and EGF (Dickson and Lippman, 1995), it also induces the secretion of the growth inhibitory factor TGF-β1 (Knabbe et al., 1987) suggesting the promotion of a inhibitory autocrine loop. However tamoxifen resistant MCF7 cells have been shown to overexpress TGF-β1 (Herman and Katzenellenbogen, 1996; Arteaga et al., 1999) and reversal of TGF-β1 activity can restore tamoxifen sensitivity in vitro (Arteaga et al., 1999). Indeed, whilst treatment with exogenous TGF-β inhibits breast cancer cell proliferation (Knabbe et al., 1987) it can also stimulate MAPK (Frey and Mulder, 1997; Visser and Themmen, 1998) associated tumour growth.

In addition in patients who do not respond to tamoxifen, TGF-β2 levels increase before clinical detection of recurrence implying resistance to any growth inhibitory effects(Kopp et al., 1995). Overexpression of TGF-β2 can suppress natural killer (NK) cell function and inhibition of TGF-β2 restores NK cell function and tamoxifen

sensitivity in vivo (Arteaga et al., 1999). This suggests TGF-β2 overexpression may affect resistance through immunological mechanisms.

Another growth factor family that has stimulated interest with respect to tamoxifen resistance is the insulin like growth factors. Both IGF-I receptors (IGF-I-Rs) and IGF-II receptors (IGF-II-Rs) are expressed in breast tumours (Papa et al., 1993) but in the context of tamoxifen resistance most attention is focussed on IGF-I-R because of evidence of crosstalk with the ER (Lee et al., 1999). More recently studies have shown an alteration of insulin-like growth factors-binding proteins (IGF-BP) that generally inhibit IGF function with IGFBP-2 demonstrated as a marker for antiestrogen resistant breast cancer cell lines, although not necessarily a contributor to the resistant cell growth (Juncker-Jensen et al., 2006). Unlike other growth factors, IGF-1R expression appears to be positively correlated with ERα expression in breast cancer and recent work has suggested that it may be involved in activation of membrane bound ERα (Razandi et al., 2003b). In addition it has been suggested that IGF-1R is a key component involved in acquisition of resistance to the anti-EGFR small molecule inhibitor gefinitib in tamoxifen resistant cells (Hutcheson et al., 2006). The HER family of tyrosine kinase growth factor receptors has also been implicated in the development of tamoxifen resistance and will be discussed in more detail in the next section.

1.4.7 Estrogen Regulated genes: TFF1 (pS2) and PR

PS2 (TFF1) and PR are both estrogen regulated genes. The expression of TFF1 is regulated through an ERE and expression PR through a Sp1 recognition site on the progesterone receptor (PR) gene. The pS2 gene is often used in breast cancer in studies of ER action/activation (Krieg et al., 2004)

In terms of predicting response to tamoxifen, cytosolic expression of trefoil protein TFF1 (previously known as pS2), has been shown to be linked to good outcomes and endocrine responsiveness (Corte et al., 2006; Gion et al., 1993) in some studies but not in others (Ioachim et al., 2003; Elledge et al., 2000).

The evidence for PR in predicting tamoxifen response has drawn conflicting conclusions over the years. Certainly PR expression has been thought to demonstrate an intact, functioning ER pathway. The large Oxford overview did not confirm PR as a predictive marker for tamoxifen response (Early Breast Cancer Trialists' Collaborative Group, 1992) however a retrospective analysis of a large dataset with standardised methodology demonstrated that ER/PR positive patients showed better outcomes on tamoxifen than ER positive/PR negative patients in multivariate analysis (Osborne et al., 2005; Bardou et al., 2003). In keeping with this, the ATAC trial suggests an increased benefit of tamoxifen over aromatase inhibitor in PR negative patients (Dowsett et al., 2005). In addition, unlike ER, sequential biopsies suggest that PR levels may decrease dramatically with up to half of tumors completely losing PR expression when resistance to endocrine therapy evolves (Gross et al., 1984) suggesting a potential model for acquired resistance. The expression of different PR isoforms as predictors of tamoxifen response may also be of importance. Overabundance of PR-A has been associated with resistance to tamoxifen (Hopp et al., 2004a) while a functional polymorphism resulting in increased production of PR-B may be associated with an increased risk of breast cancer (De, I et al., 2003).

1.5 Human Epidermal Growth Factor Receptor (HER) Family

1.5.1 HER 1-4 family: 4 closely homologous receptors

Evidence has suggested that Tamoxifen resistance may involve cross talk between the HER family and the ERα (Schiff et al., 2004). The HER family of tyrosine kinases receptors lies at the head of a complex signal transcription cascade which modulates cell proliferation, survival, adhesion, migration and differentiation. This multi-layered signalling network provides an opportunity for a potentially huge diversity of signalling messages. The family is comprised of 4 closely homologous receptors HER1 (ErbB1/EGFR), HER2 (ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4). Growth factor-induced HER signalling is essential for normal cellular processes however it also plays a key role in the aberrant development and growth of tumour cells (Yarden, 2001b).

The HER receptors are trans-membrane receptors. Each HER family member is composed of an extracellular ligand binding domain, a lipophilic transmembrane segment and cytoplasmic tyrosine kinase region with a regulatory carboxy-terminal segment (Ullrich and Schlessinger, 1990; van der Geer P. et al., 1994). Ligand binding to the extracellular domain induces the receptors to form hetero- or homo-dimers. Cross-phosphorylation of the tyrosine residues then occurs resulting in the docking sites for signalling complexes. The subsequent release of enzymes and adapter proteins into the cytoplasm stimulates multiple signal transduction cascades. Three layers of interaction have been identified as being key factors in the generation of the diverse pattern of signalling messages (Alroy and Yarden, 1997) (Figure 6).

1. Firstly, 2 main groups have been identified as HER ligands (Mendelsohn and Baselga, 2000). There are 6 direct ligands of HER1. Epidermal growth factor (EGF), amphiregulin and transforming growth factor alpha (TGFα) only bind to HER1. Heparin binding EGF (HB-EGF), betacellulin, and epiregulin can bind to both HER1 and HER4. A second class of ligand called neuregulins bind to HER3 and HER4. Neuregulin-1 and -2 bind to either receptor, while neuregulin-3 and -4 bind to HER4 only. Recent work has shown that 2 different ligands activating the same receptor can have a differential impact on transcriptional outcome (Amin et al., 2004).
2. Secondly, each of the ligands has a different preference for stabilising particular receptor dimers. Yarden et al created cell lines co-expressing a combination of HER family receptors. The mitogenic index of each dimer was compared and the most active signalling complex was between HER2 and HER3 (Yarden, 2001a). In general heterodimers generate more potent signals than homodimers (Alroy and Yarden, 1997).
3. Thirdly, each ligand receptor dimer directs its signal through a unique but overlapping set of signalling pathways (Olayioye et al., 2000). These signal transduction cascades include the MAPK pathway, PKC, phosphoinositol kinase, Akt and several transcription regulators. Ultimately these signals reach the nucleus and lead to nuclear gene activation.

Further complexity is highlighted by the fact that HER2 enhances and stabilises dimerization but apparently has no ligand (Klapper et al., 1999) whereas HER3 has no inherent kinase activity so must be dimerised with another HER family member to exert an influence on downstream signalling (Guy et al., 1994).

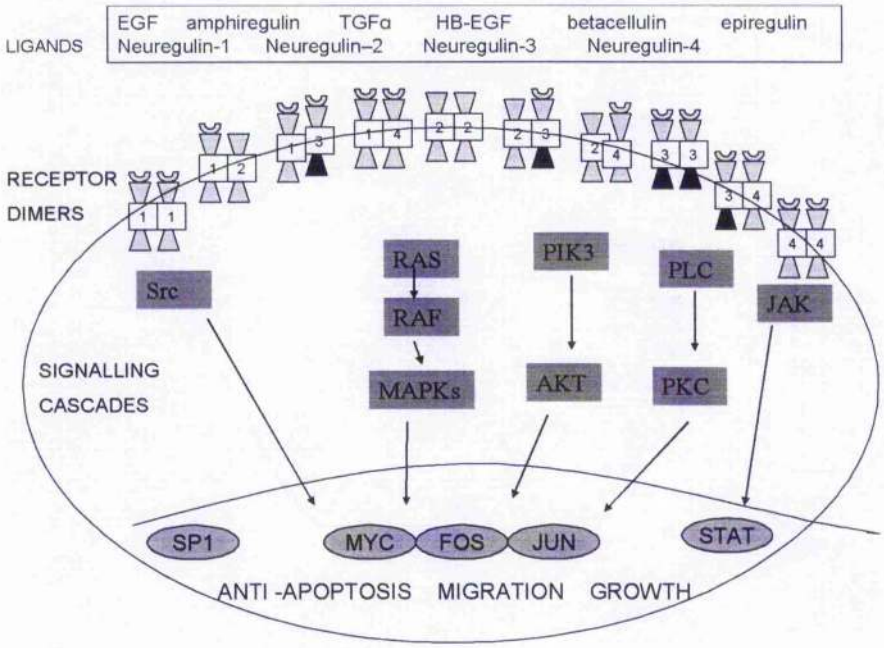

Figure 6

Multiple layers of interaction have been identified as being key factors in the generation of the diverse pattern of signalling messages.

1.5.2 Role of the HER family in normal breast.

The HER family is involved in the regulation of normal breast growth and development (DiAugustine et al., 1997; Carraway et al., 1997). Their ligands have been shown to stimulate the lobulo-alveolar development of the mouse mammary gland in explant cultures and *in vivo* (Jones et al., 1996). Identifying the individual role of each receptor is complex, especially with the presence of multiple ligands (Stern, 2003). However, in mouse, HER1 and HER2 are abundant prior to puberty and during subsequent developmental stages whereas HER3 and HER4 are at low levels prior to maturity (Schroeder and Lee, 1998; Sebastian et al., 1998). All 4 HERs are evident throughout pregnancy and lactation (Schroeder and Lee, 1998).

1.5.3 Role of the HER family in breast cancer.

Dys-regulation of all 4 members of the HER family has been demonstrated in a variety of human cancers.

1.5.3.1 EGFR/HER1

HER1 is a 170kD transmembrane receptor that is expressed on the surface of most cell types and is located on human chromosome 7q21. HER1 is expressed in a variety of human cancers, including non-small cell lung cancer, head and neck, gastric, colorectal, oesophageal, prostate, bladder, renal, pancreatic and ovarian cancers (Salomon et al., 1995). The HER1 ligand EGF was shown to be a potent mitogen in the breast cancer cell line MCF-7 (Osborne et al., 1980) and the first reports of HER1 expression in human breast cancers were in 1985 (Sainsbury et al., 1985).

Sainsbury et al were also the first to identify a link between HER1 and prognosis in breast cancer (Sainsbury et al., 1987). Since then, many studies have examined this relationship, not always with conclusive results (reviewed (Klijn et al., 1992) and (Nicholson et al., 2001a)). One of the largest studies was recently performed using immunohistochemistry in 1029 patients (Tsutsui et al., 2002). Multivariate analysis demonstrated that HER1 was an independently significant prognostic factor for disease free and overall survival. Certainly we know there is a significant negative correlation between EGFR positivity and ER status (Koenders et al., 1991).

There has also been speculation as to the future of HER1 as a predictive factor for selection of various treatment modalities. ZD1839 (Iressa) is an orally active selective HER1 tyrosine kinase inhibitor which has shown promise in preclinical trials, and is currently undergoing phase II clinical trials in breast cancer (Morris, 2002). High

expression of HER1 has been associated with resistance to radiotherapy (Sartor, 2000) and its putative role in Tamoxifen resistance will be discussed later.

1.5.3.2 HER2

The HER2 proto-oncogene encodes an 185kD transmembrane glycoprotein and is mapped to chromosome 17q21. Despite apparently having no ligand, HER2 homodimers form avidly and HER2 is the preferred dimerization partner for other HER members (Graus-Porta et al., 1997; Tzahar et al., 1996). This may help explain the high oncogenic potential of HER2. HER2 heterodimers have particularly high ligand-binding and signalling potency (Sliwkowski et al., 1994) with the most potent dimer in terms of cell growth and transformation being HER2-HER3 (Pinkas-Kramarski et al., 1996). It has been proposed (Klapper et al., 1999) that HER2 maybe the ultimate co-ordinator of the signalling network, amplifying signalling by multiple ligands.

HER2 is normally expressed in a variety of cell and tissue types and is frequently overexpressed in a number of human cancers (Hynes and Stern, 1994). HER2 overexpression and amplification is found in lung, gastric and ovarian cancers (Schneider et al., 1989; Yoshida et al., 1989; Berchuck et al., 1990).

Both preclinical and clinical studies indicate that HER2 amplification and overexpression is involved in oncogenic transformation and tumorigenesis in breast cancer. Cell lines that overexpress the HER-2 gene product display a highly transformed and tumorigenic phenotype as compared with control cells (Chazin et al., 1992; Di Fiore et al., 1987). In addition HER2 gene amplification or protein overexpression has been demonstrated in 15-30% of invasive breast cancers (Berger et al., 1988; Slamon et al., 1987). The majority of cases of HER2 overexpression

appear to be as a consequence of HER2 gene amplification (Pauletti et al., 1996). Slamon et al (Slamon et al., 1987) first linked HER2 with poor prognosis in breast cancer in 1987. Since then there have been multiple studies investigating this relationship. A meta-analysis of 47 trials (Ross and Fletcher, 1998) demonstrated HER2 was an independent predictor of prognosis in 60% of the trials involving 67% of patients. Of note all the studies that used FISH to detect gene HER2 amplification concluded that there was a clear association between HER2 status and prognosis.

Herceptin (trastzumab) is a HER2 monoclonal antibody against its extracellular domain. Patients with strongly HER2-positive metastatic breast cancer derive significant clinical benefit from single-agent and combined Herceptin therapy (Baselga, 2001) in the metastatic setting for HER2 positive patients. More recently interim results from the BIG HERA trial suggest significantly improved disease free survival with reduction in distant metastasis in HER2 positive patients given adjuvant Herceptin for 1 year (Piccart-Gebhart et al., 2005).

1.5.3.3 HER3

In 1989 a 3rd member of the HER family was identified (Kraus et al., 1989) as a 148-kDa transmembrane polypeptide mapped to chromosome 12q13. Due to substitutions in critical residues in its catalytic domain, HER3 has an impaired kinase (Guy et al., 1994), and thus can only process its signalling in the context of a receptor heterodimer. HER3 is found in cells of the developing gastrointestinal, reproductive and urinary tracts as well as the skin, endocrine and nervous systems (Prigent et al., 1992). HER3 is expressed in tumours of the skin, ovary and gastrointestinal tract (Bodey et al., 1997; Maurer et al., 1998; Simpson et al., 1995). There is also evidence for its role in breast cancer. Markedly elevated HER3 mRNA levels have been

demonstrated in certain human mammary tumour cell lines (Kraus et al., 1989). In addition HER3 has been demonstrated to be overexpressed in 15 –52% of breast cancers (Lemoine et al., 1992; Naidu et al., 1998; Travis et al., 1996).

Conflicting evidence has been produced on the prognostic significance of HER3. Some (Witton et al., 2003; Naidu et al., 1998; Tovey et al., 2004; Travis et al., 1996) have demonstrated a relationship between HER3 and markers of poor prognosis in breast cancer. Others have concluded that HER3 overexpression is consistent with a good prognostic outlook (Pawlowski et al., 2000).

1.5.3.4 HER4

HER4 deserves particular attention when examining the role of the HER family in breast cancer as there is now substantial evidence suggesting that it may play a protective role in breast cancer in contrast to the other family members. The HER4/erbB4 gene encodes a 180-kDa transmembrane tyrosine kinase (Plowman et al., 1993) on chromosome 2 (Zimonjic et al., 1995). HER4 is widely expressed in many adult and foetal tissues and is generally found in the differentiated compartments (Srinivasan et al., 1998). In normal breast tissue HER4 appears to play a critical role in the late differentiation of mammary gland function (Jones et al., 1999) especially during pregnancy and lactation (Tidcombe et al., 2003). This is consistent with data from cell lines showing that whilst HER4 can support proliferation and transformation, in some lines it has also been shown to induce growth arrest and differentiation (Sartor et al., 2001; Williams et al., 2003). Recently, evidence, again from cell lines using agonistic monoclonal antibodies, showed the HER4 antibody inhibited cell growth in contrast to the HER2 antibody which stimulated growth (Amin et al., 2004). Using a global gene-expression monitoring

system, they also reported that activation of HER2 or HER4 in the same cell line differentially activated gene transcription.

The evidence for a role for HER4 in tumorigenesis is scanty. One small study looking at solid tumours demonstrated overexpression in some adenocarcinomas and a loss of expression in squamous cell carcinomas (Srinivasan et al., 1998). Certainly HER4 is expressed in several breast cancer cell lines (Plowman et al., 1993). Studies have demonstrated the expression (29 –82%) of the HER4 protein in breast cancers (Srinivasan et al., 1998; Srinivasan et al., 2000; Suo et al., 1998; Suo et al., 2002) although they also suggested that this expression may be decreased when compared to normal breast tissue. Gene amplification has been demonstrated in 13% of breast cancers (Vogt et al., 1998).

We have recently shown that, in contrast to other HER family members, HER4 expression is associated with increased survival and lower proliferation indices (Tovey et al., 2004; Witton et al., 2003). These results are supported by data linking HER4 to established good prognostic indicators such as a lower grade of tumour (Kew et al., 2000; Srinivasan et al., 2000), ER positivity (Pawlowski et al., 2000) and low proliferation indices (Knowlden et al., 1998). However, whilst other groups have also demonstrated a link between HER4 positivity and a longer disease free interval (Suo et al., 2002) there have also been conflicting reports associating HER4 with an adverse prognostic significance (Lodge et al., 2003). More recently there has been evidence from a large series of patients suggesting that the prognostic value of HER4 overexpression is dependent on coexpression with other HER family members (Abd El-Rehim et al., 2004). In this study, when the group was looked at as a whole, HER4 status was not related to survival. However in cases showing expression of one family

member only (homodimers), they found a significant association between HER4 homodimer-expressing tumours and improved disease free survival.

There are intrinsic problems in comparing these studies and their outcomes. Different cut-off points for positivity have been chosen depending on the study and the modality of staining looked at (membrane, cytoplasm, and nuclear). Some groups have reported staining in all 3 locations, whilst others have found no membranous (Lodge et al., 2003) or no nuclear staining (Suo et al., 2002). Three different antibodies have been used in these studies. The HFR1 clone developed by the Gullick group has been the most widely used (Kew et al., 2000; Lodge et al., 2003; Srinivasan et al., 1998; Srinivasan et al., 2000; Abd El-Rehim et al., 2004). This group demonstrated the ability of this antibody to recognise HER4 by immunoprecipitation, western blotting and immuno-staining of NH3T3 cells transfected with HER4. They demonstrated no cross-reactivity with EGFR using A431 cell lysates or with HER3 or HER4 using lysates from SKBR3 or 293/HER3 cells. A SantaCruz antibody C18 has also been used by one group (Suo et al., 2002). In our previously study on frozen tissue we used a Neomarkers antibody H4.77.16.

Recent studies have substantially enhanced our understanding of the many functions of HER4. Indeed, as well as acting as a membrane signal transduction receptor, nuclear HER4 is required for mammary gland development and lactation though gene regulation in conjunction with STAT5A (Clark et al., 2005; Williams et al., 2004), and mitochondrial HER4 has been shown to mediate apoptosis in the mitochondria via BAK (Vidal et al., 2005).

Recent evidence suggests that, as with HER2 and EGFR, the HER4 protein can be enzymatically cleaved, which may markedly alter the function of the intracellular domain of the receptor. Cleavage occurs within the juxtamembrane region through the

activity of TNFα-converting enzyme (TACE) followed by further proteolytic processing by presenilin-dependent γ-secretase activity (Lee et al., 2002; Rio et al., 2000) to release the cleaved intracellular domain (4ICD). Indeed this 4ICD has been shown to harbour a BCL-2 homology 3 (BH3)-domain and independently function as a BH3-only protein (pro-apoptotic members of the BCL-2 family required to initiate mitochondria dysfunction) so mediating cellular apoptosis. However it has also been demonstrated in the nucleus acting as a chaperone for STAT5A (Williams et al., 2004) to alter gene regulation. Thus, it is essential to determine both the location and intensity of staining for HER4 in order to fully understand its' function *in vivo*. Indeed, one recent study using the HFR1 antibody demonstrated very different correlations in terms of survival depending on cellular location of HER4 staining. Whilst membranous HER4 was associated with a good prognostic outlook, nuclear HER4 was associated with significantly shorter survival times (Junttila et al., 2005). Thus one possible explanation for the conflicting reports on HER4 and its association with survival may be that the results are antibody dependent. HFR1, the antibody developed by the Gullick group recognises the intracellular domain of HER4 and is thus able to recognise both the intact receptor and the cleaved ICD as it traffics through the cell. However the H4.77.16 clone recognises an extracellular domain of HER4 and thus, on tissue sections, detects the full length receptor not the cleaved ICD. The variance in reported results for *in vivo* analysis of HER4 expression may be a reflection of differing antibody specificities, especially with respect to the intra-cellular and extra-cellular domains of the protein. Certainly we know that even for established diagnostic markers such as ER and PR within laboratories participating in external quality assessments (EQAs) there are still problems with technical validation and standardization (Rhodes et al., 2001).

1.5.3.5 HER1-4 acting in combination

As discussed with regard to HER4, one of the problems with the above analysis is the examination of these receptors in isolation, whereas in reality expression of multiple receptors may result in a synergistic or an additive response. We know that cancers that overexpress HER1 or HER2 in combination have a worse prognosis than those which are positive for only one receptor (Tsutsui et al., 2003). In contrast when HER2 and HER4 are co-expressed there is a reduced risk of relapse and death compared to tumours where HER1 and 2 are overexpressed (Suo et al., 2002; El Tanani and Green, 1997). This suggests that HER4 may be able to antagonise the HER2 effect on poor clinical outcome. In cell lines high levels of HER2 expression alters the ability of HER1 tyrosine kinase inhibitors to inhibit HER1 phosphorylation (Christensen et al., 2001). In terms of potential treatments, whilst we know HER2 amplified tumours can be successfully treated with Herceptin (Piccart-Gebhart et al., 2005; Smith et al., 2007), we don't know how co-expression of other HER family members may influence this response.

1.6 Relationship between HER1-4 and resistance to tamoxifen

Most studies in this field have investigated Tamoxifen resistance in the context of HER1 and HER2 overexpression.

1.6.1 Experimental studies

Benz et al (Benz et al., 1993) took oestrogen positive, hormonally sensitive MCF-7 cells and transfected them with very high levels of HER2. Despite the cells remaining oestrogen dependent they became resistant to Tamoxifen. Other studies (Pietras et al., 1995) using HER1 and HER2 showed similar results but found that tumour cell growth became independent of oestrogen with a downregulation of ERα. Pietras et al and others have also demonstrated that inhibitors against HER2 and HER1 can restore Tamoxifen sensitivity in HER2 overexpressing tamoxifen resistant cells (Kurokawa et al., 2000).

Furthermore MCF-7 cells which over time had become resistant to tamoxifen were demonstrated to have increased levels of both HER1 and HER2 compared to Tamoxifen responsive cells (Nicholson et al., 2001b; Knowlden et al., 2003). Again cell growth in these models was significantly reduced with the use of the HER1 inhibitor (Iressa) and HER2 antibody (Herceptin).

1.6.2 Clinical studies

There has been a multitude of studies reporting on the proposed influence of HER2 expression on hormonal resistance, but results are varied and confusing. Some studies

have demonstrated a clear link with resistance (Berns et al., 1995; Borg et al., 1994; Carlomagno et al., 1996; Houston et al., 1999; Leitzel et al., 1995; Yamauchi et al., 1997; Wright et al., 1992). Others have not demonstrated any association (Elledge et al., 1998; Soubeyran et al., 1996; Berry et al., 2000; Knoop et al., 2001; Archer et al., 1995).

The picture for HER1 is equally unclear. Some studies have indicated a positive link with Tamoxifen resistance (Newby et al., 1997; Nicholson et al., 1994) with others not showing any significant difference (Knoop et al., 2001).

Several explanations have been proposed for this conflicting evidence (Dowsett, 2001; Elledge et al., 1998).

1. Several of the studies included ERα negative or ERα unknown tumours in their analysis (Carlomagno et al., 1996; Leitzel et al., 1995) which will not respond to tamoxifen and are also more likely to be HER2 or HER1 positive. Often when the results are substratified for ERα status any significant association is lost (Nicholson et al., 1994).
2. Multiple treatment settings were analysed including neoadjuvant (Soubeyran et al., 1996), metastatic (Leitzel et al., 1995) and local recurrence (Houston et al., 1999).
3. Only a small group of patients will be both ER and HER1/2 positive which makes it difficult to achieve statistical power in a study. Some studies have shown that as little as 10% of tumours are ERα and HER2 positive with the percentage even lower for HER1 (Dowsett, 2001).
4. Chemotherapy is also routinely given to many of these patients, which may interact with HER2 status. For example it has been suggested that anthracyline therapy may overcome HER2 induced resistance.

5. Multiple methods have been used to measure HER2 status with a variety of cut-offs for positivity such as IHC (Elledge et al., 1998), FISH (Berry et al., 2000) and serum measurements (Leitzel et al., 1995).
6. The duration of Tamoxifen therapy varied. Some studies only examined the follow-up with Tamoxifen treatment after one year which may not be long enough for a resistance pattern to develop (Knoop et al., 2001).

It is likely that the limited statistical power of these studies together with the inclusion of ER negative cases explains the majority of these conflicting results and it would be important to address these points in planning a future in vivo study examining any association between the HER family and tamoxifen resistance.

There is little *in vivo* or *in vitro* evidence currently to link HER3 and HER4 to tamoxifen resistance (Larsen et al., 1999; Knowlden et al., 1998), although similar signalling pathways are activated by HER1-4.

1.6.3 Aromatase Inhibitors versus Tamoxifen

Some of the most convincing clinical evidence for the role of HER1 and 2 in tamoxifen resistance has come from neo-adjuvant trials where HER1/2 positive patients have significantly greater response to aromatase inhibitors than to Tamoxifen (Ellis et al., 2001). Whilst there is no definite evidence that this is also the case in the adjuvant setting from trials so far, this is now being built into subtrial analysis (retrospectively for the ATAC trial and prospectively for TEAM (Tamoxifen Exemestane Adjuvant Multinational)). Certainly data from the ATAC (Dowsett, 2003) and IES trials (Coombes et al., 2004a) suggest that some differences do exist in the molecular profile of patients who respond to early or to delayed treatment with

aromatase inhibitors. In the ATAC trial PR negative patients derived greater benefit from initial aromatase inhibitor treatment compared to Tamoxifen. However PR status had no impact on response when aromatase inhibitors were given as delayed treatment to patients who had been disease free on Tamoxifen for 2-3 years (IES). Whether the negative PR is acting as a surrogate marker for HER1-3 overexpression in the ATAC trial remains to be seen but we do know that, as with ERα, there is an inverse relationship between PR expression and HER2 expression (Marsigliante et al., 1993; Quenel et al., 1995).

Further evidence on the relative importance of other growth factor receptors, including HER1, 3 & 4 are required before these findings alter clinical practice, particularly in the adjuvant setting.

1.7: Cross talk between ER and HER pathways: Relationship with Tamoxifen resistance?

Whilst some have argued that the mechanisms behind the HER families' role in Tamoxifen resistance is due to a dislocation of the growth factor stimulated pathways from ER signalling, this is disputed by the fact that switching to another form of endocrine therapy can overcome resistance patterns both *in vitro* and clinically. Thus it is likely that ER signalling remains involved.

Several mechanisms have been proposed by which the type I RTKs may modify response to oestrogens and tamoxifen. There is a considerable body of biological evidence suggesting that this cross talk occurs at multiple levels and appears to be bi-directional (Nicholson et al., 1999).

The most obvious relationship between the HER family and ERα status appears to be a negative one. Certainly tumours positive for HER1-3 tend to be ERα negative (Witton et al., 2003) and, in the case of EGFR, it has also been suggested that at an individual cell level EGFR and ERα are mutually exclusive (Sharma et al., 1994). This may partially be explained by an active mechanism at a transcriptional level whereby oestrogen suppresses EGFR (Yarden et al., 2001; Wilson and Chrysogelos, 2002) or HER2 (Newman et al., 2000).

However we also know that oestrogen and EGFR can also have a stimulatory effect on each others pathways. In several ERα positive breast cancer cells lines it has been shown that oestrogen is able to transiently induce EGFR expression (Yarden et al., 1996). Some evidence has suggested that oestrogen induced growth factor pathway stimulation may be via 'non classical' pathways. For example recent studies have

demonstrated the ability of oestrogen to stimulate activation of the MAPK and PIK3 pathways independently of gene action (Migliaccio et al., 1996; Simoncini et al., 2000). Alternatively there may be interaction with sites such as Sp1 (Salvatori et al., 2003) resulting in direct transcriptional action of ERα on the EGFR gene.

In addition, the growth factors TGFα and EGF can produce oestrogenic effects in ER positive cells in the absence of oestrogenic stimuli (i.e. ligand independent activation) (Bunone et al., 1996; El Tanani and Green, 1997). Again recent evidence suggests that this interaction may be in a non-genomic manner (Razandi et al., 2003b) possibly involving interactions at the cell membrane (Chung et al., 2002). Ultimately though, irrespective of location, there still needs to be an endpoint by which the growth factors or their down stream effectors may manipulate ERα activation. As discussed earlier, oestrogen receptors are phosphoproteins (Shao and Lazar, 1999) and whilst ligand binding controls AF2 activity, AF1 activity is regulated by phosphorylation at sites including serine 104/106, S118 and S167. (Ali et al., 1993; Le Goff et al., 1994; Smith, 1998; Arnold et al., 1994). The potential of ERα to be modulated by phosphorylation provides a key mechanism by which the HER signal transduction pathways may influence ERα transcription. Multiple pathways activated by the HER receptors have been identified, which are often interlinked (Jorissen et al., 2003). These pathways may provide the means by which a growth factor signal at the cell surface results in ERα phosphorylation and subsequent modulation of its functions including DNA binding and transcriptional activation despite the presence of tamoxifen. The cellular proliferation Ras/MAPK pathway and the survival/anti-apoptosis PI3-K/AKT pathway are both generating the most interest in terms of anti-oestrogen resistance mechanisms.

1.7.1 Mitogen-activated protein kinase (MAPK) pathway

Growth factors acting via tyrosine kinase receptors activate MAPK by first the GTPase Ras then subsequent serine-theonine kinase Raf-1 activation (Hill and Treisman, 1995). The MAPK pathway is a key phosphorylation cascade by which growth factor signal transduction is conveyed from the plasma membrane to the nucleus (English et al., 1999) (figures 6 & 7). The MAPKs phosphorylate and thereby modify the function of numerous proteins including those regulating cell cycle checkpoints and gene transcription (Chang and Karin, 2001). In the nucleus MAPK activates transcription factors such as myc and Elk1. Under normal conditions activation of the MAPK pathway is transient and attenuated by many control mechanisms including inactivating phosphatases (Keyse, 2000; Pearson et al., 2001). The control however may become lost and several lines of evidence suggest that dysregulation of this pathway may have particular significance in breast carcinogenesis.

MAPK activity has been shown to elevated in breast tumours often with upregulation of its gene expression (Sivaraman et al., 1997) and has been associated with decreased survival (Mueller et al., 2000). Phosphorylated MAPK positivity (identified using IHC) has been associated with a poor response to Tamoxifen therapy (Gee et al., 2001) in ERα positive tumours. Similar results have been demonstrated *in vitro* where HER2 driven MAPK elevation has been associated with Tamoxifen resistance (Kurokawa et al., 2000; Benz et al., 1993).

The serine phosphorylation site S118 in the AF1 region of the ER has been found to be a target for phosphorylation by MAPK (Kato et al., 1995; Bunone et al., 1996). Work using cell line mutants has identified a key role for S118 in oestrogen - independent MAPK activation of ER (Atanaskova et al., 2002; Chen et al., 2002).

However there also seems to be a role for ligand dependent (via oestrogen and to a lesser extent tamoxifen) activation of S118 which does not require MAPK (Chen et al., 2002). Thus it is postulated that oestrogen and also tamoxifen activation of the AF1 region can be enhanced/circumvented by MAPK mediated growth factor signalling. This may provide a potent explanation for Tamoxifen and other anti-oestrogen resistance.

However some studies indicate this ligand-independent signalling does seem to retain the ability to be inhibited by Tamoxifen (Atanaskova et al., 2002), suggesting that the this is method of signalling may not be sufficient to induce anti-oestrogen resistance on its own.

1.7.2 Phospatidylinositol-3 kinase (PI-3K) and Akt pathway

The phospatidylinositol-3 kinase (PI-3K) and Akt pathway is known to have a key role in the cell survival (anti-apoptosis pathway) (Franke et al., 1997). Akt (protein kinase B) is a member of a conserved family of serine/theonine protein kinases that includes Akt1, Akt2 and Akt3 (Alessi and Downes, 1998). Activation of transmembrane growth factor receptors results in the recruitment of PI3 kinase to the plasma membrane (Datta et al., 1999). PI3 kinase subsequently promotes the generation of phosphorylated phosphoinositides which then bind and activate Akt (Campbell et al., 2001; Chan et al., 1999). It appears that HER3 in particular has a strong link to the Akt pathway with 6 binding sites being identified for PI3-K (Carraway, III and Cantley, 1994).

Activated Akt then plays a key role in cell survival by phosphorylating and hence modulating the activity of various transcription factors at the nucleus. Akt has been demonstrated to suppress apoptosis. Mechanisms for this include phosphorylation

(and hence inhibition of action) of the pro-apoptotic proteins BAD and capase-9 (Cardone et al., 1998; Datta et al., 1997). Thus disruption of the Akt pathway may well result in tumour growth. Indeed cell lines with constitutively activated Akt1 undergo malignant transformation (Sun et al., 2001b).

Various studies have implicated members of the AKT pathway in the pathogenesis of breast cancer. Akt1 kinase activity has been demonstrated in high grade breast cancers (Sun et al., 2001b). Akt2 gene amplification has been reported in a small percentage of breast cancers (Bellacosa et al., 1995) and Akt3 mRNA is elevated in ERα negative breast cancers (Nakatani et al., 1999).

PTEN is a phospholipid phosphatase tumour suppressor gene that normally attenuates the Akt survival signal (Cantley and Neel, 1999) by dephosphorylating phosphatidylinositol-triphosphate (PtdInsP) and suppresses cell growth through the negative regulation of cell cycle and cell survival. Downregulation of PTEN is associated with increased PI3 kinase activity with subsequently higher levels of 3'-phosphorylated phosphoinositides, which bind to and activate PK-B/Akt. Reduced expression of PTEN has been demonstrated in high grade breast cancers (Bose et al., 2002) and has recently been associated with relapse-free survival and disease-specific survival in tamoxifen treated ER positive patients (Shoman et al., 2005).

There is evidence that the Akt pathway may also mediate signalling from the growth factor receptors to the ERα (Martin et al., 2000). Constitutively active Akt has been shown to induce ER reporter activity in the absence of oestradiol (Kurokawa and Arteaga, 2003) and MCF–7 cells overexpressing AKTα were not as sensitive to inhibition by Tamoxifen compared to wild type cells (Campbell et al., 2001). Studies using cell lines with mutated serine residues have demonstrated that Akt mediates its action on the ERα by phosphorylation of serine residue Ser167 in the AF1 region.

This again occurs in a ligand independent manner (Campbell et al., 2001; Martin et al., 2000).

Thus there is evidence that both the Akt and MAPK pathways may mediate the ERα HER crosstalk leading to anti-oestrogen resistance in breast cancer. Again a limitation of examining these pathways independently is that they do not act in isolation. For example Akt has been shown to inhibit the Raf-MEK-MAPK pathway through phosphorylation of Raf-1 (Zimmermann and Moelling, 1999). Ras has also been shown to bind and activate a subunit of PI3-K (Pacold et al., 2000) and Akt activation is partially dependent on the activity of Ras

1.7.3 Continuous cycling of growth factor signalling stimulated ERα activation?

These *in vitro* results suggest the possibility that continuous cycling between a ligand-independent activated ERα and growth factor signalling could develop (Clarke et al., 2001a) so promoting unopposed tumour growth. So where would tamoxifen fit into this cycle? As discussed previously, Tamoxifen acts by targeting the AF2 but not the AF-1 regions of the ERα. Should the AF-1 region become involved in ligand independent mediated activation this would provide a mechanism by which growth factor pathway stimulation of the ERα could occur with no mechanism for Tamoxifen to antagonise it (Figure 7).

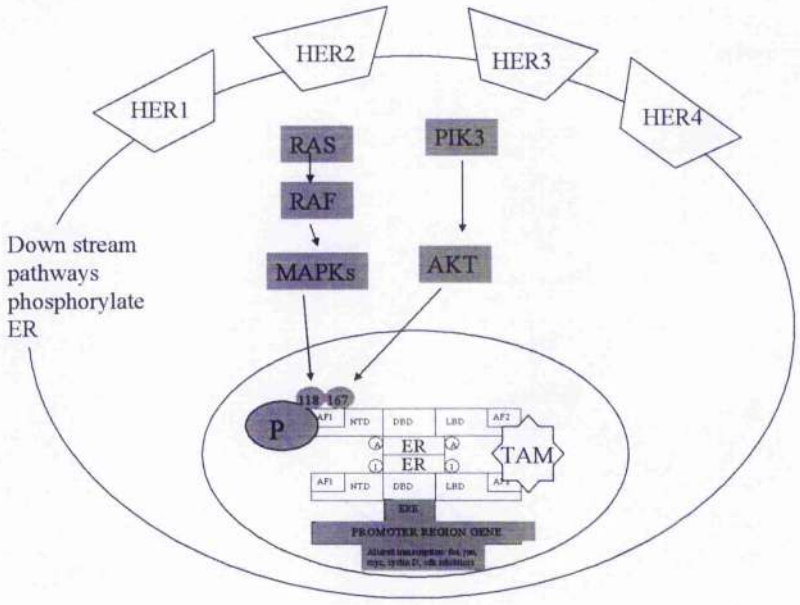

Figure 7

Cross talk between the HER family and phosphorylation sites of the AF1 region of the ER may result in ligand independent activation.

More recently it has been suggested that ERα located at the membrane may directly signal via activation/cross talk with HER1 (Razandi et al., 2003b). High levels of membranous ERα have also been associated with oestrogen-induced MAPK activation in MCF-7 cells (Zivadinovic and Watson, 2005). In addition, *in vitro* evidence has also suggested that resistance to Tamoxifen-induced apoptosis is associated with a direct interaction between HER2 and a cell membrane ER (Chung et al., 2002). If present *in vivo*, these interactions could provide a mechanism for a positive feedback loop, where following activation of the HER2-ERα axis at the cell surface, activation of downstream signalling proteins such as MAPK and Akt initiate phosphorylation of the ERα at the nucleus (Figure 8). Interesting, recent evidence has also suggested that HER2 overexpression in ERα positive breast cancer cells promoted nucleus to cytoplasm relocalisation of ERα so demonstrating further

evidence for a interaction between the HER family and ERα cellular location (Yang et al., 2004).

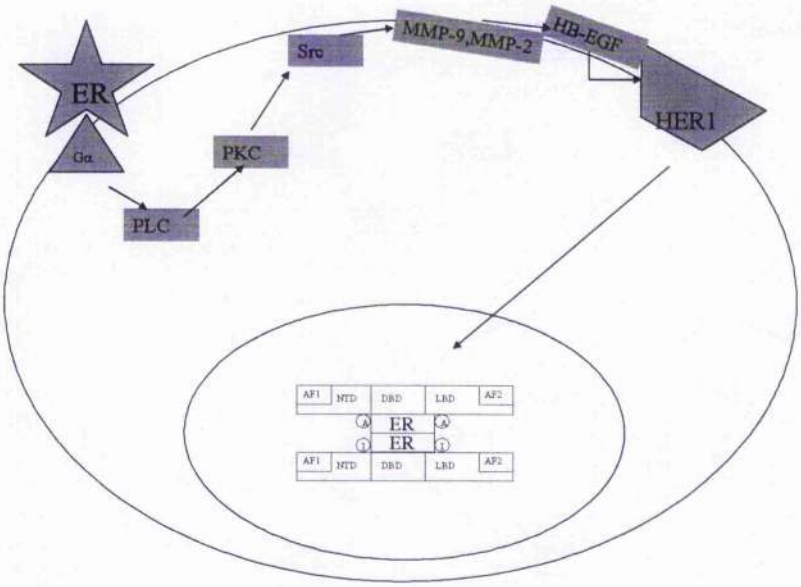

Figure 8

ER at the membrane may directly signal via interaction/crosstalk with HER1 (Razandi et al., 2003b).

1.8 Statement of Aims

A retrospective clinical follow up study has been performed on archival formalin - fixed paraffin embedded tissues, to further examine the relationship between the well demonstrated ERα-HER pathway crosstalk and Tamoxifen resistance. Tissue blocks from patients who had ERα positive cancers and who were treated with Tamoxifen have been analysed and the results correlated with clinical progression of these patients on Tamoxifen (i.e. Tamoxifen resistant disease).

The initial hypothesis under test is that the cases positive for HER1-3 (but not HER4) will exhibit *de novo* resistance to Tamoxifen treatment reflected by shortened time to relapse on Tamoxifen treatment. We also postulate that this may occur following cross talk between the HER family and the ERα and have therefore examined evidence of ERα activation in these patients by analysing the phosphorylation status. In addition, as discussed earlier, recent trial results (Coombes et al., 2004a; Dowsett, 2003) demonstrate that PR status can determine who responds to early or to delayed treatment with aromatase inhibitors. Given the known inverse relationship between PR expression and HER2 expression (Marsigliante et al., 1993; Quenel et al., 1995) we therefore also postulate that both HER1-3 and PR expression are time dependent predictors of Tamoxifen resistance (as would be predicted if these were *de novo* resistance mechanisms).

There is a need to address some of the problems we have identified previously when considering this relationship between the HER family and Tamoxifen resistance.

1. Much of the previous work has looked at the individual HER receptors in isolation. However, as explained, many of these receptors are co-expressed and certainly in the case of the potent HER2-HER3 heterodimer rely on each other for

signal transduction. Thus we will determine HER1-4 status in these tumours and identify the role that coexpression of receptors may play in determining Tamoxifen resistance.

2. The numbers involved has limited many previous studies. The small percentage of patients who are both ERα and HER positive will always limit the power of a study. We will use tissue microarray technology to increase the numbers of tumour samples we can analyse. This involves removing cores from pre-existing paraffin embedded blocks and reembedding them in an arrayed master block. Subsequent analysis of hundreds of specimens on only a few slides is therefore possible. There is also the additional advantage that all specimens are analysed under identical conditions. This technique has been validated in breast cancer (Torhorst et al., 2001) and will be discussed in more detail in the methods section.
3. During the course of undertaking this work, increasing evidence has come to light with regard to the cellular localisation of both the ERα and HER receptors. As discussed earlier ERα has been shown to act at the membrane (Razandi et al., 2002; Razandi et al., 2000) and there has also been studies reporting the existence of various members of the HER family at the nucleus (Razandi et al., 2003a; Lin et al., 2001; Ni et al., 2001; Offterdinger et al., 2002). The exact function of the receptors at these sites is not clear. Whilst scoring the TMAs for presence/intensity of receptor positivity, it will be important to also play close attention to the cellular location of any staining.
4. Although the many studies have demonstrated amplification or overexpression of the HER receptors in relation to Tamoxifen resistance, we do not know if these extra receptors are in fact activated. One way of demonstrating activation is by examination of the phosphorylation status of the receptors. Indeed phosphorylated

HER (determined using immunohistochemistry) was demonstrated in only 12% of HER2 positive patients in a recent study (Thor et al., 2000). They suggested that phospho-HER2 status provided improved prognostic information compared to HER status alone. We will therefore determine phosphorylated HER2 status in this study.

5. As discussed earlier one possible explanation for the conflicting reports on HER4 and its association with survival may be that the results are antibody dependent. We have therefore chosen to compare the staining patterns of widely used antibodies in standardised conditions which are known to target either the intracellular (HFR1) or extracellular (H4.77.16) domains of HER4.

Thus a summary of the aims would be to

1. identify the HER1-4 status of the tumours by analysis of protein expression
2. identify activation of ERα by assaying the phosphorylation of ER at the 2 key sites discussed earlier using antibodies to SER 118 and SER 167
3. identify if PR status correlates with HER family positivity
4. investigate the activation of HER2 by assaying its phosphorylation status

These results would then be analysed to:

1. identify any relationship between HER 1-4 and ERα phosphorylation
2. establish if activation of ERα by HER1-4 is associated with resistance to tamoxifen
3. identify any correlation between PR status and Tamoxifen resistance
4. determine if PR and/or HER family associated Tamoxifen resistance is time dependent.

Future Implications

This department is currently constructing a TMA bank from cases involved in the TEAM trial. Markers identified as being significant from this work will be used in the prospective translational research arm of the trial.

Chapter 2 Methods

2.1 Patient Selection

2.1.1 Ethical clearance

Ethical clearance was obtained from the Glasgow Royal Infirmary LREC committee (LREC 02SG007).

2.1.2 Patient Database

Details of sequentially diagnosed breast cancer patients, suitable for surgical excision, from years 1980 –1999 had been kept in a registered database kept within the University Department of Surgery. These patients had surgery and subsequent adjuvant therapy according to protocols at the time of diagnosis. Patients who had neoadjuvant treatment in terms of endocrine treatment, chemotherapy and radiotherapy prior to their surgery were also included in the database. The total number of patients in the database is 1116. Patient follow-up details have been entered prospectively since diagnosis. These entries include information on attendances, recurrence and metastasis details, date and cause of death and adjuvant therapy details (including tamoxifen treatment duration).

From this database, patients were selected who had been treated with adjuvant Tamoxifen therapy and who were either known to be ER positive or who did not have a record of ER status being tested. Patients with an unknown ER status were included to increase the numbers available for analysis, on the basis that all patients in the study would have their ER status re-tested. The number of Tamoxifen treated ER positive or ER unknown tumours was 685.

1.3 Patient Exclusion

From this group of 685 patients, further exclusions were made on the following basis:

1. If their follow up and full Tamoxifen history was not complete in terms of details of any relapse or duration of Tamoxifen treatment (n=52).
2. If the tumour blocks from pathology were not available or deemed unsuitable by the pathologist (B Dunne) because of limited tissue availability which may have compromised further diagnostic testing (n=177).

456 patients were therefore eligible for the study. 75 of these patients were ER nonspecified, with the remainder having being tested positive for ER by either ligand binding (130) or immunohistochemistry (251). No details about the methodology of these diagnostic tests over the years was available, and in the majority of cases it was only documented if the patient was ER positive or negative with no details on the values of ligand binding assay value or IHC score.

2.2 TMA construction

2.2.1 TMA Technology

One of the key objectives of this study was to utilise a method by which many tissue samples could be tested for multiple antibodies in a limited amount of time. Tissue microarray technology has been increasingly used as a way of achieving this objective since it was developed by Kononen et al in 1998 (Kononen et al., 1998). Since then various studies have published work validating TMA use in both breast cancer and other tissues (Camp et al., 2000; Hoos and Cordon-Cardo, 2001; Torhorst et al., 2001). The technique involves removing a representative core of tissue (0.6mm diameter) from the tumour block and inserting it into a recipient block along with cores from other donor blocks. The recipient block can then be sectioned and used for routine analysis in the same way as standard sections. Up to 300 individual tumour cores may be placed into a single recipient block thus enabling multiple tumour analysis in one IHC staining run.

The advantages of using microarray technology include bring able to perform high speed analysis, whilst minimising damage done to donor blocks so preserving valuable tissue for further research or diagnostic needs. In addition it enables standardisation of testing, with direct comparison of staining intensities between specimens so improving the subjective interpretation of results.

However there are some problems associated with this technique.

2.2.1.1 Missing samples/Loss rate

Cores within a TMA section may be damaged or lost during sectioning, or the core may not contain identifiable tumour. Core loss during sectioning and staining is a

common problem with microarrays and ranges from 10% to more than 30% (Bubendorf et al., 1999; Hoos and Cordon-Cardo, 2001; Schraml et al., 1999; Torhorst et al., 2001). Sectioning loss can be minimised when performed by a suitably trained and experienced technician, and by attempting to array uniformly long cores for each specimen. The chance of obtaining identifiable tumour in the core can be maximised by using a trained pathologist to identify representative tumour areas.

The impact of lost cases can be minimised by increasing the number of cores taken per specimen. Parallel TMAs are therefore constructed for each series of original specimens. Torhorst et al (Torhorst et al., 2001) correlated the results from standard tissue sections with the results from 4 independent TMAs. They found that the fraction of interpretable tumours was 86-93% if 2 cores were available, 92-96% if 3 cores available and 94-96% if all 4 cores available depending on antibody tested (PR, ER and p53).

2.2.1.2 Heterogeneity

Breast cancers are heterogeneous at both a morphological and genetic level (Osborne, 1985; Symmans et al., 1995). One potential drawback with TMA technology is that the 0.6mm biopsies taken from a tumour block will not be representative of the whole tissue specimen because of tumour heterogeneity. It has been suggested that optimum number of core-cut biopsies required to ensure an accurate histological diagnosis and grading of a tumour is 4 (McIlhenny et al., 2002). However this study was based on free hand core techniques and 8% of the cores had no discernable histological features at all. Tohorst et al (Torhorst et al., 2001) reported that the fraction of tumours with heterogeneous findings was 9% for ER, 29% for PR and 11% for p53. For PR they required 3 samples of each tumour to achieve the same levels of positivity as large

section analysis. However it must be remembered that a tumour block itself often represents a small fraction of the actual tumour. Camp et al (Camp et al., 2000) compared the results of expression of HER2, ER and PR in up to 10 cores per specimen with the results from standard tissue sections. They reported that results combining 2 cores demonstrated over 95% concordance between these two methods, and that the addition of a 3rd core increased concordance to over 97%.

Hoos et al (Hoos and Cordon-Cardo, 2001) analysed results found when looking at a more complex phenotype of expression of retinoblastoma (RB) protein. They demonstrated that using just 2 cores per specimen required some cases to be excluded from analysis is instances where there were contradictory results. However the addition of a 3rd core to the analysis improved concordance rates to 91%.

Based on the above results, we decided to minimise the effects of core loss and tissue heterogeneity by constructing the TMAs in triplicate. However more recent publications have suggested that at least 4 cores are required, especially for markers with a heterogeneous staining pattern such as PR (Sapino et al., 2006).

2.2.2 Technique

This department has been using TMA technology for some time in IHC and FISH analysis of breast, ovarian and prostate tissue. We are currently involved in the construction of TMAs for the TEAM and NEAT (National Epirubicin Adjuvant Trial) trials. The TMAs were constructed for this study by myself under guidance from pathology MLSOs working within the department.

Formalin fixed paraffin embedded tissue bocks, prepared after surgical excision of the tumours, were obtained from the department of pathology. A tissue section from each block was stained using haematoxylin and eosin (H&E) to identify areas of tumour.

Representative areas on the H&E were identified by a pathologist (Barbara Dunne) and marked on the slide. As discussed earlier this is essential as it ensures the core taken contains representative tumour cells.

Prior to removing the cores, 5 × 3μM sections were cut from each pathology block by a MLSO in the lab. These sections have been stored and may be used in the case of any missing samples from the TMA slides or to validate the TMAs in any cases where there are doubts about the heterogeneity of tissue staining for a particular antibody.

Subsequently 3 * 0.6mm cores of tissue were removed from each block at the area marked by BD using a tissue arrayer (Beecher instrument). This precision instrument uses 2 separate core needles for punching the donor and recipient blocks, together with a precise co-ordinate system to place and locate the tissue samples in the array. These cores of tissue were then transformed to 3 recipient blocks (80-120 cores per block) to form triplicate tissue arrays. In total we constructed 5 different arrays each in triplicate (total 15 donor blocks).

2.2.3 TMA Layout

The cores were placed at 1 mm intervals, with regular larger gaps as to ensure ease of identification of core position during analysis (Figure 9).

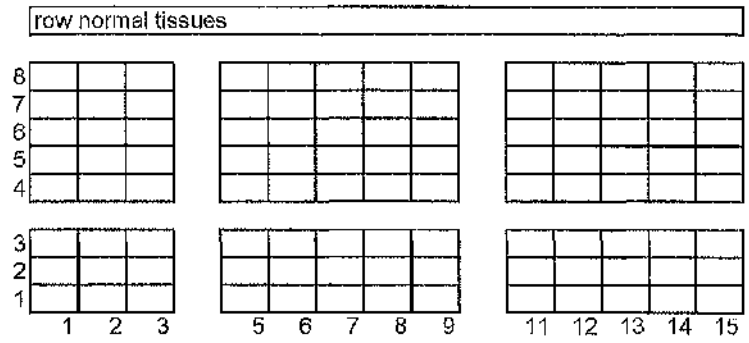

Figure 9

Illustration of typical core placement layout in TMA.

2.2.4 Control Tissues

Cores were taken from 10 blocks containing samples of normal tissue (sourced from USA commercial). These normal tissues consisted of skeletal muscle, smooth muscle, normal breast, lung, placenta, prostate, tonsil, lymph node, skin and testes. A core from each of these was placed in order, in a row, above the tumour cores in each of the 15 donor blocks. This provides the basis of controls for each of the antibodies, which have known staining patterns in normal tissues. This row of normal tissues also provided a mechanism for correct orientation of the TMA section after sectioning.

The completed tissue array blocks were heated at 37° for 10 minutes to ensure the tissue cores and paraffin stick together. Serial 3μM sections were then cut by a MLSO from the TMAs using silanised slides to improve section contact with the slide during high temperatures used in some antigen retrieval protocols.

Given the extremely valuable nature of these TMAs in terms of a future research resource we took care to establish clear criteria for the number and timing of any TMA sections cut. Cutting too few sections would result in wastage (re-aligning and facing blocks to cut into them) whilst cutting too many may result in tissue oxidation of unused sections over time.

2.3 Immunohistochemistry

Immunohistochemistry involves using an antibody to link a cellular antigen specifically to a stain that can be more readily seen with a microscope. Advantages of IHC include the ability to detect location of staining as well as providing a semi quantitative assessment of intensity. Problems however can occur in relation to specificity of the antibody as well as the need to expose antigenic sites to the antibody, particularly in formalin fixed tissues.

The 10 antibodies were chosen to achieve the aims for this study. These were antibodies against ER, PR, EGFR (HER1), HER2, HER3, HER4 (2 antibodies), phosphorylated HER2 and phosphorylated ER at 2 sites (serine 118 and serine 167). A summary of the antibodies used is attached in Appendix I.

2.3.1 Antibody Protocols: General principles

During the work up of the antibodies I followed a set of general principles described below which were subsequently tailored to each antibody as described in detail later. Both breast cancer whole sections and sections from a small practice TMA were used during the work up of all antibodies (as well as the relevant control slides).

A. Dewax, rehydration

Prior to any immunohistochemical staining using formalin fixed, paraffin embedded tissue, sections have dewaxed and rehydrated. Paraffin must be removed from the tissue to allow the water-based buffers and antibodies to penetrate.

For all antibody protocols this was done using the following regime:

1. Dewax in xylene (Fishers chemicals) 2* 4minutes

2. Rehydrate in graduated alcohols:
    - 100% alcohol 2*4minutes
    - 90% alcohol 2minutes
    - 70% alcohol 2 minutes
3. Rinse in water

B. Block of endogenous peroxidase activity

Breast tissue is known to contain endogenous peroxidase. To minimise background staining from this, sections were treated with hydrogen peroxidase (VWR International Ltd; supplied at concentration ~ 30%), which is known to irreversibly inactivate endogenous peroxidase. This was done either before or after antigen retrieval at a concentration of either 0.3% or 1% for either 10 or 20 minutes depending on the protocol determined for each antibody.

C. Antigen retrieval

To facilitate the antibody - antigens reactions in fixed tissue, it may be necessary to unmask or "retrieve" the antigens through pretreatment of the specimens. Antigen retrieval has been shown to increase reactivity of the majority of antigens in tissues. This was done by one of three methods under routine use in the lab, depending on the antibody used. These methods were:

1. Pressure microwave treatment in Tris EDTA buffer (0.37g sodium EDTA (BDH Laboratory Supplies) and 0.55g Tris base (Sigma) made up to 1 Litre with dH_20). Microwave irradiation of formalin-fixed, paraffin-embedded specimens in buffer has been found to markedly enhance the retrieval of antigens. During this procedure the energy provided helps break some of the bonds formed during fixation, thus increasing the intensity of reactions.

2. Boiling (in calibrated water bath set at 96°) in 10mmol/L citrate buffer (we used 1:10 Epitope Retrieval solution taken from DAKO Herceptest kit) for 20minutes.

3. Trypsinisation. Sections are incubated in 0.1% trypsin (Sigma) (dissolved in 0.1% calcium chloride (Sigma) solution preheated in a 37° waterbath) for 10 minutes. The conditions of concentration, time and temperature must be tightly controlled, so that the enzymes can break some of the bonds formed during fixation, uncovering antigenic sites, but the antigen should not be digested completely.

The choice of antigen retrieval method will be discussed later for individual antibodies.

D. Blocking non-specific background staining

Any non-specific background staining was blocked using one of 2 methods depending on the protocol. This was done by either:

1. Serum Free block (0.25% casein in PBS, containing carrier protein and 15mM sodium azide; DAKO) for 10 minutes
2. Normal horse serum (Vector) at concentration of 15µl/ml for 15minutes

Following incubation with blocking agent the solution was tapped off and the sections were blotted.

E. Blocking of Endogenous Biotin

Breast tissue is known to contain endogenous biotin. To minimise background staining, most of the protocols required treatment with an Avidin/Biotin Blocking agent (Vector) following the incubation with the horse serum or serum-free block. This was done by:

1. Incubation with Avidin D solution 15 minutes
2. 5 minute wash in TBS buffer
3. Incubate with Biotin solution for 15 minutes

4. 5 minute was in TBS buffer

F. Incubation with Primary antibody

Sections were incubated with the primary antibodies at optimum concentrations and conditions determined for the individual antibodies (as described later). Antibodies (including the negative control antibodies) were always diluted in a tris-HCL buffer containing carrier protein and 0.015M sodium azide (DAKO). A slide from each run was always incubated with an isotype matched control antibody whilst working up the antibodies and in the final runs to ensure no false positive staining. Slides were washed twice in TBS buffer for 5 minutes following incubation.

G. Visualisation Methods

I used the DAKO LSAB+ Kit HRP, which utilises a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. Slides were washed in TBS buffer between and after incubations with the link antibody and Strepavidin peroxidase solutions.

Detection was then completed with incubation with a 3,3'diaminobenzidine (DAB) solution (Vector Laboratories) for 2-10 minutes. The DAB solution was washed off in running water for 10 minutes.

H. Counterstaining, Dehydration and Mounting

The sections were counterstained, and then dehydrated using the following method.

- Haemoxylin 1 minute followed by rinse in running H_20
- Blue with Scotts tap water substitute (about 30 seconds) followed by rinse in running H_20
- 70% alcohol 1 minute
- 90% alcohol 1 minute
- 100% alcohol 2*1 minute

- xylene 2*1 minute
- slides were then mounted in DPX

Note: TBS buffer solution

Tris base saline buffer was made in the laboratory at a 1:10 dilution of a solution made by dissolving 60g Tris base and 87.6g NaCL per1L dH_20 (pH to 7.5).

2.3.2 IHC protocol for ER

(Dako: clone 1D5 monoclonal IgG1 mouse antibody)

To stain for ER I used a DAKO ER antibody used routinely in the diagnostic setting in our pathology department. The protocol used is given below.

*Make up Tris EDTA buffer +start warming solution while rehydrating

A) Dewax and rehydrate

- Dewax in xylene 2*4 min
- Rehydrate in 100% alcohol 2*4 min
- 90% alcohol 2min
- 70% alcohol 2min
- rinse in water
- Incubate sections in 0.3% H_2O_2 for 20 mins (8ml H_2O_2 in 400ml d H_2O)
- rinse in water

B) Antigen retrieval

- make up Tris EDTA buffer (pH8)
    - 0.37g sodium EDTA
    - 0.55g Tris
    - make up to 1 Litre with dH_2O
- microwave full power 14 min to warm solution
- add slides and lid and microwave on full power for 2 min to bring up pressure (lift yellow cap)
- microwave for 5 min under pressure
- remove weight to allow steam to escape
- cool for 20min
- wash running water

C) Staining

- DAKO pen to ring sections
- Incubate in blocking solution for 15 min (15µ horse serum per ml TBS)
- Blot serum from sections
- Incubate in primary antibody at room temp for 30min (1:50 in antibody dilutent)
- Wash TBS 5min
- Incubate in secondary antibody (DAKO yellow) for 15 min at room temp
- Wash TBS 5min
- Incubate in Streptavidin HRP (DAKO red) for 15 min at room temp
- Wash TBS 5min
- Incubate in DAB substrate until colour develops (5ml d H_2O: add 2 drops Buffer, mix, 4 drops DAB stock, mix, 2 drops hydrogen peroxide, mix) 30sec
- Wash running water 10min

D) Counterstain

- Haemoxylin for 60 sec
- Rinse running water
- Blue with Scotts tap water substitute
- Rinse running water

E) Dehydrate and mount

- 70% alcohol 1min
- 90% alcohol 1min
- 100% alcohol 2*1 min
- xylene 2*1min
- mount in DPX

2.3.3 IHC protocol for PR

The TMA sections were given to the department of pathology and processed as part of a diagnostic run for PR staining.

2.3.4 IHC protocol for EGFR

(Mouse monoclonal IgG1 Zymed 28-0005 clone 31G7)

We used a mouse anti-EGFR antibody whose use on breast cancer sections was first described in 1984 (Gusterson et al., 1984). It is a Zymed product; clone 31G7, monoclonal IgG1 antibody. It has subsequently been used in a large neoadjuvant clinical trial (Ellis et al., 2001). We also have experience in using this antibody in the laboratory in analysing overexpression of EGFR in prostate cancer, and this protocol was followed without modification as described below.

*Put 80ml 0.1% calcium chloride solution in 37° water bath

A) Dewax and rehydrate

- Dewax in xylene 2*4 min
- Rehydrate in 100% alcohol 2*4 min
- 90% alcohol 2min
- 70% alcohol 2min
- rinse in water
- Incubate sections in 0.3% H_2O_2 for 20 mins (4ml H_2O_2 in 400ml d H_2O) on stirrer
- rinse in water

B) Antigen retrieval

- Make up 0.1% trypsin in preheated 0.1% calcium chloride and allow to dissolve: (0.08g)
- Incubate slides in trypsin for 10min in 37° water bath
- Wash in running water
- Transfer slides to a staining dish with water (slides can be stored like this)

C) Staining

- DAKO pen to ring sections
- Incubate in blocking solution for 20min (15µl horse serum per ml TBS)
- Blot serum from sections
- Incubate in primary antibody for 1 hour in humidified chamber at 25° (1:50 in DAKO antibody dilutent)
- Wash TBS 5min
- Incubate in secondary antibody (DAKO yellow) for 30 min at room temp
- Wash TBS 5min
- Incubate in Streptavidin HRP (DAKO red) for 60min at room temp
- Wash TBS 5min
- Incubate in DAB substrate until colour develops (5ml d H_2O: add 2 drops Buffer, mix, 4 drops DAB stock, mix, 2 drops hydrogen peroxide, mix)
- Wash running water

D) Counterstain

- Haemoxylin for 60 sec
- Rinse running water
- Blue with Scotts tap water substitute
- Rinse running water

E) Dehydrate and mount

- 70% alcohol 1min
- 90% alcohol 1min
- 100% alcohol 2*1 min
- xylene 2*1min
- mount in DPX

2.3.5 IHC protocol for HER2

(Dako HercepTest™ Immunogen is the synthetic C-terminal fragment (intracytoplasmic part) of the HER2 protein)

This laboratory is an approved national testing centre for HER2. My slides were included in a diagnostic run using the DakoCytomation Autostainer. We also plan to use these slides in a HER2 FISH but these results will not be available for report in this thesis.

A) Dewax and rehydrate

- Dewax the slides: 2 x 5 mins in Xylene
- Rehydrate:2 x 3 mins 100% alcohol
- 3 mins 90% alcohol.
- Rinse in Water.
- Rinse in water.

B) Epitope Retrieval

- 40 mins at 96 °C in heated water bath.
- Cool for 20 mins.
- Transfer in to wash buffer for 5 mins

C) Staining

- Ring sections with DAKO pen to create a barrier
- Incubate in peroxidase blocking reagent for 5 mins.
- Rinse in water.
- Incubate in primary antibody in humidified chamber for 30 mins.
- Wash 5 mins in wash buffer
- Incubate in visualization reagent at room temp for 30 mins
- Wash 5 mins in wash buffer.

- Make DAB substrate. To 1 ml DAB substrate add 1 drop DAB chromogen
- Incubate sections with substrate until colour develops (10 mins)
- Wash in water.

D) Counterstain

- Stain in haematoxylin for 3 mins.
- Rinse in running tap water
- Blue with Scotts tap water substitute
- Rinse in running tap water

E) Dehydrate and mount

- 1 min 70% alcohol
- 1 min 90% alcohol
- 2 x 1 min 100% alcohol
- 2 x 1 min xylene
- mount in DPX

2.3.6 IHC protocol for HER4

As discussed previously we wished to compare the staining results of 2 HER4 antibodies which target either intra – or extracellular domains. Monoclonal IgG2b mouse antibody HFR1 was first used by Srinivasan et al (Srinivasan et al., 1998) in 1998 to determine the expression of HER4 in formalin fixed paraffin embedded normal tissues and cancers. This group demonstrated the ability of this antibody to recognise HER4 by immunoprecipitation, western blotting and immunostaining of cytocentrifuge preparations of NH3T3 cells transfected with HER4. There was no crossreactivity with EGFR using A431 cell lysates or with HER3 or HER4 using lysates from SKBR3 or 293/HER3 cells. We wished to compare the staining patterns of this antibody to those of a second monoclonal IgG1 antibody clone H4.77.16 also supplied by Neomarkers. Our laboratory has previous experience in using this antibody in frozen breast cancer samples (Witton et al., 2003). The HFR-1 antibody is raised against an intracellular epitope aa1249-1264 whilst the H4.77.16 antibody is raised against an extracellular fragment. Therefore HFR1 is thus able to recognise both the intact receptor and the cleaved ICD as it traffics through the cell. However the H4.77.16 clone will detect the full length receptor not the cleaved ICD.

2.3.6.1 HFR1

(Mouse monoclonal Ab Clone HFR-1#MS 637-Po)

The datasheet for this product suggested that the antibody should be used at a dilution of between 1:40 to 1:20 from the initial concentration of 200μg/ml with no antigen retrieval required. Breast cancer sections were used in the work up of this antibody together with skeletal muscle slides provided by Neomarkers used as positive control slides. Antibody concentrations from 1:10 to 1:100 incubated over periods of between

30 minutes and 2 hours were tried, with the optimum antibody concentration found to be at a 1:50 dilution (i.e. 4μg/ml) for 1 hour. Initially there was some residual mild background staining seen on the negative control slides, however this was eliminated when the hydrogen peroxide concentration was increased to 1% for 20 minutes and serum free block (DAKO) was used instead of horse serum as a blocking agent. This method provided the optimum range of staining intensity with minimal background staining and clear negative control slides.

A) *Dewax and rehydrate*

- Dewax in xylene 2*4 min
- Rehydrate in 100% alcohol 2*4 min
- 90% alcohol 2min
- 70% alcohol 2min
- rinse in water
- Incubate sections in 1% H_2O_2 for 20 mins (13.3ml H_2O_2 in 400ml d H_2O)
- rinse in water

B) No Antigen retrieval

C) Staining

- DAKO pen to ring sections
- Incubate in serum free blocking solution for 10min
- Blot serum from sections
- Avidin Biotin Block (Avidin 15min / TBS 5min / Biotin 15 min / TBS 5min)
- Incubate in primary antibody at room temp for 1 hour (1:50 in antibody dilutent)
- Wash TBS 5min
- Incubate in secondary antibody (DAKO yellow) for 30 min at room temp

- Wash TBS 5min
- Incubate in Streptavidin HRP (DAKO red) for 60min at room temp
- Wash TBS 5min
- Incubate in DAB substrate until colour develops (5ml d H_2O: add 2 drops Buffer, mix, 4 drops DAB stock, mix, 2 drops hydrogen peroxide, mix)
- Wash running water

D) Counterstain

- Haemoxylin for 60 sec
- Rinse running water
- Blue with Scotts tap water substitute
- Rinse running water

E) Dehydrate and mount

- 70% alcohol 1min
- 90% alcohol 1min
- 100% alcohol 2*1 min
- xylene 2*1min
- mount in DPX

2.3.6.2 H4.77.16

(Mouse monoclonal Ab, IgG1clone H4.77.16 #MS-270-PABX)

The datasheet for this antibody did not provide any guidance for its use in IHC and the only other published work using this antibody was for immunoprecipitation and flow cytometry (Tang et al., 1999; Witton et al., 2003) . However it had been used within our lab on frozen tissue sections (Witton et al., 2003). On frozen tissue the antibody was used a concentration of 2µg/ml overnight at 4°. No antigen retrieval had

been used and biotinylated secondary antibodies with horseradish peroxidase streptavidin-biotin complex and DAB were used for antibody detection. The initial work up of this antibody attempted to replicate this method following dewaxing and rehydration of the cut sections. However no staining was detected using this method. Therefore a modification of the protocol used with the HFR1 antibody was tried. This method using an antibody dilution 1:20 (50µg/ml) for 2 hours provided optimum range of staining intensity with minimal background staining and clear negative control slides.

A) *Dewax and rehydrate*

- Dewax in xylene 2*4 min
- Rehydrate in 100% alcohol 2*4 min
- 90% alcohol 2min
- 70% alcohol 2min
- rinse in water
- Incubate sections in 1% H_2O_2 for 20 mins (13.3ml H_2O_2 in 400ml d H_2O)
- rinse in water

B) No Antigen retrieval

C) Staining

- DAKO pen to ring sections
- Incubate in serum free blocking solution for 10min
- Blot serum from sections
- Avidin Biotin Block (Avidin 15min / TBS 5min / Biotin 15 min / TBS 5min)
- Incubate in primary antibody at room temp for 2 HOURS (1:20 in antibody dilutent)
- Wash TBS 5min

- Incubate in secondary antibody (DAKO yellow) for 30 min at room temp
- Wash TBS 5min
- Incubate in Streptavidin HRP (DAKO red) for 60min at room temp
- Wash TBS 5min
- Incubate in DAB substrate until colour develops (5ml d H_2O: add 2 drops Buffer, mix, 4 drops DAB stock, mix, 2 drops hydrogen peroxide, mix)
- Wash running water

D) Counterstain

- Haemoxylin for 60 sec
- Rinse running water
- Blue with Scotts tap water substitute
- Rinse running water

E) Dehydrate and mount

- 70% alcohol 1min
- 90% alcohol 1min
- 100% alcohol 2*1 min
- xylene 2*1min
- mount in DPX

2.3.7 IHC protocol for HER3

(Mouse monoclonal, IgG1, clone H3.105.5, #MS-303-PABX)

We used a monoclonal IgG1 antibody clone H3.105.5 supplied by Neomarkers. This antibody is raised against the extracellular domain of recombinant human HER3 oncoprotein Our laboratory has previous experience in using this antibody in frozen breast cancer samples (Witton et al., 2003). The antibody has previously been tested, within the laboratory, using an immunohistochemical immunoperoxidase technique for its ability to detect HER3 in frozen sections of pellets of cells transfected with c-erbB-3 c-DNA and expressing high levels of protein. Both antibodies labelled these cells strongly but not the untransfected parent line (data unpublished).

The datasheet for this product did not provide any guidance for its use in IHC and the only published work using the antibody for immunoprecipitation (Chen et al., 1996) . However it had been used within our lab to determine protein expression on frozen tissue sections. On frozen tissue the antibody had been used at a concentration of 2μg/ml overnight at 4°. No antigen retrieval was used and biotinylated secondary antibodies, horseradish peroxidase streptavidin-biotin complex and DAB were used for antibody detection. My initial work up of this antibody attempted to replicate this method following dewaxing and rehydration of the cut sections. However no staining was detected using this method. Therefore a modification of the protocol used for the HER4 antibodies was tried with no antigen retrieval and room temperature incubations. This method demonstrated some staining. The protocol was then run at antibody concentrations between 1:10 and 1:100 dilutions for between 30 minutes and 2 hours. These titrations demonstrated that the antibody dilution 1:20 (50μg/ml) for 2 hours provided the optimum range of staining intensity with minimal background staining and clear negative control slides.

A) Dewax and rehydrate

- Dewax in xylene 2*4 min
- Rehydrate in 100% alcohol 2*4 min
- 90% alcohol 2min
- 70% alcohol 2min
- rinse in water
- Incubate sections in 1% H_2O_2 for 20 mins (13.3ml H_2O_2 in 400ml d H_2O)
- rinse in water

B) No Antigen retrieval

C) Staining

- DAKO pen to ring sections
- Incubate in serum free blocking solution for 10min
- Blot serum from sections
- Avidin Biotin Block (Avidin 15min / TBS 5min / Biotin 15 min / TBS 5min)
- Incubate in primary antibody at room temp for 2 HOURS (1:20 in antibody dilutent)
- Wash TBS 5min
- Incubate in secondary antibody (DAKO yellow) for 30 min at room temp
- Wash TBS 5min
- Incubate in Streptavidin HRP (DAKO red) for 60min at room temp
- Wash TBS 5min
- Incubate in DAB substrate until colour develops (5ml d H_2O: add 2 drops Buffer, mix, 4 drops DAB stock, mix, 2 drops hydrogen peroxide, mix)
- Wash running water

D) Counterstain

- Haemoxylin for 60 sec
- Rinse running water
- Blue with Scotts tap water substitute
- Rinse running water

E) Dehydrate and mount

- 70% alcohol 1min
- 90% alcohol 1min
- 100% alcohol 2*1 min
- xylene 2*1min
- mount in DPX

2.3.8 IHC protocol for phospho specific HER2 (pHER2)

(Mouse monoclonal Ab IgG1, Ab-18 (clone PN2A), #1072 Neomarkers)

HER2 has at least 4 tyrosine autophosphorylation sites including Tyr1023 and Tyr1248 on the C-terminal (Hazan et al., 1990). Antibodies against the Tyr1248 site have been developed (DiGiovanna and Stern, 1995; Epstein et al., 1992) by raising antisera against the tyrosine-phosphorylated c-terminal (residues 1243-1255) HER2 peptide sequence and the resultant clone PN2A is commercially available from Neomarkers. Specificity of this clone was demonstrated, by its' developers, by its ability in immunoblotting to recognise p185 in a tyrosine-phosphorylation dependent manner without recognising the closely related phosphorylated EGFR or the highly homologous COOH terminus of HER4 (DiGiovanna and Stern, 1995; Bangalore et al., 1992).

We used the Neomarkers PN2A clone (monoclonal IgG1). The datasheet for this product suggested antigen retrieval was optimised by boiling sections in 10mM citrate buffer followed by incubation of the antibody diluted to between 1:10 and 1:20 times the original concentration of 200μg/ml for 2 hours at room temperature. Positive control slides supplied by Neomarkers and sections from paraffin embedded SKBR-3 cells (known to express phosphorylated HER2 (DiGiovanna and Stern, 1995) were used a positive controls to work up antibody.

Antibody retrieval was performed by boiling (in calibrated water bath set at 96°) the sections in 10mmol/L citrate buffer (1:10 Epitope Retrieval solution taken from DAKO Herceptest kit) for 20minutes.

Antibody dilution titrations were performed for 1:100, 1:50, 1:20 and 1:10 concentrations. I also compared the results of incubating the antibody overnight for 4° to incubation at room temperature from between 2 hour and 8 hour durations.

Incubation with the antibody at 1:10 concentration for 6 hours at room temperature provided the optimum results in terms of range of intensity staining combined with minimal background staining and clear control slides.

A) Dewax and rehydrate

- Dewax in xylene 2*4 min
- Rehydrate in 100% alcohol 2*4 min
- 90% alcohol 2min
- 70% alcohol 2min
- rinse in water
- Incubate sections in 1% H_2O_2 for 10 mins (13.3ml H_2O_2 in 400ml d H_2O)
- Rinse in water

B) Antigen retrieval

- 1:10 HER2 antigen retrieval solution used
- 20min 96°
- 20min cool
- rinse in water

C) Staining

- DAKO pen to ring sections
- Incubate in serum free blocking solution (DAKO) for 10min
- Blot serum from sections
- Avidin Biotin Block (Avidin 15min / TBS 5min / Biotin 15 min / TBS 5min)
- Incubate in primary antibody at room temperature for 6° (1:10 in antibody dilutent)
- Wash TBS 5min
- Incubate in secondary antibody (DAKO yellow) for 30 min at room temp

- Wash TBS 5min
- Incubate in Streptavidin HRP (DAKO red) for 60min at room temp
- Wash TBS 5min
- Incubate in DAB substrate until colour develops (5ml d H_2O: add 2 drops Buffer, mix, 4 drops DAB stock, mix, 2 drops hydrogen peroxide, mix)
- Wash running water

D) Counterstain

- Haemoxylin for 60 sec
- Rinse running water
- Blue with Scotts tap water substitute
- Rinse running water

E) Dehydrate and mount

- 70% alcohol 1min
- 90% alcohol 1min
- 100% alcohol 2*1 min
- xylene 2*1min
- mount in DPX

2.3.9 IHC protocol for Phospho specific ER

The oestrogen receptor has multiple phosphorylation sites, which modulate the function of the receptor as discussed previously. We wished to concentrate on the phosphorylation sites of Serine 118 (the putative target of the MAPK pathways) and Serine 167 (the putative target of the Akt pathway). Antibodies to each of these sites are commercially available. There is no published literature regarding either of these antibodies.

2.3.9.1 Phospho ERα Serine 118

(Mouse monoclonal, IgG2b 16J4 antibody #2511 cell signalling)

The Phospho-oestrogen receptor (ser118) 16J4 antibody was purchased from Cell signalling. It is a 65.5 kDa monoclonal antibody, (isotype IgG2b) produced by immunising mice with a synthetic phosphopeptide corresponding to residues surrounding Ser118. Product literature for the antibody demonstrates its ability to detect ER only when phosphorylated at Ser118, with no cross reactivity with ERβ.

Breast cancer sections were used in the work up of this antibody. The datasheet for this antibody suggested heating the sections in 10mM sodium citrate buffer for 10 minutes. It suggested overnight incubation at 4° at 1:50 dilution (in blocking solution). However using this method, there was some slight background staining on some sections treated with an isotype matched control antibody. By changing the antigen retrieval time to 20 minutes (as per Herceptest), diluting the antibody in DAKO antibody dilutent and using protein free block instead of horse serum, this background was eliminated. Using this method at 1:50 the staining pattern was generally strong throughout the majority of positive sections, therefore antibody titration was performed using dilutions from 1:50 to 1:500. The optimum

concentration in terms of range of intensity staining and minimal background staining was found to be 1:300.

A) *Dewax and rehydrate*

- Dewax in xylene 2*4 min
- Rehydrate in 100% alcohol 2*4 min
- 90% alcohol 2min
- 70% alcohol 2min
- rinse in water

B) Antigen retrieval

- 1:10 HER2 Ag retrieval sol used
- 20min 96°
- 20min cool
- rinse in water
- Incubate sections in 1% H_2O_2 for 10 mins (13.3ml H2O2 in 400ml d H_2O)

C) Staining

- DAKO pen to ring sections
- Incubate in serum free blocking solution for 10min
- Blot serum from sections
- Avidin Biotin Block (Avidin 15min / TBS 5min / Biotin 15 min / TBS 5min)
- Incubate in primary antibody at 4° overnight (1:300 in antibody dilutent)
- Wash TBS 5min
- Incubate in secondary antibody (DAKO yellow) for 30 min at room temp
- Wash TBS 5min
- Incubate in Streptavidin HRP (DAKO red) for 60min at room temp
- Wash TBS 5min

- Incubate in DAB substrate until colour develops (5ml d H_2O: add 2 drops Buffer, mix, 4 drops DAB stock, mix, 2 drops hydrogen peroxide, mix)
- Wash running water

D) Counterstain

- Haemoxylin for 60 sec
- Rinse running water
- Blue with Scotts tap water substitute
- Rinse running water

E) Dehydrate and mount

- 70% alcohol 1min
- 90% alcohol 1min
- 100% alcohol 2*1 min
- xylene 2*1min
- mount in DPX

2.3.9.2 Phospho ERα Serine 167

(Rabbit polyclonal Ab, #2514 cell signalling)

The phospho-oestrogen receptor (ser167) polyclonal antibody (Cell Signalling) was produced by immunising rabbits with a synthetic phospho-ser167 peptide corresponding to residues surrounding Ser167 of human ER. Product literature for this antibody states that the antibody detects ER only when phosphorylated at Ser167 with no cross-reaction with phosphorylated isoform ERβ.

Breast cancer sections were used in the work up of this antibody. The product datasheet for this antibody suggested heating the sections in 10mM sodium citrate buffer for 10minutes. It suggested overnight incubation at 4° at 1:50 dilution (in

blocking solution). However we decided to follow the protocol already described above as used for Ser118 antibody. This produced some faint staining at the 1:50 concentration. Antibody concentrations at 1:10, 1:20 were then tried with the 1:10 concentration demonstrating a good range of staining patterns between sections.

A) *Dewax and rehydrate*

- Dewax in xylene 2*4 min
- Rehydrate in 100% alcohol 2*4 min
- 90% alcohol 2min
- 70% alcohol 2min
- rinse in water

B) Antigen retrieval

- 1:10 HER2 Ag retrieval used
- 20min 96°
- 20min cool
- rinse in water
- Incubate sections in 1% H_2O_2 for 10 mins (13.3ml H_2O_2 in 400ml d H_2O)

C) Staining

- DAKO pen to ring sections
- Incubate in serum free blocking solution for 10min
- Blot serum from sections
- Avidin Biotin Block (Avidin 15min / TBS 5min / Biotin 15 min / TBS 5min)
- Incubate in primary antibody at 4° overnight (1:10 in antibody dilutent)
- Wash TBS 5min
- Incubate in secondary antibody (DAKO yellow) for 30 min at room temp
- Wash TBS 5min

- Incubate in Streptavidin HRP (DAKO red) for 60min at room temp
- Wash TBS 5min
- Incubate in DAB substrate until colour develops (5ml d H_2O: add 2 drops Buffer, mix, 4 drops DAB stock, mix, 2 drops hydrogen peroxide, mix)
- Wash running water

D) Counterstain

- Haemoxylin for 60 sec
- Rinse running water
- Blue with Scotts tap water substitute
- Rinse running water

F) Dehydrate and mount

- 70% alcohol 1min
- 90% alcohol 1min
- 100% alcohol 2*1 min
- xylene 2*1min
- mount in DPX

2.4 Scoring Principles

During the work up of the antibodies, my skills at semiquantitative immunohistochemistry scoring were developed under the guidance of my supervisor (JB). Scoring was performed on a light microscope (Laborlux S, Leitz). Prior to any final scoring of the TMAs, a series of TMA slides (including the Herceptest and ER slides) were double scored, achieving an ICCC (Intra-class correlation coefficient) of 0.94 (n=890) for membrane staining and 0.84 (n=827) for nuclear staining. These results indicated excellent correlation between scores. All subsequent cases were scored by ST alone. In addition intra-observer correlation was also performed by selecting a TMA slide from 6 separate antibody runs (for phospho HER2, phospho ER(167), ER, PR, HFR1 and H3.105.5) and scored blind by ST on 2 separate occasions for each relevant staining modality (membranous, cytoplasmic, nuclear). The Intra-class coefficient correlation (ICCC) score was 0.8282 for membrane scoring (n=274), 0.9431 for cytoplasmic scoring (n=345) and 0.9327 (n=422) for nuclear staining.

As discussed previously, there have been numerous publications describing the discovery of protein expression out-with classically expected areas. For this reason, for each core scored, we recorded a separate score for any membrane, cytoplasmic or nuclear staining seen.

In the absence of any consistent method of scoring most of the antibodies used, we elected to use a uniform approach for each staining location.

1. *Membrane staining*. For any membrane staining demonstrated, I opted to use the well described Herceptest system. Cores with over 10% of strong

membrane staining were assigned 3+; cores with over 10% moderate staining were assigned 2+. Cores with over 10% weak staining were assigned 1+.

2. *Cytoplasmic staining*. For any cytoplasmic staining seen a Histoscore was given. This method is well described for cytoplasmic staining and involves giving a weighted score for percentages of staining seen. For example, a core demonstrating 50% of cells with weak (1+) staining intensity, 20% of cells at a moderate (2+) and 20% of cells at a strong (3+) intensity would be given a histoscore of 150 (50*1 + 20*2 + 20*3). Thus using this method the maximum histoscore that can be achieved is 300.
3. *Nuclear staining*. Traditionally in the clinical diagnostic setting ER and PR scoring has been done using the Allred system (Harvey et al., 1999). This method involves assigning a proportion score (the estimated proportion of positive-staining tumor cells: 0, none; 1, < 1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3; to 2/3; and 5, > 2/3) and an intensity score (the average intensity of positive tumor cells: 0, none; 1, weak, 2, intermediate; and 3, strong). The proportion and intensity scores are added to obtain a total score (range from 0 to 8). An alternative but similar method is the QuickScore (Detre et al., 1995) which is calculated in a similar way but with different cut-offs for the proportion score (1=0-4%; 2=5-19%; 3=20-39%; 4=40-59%; 5=6-=79% and 6=80-100%). However we wished to use a continuous variable method of scoring which would also enable ease of correlation with cytoplasmic scoring results. We therefore also scored all the nuclear staining using the Histoscore method.

Once individual cores had been individually scored, we then needed to decide on how the triplicate cores would be combined to provide one single score for each tumour when there was any discrepancy between results. There was little guidance in the published literature on how this was best achieved. Therefore we developed the following approach:

1. *Membrane scores*. When there was any discrepancy between cores, then the percentages stained at each intensity level were combined. For example where 3 cores were available there would need to be at least 30% combined percentage of strong membranous staining for the combined score to be given a 3+. If just 2 cores were available the combined percentage would have be at least 20% to achieve an intensity level.
2. *Cytoplasmic scores*. The average histoscore for the available cores was taken.
3. *Nuclear scores*. The average histoscore for the available cores was taken.

2.5 Statistical analysis

Survival analysis can be performed using a variety of endpoints. Traditionally breast cancer related death has been used for analysis of prognostic markers as it is a fixed, 'hard' endpoint. However it can be lead to problems when analysing response to a particular therapy as patients relapsing on Tamoxifen would normally be switched to alternative endocrine therapies such as aromatase inhibitors, which would confuse any data looking at Tamoxifen use only. In addition, for this analysis, we decided to terminate follow-up duration after Tamoxifen use was stopped. The advantage of this method is that it focuses on patients with disease recurrence whilst *on* Tamoxifen (i.e. '*Tamoxifen resistant*') and excludes patients who relapse *after* Tamoxifen use. This second set of patients could be theoretically be considered as having potentially tamoxifen '*sensitive*' tumours, as they were disease free whilst on adjuvant Tamoxifen but relapsed when their treatment came to an end.

The statistical software package SPSS version 9 was used for all analysis. The Kaplan Meier life table statistical analysis was used for analysis of disease free survival whilst on Tamoxifen. This was performed over two time periods with respect to HER and PR status: throughout the duration of Tamoxifen treatment (to parallel ATAC) or after 3 years of Tamoxifen treatment (to parallel the IES trial). Note again that patients recurring after termination of adjuvant treatment were classed as censored events, as these patients could be classed as relapses because of treatment withdrawal rather than because of resistance to Tamoxifen treatment. Cox Regression and Hazard Ratio analysis was performed with inclusion of the biological marker alongside known prognostic factors size, nodal status and grade. Because of the variety of chemotherapy regimes used over the time period of this study, the influence of

chemotherapy treatment on the relationship between biomarker and tamoxifen resistance was not statistically examined. In addition, menopausal status was not documented for these patients, however age (above or below 50) was incorporated as a variable in statistical analysis.

Chapter 3: Results

3.1 ERα status and patient characteristics of ERα positive group

3.1.1 ERα Status

It was particularly important to confirm ERα status in our cohort as in the period over which these patients were diagnosed ERα testing moved from ligand binding assays to conventional ICH testing. The 15 TMA slides were stained alongside a control slides which had been provided by the department of pathology. There was an example of a strongly positive, weakly positive and negatively staining breast cancer section on the slide and this provided a reference for subsequent scoring. One control slide was incubated with an isotype matched antibody and showed no staining.

3.1.2 ERα Staining and scoring

For 34/456 (7.5%) cases there was insufficient material for ERα analysis, either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Further results and discussion relating to missing/non-valid cores will take place later.

Both nuclear and cytoplasmic staining was noted (Appendix IIa). The nuclear staining was scored using the Histoscore system as described previously and scores for each core were then averaged. ERα slides were double scored for nuclear staining by ST and JB and the results averaged. All cases with a nuclear Histoscore 10 or above were considered ERα positive (based on current departmental pathology guidelines for suitability for endocrine treatment). 20/422 (4.74% of valid cases) were therefore ERα

negative leaving 402 cases classed as being ERα positive. The 54 ERα negative or ERα unknown patients were excluded from any further analysis relating to this study with regard to Tamoxifen resistance.

The mean ERα histoscore of the ERα positive (n=402) tumours was 150.46 (S.D. 57.89) and the median was 153.00 (range 10-300) (Figure 10).

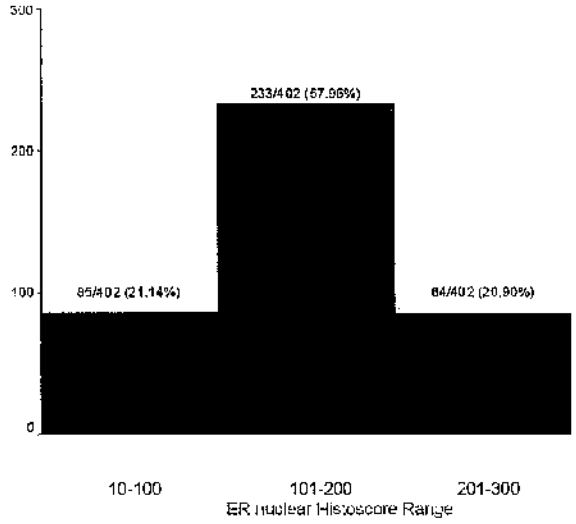

Figure 10

ER nuclear staining: Histoscore intensity and frequency.

These ERα results were compared to the original results from pathology (performed at time of diagnosis, either by IHC or ligand binding) (Table 1). There is a significant correlation demonstrated between our TMA results and the pathology results. With incorporation of cases labelled weakly positive by pathology into the pathology ER positive group, the concordance between TMA and pathology results is 98% (kappa .487). Note that 3 cases labelled positive by pathology (all by IHC) were negative within the TMA sections. Of those cases labelled weak (all by IHC) by pathology 4/20 were negative on the TMA sections.

Table 1
Comparison to pathology ERα status

		TMA ERα		Total
		NEG	POS	
PATH ERα status	neg	3		3
	ns	10	61	71
	pos	3	325	328
	wk	4	16	20
Total		20	402	422

Comparison of TMA obtained ERα status with pathology department determined ERα status

3.1.3 Patient Characteristics: ER positive only group (n=402)

The original group of ER positive/unknown patients consisted of 686 patients prior to exclusions for block unavailability or missing follow-up. The final dataset of our TMA classified ER positive patients (n=402) was compared to this original group in terms of grade, nodal status, histology, size, Nottingham Prognostic Index (NPI) (Galea et al., 1992) and age (Table 2). As can be seen no significant difference can be seen between the 2 groups in terms of these clinico-pathological variables (χ^2).

In addition to tamoxifen, 99/399 (24.8%) patients had chemotherapy (3 unknown) and 110/399 (27.57%) had radiotherapy (3 unknown). The median duration of tamoxifen therapy was 5 years (range 0.3-18yrs). The mean follow-up duration is 6.91 years (SD 3.34 years) and median 6.45 years (range 0.64-18.42 years). There were seventy four breast cancer specific deaths. There were one hundred breast cancer relapses, seventy eight of which were whilst on Tamoxifen.

Table 2
Comparison between final data set (n=402) and original pre-excluded group (n=686)

		final data set		Pre-excluded group		χ2
		number/total	valid %	number/total	valid%	p value
Grade	1	82/310	26.45	142/569	24.96	
	2	154/310	49.68	270/569	47.45	p=0.4868
	3	74/310	23.87	157/569	27.59	
	missing	92		117		
nodal status	0	193/369	52.30	340/612	55.6	
	0-3	107/369	29.00	176/612	28.8	p=0.7596
	4+	62/369	18.70	96/612	15.7	
	missing	33		74		
histology	ductal	322/397	81.11	522/659	79.2	
	lobular	45/397	11.33	74/659	11.2	p=0.5371
	other	30/397	7.56	63/659	9.6	
	missing	5		27		
size	T1	154/380	40.53	252/630	40	
	T2	204/380	53.68	339/630	53.8	p=0.9601
	T3	22/380	5.79	39/630	6.2	
	missing	22		56		
npi	<3.5	105/284	36.97	145/442	32.8	
	3.5-5.5	135/284	47.18	213/442	48.2	p=0.3956
	5.5+	45/284	15.85	84/442	19	
	missing	118		244		
age	<50	65	16.17	123/686	17.9	p=0.6828
	>50	337	83.83	563/686	82.1	

The final dataset of our TMA classified ER positive patients (n=402) was compared to the original pre-excluded group in terms of grade, nodal status, histology, size, Nottingham Prognostic Index (NPI) and age. The p value was calculated from chi-square χ2 analysis

The number and timing of recurrences is shown in Table 3. The average time to recurrence on Tamoxifen (n=68) is 2.5 years (SD 1.89 years) with median 2.15 years (0.1-8.1 years). The median time on Tamoxifen (for those without recurrence) was 5yrs.

Table 3
Recurrence Type

Recurrence type	Frequency	Percentage
None	283	70.40
On tamoxifen early (years 0-3)	53	13.18
On tamoxifen late (>3years)	25	6.22
After tamoxifen completion	34	8.46
Second Primary	7	1.74
Total	402	100.00

Frequency and percentage of type and timing of breast cancer recurrence in relation to tamoxifen use.

3.1.4 ER cytoplasmic staining

The mean cytoplasmic histoscore for the ER positive group was 49.57 (S.D. 36.54) and the median 50 (0-150). ER cytoplasmic staining intensity was correlated with ER nuclear staining intensity ($p<0.001$ Kendall's tau-b).

3.1.5 Disease Free Survival on Tamoxifen: Relationship to intensity of ER staining

When the ERα positive cohort was split into groups depending on intensity of ERα nuclear staining (i.e. above and below median value) there was no significant differences in recurrence on Tamoxifen that could be related to intensity of the nuclear staining ($p=0.2327$). This was also the case when only relapses occurring in the first 3yrs where analysed.

Interestingly however, cases without any cytoplasmic staining ($n=83$) were significantly more likely to recur on Tamoxifen (Figure 11, $p=0.0127$). Note that for this, and all following survival curves the time on tamoxifen extends beyond 10yrs as several patients in our cohort (especially those on diagnosed earlier) did stay on tamoxifen long after the now established 5yr treatment duration.

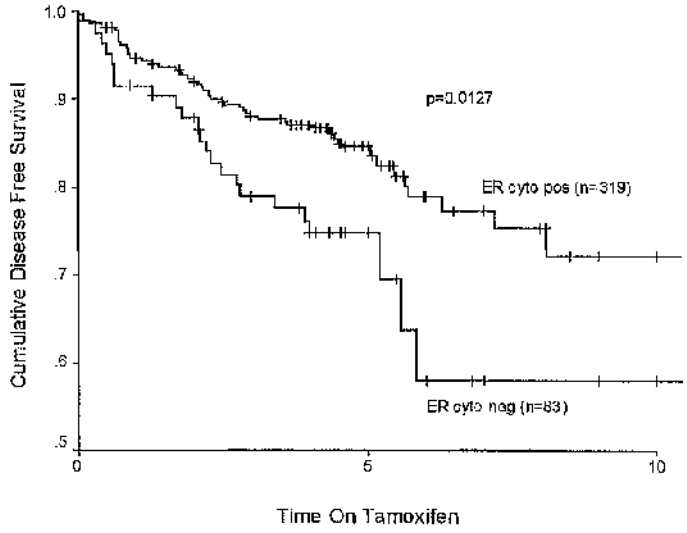

Figure 11

Kaplan-Meier survival curves demonstrating cumulative disease free survival differences (endpoint of breast cancer disease relapse whilst on Tamoxifen) between patients positive or negative for cytoplasmic ERα staining. Cutoffs for positivity for variables are defined in the text. P values represent log rank testing of the difference in cumulative disease free survival.

3.2 Core availability

3.2.1 Missing cores/cores with no tumour

Cores may be invalid because of either core dropout (missing cores) or because the core contains no respresentative tumour. An example of the number and percentages of cores that are valid/missing can be seen in Table 4 which has been taken from results for the ERα antibody. The percentage of cores missing ranged frrom 0-30% (average 11.15%) and the percentage with no readily identifable tumour present ranged from 16-28.3% (average 22.7%). This meant that the number of valid cores ranged from 54-79% (average 66.69%). Cores A to E were constructed chronologically and it can be seen that with regards to the core drop out rate this improved markedly from over 30% loss for TMA A to less than 3% for TMA E as experience of core construction was obtained.

Table 4
Core availability for ERα TMAs

		missing	no tumour	valid
A (n=100)	1	30 (30)%	16 (16%)	54 (54%)
	2	30(30%)	23 (23%)	47 (47%)
	3	43 (43%)	21 (21%)	43 (43%)
B (n=119)	1	13 (10.9%)	24 (20.2%)	82 (68.9%)
	2	21 (17.6%)	23 (19.3%)	75 (65%)
	3	17 (14.3%)	21 (17.6%)	81 (68.1%)
C (n=100)	1	2(2%)	19 (19%)	79 (79%)
	2	2(2%)	19 (19%)	79 (79%)
	3	4 (4%)	22 (22%)	74 (74%)
D (n=60)	1	3(5%)	17 (28.3%)	40 (66.7%)
	2	2(3.3%)	18 (30%)	40 (66.7%)
	3	0 (0%)	19 (31.7%)	41 (68.3%)
E (n=77)	1	2(2.6%)	16 (20.8%)	59 (76.6%)
	2	1(1.3%)	19 (24.7%)	57 (74%)
	3	1 (1.3%)	22 (28.6%)	54 (70.1%)

Frequency and percentage of missing cores, cores with no tumour and valid cores for each TMA (A-E). TMAs were constructed in triplicate (1-3).

As the TMAS are constructed in triplicate most cases had a least one valid core available for analysis. Again using the ERα TMA as an example, Table 5 shows that whilst 181/456 (39.7%) had all 3 cores available, only 34/456 had no valid cores. Table 6 shows the percentage of cases with one or more valid cores available for analysis for each of the antibodies used when analysing results for the ERα positive cohort only (n=402). The percentages of cases available for analysis ranges from 84.83 to 98.88%.

Table 5
Valid cores for ERα TMAS

Valid cores	Frequency	Percent
0	34	7.50
1	130	28.51
2	111	24.34
3	181	39.69
Total	456	100

Frequency and percentage of number of valid cores (0-3) for the TMAs stained for ERα.

Table 6
Percentage of cases with valid core(s) for each antibody

Antibody	Percentage
PR	96.52
HER1	98.88
HER2	97.5
HER3 H3.105.5	87.81
HER4 H4.77.16	84.83
HER4 HFR1	89.3
pER118	95.52
pER167	90.8
pHER2	93.78

Percentage of cases with one or more valid cases available for analysis for each of the antibodies when analysing the ERα positive cohort only (n=402)

3.2.2 Correlation between core results is marker/antibody dependent

Whilst we have not compared the TMA core results with those from whole sections, Table 7 provides some idea of correlations of scores between the cores for each antibody. It can be seen that there is a noticeable difference between known, well

validated antibodies such as ER, PR and HER2 (which demonstrate higher ICCC scores) and the phosphorylated antibodies such as pER118, pER167 and pHER2 (which show lower ICCC scores). Reasons for this may include the heterogeneity of the tissue for these particular receptors in their activated state or may reflect problems with pick-up of the phosphorylated state in formalin fixed tissue. Certainly for some of these phosphorylated antibodies comparison and correlation with whole tissue sections may well be important.

Table 7
Correlation between cores for each antibody
ICCC between cores for each antibody for those with all 3 valid cores

Antibody	n	ICCC MEMB	ICCC CYTO	ICCC NUC
ER	181		0.5956	0.7156
PR	228			0.7640
HER2	198	0.7911		
HER4 H4.77.16	130	0.2714	0.1746	0.1091
HER4 HFR1	145	0.3090	0.3274	0.2920
pER118	69	0.0961	0.4766	0.4668
pER167	173	0.2649	0.5570	0.2626
pHER2	169	0.2667	0.4376	-0.0061

Intra-class coefficient correlation (ICCC) scores for each antibody, in each cellular location, for those cases with all 3 cores valid.

3.3 Progesterone (PR)

3.3.1 PR Expression

Both nuclear and cytoplasmic staining was seen (Appendix IIb). All cases with a nuclear Histoscore 10 or above were considered PR positive (based on current departmental pathology guidelines for reporting PR positivity). There were 241/388 (62.1%) PR positive patients and 147/388 (37.9%) PR negative cases. The mean PR nuclear histoscore is 75.38 (S.D. 87.15) and median 33.75 (range 0-300) (Figure 12). There was no significant correlation seen between PR and ER nuclear Histoscore values (p=0.309, Kendall's tau-b).

3.3.2 Survival data

Patients negative for PR were significantly more likely to relapse whilst on Tamoxifen (Figure 13, p=0.0017).

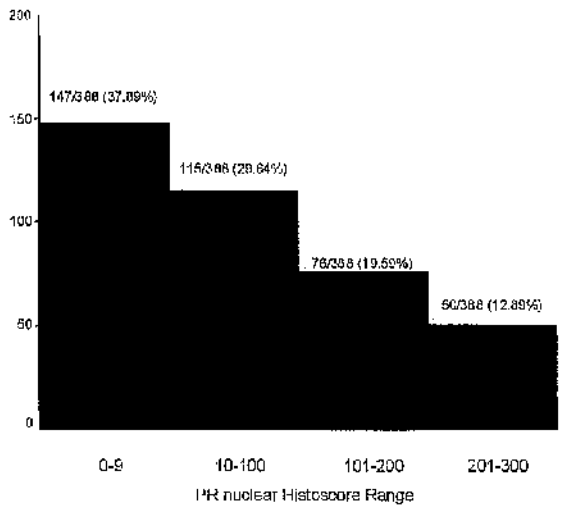

Figure 12

PR nuclear staining: Histoscore intensity and frequency.

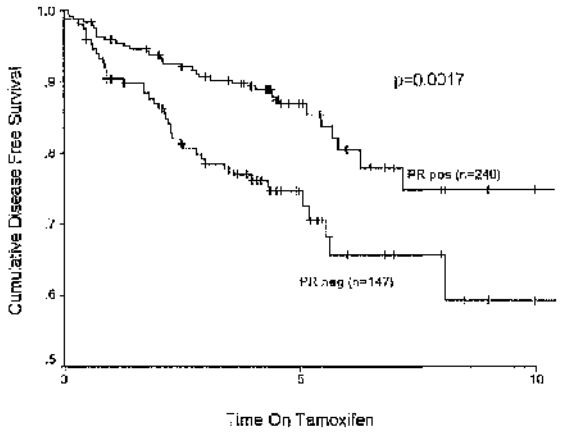

Figure 13

Kaplan-Meier survival curves demonstrating cumulative disease free survival differences (endpoint of breast cancer disease relapse whilst on tamoxifen) between patients positive or negative for nuclear PR staining. Cut-offs for positivity for variables are defined in the text. P values represent log rank testing of the difference in cumulative disease free survival.

3.4 HER 1-4

3.4.1 HER 1-4 Expression and staining patterns

EGFR/HER1

Only membranous staining was demonstrated for HER1 (Appendix IIc). The frequency and intensity of staining is shown in Figure 14a. Cases with *any* membranous HER1 staining were classed as being positive for HER1 overexpression (6/393, 1.5%).

HER2

Both membranous and cytoplasmic staining was seen (Appendix IId). The slides were scored independently by 2 scorers with an ICCC of 0.8863. The mean Herceptest score from the 2 scores was taken to the nearest integer. The frequency and intensity of HER2 staining are demonstrated in Figure 14b. Patients were considered positive for HER2 if they had at least 2+ staining intensity (i.e. at least 10% tumour cells were scored as being moderately positive). 51/397 (12.8%) cases were therefore positive for HER2.

HER3

Membranous, cytoplasmic and nuclear staining was seen using the H3.105.5 antibody (Appendix IIe). The membrane score frequency for ERα positive patients is shown in Figure 14c. Patients were considered positive for HER3 if they had at least 2+ staining intensity at the membrane (i.e. at least 10% tumour cells were scored as being moderately positive) resulting in 56/353 (15.9%) cases considered positive. The median cytoplasmic histoscore was 83.33 (range 0-265) with mean 93.99 (SD 63.53) and the median nuclear score was 58.33 (range 0-250) with mean 61.21 (SD 36.05).

HER4

Membranous, cytoplasmic and nuclear staining was seen using the H4.77.16 antibody (Appendix IIf). Membrane score frequency for ERα positive patients is shown in Figure 14d. Patients were considered positive for HER4 if they had at least 2+ staining intensity (i.e. at least 10% tumour cells were scored as being moderately positive). 46/341 (13.5%) cases were positive for HER4. The median cytoplasmic score was 38.33 (range 0-250) with mean 48.57 (SD 50.83). The median nuclear score was 0 (range 0-200) with mean 15.01 (SD 26.42).

These findings correlate with our previously published results in ER positive cancer where we demonstrated 3.0% patients were EGFR positive, 11.2% HER2 positive, 11.0% HER3 positive and 16.5% HER4 positive (Witton et al., 2003). A recent large study showed generally higher rates of positivity throughout with 14.0% positive for EGFR, 29.3% for HER2, 45% for HER3 and 44.9% for HER4 (Abd El-Rehim et al., 2004). This study used different antibodies and a different method of identifying cutoff levels than our study.

3.4.2 Survival and disease free analysis

Despite a marked separation of the relapse free survival curves for HER1 positive patients relapsing on Tamoxifen, statistical significance in this small group was not reached (Figure 15a p=0.4739). HER2 and HER3 positive patients were significantly more likely to relapse on Tamoxifen (Figure 15b & 15c, p=0.0280, p=0.0278 respectively). However HER4 positive patients showed no significant difference in survival on Tamoxifen (Figure 15d, p=0.2159).

Figure 14a

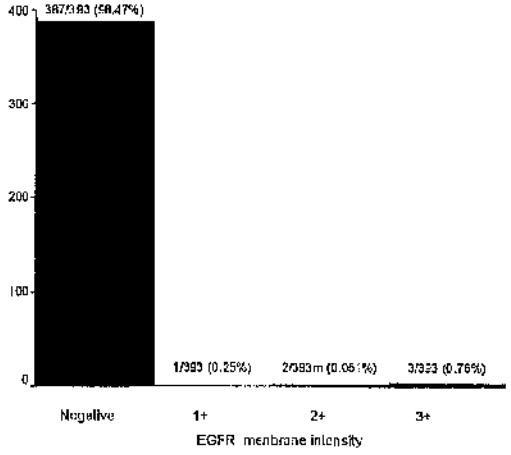

Figure 14b

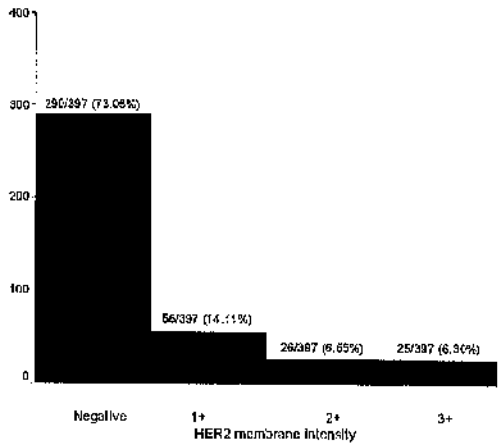

Figure 14c

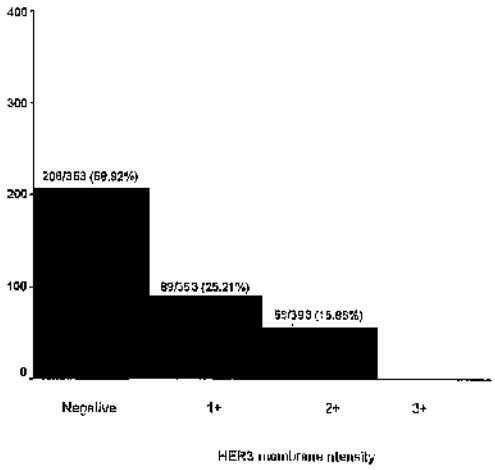

Figure 14d

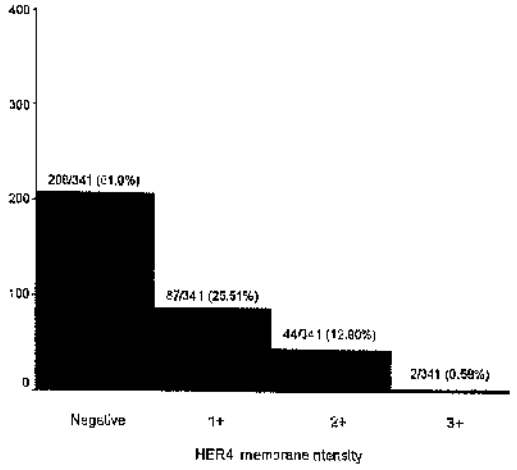

Figure 14

Frequency histograms for membrane staining intensity for a) HER1, b) HER2, c) HER3 and d) HER4.

Figure 15a

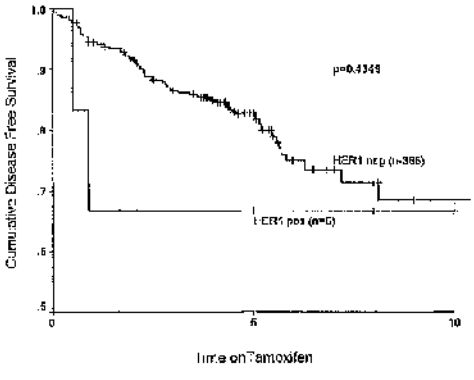

Figure 15b

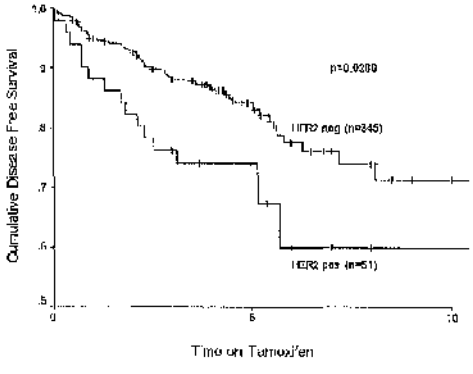

Figure 15c

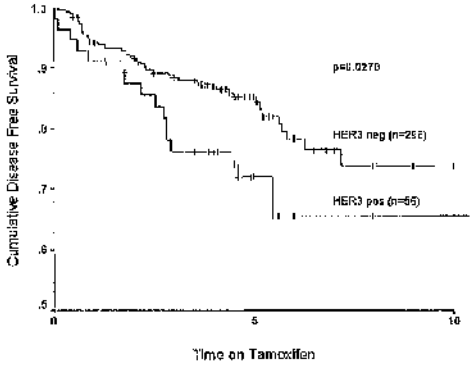

Figure 15d

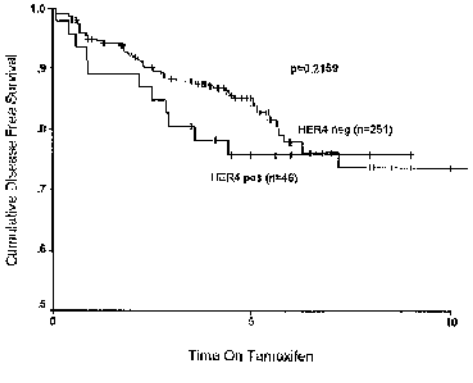

Figure 15

Kaplan-Meier survival curves demonstrating cumulative disease free survival differences (endpoint of breast cancer disease relapse whilst on tamoxifen) between patients positive or negative for a) HER1, b) HER2, c) HER3 and d) HER4. Cut-offs for positivity for variables are defined in the text. P values represent log rank testing of the difference in cumulative disease free survival.

3.4.3 Coexpression of HER1-4 antibodies.

The following illustrates the co-expression patterns of the HER1-4 family.

1.HER1: 6/ 393(1.5%) positive

- 1/6 also positive for HER4
- 1/6 also positive for HER3 and HER4
- 1/6 also positive for HER2, HER3 and HER4
- 3/6 positive for HER1 alone

2.HER2: 51/397 (12.85%) positive

- 7/51 also positive for HER3
- 3/51 also positive for HER4
- 5/51 also positive for HER3 and HER4
- 1/51 also positive for HER1, HER3 and HER4
- 35/51 positive for HER2 alone

3.HER3 56/353 (15.86%) positive

- 7/56 positive for HER2
- 17/56 also positive for HER4
- 1/56 also positive for HER1 and HER4
- 5/56 also positive for HER2 and HER4
- 1/56 also positive for HER1, HER2 and HER4
- 25/56 positive for HER3 alone

4. HER4 46/341 (13.5%)

- 1/46 also positive for HER1
- 3/46 also positive for HER2
- 17/46 also positive for HER3
- 1/46 also positive for HER1 and HER3

- 5/46 also positive for HER2 and HER3
- 1/46 also positive for HER1, HER2 and HER3
- 18/46 positive for HER4 alone

There were significant correlations between expression of EGFR and HER4 (p=0.019, Fishers exact test), between HER2 and HER3 (p=0.030, χ^2) and between HER3 and HER4 (p<0.001, χ^2).

98/350 (28%) patients were positive for either one of HER1, HER2 or HER3 (HER1-3), and HER1-3 positivity predicted for early relapse on Tamoxifen (p=0.0060, Fig 16a). Interestingly the trend for relapse on Tamoxifen appears to increase if patients are positive for more than one HER family member (p=0.0093, Figure 16b).

Figure 16a

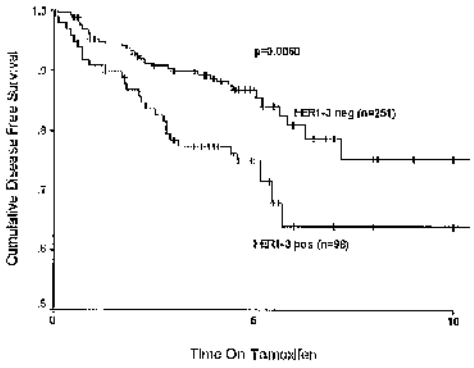

Figure 16b

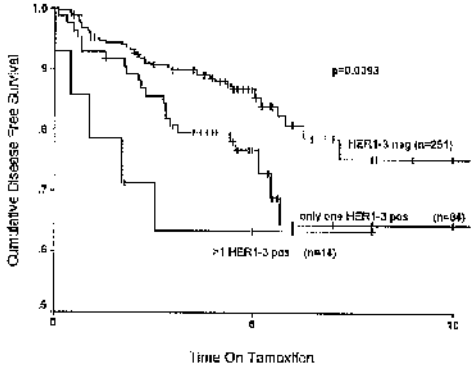

Figure 16

Kaplan-Meier survival curves demonstrating cumulative disease free survival differences between a) patients positive or negative for HER1-3 (positive for either one of HER1, HER2 or HER3). b) patients positive for more than one HER1-3 family member, patients positive for only one type of HER receptor and patients negative for all HER family members. Cutoffs for positivity for variables are defined in the text. P values represent log rank testing of the difference in cumulative disease free survival.

3.4.4 Correlation with PR status and relationship with survival

Table 8 shows the relationship between PR and each HER1-3 member. HER3 shows a significant inverse relationship with PR (p=0.001, χ^2). There is no significant relationship between PR and either HER1 or HER2, although when the group is combined as HER1-3 the significant inverse relationship persists (p=0.001, χ^2).

Table 8
Relationship between PR and HER status.

		HER1		HER2		HER3		HER1-3	
		neg	pos	neg	pos	neg	pos	neg	pos
PR	neg	141	2	120	22	94	31	74	48
	pos	236	4	213	28	197	25	174	49
Total		377	6	333	50	291	56	248	97

Patients positive for HER1, HER2, HER3 or HER1-3 combined (cases positive for one or more of HER1 HER2 or HER3) with respect to PR status. Cut-offs for positivity are defined in the text.

Figure 17 shows the significantly increased rate of relapse on Tamoxifen (p<0.0001) when HER1-3 positive *and/or* PR negative patients are combined as a poor prognostic group. This 'high risk' HER1-3 positive/PR negative group also predicted for increased relapse in Cox's multivariate analysis (p=0.0069) when analysed alongside known prognostic variables size, grade and nodal status.

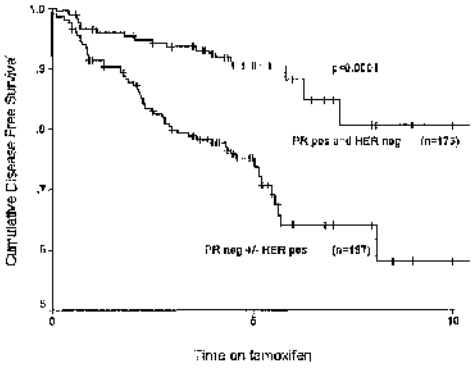

Figure 17

Kaplan-Meier survival curves demonstrating cumulative disease free survival differences between patients either HER1-3 positive and/or PR negative and patients HER1-3 negative and PR positive. Cutoffs for positivity for variables are defined in the text. P values represent log rank testing of the difference in cumulative disease free survival.

3.4.5 Analysis of molecular markers as time dependent variables

Kaplan-Meier survival analysis was subsequently performed following exclusion of those patients who relapsed during the first 3 years of treatment, with the aim of focussing on those patients who had later relapses. Using this method of analysis the PR negative/HER1-3 positive group no longer demonstrated any significant difference in recurrence rates on Tamoxifen (p=0.0858).

Table 9 shows the number (and percentages) of recurrences on Tamoxifen in years 1-3 compared to years 4-5 in the high/low risk groups as identified by the relevant molecular markers. (Note that only patients remaining *disease free* at 3 years are included in the year 4-5 group). It also shows the Hazard ratios (95% CI) from Cox's multiple regression with the relevant factors analysed alongside size, nodal status and grade. The *p-values* from this analysis demonstrate a significant difference between

recurrences in the high versus low risk groups when analysed for the years 1-3, whilst PR and HER1-4 status has no significant impact on recurrences in years 4-5.

Table 9
Time dependent analysis of variables.

	HER1-3			PgR			HER1-3 pos/PR neg		
Years	Positive	Negative	Hazard Ratio	Positive	Negative	Hazard Ratio	High risk	Low Risk	Hazard Ratio
1-3	21/98 21.4%	25/251 10.0%	2.18 (1.13-4.21) p=0.0189	22/240 9.2%	31/147 21.1%	0.48 (0.26-0.92) p=0.0254	39/197 19.8%	11/173 6.4%	3.08 (1.40-6.8) p=0.0052
4-5	3/70 4.3%	7/204 3.4%	0.96 (0.18-5.05) p=0.9611	8/204 3.9%	5/108 4.6%	1.62 (0.39-6.68) p=0.5016	8/147 5.4%	5/152 3.3%	0.91 (0.23-3.64) p=0.8990

HER1-3 positive = cases expressing one or more of HER, HER2, HER3 above the cut offs definod in the text. HER1-3 negative = cases negative for all three receptors. PR positive = cases expressing progesterone receptors. PR negative = cases lacking progesterone receptor expression. Combined High Risk = cases either HER1-3 pos and/or PgR negative, Low risk = cases both HER1-3 negative and PgR positive. Hazard Ratio = relative increased hazard (with 95% confidence intervals in brackets) from Cox's regression analysis for HER1-3 positive versus HER1-3 negative cases, PgR positive versus PR negative and High versus Low risk cases respectively. Years 1-3 (4-5) = patients at risk (denominator) and numbers of relapses (numerator) within different subgroups during years 1-3 (4-5 = in years 4 and 5) of tamoxifen treatment only. Percentages = percentage relapse rates in at risk population during time period in question. P values derived from Cox's multiple regression analysis

3.5 Phosphorylated ER

3.5.1 pERαSer118 and pERαSer167 expression

Nuclear, cytoplasmic and membranous staining was observed for both pERαSer118 and pERαSer167 (Appendix IIg and IIh and Figures 18 a-f). For pERαSer118, 12.5% (48/384) showed membranous staining, 94.3% (362/384) exhibited cytoplasmic staining (median histoscore 175 (range 0-300), mean 163.69 (SD 81.60)) and 98.4% (378/384) exhibited nuclear staining (median score of 145 (range 0-270), mean 141.77 (SD 47.12)). For pERαSer167, 20.8% (76/365) showed membranous staining, 70.68% (258/365) exhibited cytoplasmic staining (median histoscore 50 (range 0-250), mean 60.42 (SD 55.71)) and 95.6% (349/365) demonstrated nuclear staining (median histoscore 96.70 (range 0-250), mean 102.16 (SD 55.45)).

There were significant correlations between the intensity of nuclear pERαSer118 and nuclear pERαSer167 staining ($p<0.001$, Kendall's tau-b) and between cytoplasmic pERαSer118 and cytoplasmic pERαSer167 staining intensity ($p<0.001$, Kendall's tau-b). There was also overlap between membranous staining with 25/357 cases exhibiting staining for both pERαSer118 and pERαSer167 at the membrane.

Figure 18a

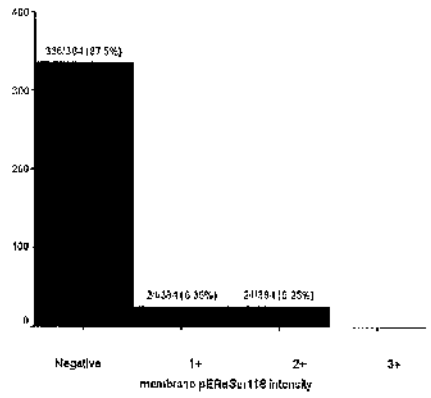

Figure 18b

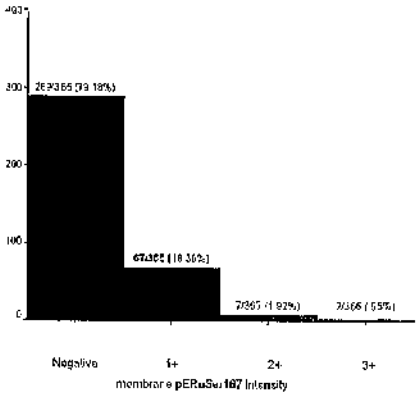

Figure 18c

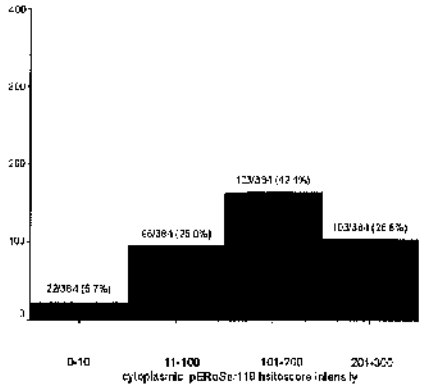

Figure 18d

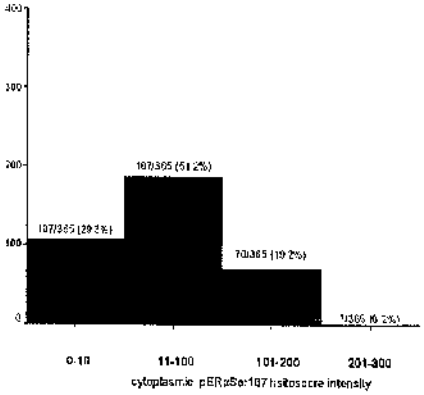

Figure 18e

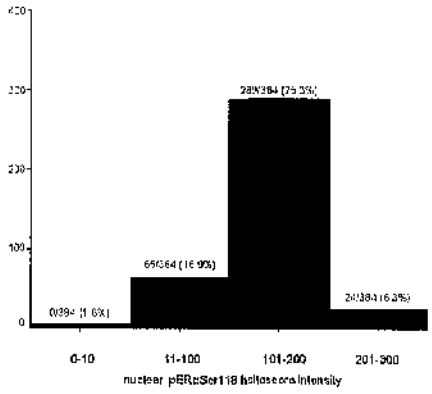

Figure 18f

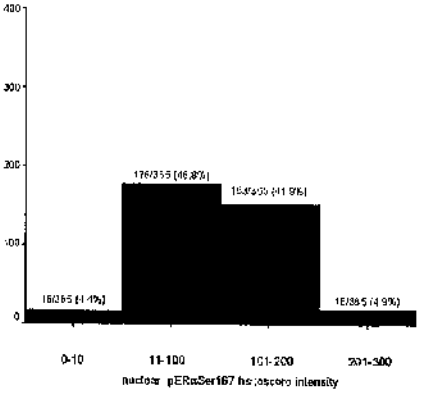

Figure 18

Frequency histograms for membranous a) pERαSer118 and b) pERαSer167 staining; cytoplasmic c) pERαSer118 and d) pERαSer167staining; and nuclear e) pERαSer118 and f) pERαSer167 staining

3.5.2 Relationship between phosphorylated ERα and ERα status

Nuclear and cytoplasmic pERαSer118 staining correlated significantly with the intensity of ERα *nuclear* staining ($p<0.001$ and $p=0.006$ respectively, Kendall's tau-b, Table 10) as did nuclear and cytoplasmic pERαSer167 staining (both $p<0.001$; Kendall's tau-b).

In addition nuclear and cytoplasmic pERαSer118 staining correlated significantly with the intensity of ERα *cytoplasmic* staining ($p=0.004$ and $p<0.001$ respectively, Kendall's tau-b) as did nuclear and cytoplasmic pERαSer167 staining ($p=0.003$ and $p<0.001$; Kendall's tau-b).

However there was no correlation between the intensity of nuclear ERα staining and the presence of either membranous pERαSer118 or pERαSer167 expression (Mann-Whitney, Table 10). There was also no correlation between cytoplasmic ERα staining and the presence of membranous pERαSer118 although there was a significant correlation with the presence of membranous pERα167 (Mann Whitney, $p<0.001$)

3.5.3 Relationship between phosphorylated ERα and PR status

There was no correlation between PR positivity and either nuclear or cytoplasmic pERαSer118 or pERαSer167 histoscores (Mann Whitney) or presence of membranous pERαSer118 ($p=0.082$, χ^2) or pERαSer167 ($p=0.139$, χ^2).

Table 10
Relationship between pERSer118, pERSer167 and nuclear ER and HER1-3 expression

		ERα		HER1-3
		nuclear ER	cytoplasmic ER	
pERαSer118	nuclear	**p<0.001***	**p=0.004***	*p=0.006***
	cytoplasmic	**p=0.006***	**p<0.001***	p=0.294**
	membrane	p=0.407**	p=0.496**	**p<0.001*****
pERαSer167	nuclear	**p<0.001***	**p=0.003***	p=0.522**
	cytoplasmic	**p<0.001***	**p<0.001***	p=0.229**
	membrane	p=0.133**	**p<0.001****	**p=0.002*****

*Kendall's tau-b, **Mann-Whitney, ***χ2
bold=positive correlation, *italic=negative correlation*

pERαSer118 represents phosphorylated ERα at Ser118 at nucleus,cytoplasm and membrane. pERαSer167 represents phosphorylated ERα at Ser167 at nucleus, cytoplasm and membrane. ERα represents nuclear or cytoplasmic staining ERα staining. HER1-3 represents patients positive for one of HER1, HER2 and/or HER3 at the membrane.

3.5.4 Relationship between phosphorylated ERα and HER1-3 status

Nuclear pERαSer118 staining was negatively correlated with HER 1-3 overexpression (p=0.006, Mann-Whitney) but there was no association between nuclear pERαSer167 and HER1-3 expression (p=0.522). In addition there was no correlation between cytoplasmic pERαSer118 or pERαSer167 and HER1-3 expression (Mann Whitney). However, the presence of both *membranous* pERαSer118 and *membranous* pERαSer167 was significantly correlated with HER1-3 positivity (p<0.001 and p=0.002 respectively; Pearsons χ2, Table 10).

3.5.5 Correlation with relapse on tamoxifen and known clinical prognostic markers

Increased nuclear pERαSer167 correlated with smaller size and lower grade of tumour (p<0.001 and p=0.001 respectively, Kendall's tau-b) and cytoplasmic pERαSer167 intensity correlated with small tumour size (p<0.001). However the presence of membranous pERαSer118 was associated with larger tumours (p=0.009, Pearsons χ2).

Nuclear and cytoplasmic pERαSer118 and membranous pERαSer167 did not correlate with nodal status, grade or size of tumour.

Whilst there was no significant association between phosphorylated ERα at the nucleus or cytoplasm and relapse on tamoxifen, patients positive for membranous pERαSer118 were significantly more likely to have disease relapse (p=0.0412, Figure 19). No such association was observed with membranous pERαSer167 (p=0.8472).

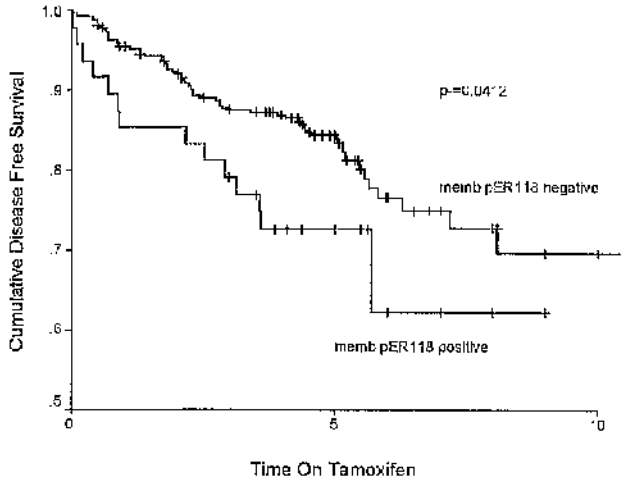

Figure 19

Kaplan-Meier survival curves demonstrating cumulative disease free survival differences (endpoint of breast cancer disease relapse whilst on tamoxifen) between patients positive or negative for membranous pERαSer118. Cut-offs for positivity for variables are defined in the text. P values represent log rank testing of the difference in cumulative disease free survival.

3.6 Comparison of HER4 antibodies.

3.6.1 Cut-offs determining membranous, cytoplasmic and nuclear staining status

Membranous, cytoplasmic and nuclear staining was seen using both H4.77.16 (Appendix IIg) and HFR1 (Appendix IIi) antibodies. To compare the respective staining of the two antibodies, cutoffs were chosen to determine positive or negative staining for the respective modalities. For membranous HER4 any staining at the 2+ or higher level was chosen as described previously. Cases were considered positive for cytoplasmic or nuclear staining if the average histoscore for the respective modality was over 10. Concordance between two antibodies, with regard to staining at the membrane, cytoplasm and nucleus, was evaluated using chi-square kappa value where a value of 1 indicates perfect agreement and a value of 0 indicates that agreement is no better than chance.

3.6.2 Membrane staining

Membrane staining intensities for each antibody are shown (Figure 20a & b). 46/341 (11.4%) patients were classed as positive using the H4.77.16 antibody and 28/359 (7.0%) using the HFR1 antibody. The concordance between the 2 antibody results is 88.44% (n=329, Table 11) with a kappa value of 0.426 (where a value of 1 indicates perfect agreement and a value of 0 indicates that agreement no better than chance). It can be seen that the percentage of tumours that are negative is similar between the 2 antibodies but that the H4.77.16 antibody appears to detect a stronger intensity of staining at the membrane. This may reflect a difference in sensitivity between the antibodies or alternatively reflect a difference between TACE cleaved but γ-secretase intact HER4.

3.6.3 Cytoplasmic staining

The median cytoplasmic histoscore for H4.77.16 antibody was 36.67 (range 0-250) and mean 48.21 (SD 50.32). The median cytoplasmic histoscore for HFR1 was 75 (range 0-253) and mean 78.75 (SD 61.10). The HFR1 antibody therefore has generally has higher levels of cytoplasmic staining (Figures 120c & d). 225/341 (66.0%) patients were classed as positive using the H4.77.16 antibody and 293/359 (81.6%) using the HFR1 antibody. The concordance between the 2 antibody results is 74.77% (n=329, Table 11) with a kappa value of 0.351. This difference may reflect the fact that HFR1 can recognise both cleaved 4ICD and the intact (recycling) HER4 whilst H4.77.16 will recognise the intact form only.

3.6.4 Nuclear staining

The median nuclear score for H4.77.16 antibody was 0 (range 0-200) and mean 15.01 (SD 26.42). The median nuclear score for HFR1 was 63.33 (range 0-200) and mean 64.83 (SD 38.65). The antibodies showed very different staining patterns in the nucleus (Figures 20e & f). 116/341 (34.0%) patients were classed as positive using the H4.77.16 antibody and 332/359 (89.3%) using the HFR1 antibody. Whilst over 60% of patients stained with the H4.77.16 had no nuclear staining, the vast majority showed some staining with the HFR1 antibody. Once split into positive and negative groups the concordance between the 2 antibody results is 40.12% (n=329, Table 11) with a kappa value of 0.051. This lack of agreement may well reflect the fact that the cleaved 4ICD (recognised by HFR1) is much more likely to be found in the nucleus than the intact form.

Figure 20a

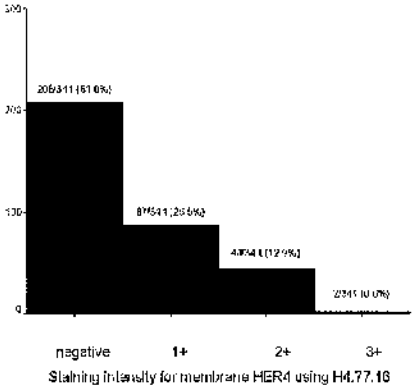

Figure 20b

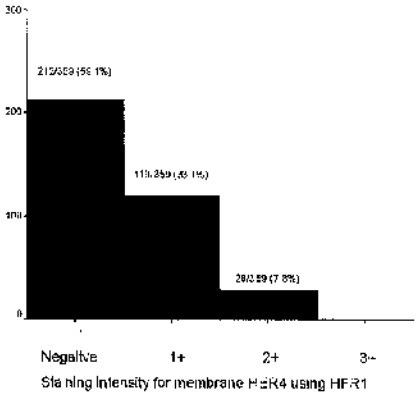

Figure 20c

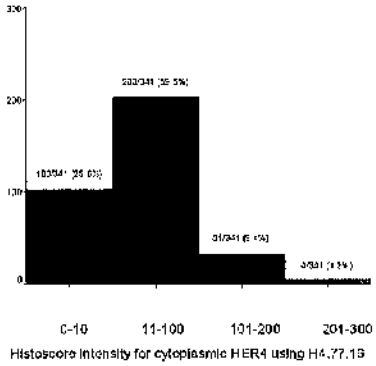

Figure 20d

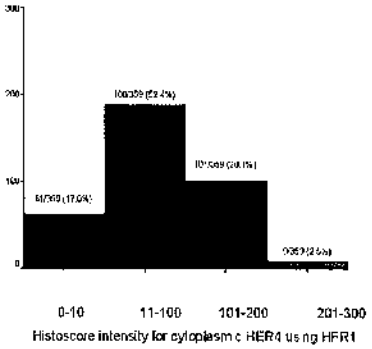

Figure 20e

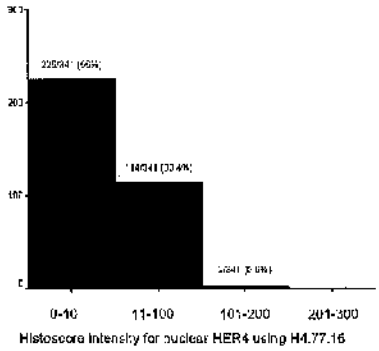

Figure 20e

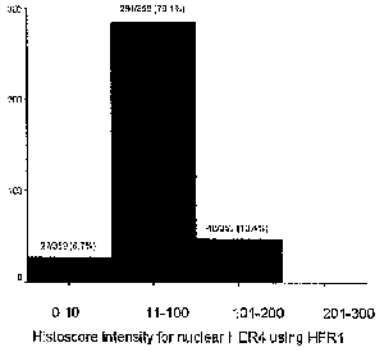

Figure 20

Frequency histograms for membranous (a & b), cytoplasmic (c & d) and nuclear (e & f) staining intensity using the H4.77.16 and HFR1 antibodies.

Table 11
Comparison of H4.77.16 and HFR1 staining patterns in the membrane, cytoplasm and nucleus

Membrane

		H4.77.16 neg	pos	total	kappa value
HFR1	neg	273	28	301	
	pos	10	18	28	0.426 (p<0.001)
	total	283	46	329	

Cytoplasmic

		H4.77.16 neg	pos	total	kappa value
HFR1	neg	41	15	56	
	pos	68	205	273	0.351 p<0.0001
	total	109	220	329	

Nuclear

		H4.77.16 neg	pos	total	kappa value
HFR1	neg	21	3	24	
	pos	194	111	305	0.051 (p=0.018)
	total	215	114	329	

Chi- square where numbers of cases negative or postitive for each antibody in at either the membrane, cytoplasm or nucleus are shown using cutoffs defined in the text. Cohen's Kappa value measures the agreement between the 2 antibodies where a value of 1 indicates perfect agreement and a value of 0 indicates that agreement is no better than chance

3.6.5 Relationship with clinicopathological variables and the other HER family members.

Both antibodies demonstrated a significant relationship between membranous HER4 and increasing size and HER3 positivity (Table 12). For the patients positive for HER4 using the HFR1 antibody; 22/28 (78.57%) were also positive for another member of the HER family. For the H4.77.16 this figure was 28/46 (60.87%).

Table 12
Membranous HER4:Correlation with pathological variables and HER family

	JR H4.77.16 membrane			HFR1 membrane		
	x^2	p value	correlation	x^2	p value	correlation
NPI	4.799	0.091		6.095	0.047	pos
size	8.652	0.013	pos	8.608	0.008	pos
grade	2.453	0.293		0.75	0.687	
nodal	1.769	0.413		3.202	0.202	
egfr	Fishers	0.019	pos	Fishers	0.074	
her2	1.657	0.243		Fishers	0.314	
her3	53.67	<0.001	pos	Fishers	<0.001	pos

*Grade = Bloom and Richardson grade. Nodal status = number of positive nodes. NPI = Nottingham Prognostic Index (grade+nodal status+ 0.02*size in mm).*

Both antibodies showed a correlation between HER4 cytoplasmic staining and increasing NPI, nodal status, size and HER3 positivity (Table 13). Neither antibody showed any significant correlations between nuclear HER4 staining and pathological variables or HER1-3 status.

Table 13
Cytoplasmic HER4:Correlation with pathological variables and HER family

		H4.77.16 histoscore		HFR1 histoscore	
	test	p value	correlation	p value	correlation
NPI	Kendall's tau-b	0.004	pos	<0.001	pos
size	Kendall's tau-b	<0.001	pos	0.021	pos
grade	Kendall's tau-b	0.076		<0.001	pos
nodal	Kendall's tau-b	0.004	pos	<0.001	pos
egfr	Mann-Whitney	0.035		0.436	
her2	Mann-Whitney	0.381		0.771	
her3	Mann-Whitney	<0.001	pos	0.001	pos

*Grade = Bloom and Richardson grade. Nodal status = number of positive nodes. NPI = Nottingham Prognostic Index (grade+nodal status+ 0.02*size in mm).*

3.6.6 Survival and disease free analysis.

For both antibodies there was no relationship between disease free or overall survival and membranous HER4 staining. Cases who were positive for HER4 only (and not for any other members of the HER family) where identified (n=6 for HFR1 and n=18 for H4.77.16) but these patients again did not have significantly different rates of survival. Cytoplasmic staining was not correlated with disease free or overall survival using either antibody. However cases demonstrating nuclear HER4 staining using the H4.77.16 antibody were significantly more likely to have poorer overall survival (p=0.0124, Figure 21). There was no such correlation with survival with the HFR1 antibody.

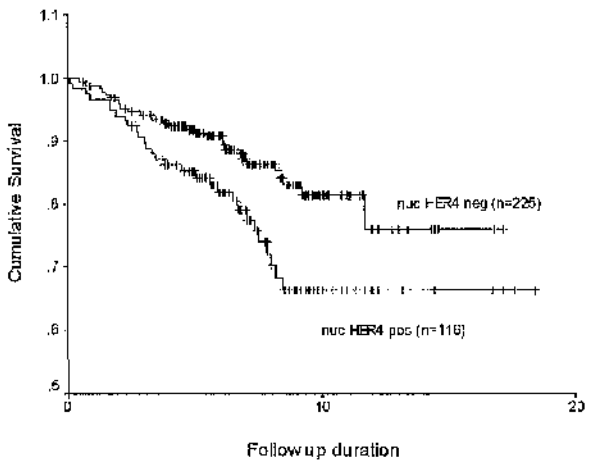

Figure 21

Kaplan-Meier survival curves demonstrating cumulative disease free survival differences (endpoint of breast cancer disease relapse whilst on tamoxifen) between patients positive or negative for nuclear HER4 using the H4.77.16 antibody. Cutoffs for positivity for variables are defined in the text. P values represent log rank testing of the difference in cumulative disease free survival.

3.7 pHER2 antibody

3.7.1 pHER2 staining

Membranous, cytoplasmic and nuclear staining was seen (Appendix Ij). Intensity of membranous staining can be seen in Figure 22. Using 2+ scoring intensity as a cutoff there are 24/366 (6.37%) positive for phosphorylated HER2.

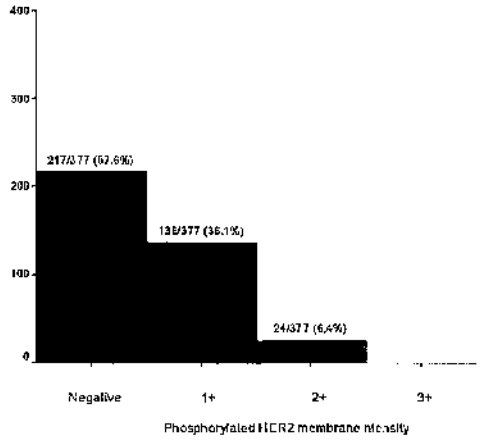

Figure 22

Frequency histogram showing intensity of membranous phosphorylated HER2 staining

3.7.2 Survival and disease free analysis

Neither membranous, cytoplasmic nor nuclear staining positivity was correlated with disease free survival on tamoxifen. In addition there was no correlation with known prognostic variables, size, grade and nodal status.

3.7.3 Relationship between pHER2 and HercepTest scores.

There is no significant relationship ($\chi 2$ p=0.938, Table 14) between the intensity of membrane staining for HER2 and phosphorylated HER2 staining. Indeed many cells negative for Herceptest showed staining for phosphorylated HER2.

Table 14
Relationship between HER2 and Phospho-HER2 scoring

		Phospho-HER2			Total
		0	1+	2+	
Herecepest	0	157	100	16	273
	1+	31	19	3	53
	2+	15	8	2	25
	3+	14	8	3	25
Total		217	135	24	376

Relationship between membranous HER2 and membranous Phospho-HER2 scoring. Intensity scored as 1+(weak), 2+(moderate) or 3+ (strong) staining.

Our results did not support the previously published reports suggesting that that pHER2 status provided improved prognostic information compared to HER2 status alone (Thor et al., 2000). Therefore no further analysis was performed using these results.

Chapter 4 Discussion

4.1 TMA validity and Limitations of the study

We have demonstrated that TMA technology can be used successfully to quickly process large numbers of cases though immunohistochemistry protocols in a limited amount of time. In addition we were able to perform direct comparison of staining intensities between specimens so improving the subjective interpretation of results. However as other studies have shown, we also had significant rates of core loss.

The overall percentage of cores missing was 11.15% and the percentage with no readily identifable tumour present was 22.7%. This meant that the number of valid cores ranged from 54-79% (average 66.69%). This is in keeping with published results (Bubendorf et al., 1999; Hoos and Cordon-Cardo, 2001; Schraml et al., 1999; Torhorst et al., 2001). Of note, the core drop out rate improved markedly as experience of TMA construction was obtained suggesting that core loss is dependent on operator experience. As predicted, constructing the TMAS in triplicate minimised the impact seen because of core loss. Depending on the antibody, the percentages of cases with at least one valid core ranged from 84.83 to 98.88 %. These figures suggest that using 4 cores per case may have improved the availability of valid cores for analysis.

Concerns have also been raised with regard to the impact of tissue heterogeneity on TMA results although it must be remembered that a tumour block itself often represents a small fraction of the actual tumour. Evidence comparing core and whole section results has suggested that this problem is minimised by having more than one core available for analysis (Hoos and Cordon-Cardo, 2001; Camp et al., 2000). We have not compared TMA results to those of whole sections; however we did

determine the correlation between core results in those cases that had all 3 cores available. This demonstrated a noticeable difference between known, well validated antibodies such as ER, PR and HER2 which demonstrate higher ICCC scores, and the phosphorylated antibodies such as pER118, pER167 and pHER2 which show lower ICCC scores. The discrepancy in these results may result from the heterogeneity of the tissue for these particular receptors in their activated state or may reflect problems with pick-up of the phosphorylated state in formalin fixed tissue. In addition some of the less well validated antibodies had more evidence of background staining compared to Herceptest staining for example. Certainly for some of these phosphorylated antibodies, correlation with whole tissue sections may well be important.

One concern with regard to analysis of multiple testing of variables is the possibility that "significant" associations are being demonstrated due to 'chance' rather than being due to a true correlation. For an acceptable significance of $p=0.05$ this would occur in 1 in 20 tests. Whilst the majority of this study is based on survival analysis, multiple correlations have been performed particularly in comparison of HER4 antibody staining. One method of adjusting for this would be to perform the Bonferroni test, based on Student's t statistic, which adjusts the observed significance level for the fact that multiple comparisons are made (Bland and Altman, 1995). However it must also be noted that all analysis performed in this study were on a pre-planned basis and in addition the majority of correlations analysed have been statistically significant.

The findings in this thesis highlight the problems of comparative analysis within the literature with regard to identification of prognostic/predictive markers. The use of different antibodies, scoring systems, conditions and comparison of TMAs with whole

sections all make comparative analysis difficult. A recent meeting on Cancer Diagnostics (NCI-EORTC) has led to the development of REMARK guidelines (McShane et al., 2005) which provide suggestions on how both the trial design and reporting of results of marker based studies can be standardised to improve the ability to compare results across studies.

One recent advance in immunohistochemical based marker studies has been the use of automated image analysis (Camp et al., 2002). There are various systems on the market including ACIS (ChromoVision) and AQUA (HistoRx). Whilst automated image analysis is becoming more commonplace in pathology laboratories it has its drawbacks. Whereas an experienced pathologist can score only tumour cell immunoreactivity, this may not be true of a machine, and overestimates of postivity might ensue. This is particularly important when considering location of staining, where detection of staining at the nucleus, cytoplasm or membrane may have very different prognostic implications. However use of image analysis technology is particularly attractive in terms of image capture for storage of the image for future analysis and for ease of inter-scorer comparision.

Whilst IHC based marker studies continue to be used to identify new prognostic markers, other emerging approaches such as reverse transcription polymerase chain reaction and DNA microarrays are increasingly used as prognostic or predictive tests. For example Oncotype DX (Genomic Health Ltd) represents an important conceptual advance in the diagnosis of ER positive cancers. This RT-PCR based assay measures ER mRNA expression as well as the expression of several downstream ER-regulated genes (PR, BCL-2, SCUBE-2) that may represent a functional ER (Paik et al., 2004).

The use of high throughput gene-expression profiling techniques has lead to the identification of subsets of breast cancers with different molecular signatures. One

such comprehensive study using hierarchical clustering has suggested that gene-expression patterns of breast cancer can be split into 4 major classes: luminal-like (usually ER positive), basal-like (mostly ER negative), normal-like and HER2-positive (Perou et al., 2000). This technique has also been used to identify a 70-gene prognostic predictor for increased chance of relapse (van de Vijver et al., 2002; 't Veer et al., 2002). However with all new technologies, reproducibility of results and standardisation of methodology must be determined. Promising markers identified using these methods must investigated in laboratory to gain insight into their function before being considered as new diagnostic markers (Pusztai et al., 2006).

4.3 HER1-3 positive and/or PR negative patients are associated with early relapse on tamoxifen.

We have demonstrated results which confirm the significant role played by the type I receptor tyrosine kinases HER1, HER2 & HER3 in promoting tamoxifen resistance in hormone responsive breast cancers. The results also emphasise the different role of HER4 in this context. In addition, these data demonstrate that PR negative tumours have a reduced responsiveness to tamoxifen which is only weakly related to expression of HER1-3. Therefore a phenotype of either HER1-3 positivity or PR negativity in ER positive breast cancers appears to be linked to tamoxifen resistance - as defined by elevated risk of disease relapse despite endocrine therapy. Strikingly, however, it appears that this elevated risk applies in the first 3 years of tamoxifen therapy only. Most interestingly, these data appear to explain recently reported discrepancies relating to PR status and aromatase inhibitors in clinical trials (Coombes et al., 2004a; Dowsett, 2003).

Previously we have demonstrated that expression of HER1-3 is linked to high proliferation indices in breast cancer, whilst HER4 is associated with a low proliferation index (Tovey et al., 2004). Furthermore, preliminary data suggested that patients with ER positive disease were at greater risk of early relapse due to HER1-3 expression than ER negative cases (Witton et al., 2003). Now we have analysed the expression of the type I receptor tyrosine kinases (HER1-4) in a large retrospective group of tamoxifen treated, ER positive cases. Patients whose tumours were positive for HER1-3 were shown to be at a significantly greater risk of relapse whilst on adjuvant tamoxifen, supporting a role for the HER1-3 receptors in tamoxifen

resistance (figures 15 & 16). Conversely, we show no relationship between the expression of HER4 and early relapse on tamoxifen supporting growing evidence that this receptor type plays a distinct role to other members of the type I RTK family. Further discussion with regard to HER4 and its role in breast cancer will occur later.

Our results also suggest that the overexpression of more than one HER family member significantly increases the likelihood of relapse on tamoxifen (figure 16). Similar evidence from a recent report (Abd El-Rehim et al., 2004) and our earlier data (Witton et al., 2003) supports an additional negative effect on outcome when multiple receptors are co-expressed. The ability of HER2 and HER3 to potentiate signalling through other receptors has been demonstrated in vitro (Yarden, 2001b) suggesting this is a possible mechanism by which heterodimers may produce a more marked effect than homodimers. We know that HER2 enhances and stabilises dimerization but apparently has no ligand (Klapper et al., 1999) whereas HER3 has no inherent kinase activity so must be dimerised with another HER family member to exert an influence on downstream signalling (Guy et al., 1994). Evidence has suggested that this HER2-HER3 heterodimer is the most potent combination of all (Yarden, 2001a).

Whilst some of the most convincing published clinical evidence for the role of HER1 and 2 in tamoxifen resistance has come from neo-adjuvant trials (Ellis et al., 2001) the major adjuvant trials comparing tamoxifen to aromatase inhibitors have largely failed to build this in to planned prospective analysis. However there has been some data from the ATAC (Dowsett, 2003) suggesting that differences do exist in the molecular profile of patients who respond to early aromatase inhibitors in preference to tamoxifen when analysed in regard to the PR status of the patients. Our results confirm suggestions that ER positive/PR negative tumours are significantly less likely to respond to tamoxifen treatment than ER positive/PR positive tumours (Coombes et

al., 2004a; Dowsett, 2003).This is of particular current interest given the ATAC and IES evidence with regard to PR. The ATAC trial was designed to compare the efficacy of tamoxifen versus the aromatase inhibitor Arimidex (either alone or in combination) in ER positive post menopausal women. Early results from this trial demonstrate a superiority of Arimidex over tamoxifen in terms of disease free survival. However, retrospective sub analysis from this trial also suggested that this increased clinical benefit derived from Arimidex was particularly evident in the PR negative tumour patients (Dowsett, 2003).

The inverse relationship demonstrated here between PR and HER 1-3 expression (Table 8) might support supposition that PR negativity may be acting as a surrogate marker for HER1-3 overexpression in the ATAC trial. This would link the ATAC data with data from the neoadjuvant trials (Ellis et al., 2001; Ellis et al., 2003) demonstrating that aromatase inhibitors circumvent HER1/2 mediated tamoxifen resistance in vivo. However, closer examination of the evidence presented here confirms that ER positive/PR negative tumours and HER1-3 positive tumours represent distinct patient sub-groups with relatively little overlap. Only 12% (42) of tumours were both PR negative and HER1-3 positive with 19.1% PR negative/HER1-3 negative and 15.3% PR positive/HER1-3 positive. This suggests that the association between HER1-3 expression and loss of PR expression, whilst real, is weak and that alternative, as yet unexplained, mechanisms must underpin tamoxifen resistance in PR negative tumours. PR expression has long been thought to reflect a functional, ER transcription pathway. It is possible that the ER in these PR negative patients is not functioning as a nuclear receptor, but instead is active through non genomic mechanisms either in the cytoplasm or at the membrane where it may interact with the HER family or other active signal transduction pathways. However, evidence relating

to the expression of different PR isoforms, and potentially ERβ, as predictors of tamoxifen response may also be of importance (Bardou et al., 2003; Isaksson et al., 2003; Iwase et al., 2003). Further work is required to investigate these potential explanations. However, our data suggests that in both HER1-3 positive and PR negative breast cancers there is evidence of resistance to tamoxifen (this group represents almost 50% of all ER positive cancers in this study). In these patients, based on published evidence from the ATAC trial and from Ellis' group, we hypothesise that enhanced response rates could be obtained by use of aromatase inhibitors in place of tamoxifen at an early stage. Conversely, we hypothesise, many other patients, particularly those with HER1-3 negative and PR negative tumours, may continue to derive significant benefit from tamoxifen without the need to switch to aromatase inhibitors.

However, this conclusion would initially appear to be contradicted by data from the IES study (Coombes et al., 2004a), in which patients were randomised to continued treatment with either the aromatase inhibitor Exemestane or tamoxifen following completion of 2-3 years of tamoxifen. This trial also showed a benefit in disease free survival in those patients switched to Exemestane. This benefit was shown equally in both PR negative and PR positive patients. We hypothesised that in this scenario many patients with *de novo* tamoxifen resistance would in effect be excluded from this trial as many recurrences would have occurred prior to randomisation. Perhaps most interestingly then, our results also show that HER and PR status do not appear to influence relapse on tamoxifen after 3 years of tamoxifen treatment which parallels these recently published IES results (Coombes et al., 2004a).

We showed no significant differences in relapse rates on tamoxifen relative to PR status in ER positive patients who had already survived disease free for three years.

These results support our conclusion that PR negativity identifies a group of tumours with *de novo* tamoxifen resistance, who may therefore respond to aromatase inhibitors if treated early, i.e. from time of diagnosis (as per the ATAC trial). We suspect these patients are in effect excluded from the IES study by virtue of their having relapsed prior to the randomisation point of this study. This suggests that PR status is a time dependent predictor of early relapse due to *de novo* tamoxifen resistance. Further evidence for this supposition is provided for the moderate association between PR status and outcome in the ARNO 95/ ABCSG 8 Trials (Jakesz et al., 2004) which randomised patients to either tamoxifen or Anastrasole following only 2 years of tamoxifen treatment. Taken together, these data strongly support the conclusion that the predictive value of PR, and possibly HER1-3, expression is time dependent and hence identifies patients at high risk of *de novo* tamoxifen resistance. However, data from the BIG1-98 study, which is comparable to ATAC in design, does not appear to support this argument (Jakesz et al., 2004). Whilst the most likely explanation of this is under-powering of the sub-analysis relating to PR status, caution should be exercised before extrapolating results from ATAC and those presented here to treatment of patients. These results also highlight the importance of distinguishing clearly between the molecular pathways involved in *de novo* and acquired resistance, particularly in the clinical setting in terms investigating any changing molecular profile of recurrent tumours. Ultimately investigation of the underlying mechanisms behind later recurrences may demonstrate up-regulation of previously dormant HER1-3 pathways or alternatively it may involve other, as yet unknown pathways, which may also be detectable at diagnosis.

From a clinical perspective, data from the ATAC trial, if confirmed, may provide sufficient support to encourage early switching of ER positive patients from

tamoxifen to aromatase inhibitors if their tumours do not express PR. Despite the importance of these findings however, additional information from prospective clinical trials is required before recommendations relating to patient management can be made. In this context it is particularly important that translational science tumour banks are being collected in the context of current clinical trials. The value of such tumour banks is clearly demonstrated by the ATAC data, despite the retrospective nature of the analysis. The data presented here supports extending pre-planned prospective analysis, such as that defined within the multinational TEAM trial (prospectively testing for interactions between HER1-3 and outcome relating to tamoxifen or Exemestane treatment), to incorporate measurement and reporting of PR status. Such analyses should, in the light of ongoing variations in PR and ER measurement (Chebil et al., 2003), be performed centrally. The recent redesign of the TEAM trial to allow comparison of "switching" from Tamoxifen to Exemestane after 2-3 years directly with continuous Exemestane treatment provides an ideal platform for this hypothesis to be tested. The ability to select, at diagnosis, patients at high risk of early relapse on tamoxifen could provide the opportunity to tailor their adjuvant therapy differently, either in terms of an aromatase inhibitor or by supplying them with a HER family inhibitor such as Iressa or Herceptin to be used in conjunction with their endocrine treatment (particularly if they are pre-menopausal and hence not suitable for aromatase inhibitors).

4.4 HER1-3 activates membrane ER via phosphorylation. A role for phosphorylated ERα in tamoxifen resistance?

Having provided evidence that HER1-3 overexpression is associated with de novo tamoxifen resistance, attention must be focussed on the mechanisms and pathways involved. Several mechanisms have been proposed by which the type I RTKs may modify response to oestrogens and tamoxifen. As discussed previously there is a considerable body of biological evidence suggesting that cross talk between the HER family signalling pathways and the ER at several levels may be responsible. Signalling pathways activated by the type I RTKs have been shown to activate the ER by phosphorylation at sites including those on the AF1 region of the ER. As discussed previously, the serine phosphorylation site S118 on the AF1 region of ER is a target for phosphorylation by MAPK (Kato et al., 1995). MAPK is a component of the Ras-Raf-MAPKs pathway and over-expression or activation of this pathway has been associated with a poor clinical response to tamoxifen therapy (Gee et al., 2001) and to tamoxifen resistance *in vitro* (Benz et al., 1993; Kurokawa et al., 2000). In addition the phosatidylinositol-3 kinase (PI-3K) - Akt pathway has been also shown to mediate activation of the ER at serine 167 (also in the AF1 region) (Campbell et al., 2001) resulting in reduced sensitivity to tamoxifen *in vitro*. The consequence of phosphorylation of the ER in breast cancers may be to promote the known receptor agonistic activity of tamoxifen and to enhance transcription of genes involved in proliferation and genes which block apoptosis leading to tumour growth. Therefore our initial hypothesis was that we would be able to demonstrate a relationship between overexpression of the type 1 RTKS at the membrane and phosphorylated ER

at the nucleus, and that these patients would also be more likely to relapse on tamoxifen therapy.

Our results demonstrated the presence of phosphorylated ERα within the nuclear, cytoplasmic and membrane components of breast cancer cells. In terms of the nuclear staining we demonstrated results that initially may be thought to be out of keeping with our original hypothesis. We demonstrated no correlation between HER1-3 and phosphorylated nuclear ERα (ser167) and nuclear expression of pERαSer167 was associated with smaller tumour size and lower grade. In addition, HER1-3 was inversely related to expression of pERα at the Ser118 site. In fact, these results are in keeping with other recently published results using the same pERα118 antibody. Murphy et al determined pERα118 staining in 117 primary breast tumours from node-negative patients who were subsequently treated with adjuvant tamoxifen. They also demonstrated a positive correlation of nuclear pERα118 with expression of total ERα, PR positivity, lower grade and longer disease free survival (Murphy et al., 2004a; Murphy et al., 2004b). In addition, Gee et al have published preliminary results using this antibody (cohort size not described) which also demonstrated associations with improved disease free survival and PR status. (Gee et al., 2005). These studies, when combined with data presented here, would suggest that the effects of HER1-3 in promoting tamoxifen resistance may be mediated through mechanisms other than direct phosphorylation of the ER, at these specific sites, within the nucleus of breast cancer cells and that phosphorylated nuclear ER acts as a marker of an intact, functional, ERα signalling pathway. However these results are also complicated by the fact that Murphy et al reported an association between nuclear pERα118 staining and MAPK overexpression and Gee et al noted a direct association with Akt activity which has also been demonstrated by ourselves (Kirkegaard et al., 2005). The

possibility that ER function may be modified by either phosphorylation at other sites, or via phosphorylation of co-factors, cannot be currently excluded.

In addition to the widespread nuclear phosphorylated ER staining, we also found that a small but significant subset of patients demonstrated either pERα118 or pERα167 staining at the membrane. Whilst the Murphy group did not comment on the presence of membrane staining (Murphy et al., 2004a), Gee et al commented on its presence but failed to link it with any meaningful clinical relationships (Gee et al., 2005). In contrast to our results found with nuclear staining we have also demonstrated novel findings associating phosphorylated ERα at the *membrane* with HER1-3 overexpression and increased tumour size, which may indicate a different function for the ERα at this site.

In the late 1970s Pietras & Szego (Pietras and Szego, 1977) reported the presence of high affinity binding sites for oestrogen associated with the plasma membranes of the MCF7 human breast cell line. This observation was largely ignored until recently, when evidence for non-genomic actions of ERα increased, particularly as a postulated explanation for the more rapid signalling via signal transduction pathways normally activated by growth factor receptors, produced by oestrogen stimulation (Simoncini et al., 2000; Kousteni et al., 2001; Castoria et al., 1999). This non genomic signalling appears to take place outside the nucleus and now it appears that oestrogens are able to mediate some of these actions through a membrane associated oestrogen receptor (Watson and Gametchu, 1999; Simoncini et al., 2000; Pappas et al., 1995).

Recent work has suggested that these membrane receptors may be particularly important in the role oestrogen plays in 'survival' or anti-apoptotic mechanisms (Marquez and Pietras, 2001; Razandi et al., 2000). Oestrogenic stimulation of

membrane ERα has been shown to result in rapid stimulation of G proteins, protein kinase C, protein kinase A, MAPK and PI3K activation *in vitro* (Migliaccio et al., 1996; Simoncini et al., 2000; Zivadinovic and Watson, 2005; Razandi et al., 2003b; Filardo et al., 2002; Kelly et al., 1999). These kinase signals have been shown to activate nuclear ERα transcriptional activity (Sun et al., 2001a). Therefore the genomic and non genomic actions of ERα seem to be complementary and may even synergise via cross-regulatory interactions mediated by cross talk with growth factor pathways (Pedram et al., 2002). The physiological relevance of rapid extranuclear signalling has been provided by experiments showing that these actions contribute to the anti-apoptotic effect of oestrogen in bone (Kousteni et al., 2002) and to the rapid effects of oestrogen on vasodilation and protection of endothelial cells against injury (Figtree et al., 2003). Whilst in vitro evidence for this association between membranous ERα and the HER family and its downstream pathways has been shown in an increasing number of cell types, our results demonstrate for the first time a potential link between the 2 pathways in human breast cancer specimens.

In addition to this we also show a correlation between membranous phosphorylated ERα and disease relapse on tamoxifen at Ser118 but not at Ser167. There is some evidence that, in contrast to genomic activity (i.e. action of the ERα to promote gene transcription), non-genomic ERα activity can be stimulated by SERMS such as tamoxifen (Zivadinovic and Watson, 2005; Aronica et al., 1994; Cato et al., 2002). Therefore tamoxifen may be incapable of breaking (or even may stimulate) any cycle linking non-genomic and genomic ERα the growth factor pathways. Thus membrane bound ERα (in conjunction with the HER family) may be responsible for initiating tumour cell proliferation even in the presence of tamoxifen resulting in de novo tamoxifen resistance. Aromatase inhibitors however, act by depriving the

environment of oestrogen which would shut off both the nuclear and membranous ER activity resulting in termination of this cycling.

It is not clear from our results if the interaction demonstrated between phosphorylated ERα at the membrane and HER1-3 is true for pERα only or whether it represents an interaction with membranous ERα in general, regardless of phosphorylation status. Membranous ER staining has not traditionally been detected in routine diagnostic ERα immunohistochemistry staining and indeed none was seen in our study. This may reflect an antibody concentration effect whereby the much smaller numbers of ERα receptors at the membrane are overshadowed by the greater intensity of ERα nuclear staining. By detecting phosphorylated ERα only, this concentration effect may become less prominent, enabling detection of staining of pERα at the membrane.

These findings emphasise the importance of determining the non-classical actions of ERα particularly with respect to interactions with cell survival pathways. However further work is needed to confirm any correlation that HER1-3 associated membranous ERα may have with tamoxifen resistance. These results also have implications for establishing ERα status, particularly in the clinical diagnostic setting. Whilst, over the years, ERα detection has progressed from ligand binding assays to standardised IHC testing, more detailed analysis in terms of location and function of the receptor may become the norm for diagnostic testing in the future. As we become increasingly more aware of the complex nature of ERα and its interactions, these results only serve to highlight the difficulties involved in identifying the mechanisms behind tamoxifen resistance and subsequently in developing treatment strategies to overcome them.

4.5 HER4

We have demonstrated for the first time that the H4.77.16 antibody can be used successfully in formalin fixed tissue. In keeping with previous reports we also have found membranous, cytoplasmic and nuclear staining. The HFR-1 antibody is raised against an intracellular epitope aa1249-1264 (therefore will detect both the intact and cleaved form of 4ICD) whilst the H4.77.16 antibody is raised against an extracellular fragment (hence will detect only the full length HER4 protein or the cleaved extracellular domain at the cell surface). This difference in antigen site may explain the different staining patterns we have seen in terms of location; with each antibody appearing to select for distinct compartments. Thus HFR1 may select for cytoplasmic and nuclear HER4 ICD whilst H4.77.16 selects for membranous HER4 and possibly also HER4 being recycled in cytoplasm or nucleus. This ability to distinguish between site and function of HER4 and its fragments is particularly important with recent evidence highlighting the different functions of nuclear and mitochondrial HER4. We now know that whilst HER4 at the membrane is accountable for signal transduction, mitochondrial 4ICD nuclear HER4 appears to be involved in apoptosis mediation (Vidal et al., 2005) and nuclear HER4 is required for mammary gland development and lactation.

Despite the differences seen in staining location, we demonstrate that in terms of relationships with pathological variables, HER family members and prognostic importance, when tested under standardised conditions on the same set of tumours; both antibodies provide generally similar results. The exception to this is the association that H4.77.26 detected nuclear HER4 has with poorer survival. This

correlates with recently published results demonstrating that whilst membranous HER4 was associated with a good prognostic outlook, nuclear HER4 was associated with significantly shorter survival times (Junttila et al., 2005). Interestingly though this study was using the HFR1 antibody. Clearly this demonstrates that despite strong evidence for the role of the cleaved 4ICD, intact HER4 may also have a significant role to play.

We have not demonstrated any association between membranous HER4 and survival when considered alongside other HER family members or alone. However we did demonstrate an association between membranous and cytoplasmic HER4 and known poor prognostic variables. These results have not helped clarify the ongoing debate on the role of HER4 in breast cancer but may be explained by several factors. Our patients are a tamoxifen treated group, the majority of which are ER positive. Previous studies, including ours, have suggested a greater tendency for HER4 to be associated with ER positive tumours (Tang et al., 1999; Wright et al., 1992; Pawlowski et al., 2000). Within this generally less aggressive set of cancers, the effect of HER4 may be less pronounced.

The patterns of coexpression reported between HER4 and other family members may also explain some of the conflicting reports here and in published studies. In contrast to this study, in our previously reported series where HER4 was associated with good prognosis, we found minimal overlap between HER4 positive patients and other HER family members (Witton et al., 2003). In addition a recent large study has suggested that the "protective" effect of HER4 may be abrogated if expression of other members of the type I RTKs are also present (Abd El-Rehim et al., 2004). That may explain why on this occasion, where we demonstrate higher levels of co-expression, we no longer see the beneficial results on survival reported previously.

This is also consistent with data from cell lines showing that whilst HER4 can induce growth arrest and differentiation (Sartor et al., 2001; Williams et al., 2003), when co-expressed with other receptors, such as HER2 & HER3, signalling through these receptors promotes proliferation and overrides the effects seen when HER4 is expressed in isolation (Yarden, 2001b).

In conclusion, we have demonstrated that antibodies against 2 different HER4 receptor antigen sites identify clear differences in staining patterns. The differences in published reports may well reflect the differing abilities of antibodies to detect distinctly different HER4 functions. In the future careful attention to the location and consequent function of the HER4 fragment targeted is needed. It is possible that antibodies more specifically targeting the TACE or BH3 domain may prove valuable in further elucidating the functions of HER4 particularly in regard to impact on clinical outcome in breast cancer.

Chapter 5 Conclusion

The research presented here is particularly important because it demonstrates a time dependent element to tamoxifen resistance which is likely to reflect *de novo* resistance. Both HER1-3 and PR expression, in ER positive primary breast cancers, could now be used to identify patients who may exhibit *de novo* tamoxifen resistance. Furthermore both PR and HER1-3 appear to be time dependent predictors of risk of relapse with risk declining markedly after 3 years of tamoxifen treatment. These data, taken in context with data from the recent ATAC and IES studies, strongly support the hypothesis that HER1-3 and PR status identifies separate tamoxifen resistant tumour subsets, and that patients with HER1-3 expression or lacking PR expression are likely to benefit from early implementation of therapy with aromatase inhibitors. Conversely, these data would also suggest that ER/PR positive tumours lacking HER1-3 expression (over 50% of cases), could be treated by switching to an aromatase inhibitor following 2-3 years of tamoxifen treatment. Further research to determine the mechanisms relating to tamoxifen resistance and to test this hypothesis in the context of the redesigned TEAM trial will provide a valuable insight into the most appropriate future therapeutic options for differing sets of breast cancer patients. In addition to reinforcing the concept that members of the HER family are involved in tamoxifen resistance, we have also produced novel evidence with regard to how this may be taking place. Phosphorylated ER at the nucleus was not linked with the HER family or with tamoxifen resistance and instead appears to indicate a functional responsive ER. Detection of the presence of phosphorylated ER at the nucleus may in fact be more relevant if tested for after the commencement of tamoxifen therapy, with those still expressing it at that time being possible candidates for developing clinical tamoxifen resistance. Detecting the presence/absence of this marker during

neoadjuvant endocrine trials may shed more light on this in the future. With regard to the membranous phosphorylated ERα staining we have demonstrated relationships with both the HER family and with resistance to tamoxifen. This comes at a time when much attention has been focussed on membranous ERα signalling mechanisms and our results should contribute to this debate as it is the first time this relationship has been demonstrated in human breast cancer specimens. Further work is required to more closely examine the nature of this link between ERα at the membrane and the HER family. Particularly interesting are the Src family kinases which are known to be involved in cancer cell adhesion and migration. There is evidence suggesting that specific G proteins mediate the ability of oestrogen receptors at the membrane to activate matrix metalloproteinases via Src to lead to transactivation of EGFR. Analysis of expression of Src within our database has been planned.

While clinical knowledge with regard to the mechanisms behind *de novo* resistance is rapidly progressing, further attention should be directed towards identifying the causes of acquired resistance. This would include for example the scenario of PRpos/HERneg patients who initially do well on tamoxifen but who develop resistance at a later date. Do these patients develop secondary stimulation of the HER pathways, or do they have an unidentified marker that could have been identified at the outset? Whilst it appears that fewer ER positive patients have *de novo* resistance to aromatase inhibitors, will these agents also be linked to a later acquired resistance pattern as demonstrated in *in vitro* with regard to long term oestrogen deprivation (Chan et al., 2002)? Traditionally it has been difficult to obtain histological tissue from breast cancer recurrences (many metastasis occur in bone for example which is difficult to biopsy). However information gained from these samples may be invaluable with regard to determining causes of acquired resistance. A recent study

using detecting serum HER2 showed that 25% of patients treated with tamoxifen and 26% of patients treated with letrozole in a metastatic or advanced disease setting converted from serum HER2 negative to HER2 positive at the time of progression (Lipton et al., 2005). This switch in HER status has also has been detected in formalin fixed tissue (Gutierrez et al., 2005) (in very small numbers).

Further research into causes of tamoxifen resistance (both *de novo* and acquired) is particularly important for younger women with breast cancer. Whilst postmenopausal HER positive women have the advantage of being able to switch to aromatase inhibitors, premenopausal women may not. If the aetiology behind tamoxifen resistance in these younger women is clearly identified as being due to HER1-3 overexpression, then targeted therapy such as Herceptin or Iressa can be used. Many clinical trials using combinations of either aromatase inhibitors or tamoxifen with a tyrosine kinase inhibitors have begun in the metastatic setting and may soon be extended to the adjuvant treatment (Johnston, 2005). There are also other emerging dual-inhibitor therapies (Reid et al., 2007) such as Lapatinib, a tyrosine kinase inhibitor of both EGFR and HER2, which has shown clinical benefit in trastuzumab refractory metastatic breast cancer.

Breast cancer survival rates have improved markedly over the years largely because of better directed therapies based on clinical trial evidence. However continued improvements are likely when treatment can be initiated on individual, rather than population based, characteristics. The recent HERA trial results are evidence of targeted individual treatment based on a single biological marker. Identification of further markers will be able to help clinicians give detailed prognostic and treatment advice tailored to the individual cancer patient.

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Appendix I

Antibody	clone	isotype	species	source	specificity	dilution	antigen retrieval	incubation
ER	1D5	IgG1	mouse	DAKO	Western Blotting, no cross reactivity with ERbeta.(El Saati et al 1993)	01:50	Microwave tris EDTA buffer	30 min room temp
EGFR	31G7	IgG1	mouse	Zymed 28-0005	Western blotting. Recognises Variant III form of EGFR in addtion to wild type but not HER2		Trypsin/0.8% calcium chloride	1hr 25deg
HER2	Herceptest			DAKO	Validated by comparison to FISH amplification (Berner et al 2001)		citrate buffer 40min 96deg	30min 25deg
HER3	H3.105.5	IgG1	mouse	Neomarkers #MS-303-PABx	IHC frozen sections of pellets of MCF7/transfected HER3 cells. No cross-reactivity with HER4	1:20 dilution (50µg/ml)	none	2hrs room temp
HER4	HFR1	IgG1	mouse	Neomarkers #MS637-Po	Srinivasan et al 1998 Immunoprecipitation, western blotting and immunostaining. NH3T3/transfected HER4 cells. No crossreactivity with EGFR(A431 cells), HER3(3293/HER3 cells)	1:50 dilution (4µg/ml)	none	1hr room temp
HER4	H4.77.16	IgG1	mouse	Neomarkers #MS-270-PABX	IHC frozen sections of pellets of MCF7/transfected HER4 cells. No cross-reactivity with HER3 transfected cells	1:20 dilution (50µg/ml)	none	2hrs room temp
Phosphorylated HER2	PN2A pTyr1248	IgG1	mouse	Neomarkers Ab-18 #1072	DiGiovanna et al 1995 Immunoblotting of EGF stimulated SKBR3 cells. No cross reactivity with phospho EGFR	1:10 dilution	citrate buffer 20min 96deg	6hours room temp
Phosphorylated ER (ser118)	16J4	IgG2b	mouse	Cell signalling #2511	Western blotting. Band detected with E + Heregulin stimulated MCF7 cells only	1:300 dilution	citrate buffer 20min 96deg	overnight 4deg
Phosphorylated ER (ser167)		polyclonal	rabbit	Cell signalling #2514	Western blotting. Band detected with E + Heregulin stimulated MCF7 cells	1:10 dilution	citrate buffer 20min 96deg	overnight 4deg

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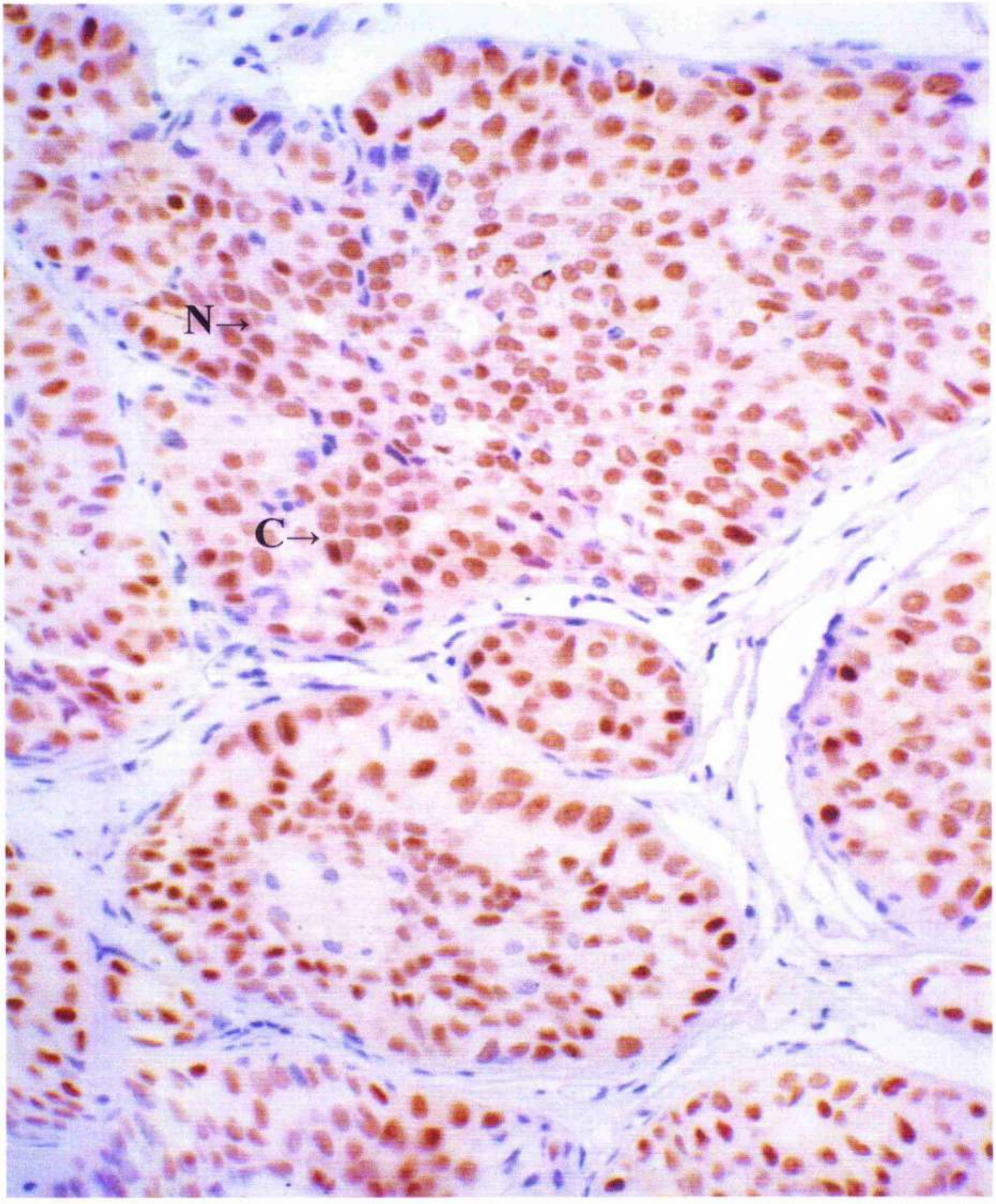
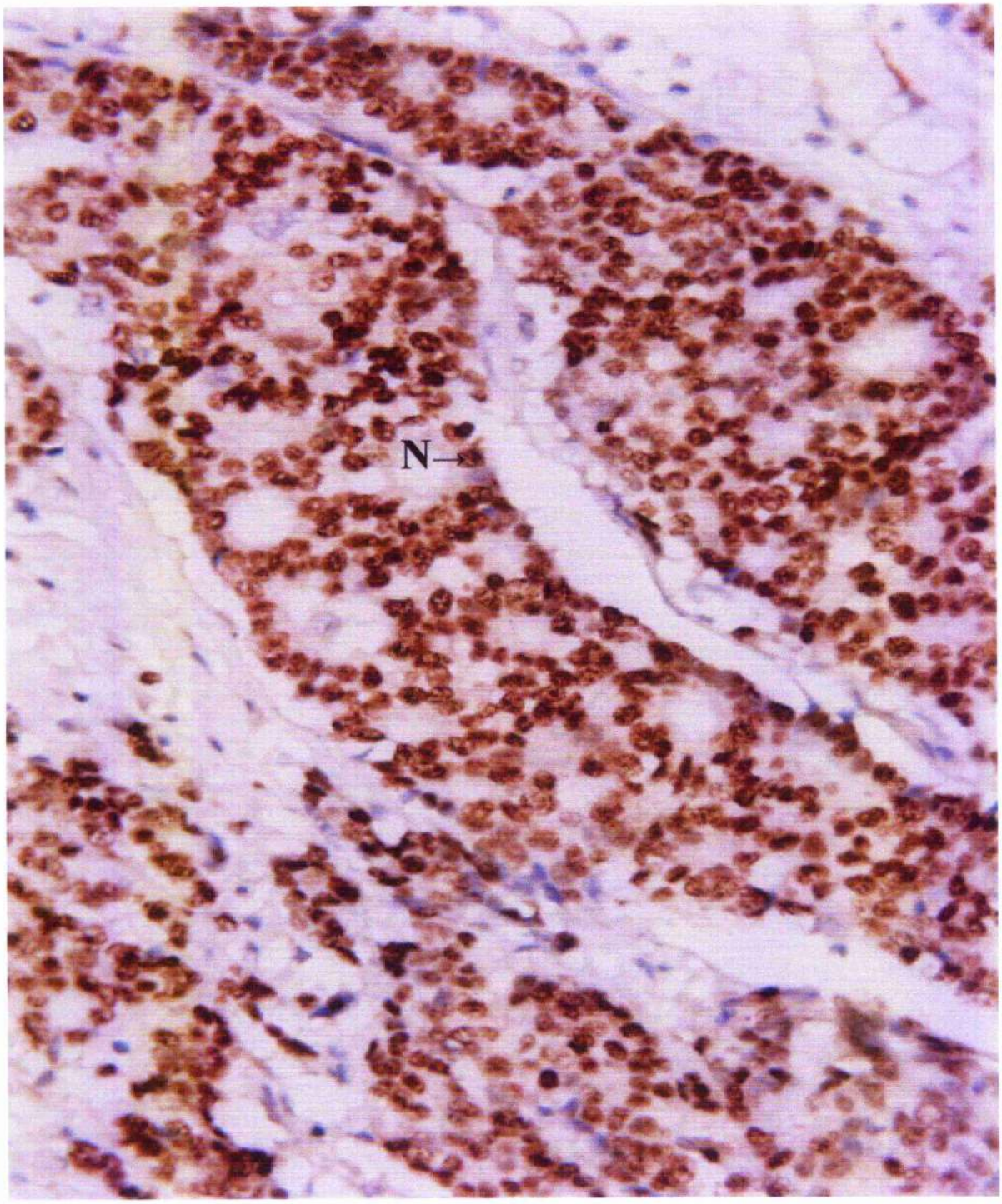
Appendix II

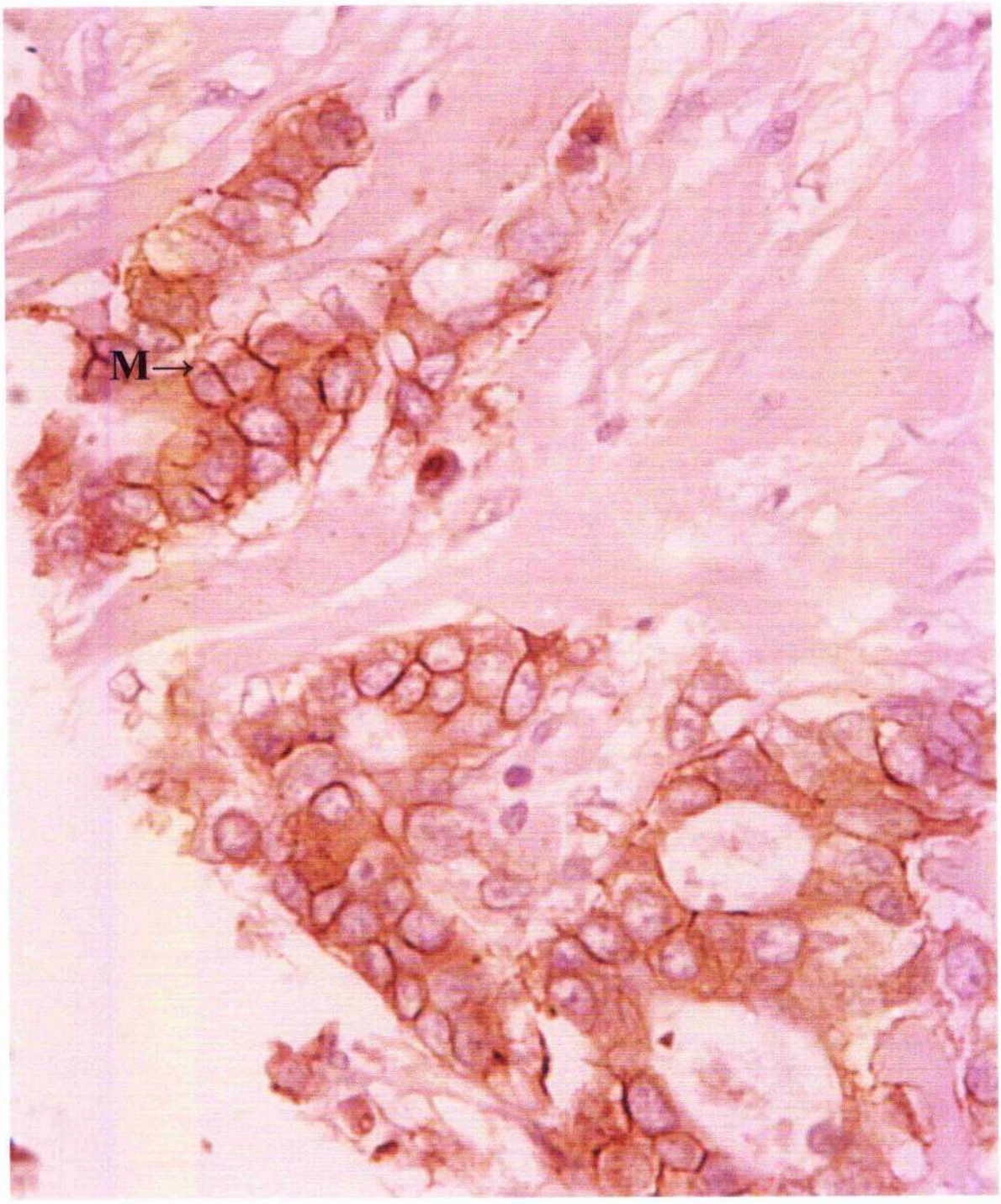
Photomicrographs of immunohistochemical TMA staining



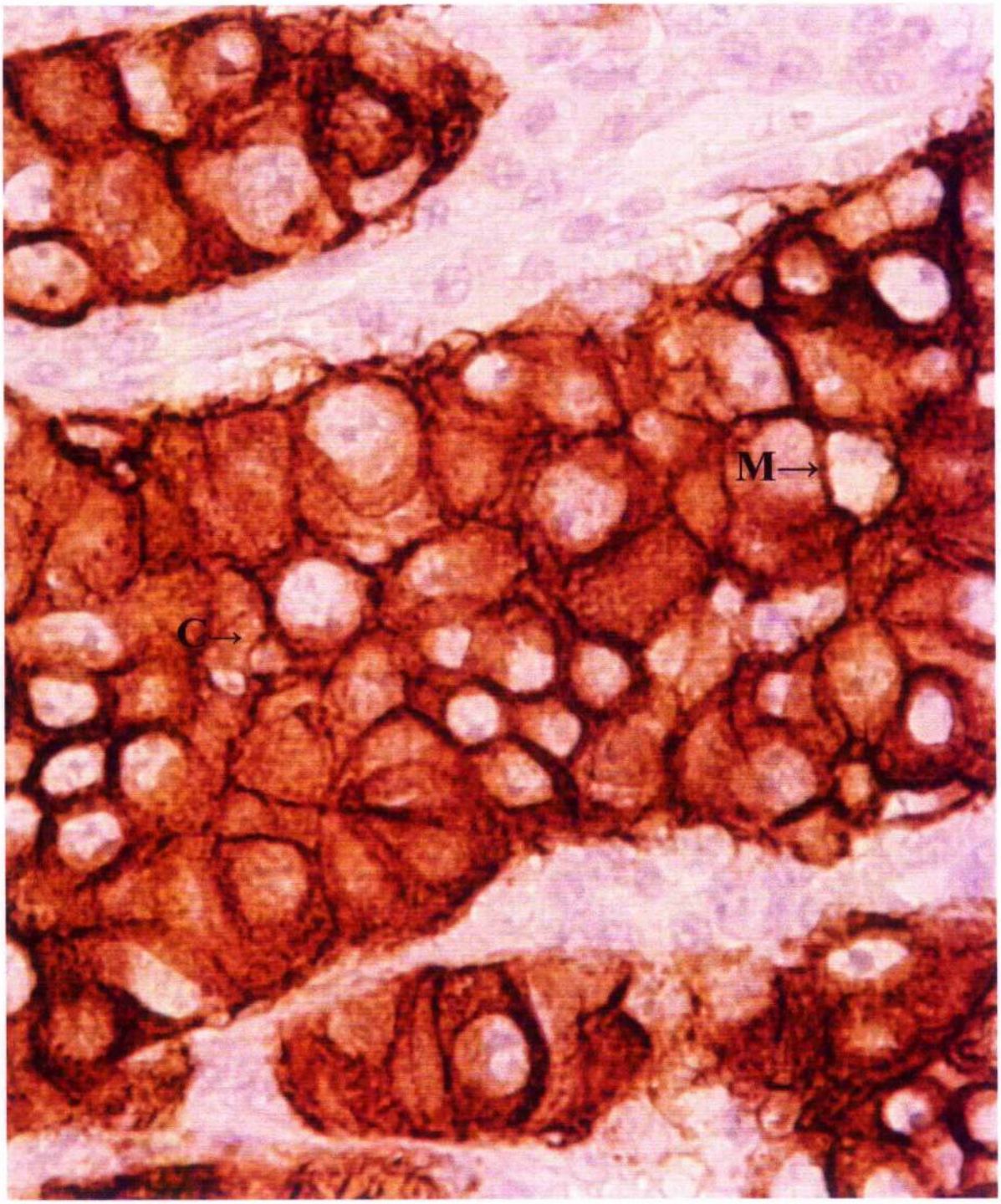
*I.a. Nuclear (N) and weak cytoplasmic (C) ER staining (magnification *100)*



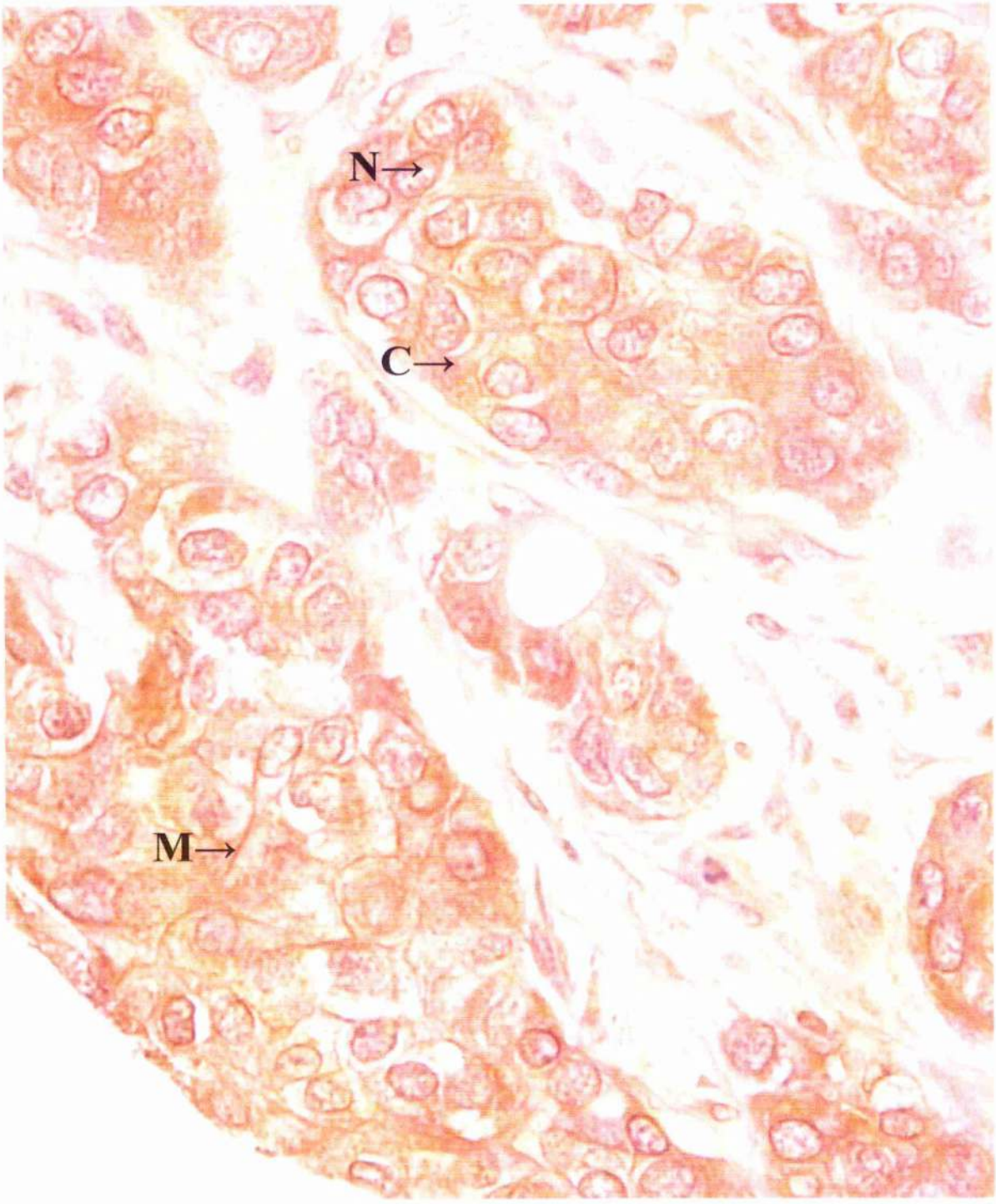
*I.b Nuclear (N) PR staining (magnification *100)*



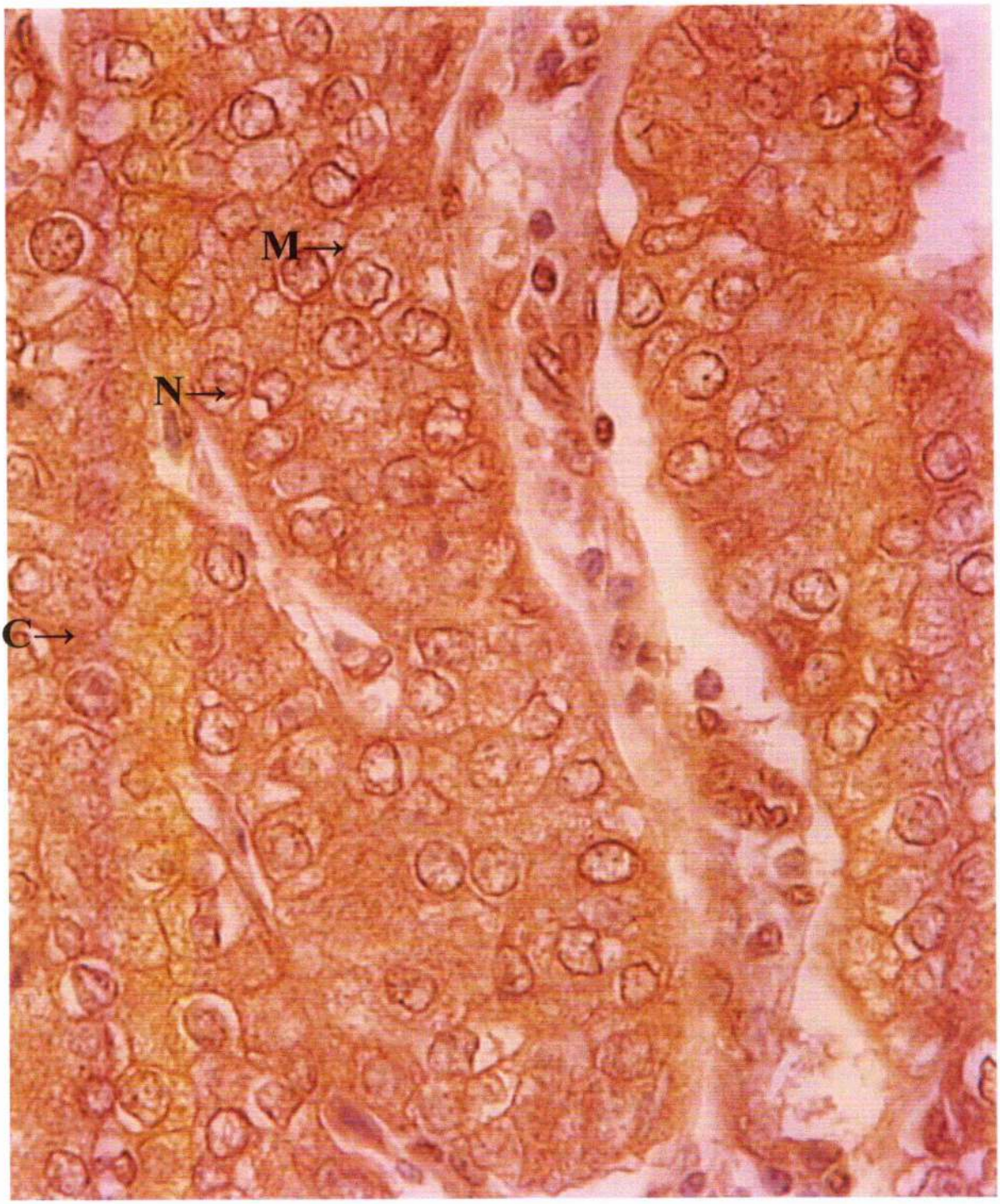
*1.c Membranous (M) HER1 staining (magnification *200)*



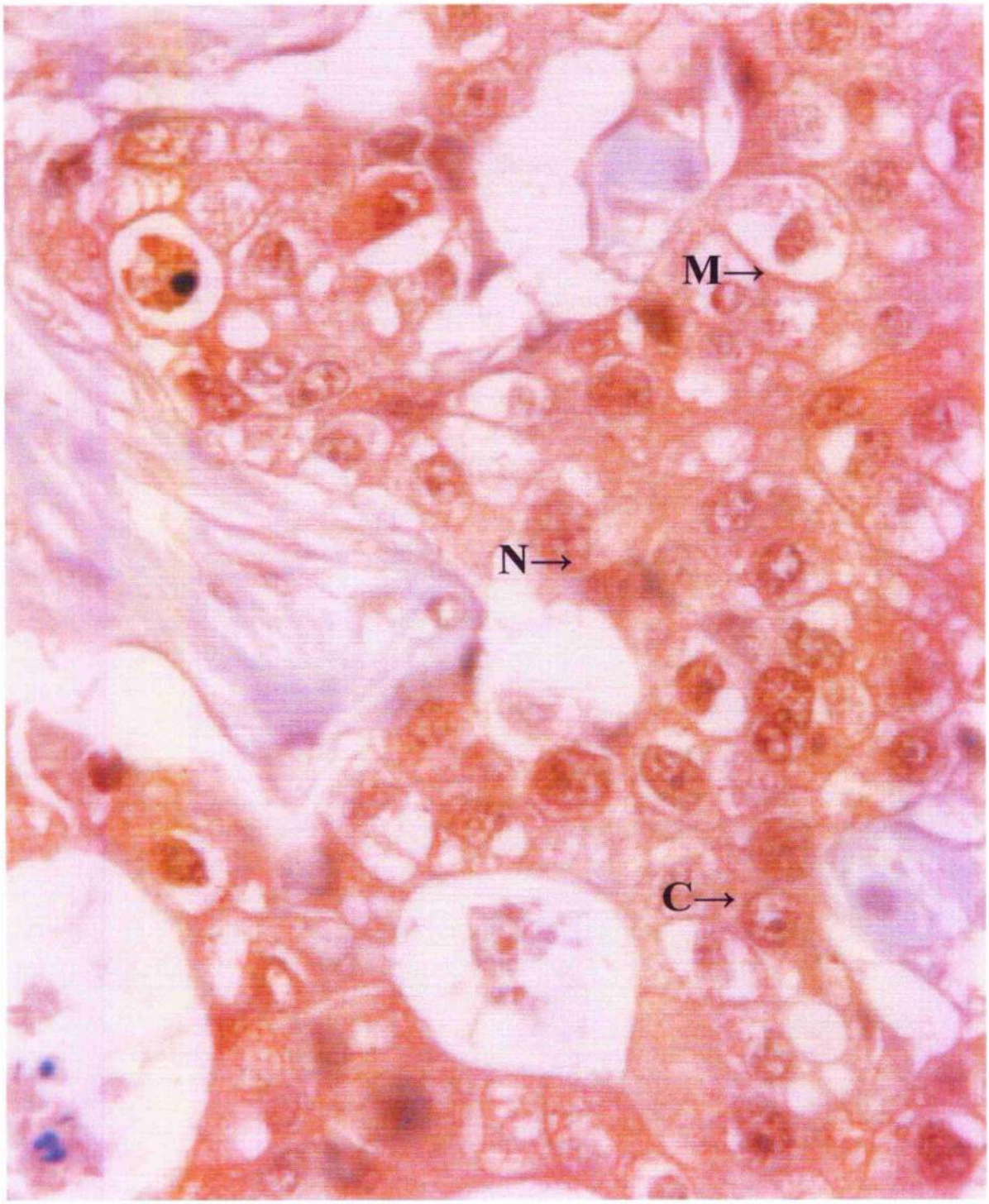
*I. d Membranous (M) and cytoplasmic (C) HER2 staining (magnification *200)*



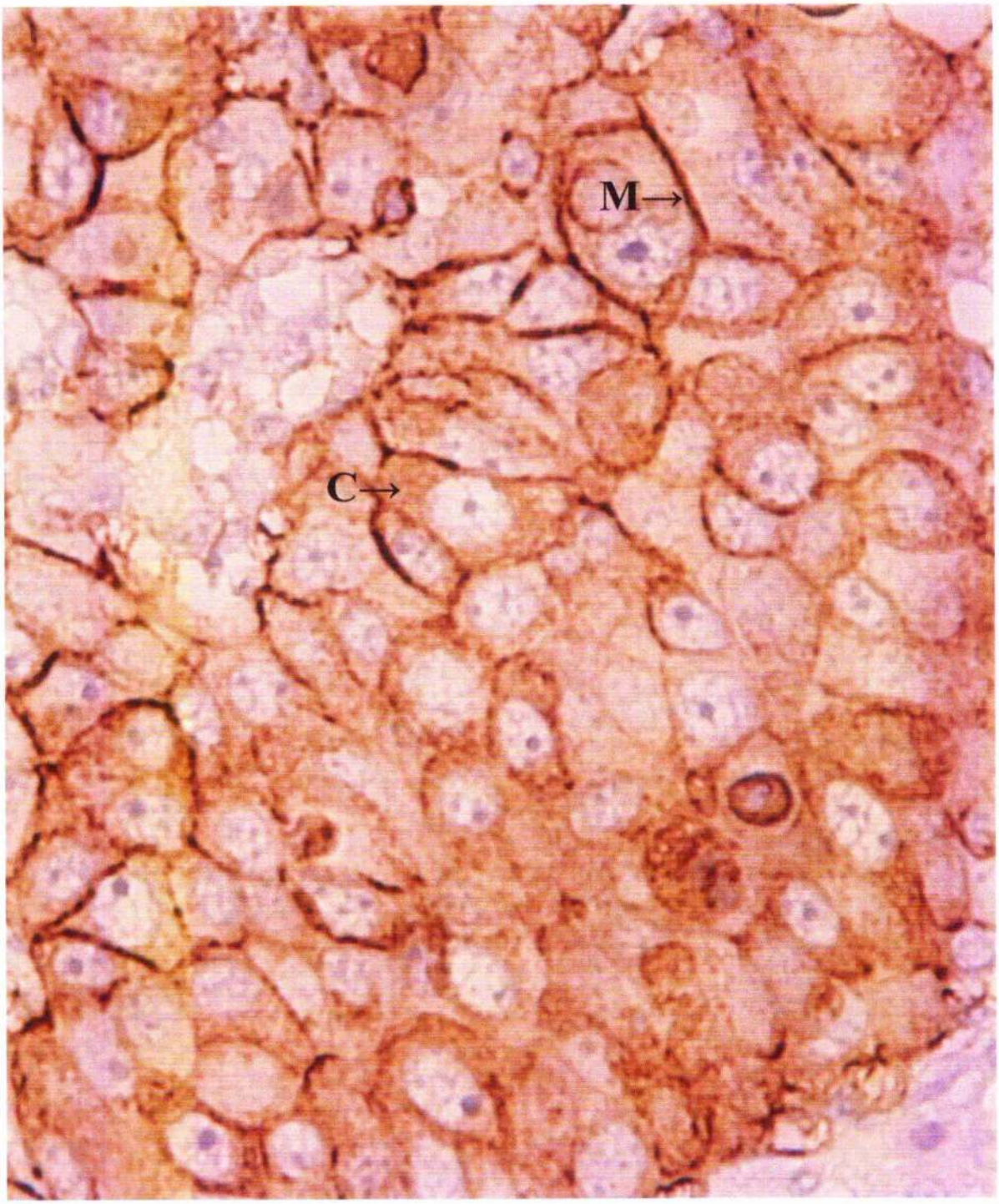
*I. e Membranous (M), cytoplasmic (C) and weak nuclear (N) HER3 staining (magnification *200)*



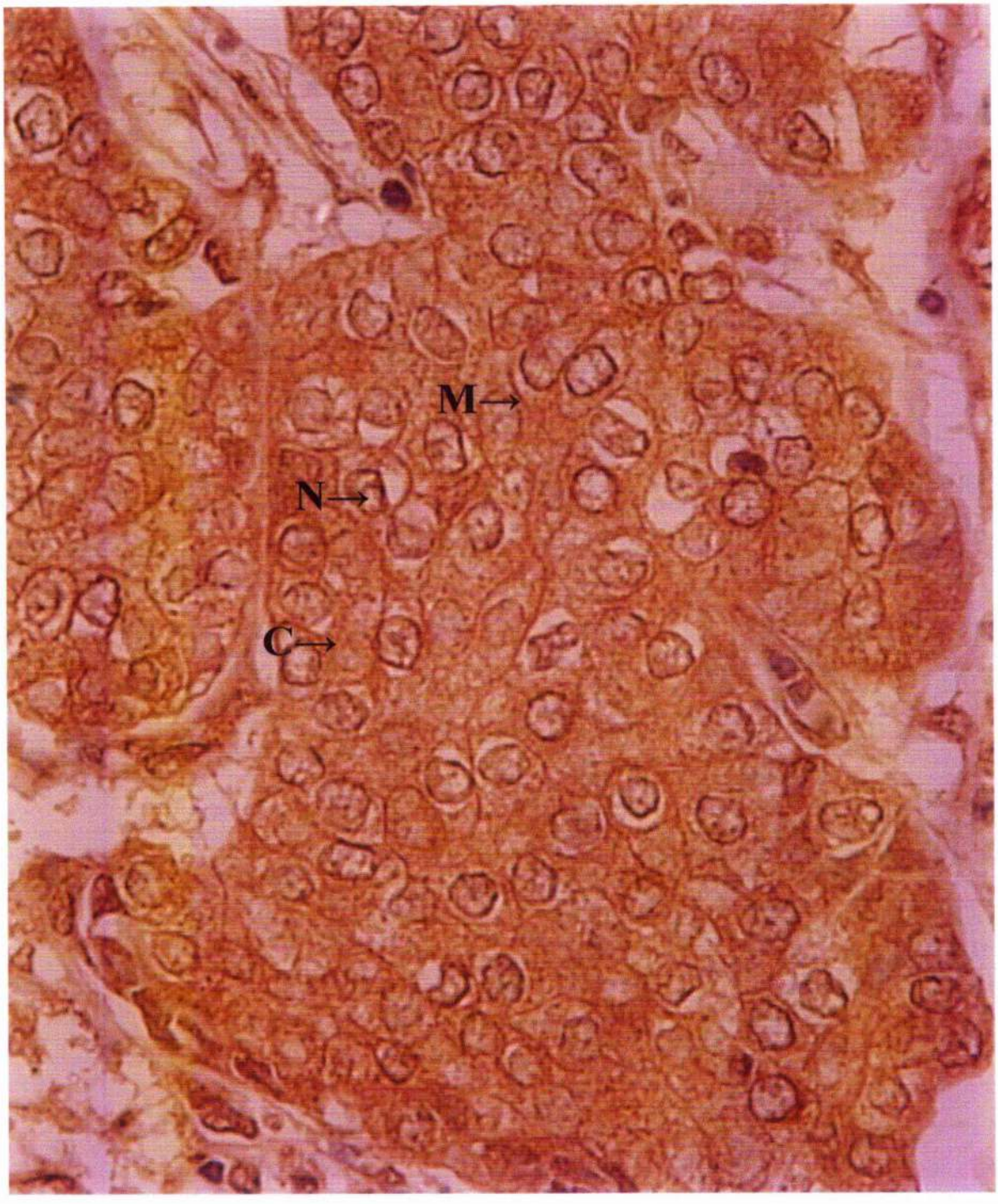
*I. f Membranous (M), cytoplasmic (C) and nuclear (N) HER4 staining (H4.77.16 antibody) (magnification *200)*



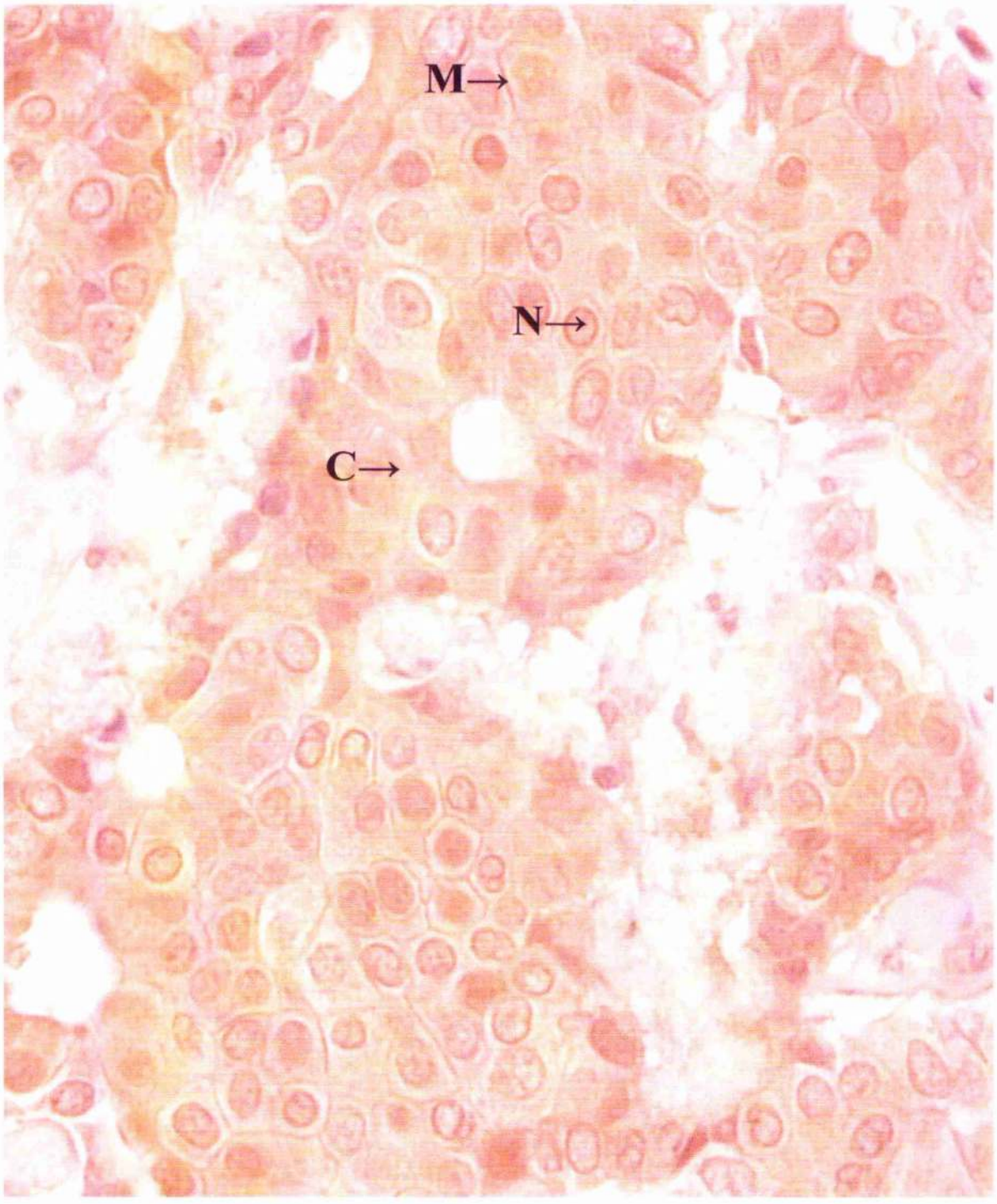
*I. g Membranous (M), cytoplasmic (C) and nuclear (N) staining for phosphorylated ER (Serine 118) (pERαSer118) (magnification *200)*



*I .h Membranous (M) and cytoplasmic (C) staining for phosphorylated ER (Serine 167) (pERαSer167) (magnification *200)*



*I.i Membranous (M), cytoplasmic (C) and nuclear (N) staining for HER4 (HFR1) (magnification *200)*



*I.j Membranous (M), cytoplasmic (C) and nuclear (N) staining for Phosphorylated HER2 (pHER2) (magnification *200)*