Putative biomarkers of tamoxifen response in breast cancer

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Abbreviations

aCGH: array comparative genomic hybridization

ASCO: American Society of Clinical Oncology

BAC: bacterial artificial chromosome

ECOG status: Easter Cooperative Oncology Group status

ER: estrogen receptor-α

FISH: fluorescence *in situ* hybridization

IHC: immunohistochemistry

LB: lysogeny broth

NCIC: National Cancer Institute of Cancer

P: placebo

PR: progesterone receptor

RCT: randomized controlled trial

T: tamoxifen

TF: transcription factor

TMA: tissue microarray

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Abstract

Several molecular factors have been associated with the sensitivity of breast cancer to tamoxifen (T): Pax2 can mediate the repression of ERBB2 expression by the tamoxifen-ER complex; TC21 is a member of the Ras superfamily associated with increased recurrence in tamoxifen-treated patients; CCND1 (Cyclin D1) is frequently amplified in breast cancers resistant to this drug; and RSF1 is often co-amplified with CCND1 on chromosome 11q. Here, validation of the predictive value of these biomarkers is explored in the MA.12 trial, in which premenopausal women with node-positive/high-risk node-negative early breast cancer, of any hormonal status, were randomized to T (20 mg/day) or P for 5 years after receiving adjuvant chemotherapy. Overall survival (OS) and relapse-free survival (RFS) were evaluated. Pax2 and TC21 (measured via IHC) expression were not associated with OS or RFS. However, in the ER+ subgroup, patients with low TC21 expression derived greater benefit from T compared with P than patients with high TC21 expression, as measured by RFS. No significant interaction with treatment was observed with CCND1 or RSF1 amplification (measured via FISH), although patients with high RSF1 copy number showed a trend toward no benefit from T. Although none of the 4 biomarkers for T response were completely validated in this clinical trial, the predictive effect of TC21 expression and RSF1 amplification deserve further study.

Abrégé

Plusieurs facteurs moléculaires ont été associés à la réponse du cancer du sein au tamoxifène (T): Pax2 est impliqué dans la répression de l'expression de ERBB2 par le complexe tamoxifène-recepteur des oestrogènes (ER); TC21 fait partie de la super-famille des protéines Ras, dont l'expression a été associée à un taux de récidive de cancer du sein plus élevé chez les patientes traitées avec le T ; CCND1 (cycline D1) est fréquemment amplifié dans les cancers du sein resistants au tamoxifène ; et RSF1 est souvent co-amplifié avec CCND1 sur le chromosome 11q. J'ai entrepris la validation de la valeur prédictive de ces biomarquers dans l'étude MA.12, dans laquelle des femmes préménopausées avec un cancer du sein primaire, avec des metastases axillaires ou avec un cancer du sein à haut risque mais sans metastases axillaires, étaient randomisées soit au T (20 mg/jour) ou à la prise de Placébo pour 5 ans après avoir reçu la chimiothérapie adjuvante. La survie globale (OS) et la survie sans récidive (RFS) ont été évaluées. L'expression de Pax2 et de TC21 (mesurée par l'immunohistochimie) n'était pas associée avec OS ou RFS. Cependant, parmi les patientes avec des tumeurs positives pour le récepteur d'estrogène, celles dont la tumeur avait une expression réduite de TC21 avaient une meilleure survie avec T qu'avec le P comparé aux patientes avec une tumeur ayant une expression élevée de TC21. Il n'y avait pas d'interaction statistiquement

signicative avec l'amplification de CCND1 ou du RSF1 (mesurées par FISH), mais les patientes porteuses de tumeurs avec une amplification du gene RSF1 élevée avaient une tendance à ne pas bénéficier du traitement avec le tamoxifène. Bien qu'aucun des biomarqueurs n'ait été totalement validé dans cette étude, l'effet prédictif de l'expression de TC21 et de l'amplification de RSF1 méritent des recherches plus approfondies.

1. Introduction

Breast cancer is the most common cause of cancer deaths in Canadian women and is the most frequently diagnosed cancer in women in both the developed and the developing worlds, with 1.5 million women diagnosed with this cancer annually (Anderson et al., 2008). 50-80% of breast cancers are estrogen receptor-positive (ER+) and rely on its ligands, the estrogen hormones estradiol and estrone, to grow (Neubauer et al., 2008). Tamoxifen, an adjuvant endocrine therapy that blocks the ability of the estrogen receptor to bind estrogen, has thus become an important drug in combatting this disease. In women with early stage ER+ breast cancer, tamoxifen reduces the risk of recurrence by about half and the risk of breast cancer death by about a third. These benefits are largely independent of chemotherapy, age, progesterone receptor status, or other tumour characteristics (C. M. Kelly et al., 2010). However, not all patients benefit from tamoxifen. In advanced disease, only 50% of the patients with ER+ primary disease respond to tamoxifen, and eventually, all patients experience progression (van Agthoven et al., 2009). While tamoxifen has been the gold standard for hormonal therapy and has saved an estimated half a million women (W. Schroth et al., 2009), ER positivity is far from a perfect predictor of the efficacy of this treatment.

It is well established that breast cancers progress through the accumulation of genomic and epigenomic aberrations (K. Chin et al., 2006). These include gene amplifications and dysregulated protein expression, and many candidate genes and proteins involved in breast tumour progression have been explored in the literature. The 11q13 locus has been shown to be amplified in about 15% of breast cancers and is associated with a decreased relapse-free survival (RFS) in patients treated with tamoxifen as compared to placebo. Of the genes found in 11q13, CCND1, the gene encoding the G1/S protein cyclin D1 is the most validated predictor of tamoxifen response (Jirstrom et al., 2005). It is commonly assumed to be the driver gene of this amplicon, meaning that its amplification contributes to cancer initiation and/or progression (Ji et al., 2010). PAK1, spanning 11q13-14 and encoding the p21-activated kinase-1 protein involved in cell motility and morphology (Bostner et al., 2007), has also been explored as a possible driver of amplification of the long arm of chromosome 11. However, array comparative genomic hybridization (aCGH) performed in our lab on 90 breast tumours showed tight 11q14 amplifications that excluded PAK1. Expression data of the genes in the amplified region revealed that RSF1 may instead be the intended target of this amplification. In ovarian cancer, amplification of this gene confers poor survival (Brown et al., 2008).

The protein PAX2 has also shown promise in predicting tamoxifen response in breast cancer. PAX2 is part of the PAX family of proteins that encompasses transcription factors that regulate tissue development and cellular differentiation in embryos (Silberstein, Dressler, & Van Horn, 2002). It is expressed in 40-60% of tumours and tamoxifen-treated patients with PAX2+ tumours have been shown to have a significantly increased RFS (Hurtado et al., 2008).

Ras proteins are suggested to be involved in tamoxifen resistance, and R-Ras2, or TC21, is overexpressed in about 45% of breast cancers (Clark, Kinch, Gilmer, Burridge, & Der, 1996). A GTPase, TC21 is important in signal transduction pathways controlling cellular proliferation (Erdogan, Pozzi, Bhowmick, Moses, & Zent, 2007). Patients with high cytoplasmic TC21 expression have been shown to have increased recurrence rates when treated with tamoxifen as compared to placebo (Rokavec et al., 2008).

Additional biomarkers predicting response to tamoxifen are required so that women can be directed to the most effective treatment. As more therapeutic options become available to women with breast cancer, the choice of whether to choose tamoxifen as an adjuvant therapy becomes more complex. The information that these putative biomarkers can provide would be valuable to avoid unnecessary side effects and to personalize patient care.

2. Literature Review

2.1 Cancer

Cancer refers to a group of diseases in which abnormal cells divide uncontrollably and are able to invade other tissue (Institute, 2010). They are named for the organ or type of cell in which they originate (e.g. colon cancer) and can be further classified according to their histological type (e.g. epithelial) and molecular characteristics (e.g. HER2+).

Normal cells of the body grow and divide in a tightly controlled and finite manner. Cells are programmed so that damage induces apoptosis or signals of dysfunction to be presented to immune cells. If the pathways that regulate a cell's proliferation are affected and the mechanisms to fix these errors are impaired, the cell may begin to divide uncontrollably.

The change from a normal to a malignant tumour, with the possibility of a benign lesion in between, occurs in a multi-step clonal selection process. Genetic alterations can be inherited as germline mutations or be somatic (acquired) as a result of cumulative exposure to physical and chemical carcinogens, which can also cause DNA damage or act as epigenotoxic agents and affect DNA methylation, mRNA stability, or protein phosphorylation. Most of these mutations will kill the cell or be repaired, but those mutations that cause uncontrolled cellular proliferation and/or aberrant apoptosis (Russo, 2010) are passed on to

progeny, and it is estimated that an average of 11 mutations are required to form the malignancy known as cancer (Sjöblom et al., 2006). These mutations either inactivate tumour suppressor genes, also known as “caretaker” genes, or inappropriately activate proto-oncogenes (Lodish, 2007).

Oncogenes are usually kept under tight control and used at very specific times in a cell’s life (Crocker & Murray, 2003). In cancer, they are either activated via point mutations (an alteration of a single nucleotide base pair), chromosomal translocations (a rearrangement of genes between non-homologous chromosomes), or gene amplification (an increase in the number of copies of a gene) (Braun & Anderson, 2007). For example, in 50% of colorectal cancers and 95% of pancreatic cancers, there is a RAS point mutation (Nakano et al., 2001); >95% of chronic myelogenous leukemia patients have the Philadelphia chromosome, a translocation between chromosomes 9 and 22, that brings ABL and BCR together, removing ABL from its suppressive promoter and creating chimeric RNA and protein (Mühlmann, 1998); and amplifications of ERBB2 are found in up to 20% of breast cancers (Oncology, 2006).

Amplification represents a defect in DNA replication. In cancer, DNA amplification is thought to be initiated by a chromosome break and followed by inappropriate cell-cycle progression in the presence of this damaged DNA. One proposed mechanism of DNA amplification is based on double minute (small

extrachromosomal DNA fragments) formation, possibly resulting from a collapsed replication bubble that causes an inability to progress past a lesion in the DNA (Albertson, 2006). The episome undergoes extrachromosomal replication and may subsequently reintegrate into a chromosome. Another possible mechanism of DNA amplification is based on breakage-fusion-bridge (BFB) cycles, initiated when broken sister chromatids fuse, leading to a dicentric chromosome that then breaks apart when the two centromeres are pulled to opposite spindle poles in anaphase. Depending on the site of the break, the result is a chromosome with an inverted duplication at the terminus. Because this chromosome has a broken end, the amplification process can repeat in subsequent cell divisions (Kwei, Kung, Salari, Holcomb, & Pollack, 2010).

2.2 Breast cancer

Breast cancer is the most prevalent cancer in the world, and it is estimated that more than one million women are diagnosed with breast cancer every year and more than 410,000 die from the disease (Coughlin & Ekwueme, 2009). In Canada alone in 2008, 431 women were diagnosed with breast cancer and 102 women died of it every week (Canadian Cancer Society, 2007). Breast cancer alone directly costs the Canadian health care system $309 million per year (Patra, 2007).

Breast cancer is a collection of breast diseases that have diverse histopathologies, genetic and genomic variations, and clinical outcomes (Vargo-Gogola & Rosen, 2007). Cells within the breast, most commonly in the lining of the milk ducts or in the milk-producing glands, become abnormal and divide without control or order (N. L. o. Medicine, 2010).

The female breast is composed of lobules (milk-producing glands at the ends of the lobes) and ducts (milk passages that transfer milk to the nipple) interspersed with fibro-adipose connective tissue (Gudi, 2007; Imaginis, 2010). Within the lobules are the functional units of the breast, the terminal duct-lobular units (TDLUs) (U. o. V. S. o. Medicine, 2010), where the majority of cancers arise (Kopans, 2007). These are completely lined by two cell types: the inner epithelium, with secretory and absorptive functions, and the outer myoepithelial cell layer, both of which adhere to a basement membrane (Gudi, 2007). The TDLUs are embedded in specialized, hormonally responsive connective tissue stroma, the intralobular stroma (U. o. V. S. o. Medicine, 2010). Fibrocystic disease, hyperplasias, and *in situ* and invasive carcinomas occur in the TDLU, while papillomas, duct ectasia, and a small proportion of carcinomas occur in the large duct system (Gudi, 2007).

2.2.1 Benign breast disease

The vast majority of the lesions that occur in the breast are benign (Guray & Sahin, 2006). Benign proliferative breast lesions (Institute, 2010) are mostly dysplastic (abnormally proliferative and atypical in appearance) and can be pre-malignant and increase breast cancer risk by 4 to 5 times, but benign disease otherwise has little to no effect on the risk of future malignancy (Society, 2009). Benign tumour cells do not spread by invasion or metastasis and are separated into three major pathological categories (Hartmann et al., 2005):

- Non-proliferative lesions – the most common benign disease (Vaidyanathan, Barnard, & Elnicki, 2002) and not linked with the overgrowth of breast tissue (Society, 2009)
- Proliferative without atypia – linked with cellular growth in the ducts or lobules and may raise the risk of breast cancer slightly (by 1.5 to 2 times) (Society, 2009), especially with family history (Hartmann, et al., 2005)
- Atypical hyperplasias – linked with excess growth of cells in the ducts or lobules with abnormal cellular morphology and raise breast cancer risk by 4 to 5 times (Society, 2009)

Depending on the benign disease, antibiotics can be prescribed, it may be removed by fine needle aspiration or surgery, or it can be left in the breast and therapy recommended for symptom relief, if necessary.

2.2.2 Malignant breast disease

Malignant tumours differ from their benign counterparts in that they resemble less the normal tissue in terms of growth rate, systemic effects, macro- and microscopic appearance, histology, ploidy, and protein expression. Cancers can invade and metastasize, and sustained angiogenesis is required in most malignant lesions. They are self-sufficient in growth signals and insensitive to inhibitory stimuli. They evade apoptosis, have unlimited replicative potential, and have defects in genetic repair, which leads to further genetic instability. If in the duct, the stage after benign atypical hyperplasia is ductal carcinoma *in situ* (DCIS). If in the lobule, and without breaching the basal membrane, this stage is considered pre-malignant and therefore not cancer (Research, 2009). DCIS is defined as a tumour that fills at least two ducts completely without breaching the myoepithelium of the duct and is at least 2mm in size (Medicine, 2011). Subsequently, invasive carcinoma can be ductal or lobular, with the latter being less common. These cells have grown through the walls of the milk ducts or glands into the adjacent fatty tissue (Agency, 2010). Lastly, cancer cells from the primary site can migrate to create further tumours in distant organs to create metastases (Fidler, 2003).

Most deaths from cancer are due to metastases that are resistant to conventional therapies (Fidler, 2003) rather than the burden of the primary neoplasm. Breast

cancer cells are most likely to metastasize to the bone, lungs, or liver. Patients with breast cancer are at risk for metastasis for their entire lifetime, and the heterogeneous nature of breast cancer metastasis makes it difficult not only to determine a cure, but also to assess risk factors for metastasis (Weigelt, Peterse, & van't Veer, 2005).

2.2.3 Diagnosing breast cancer

Diagnosing the type of breast cancer is important to direct treatment and determine prognosis. Many women may be able to palpate a lump or notice breast changes, including skin irritation of the breast or under the arms or changes in breast size or shape. The colour or feel of the skin of the breast, areola, or nipple (dimpled, puckered, or scaly) can change. Women can also have discharge from, or erosion, inversion, or tenderness of, the nipples (Consult, 2010). A physician will send a patient presenting these symptoms for a mammogram or, if particularly high-risk, an MRI in order to make a diagnosis.

However, by the time these symptoms are noticed, micrometastases may have begun to spread. All provinces and territories in Canada except for Nunavut have organized breast cancer screening programs to try to detect asymptomatic breast cancer via bilateral, two-view screening mammograms. Half of all new breast cancer cases are diagnosed among women aged 50 to 69, and delivery of

routine, high-quality breast screening to this group can reduce breast cancer mortality rates by as much as one-third (Canada, 2006).

Depending on the results of these imaging tests, a breast biopsy may be recommended. Biopsy is the only definitive way to determine whether cancer is present (Imaginis, 2011a). A palpable lesion can be excised surgically via a lumpectomy or (modified) mastectomy or biopsied with fine-needle aspirate or core needle biopsy. Nonpalpable lesions can be excised under x-ray or ultrasound guidance (Institute, 2011). Nipple aspiration and ductal lavage can also be used to sample epithelial cells from the ducts (Visvanathan et al., 2007).

If the cancer is invasive or the patient presents with an exceptionally large or aggressive DCIS tumour, an axillary (underarm) node dissection or the newer sentinel lymph node biopsy is performed to determine the extent to which the disease may have metastasized. Because drainage of the lymph from the breast travels first to the axillary lymph nodes, breast cancer often first spreads to that site (Veronesi et al., 1999). The disease status of the axillary lymph nodes is the most significant prognostic factor for patients with early-stage breast cancer (Lyman et al., 2005).

A pathologist will analyze the histologic section or smear that was taken from the biopsy, tumour, and/or the lymph nodes to characterize the specimen and help determine treatment. The pathology report covers stage (Appendix 1) and grade

(Appendix 2), which is comprised of the TNM staging system (based on the extent of the tumour [T], whether cancer cells have spread to regional lymph nodes [N], and whether distant metastasis [M] has occurred, described as stage 0, stage I, stage II, stage III, or stage IV) (Edge, Cancer, & Society, 2010) and of the Elston and Ellis modification of the Bloom-Richardson histologic grading scheme (Radiology, Surgeons, Pathologists, & Oncology, 2007) (based on the frequency of cell mitosis [rate of cell division], tubule formation [percentage of cancer composed of tubular structures], and nuclear pleomorphism [change in cell size and uniformity]) (Singh et al., 2007), respectively. The report will also describe the patient's hormone receptor status.

2.3 Hormones and Hormonal Therapy in Breast Cancer

Hormones are chemical messengers meant for long-distance signalling. Hormones are secreted by endocrine glands and travel through the bloodstream to their target cells. Steroid hormones, one of the three hormonal classes, are made from cholesterol in the gonads and adrenal glands. They are fat-soluble and can therefore pass through the cell membrane to bind with a steroid hormone receptor (Losel & Wehling, 2003).

Normal breast tissue depends on steroid hormonal signalling by estrogen for growth in preparation for and during pregnancy. Estrogen signalling is likewise required for uterine epithelial proliferation (Zhou, Ng, Adesanya-Famuiya,

Anderson, & Bondy, 2000). The dependency on estrogen for growth often persists in breast tumours, and this requirement is exploited to treat cancer.

2.3.1 Discovery of Estrogen and Estrogen Receptor (ER) in Breast Cancer

In the 1890s, before estrogen was discovered, it was observed in premenopausal women with breast cancer that, during the menstrual cycle, there were changes in the size of metastases. It was found that removing the ovaries of premenopausal women with breast cancer caused about one third of patients to have significant shrinking of the tumour despite having metastatic disease (Beatson, 1983). By 1900 it was estimated that, after analysis of patients treated with oophorectomy for breast cancer in Great Britain, only one third of metastatic tumours responded to oophorectomy (Jordan, 2009). It was then shown that oophorectomy could also prevent the development of breast cancer in mice (Lathrop & Loeb, 1916). The method by which oophorectomy treated and prevented breast cancer was revealed when estrogen was discovered in ovarian follicular fluid (Allen & Doisy, 1983) and that estrogen could induce mammary tumours in mice (Lacassagne, 1936).

In the 1950s and 1960s, treatments for breast cancer focused on removing the ovarian source of hormones in premenopausal women and among postmenopausal women, the adrenal glands and/or the pituitary controlling them. Again, about one third of patients responded favourably, but the other two thirds

that had undergone these serious operations were not helped by them (E. V. Jensen, Jacobson, Walf, & Frye, 2010). ER was the first receptor to be extracted and assayed by radioligand binding assays in 1962. Elwood Jensen was able to used radiolabelled estradiol to follow the fate of the hormone in rats, noting that it accumulated in target tissues (uterus and vagina) but not in non-target tissues (muscle, kidney, liver), and that it was chemically unaltered. This disproved the notion that oestrogens were metabolised to somehow provide energy for the biological response provoked (i.e., growth stimulation of breast cancer) (Elwood V. Jensen & Jordan, 2003). Finally, it was found that ER levels in tumour biopsies correlated with clinical outcomes in breast cancer: breast tumours without ER were unlikely to respond to endocrine ablation, and so the ER assay became the standard of care in the 1970s to predict response to surgical castration (Jordan, 2009).

2.3.2 ER Mechanism of Action

The ER acts primarily as a nuclear transcription factor (TF), located mostly in the nucleus regardless of the presence of estrogen. This is in contrast to most steroid receptors, which translocate from the cytoplasm to the nucleus upon activation by their ligand (Conzen, 2010). The key components of the ER are the DNA-binding domain, which binds with high affinity and specificity to DNA sequences (estrogen response elements [EREs]) in the promoter regions of

target genes, and the ligand-binding domain, which binds estrogens and estrogen analogues. Gene transcription is activated through two separate transactivation domains within ER: activating function (AF)-1 in the amino-terminal region, which is ligand-independent, and AF2 in the carboxy-terminal region, which is ligand-dependent, (Hewitt, 2010; Johnston & Dowsett, 2003). Coregulator molecules modulate receptor-mediated transcription by interacting with both the receptor AF sequences and molecules associated with RNA polymerase II (Edwards, 2000).

When an estrogen reaches the cell nucleus and binds to an estrogen receptor, the conformation of the ligand-binding domain of the receptor is altered, allowing interaction with coactivator molecules. Dimers of estrogen-estrogen receptor complexes bind with high affinity and specificity to EREs, 13-base pair inverted-repeat DNA sequences (GGTCAnnnTGACC) within the promoters of estrogen's target genes, with one receptor in contact with each five base-pair segment of the response element (Klinge, 2001). Transcription is induced through interaction with, and activation of, the necessary components of the transcriptional apparatus, including cis-regulatory elements (Carroll & Brown, 2006) and histone acetyl transferases (HATs), which weaken the interaction between histones and DNA thereby opening up the chromatin for interaction with TFs (Vande Woude & Klein, 2010). ER rarely binds to promoter regions, instead using pioneer factors

required to load ER on the chromatin at sites often located distally from the transcription initiation site (Hurtado, et al., 2008). The presence of the active estrogen receptor complex on the response sequence of target genes places this sequence in proximity to the RNA polymerase II transcription complex, allowing physical interaction and changes in the rate of transcription (Hewitt, 2010). Transcription can also be activated through interactions with other TFs (such as members of the activation protein 1 [Ap1] and specificity protein 1 [Sp1] families) to facilitate binding to serum response elements (Figure 1, a) (Musgrove & Sutherland, 2009).

If the ligand binding to ER is an antagonist, the conformation change induced is one that prevents interaction with coactivators (Edwards, 2000).

Estrogen can also mediate effects at the cell membrane or in the cytoplasm in what is termed a “nongenomic” fashion. Estrogen receptors can interact with membrane proteins to induce, for example, MAP kinase (M. J. Kelly & Levin, 2001). Estrogens may bind to the ER and induce the assembly of functional protein complexes. These complexes activate signalling cascades that in turn activate TF (Figure 1, c, d and e) (Musgrove & Sutherland, 2009).

Finally, ER can be phosphorylated by Erk or Akt as a consequence of signalling events downstream of receptor tyrosine kinases (RTKs) like epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), or

insulin-like growth factor receptor (IGFR) (Figure 1, b) (Musgrove & Sutherland, 2009).

A number of genes, including those for trefoil factor 1/pS2, cathepsin D, cyclin D1, c-Myc and progesterone receptor, are positively regulated by ER (Lin et al., 2004). Estrogens act most importantly on the reproductive organs but also act on other organ systems, including cardiovascular, skeletal, immune, gastrointestinal and neural sites (Hewitt, 2010).

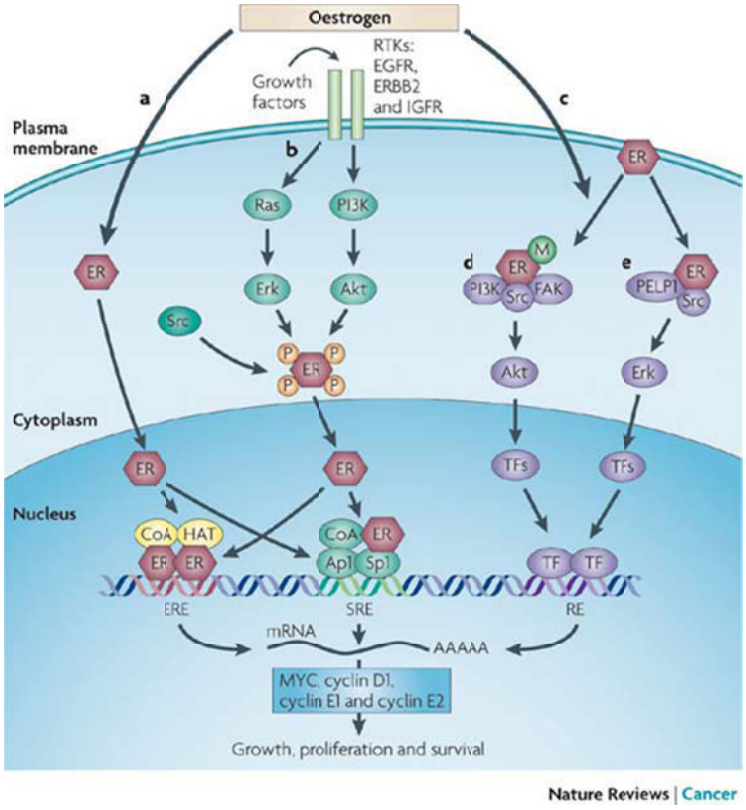

Figure 1: Estrogen action.

Three distinct pathways of oestrogen regulation of gene expression are shown.

a) First, in classic estrogen signalling, ligand-bound ER activates gene

expression, either through direct binding of dimeric ER to ERE, in complexes including co-activators and HATs, or through protein–protein interactions with other TFs – particularly members of the Ap1 and Sp1 families — to facilitate binding to serum response elements (SREs) and activation of transcription. b) Second, ER can also be activated as a consequence of signalling events downstream of RTKs such as EGFR, HER2, and IGFR. Phosphorylation (P) by the Erk or Akt serine/threonine kinases leads to ligand-independent activation of the ER. c) Third, signalling can be mediated through non-genomic mechanisms by ER that is localized at the cell membrane or in the cytoplasm. Ligand binding induces the assembly of functional protein complexes that involve other signalling molecules and that activate intracellular signalling cascades, resulting in TF activation. Two recently characterized mechanisms that ultimately activate transcription independently of ER binding to DNA are illustrated: d) ligand-induced methylation (M) of ER and formation of an ER–PI3K–Src–focal adhesion kinase (FAK) complex that activates Akt, and e) activation of Erk by ER–Src–PELP1 complexes (Musgrove & Sutherland, 2009).

2.3.3 Discovery of Tamoxifen

Researchers at the Merrell Company in Cincinnati began to study ethamoxytriphetol in a program evaluating synthetic estrogens. Also known as

MER25, this compound had a structural similarity to the known estrogen trianisylchlorethylene. It was found, however, that MER25 was anti-estrogenic, and that this non-steroidal anti-estrogen and the similar clomiphene were effective post-coital contraceptives in lab animals. MER25 was too toxic to move on to the clinic and clomiphene had the exact opposite effect in humans: it induced ovulation and enhanced fertility (Holtkamp, Greslin, Root, & Lerner, 1960). MER25 and clomiphene were not extensively studied in breast cancer because of side effects and the fear of unforeseen toxicity (Bloom & Boesen, 1974).

The failure of these anti-estrogens as contraceptives caught the attention of researchers at Imperial Chemical Industries (ICI), of which the biosciences division is now AstraZeneca (Jordan, 2008). The goal was to synthesize a potent non-steroidal anti-estrogen with reduced side effects that could be marketed as a contraceptive. Through research in rats, the scientists identified ICI46,474, or tamoxifen (Harper & Walpole, 1967). However, while acting as an anti-estrogen in rats, tamoxifen had full estrogenic effects in mice, so its action in humans was uncertain (Jordan, 2003).

Despite demonstrating potent anti-fertility and anti-estrogen action in the uterus, tamoxifen was shown to have a very low affinity for the estrogen receptor in vitro (Skidmore, Walpole, & Woodburn, 1972). This apparent anomaly was resolved

when it was found that tamoxifen was a pro-drug for 4-hydroxy(4-OH)tamoxifen, an anti-estrogenic metabolite with high affinity for the estrogen receptor (Sun et al., 2006). Thereafter, studies in DMBA-induced rat mammary tumours showed that tamoxifen inhibited estradiol binding to tumour ERs (Jordan, 2003) and reduced tumour growth (E. V. Jensen, et al., 2010), and the ER assay predicted tamoxifen response. These results were replicated in women with advanced breast cancer (Kiang & Kennedy, 1977) and tamoxifen treatment produced similar remission rates to adrenalectomy and/or ovariectomy. Again, about one third of patients responded and the major surgery was not needed (E. V. Jensen, et al., 2010).

The success of tamoxifen was the catalyst for the discovery of numerous new breast cancer treatments. These adjuvant treatments are collectively termed hormonal or endocrine therapies.

2.3.4 Tamoxifen: Mechanism of Action

Tamoxifen competes with estrogen for ER binding (Johnston & Dowsett, 2003). An estrogen receptor antagonist will bind to ER and cause a conformation change in the receptor and inhibit the ligand-dependent AF2 domain (Hewitt, 2010). Co-activators are sensitive to this change, and the abnormal configuration instead recruits co-repressors (Shibata et al., 1997). Tamoxifen-bound ER, while it does dimerize and bind to DNA, elicits different downstream effects because of

its altered conformation and co-activator/co-repressor balance (Johnston & Dowsett, 2003).

Tamoxifen's side effects, including increased risk of endometrial cancer and thromboembolism, are attributed to the drug not being a pure antagonist (Johnston & Dowsett, 2003). It is likely that conformational change induced by tamoxifen results in variable interactions with co-factors (Conzen, 2010) and that cross-signalling growth-factor transduction pathways also play a part in the designation of tamoxifen as a selective estrogen receptor modulator (SERM) (Johnston & Dowsett, 2003).

I. Tamoxifen as a SERM

Multiple methods by which tamoxifen may confer differential responses among patients, over time, and between tissues have been proposed. As a SERM, tamoxifen elicits different responses depending on the tissue on which it is acting: in the breast, tamoxifen blocks estrogen effects, but, in tissues such as the bone and uterus, functions similarly to it (Musgrove & Sutherland, 2009).

Tamoxifen elicits its antagonist function through the recruitment of co-repressors. The balance of co-activators and co-repressors available in the tissue is imperative to antagonism: in the case that co-activators are much more available than co-repressors, recruitment of co-activators to the ligand-independent AF1 may bypass the tamoxifen-induced AF2 inhibition (Musgrove & Sutherland,

2009). In the endothelium, tamoxifen-ER complexes have been known to recruit co-activators to target genes, such as MYC and IGF-1, that do not contain classical EREs (Conzen, 2010). Overexpression and increased phosphorylation of ER co-activators is known to lead to constitutive ER-mediated transcription (Musgrove & Sutherland, 2009). ER activity can also be modulated through increased growth factor expression, like EGF, or increased growth factor receptor expression, like EGFR or HER2 (Conzen, 2010).

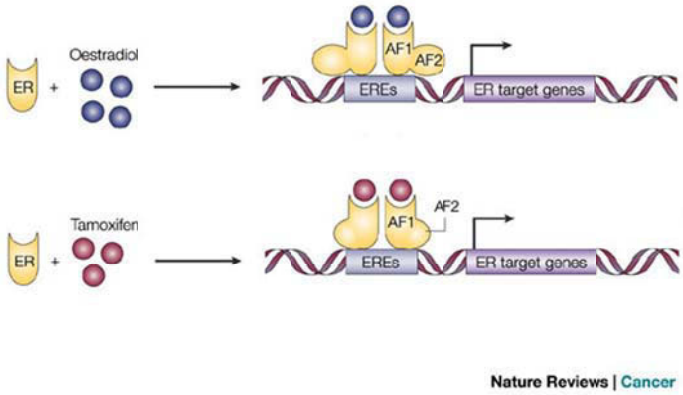

Figure 2: Tamoxifen action.

In the top panel, estrogen and ER are shown with functional AF1 and AF2 domains, leading to ER target gene transcription. In the lower panel, changes in AF2 inhibit ER action. However, transcription may still occur ligand-independently and less efficiently through the AF1 domain (adapted from Johnston & Dowsett, 2003).

2.3.5 Tamoxifen in the Clinic

Adjuvant (i.e., given after the primary therapy, be it surgery or radiation) (Musgrove & Sutherland, 2009) therapy began to be evaluated to determine whether cytotoxic chemotherapy would destroy micrometastases and cure patients after removal of the primary tumor. Tamoxifen, having been approved for use in a palliative treatment, was also evaluated in the adjuvant setting. Tamoxifen was given to all patients, regardless of ER status: outside the United States, standardized ER assays were not generally available. In addition, only one year of tamoxifen was used: this was the length of treatment in advanced breast cancer and there was a fear that longer exposure would result in earlier resistance.

In further experiments with DMBA-induced rat mammary tumours, the short-term administration of high concentrations of tamoxifen delayed rather than prevented the development of tumours. Long-term, small daily doses resulted in 80% of the rats remaining tumour-free (Jordan, Allen, & Dix, 1980). The EBCTCG meta-analysis of clinical trials definitively established that a five-year protocol of daily low-dose tamoxifen led to increased survival and decreased risk of contralateral breast cancer in ER+ pre-menopausal women. It also showed a 41% reduction in annual risk of relapse, a 34% reduction in the annual death rate in ER+ patients,

a 12% reduction in 15-year recurrence, and a 9% reduction in breast cancer mortality with tamoxifen treatment (Group, 1998).

Treatment exceeding five years does not seem to confer further benefit to patients. Tumour control effects may persist for ten years after tamoxifen cessation, and while there is no detectable survival advantage, side effects continue to worsen (Group, 1998). If treatment were continued indefinitely, there is a possibility that tamoxifen may stimulate tumour growth, as demonstrated in xenograft mice (Osborne, Coronado, & Robinson, 1987).

The fact that tamoxifen could prevent the induction and growth of ER+ carcinogen-induced rat mammary carcinomas (Jordan, 1976) begged the question of whether tamoxifen could prevent the majority of breast tumours – ones that expressed ER. The Breast Cancer Prevention Trial conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP) showed 43% fewer cases of invasive breast cancer diagnosed among women assigned to tamoxifen compared to placebo and 27% fewer diagnoses of non-invasive breast tumours at 7 years of follow-up (Fisher et al., 2005). The NSABP indeed stopped the trial 16 months early so that women on placebo could choose to switch to tamoxifen. The International Breast Cancer Intervention Study (IBIS), which went to completion, similarly found that the risk of ER-positive invasive breast cancer was 34% lower in the tamoxifen arm after 8 years of follow-up (Cuzick et al.,

2007). Tamoxifen is currently used to prevent breast cancer in women at high-risk for the development of breast cancer, i.e. having a breast biopsy showing high-risk benign breast disease, a family history consistent with high risk, or modified Gail score (Appendix 3) (Ropka, Keim, & Philbrick, 2010).

I. Predicting Tamoxifen Response

A lack of ER expression is the best predictor of response to tamoxifen, and is the primary mechanism of *de novo* resistance. *De novo* or intrinsic resistance is defined as the failure to respond to initial drug therapy (Musgrove & Sutherland, 2009). To screen patients for the presence of ER, breast biopsies are stained for ER and those that are positive are treated with tamoxifen for five years. This screening is most commonly done via immunohistochemistry, which has been shown to be superior to ligand-binding assays (Harvey, Clark, Osborne, & Allred, 1999) and is simpler and, compared also to enzyme immunoassays, uses a small amount of tissue (Elledge, 2010). A patient is considered ER+ if at least 1% of the tumour in the sample tests positive (Hammond et al., 2010), although this definition is still under discussion.

While this intrinsic resistance, due to a lack of the protein on which tamoxifen acts, is expected, more puzzling is the observation that 40% of patients with ER+ breast cancers will not respond to tamoxifen (Chanrion et al., 2008). Almost all patients with metastatic disease and up to 40% of the patients that receive

tamoxifen as adjuvant therapy have been reported to experience tumour relapse (Normanno et al., 2005). Disease progression in those patients who were initially responsive to therapy is termed acquired resistance (Musgrove & Sutherland, 2009).

To supplement the required but limited information that ER status can provide on tamoxifen response, breast tumours are also screened for the presence of progesterone receptor (PR).

II. Progesterone Receptor

PR is also a ligand-dependent nuclear hormone receptor, activated by progesterone. This steroid hormone is also essential for breast development and reproductive organ changes during the menstrual cycle and pregnancy (Lange & Yee, 2008). For example, a rise in progesterone levels is required after ovulation to allow embryo implantation in the endometrium (Hewitt, 2010). Established target genes of progesterone receptor include RANKL, Wnt4, amphiregulin, and TGFβ (Brisken & Duss, 2007).

The predictive value of PR status has long been attributed to the dependence of PR expression on activity of the ER. Since the synthesis of PR is positively regulated by ER, its absence was hypothesized to be a marker of a nonfunctional ER pathway. The hypothesis would suggest that ER+/PR- patients would not respond to tamoxifen, but this is untrue in the clinic: 1/3 of patients with this

hormone receptor expression respond to tamoxifen (Conzen, 2010). This discrepancy could be due to false-negative PR tests, as PR screening is much less standardized and there is disagreement about what should be considered a positive result (Duffy, O'Donovan, & Crown, 2011). Newer information suggests additional mechanisms by which PR may be expressed. Emerging data suggest that a lack of PR expression in ER+ tumours might be a surrogate marker for hyperactive growth factor signalling, which downregulates PR and contributes to tamoxifen resistance: indeed, compared to ER+/PR+ tumours, a greater number of ER+/PR- tumours contain overexpressed and amplified levels of HER2 and EGFR1 (Elledge, 2010).

Tamoxifen plays an important direct role in progesterone receptor-dependent proliferation inhibition. As previously explored, estrogens activate the Erk and Akt pathways via direct interaction of ER with Src and PI3K, respectively. In ER+ breast cancer cells, progesterone effects on these pathways are mediated by an interaction of two domains of PR with the ligand-binding domain of ER (Ballaré et al., 2003), which is activated in the absence of estrogens and triggers activation of the cascades. Consequently, progesterone induction of cell proliferation is inhibited, not only by antiprogestins but also by antiestrogens, as well as by inhibitors of kinase activation (Vicent et al., 2006).

Expression of ER and PR are positively correlated (Grann et al., 2005), and compared to ER+/PR- patients, those positive for both hormone receptors have better outcomes and respond better to tamoxifen (Elledge, 2010). The extent of PR's predictive value in the context of tamoxifen effect is disputed: patients that present with a tumour of the 3% of breast cancers that are ER-/PR+ may respond to tamoxifen, but there are conflicting results as to whether PR positivity confers an increased predictive value in ER+ patients (Duffy, et al., 2011). While a complete lack of ER expression does identify a group of breast cancer patients that do not benefit from endocrine therapies, only a fraction of patients whose tumours express ER will benefit from endocrine therapy. 75% of tumours positive for ER and PR respond to tamoxifen, but 25% fail to respond or develop early resistance (Conzen, 2010). ER and PR are clearly less-than-perfect predictors of response to tamoxifen and thus, there is a search for better predictive biomarkers of response to this drug.

2.4 Predictive and Prognostic Biomarkers

A search for new tumour biology-driven therapeutics has raised an intense interest in elucidating corresponding prognostic and predictive factors in order to improve patient outcome (Oldenhuis, Oosting, Gietema, & de Vries, 2008). A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic

responses to a therapeutic intervention. At the broadest level, they can provide insight into biological pathways and networks. They can also be used to monitor the effects of medical interventions, in diagnostic and prognostic tests, and to define the individuals and populations most likely to respond to therapy (Institute of Medicine (US) Forum on Drug Discovery, 2009).

Biomarkers can give prognostic and/or predictive information about a patient. A prognostic biomarker, associated with the metastatic and/or growth rate potential of the primary tumour (Hayes, Trock, & Harris, 1998), provides information about the overall cancer outcome, regardless of therapy. Nodal status is generally accepted as the best prognostic marker available in breast cancer (Bay, Jin, Huang, & Tan, 2006). They generally reflect the inherent biologic aggressiveness of a tumour, including its ability to proliferate, invade, and/or spread (Elledge, 2010). A biomarker with predictive value gives information on the effect of a therapeutic intervention in a patient, and can also be a target for therapy (Oldenhuis, et al., 2008).

Biomarker presence or level can be obtained in numerous ways, such as in easily-obtainable bodily fluids like plasma, serum, or urine. More invasive techniques requiring tumour tissue for protein, DNA, and RNA analyses are also used (Oldenhuis, et al., 2008).

2.4.1 ER and PR as Biomarkers

Hormone receptor status provides both predictive and prognostic information (Elledge, 2010). High cellular expression of ER and PR predicts benefit from endocrine therapy in the adjuvant and metastatic setting (Oldenhuis, et al., 2008), and ER was the first and continues to be one of the best predictive markers in oncology. As previously stated, the original rationale for investigating ER as a predictive marker for hormone therapy was based on the fact that the growth of at least some breast cancers was dependent on estrogens. Since estrogens promoted tumour growth via the ER, it was hypothesised that levels of this receptor in breast cancers would correlate with benefit from anti-estrogenic therapy. Support for this hypothesis emerged in the early 1970s when it was shown that approximately 50% of ER-positive patients with advanced breast cancer had objective regression when treated with endocrine ablative therapy. In contrast, patients with ER-negative tumours rarely experienced regression with these therapies. ER was later shown to be associated with benefit from adjuvant hormone therapy in patients with early invasive breast cancer (Duffy, et al., 2011). In 1998, the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) published a meta-analysis of randomized trials comparing tamoxifen vs. placebo for the adjuvant treatment of early invasive breast cancer showing that tamoxifen prolonged both disease-free survival (DFS) and overall survival (OS) in patients

with ER-rich tumours but had little benefit in patients who had ER-poor cancers. For women with ER-rich breast tumours, treatment with adjuvant tamoxifen for 1 year, 2 years and approximately 5 years resulted in proportional recurrence reduction of 21%, 28% and 50%, respectively. The proportional mortality reductions in patients with ER-rich tumours were 14%, 18% and 28% following treatment for 1 year, 2 years and about 5 years, respectively. In contrast to ER-rich patients, women with ER-poor tumours derived no benefit from adjuvant tamoxifen (Early Breast Cancer Trialists' Collaborative Group, 1998). The 15-year update of this meta-analysis confirmed these findings by showing that administration of adjuvant tamoxifen for about 5 years reduced annual breast cancer mortality by 31% in ER-positive patients but was ineffective in ER-negative patients. In this updated study, the absolute benefit of tamoxifen at 15 years was more than double that at 5 years (Early Breast Cancer Trialists Collaborative Group, 2005).

The negative predictive value of ER is high: ER-negative patients almost never derive benefit from hormone therapy. ER's positive predictive value, however, is less accurate: in patients with advanced breast cancer, only about 50% of ER-positive patients undergo objective response following treatment of hormone therapy. Furthermore, in patients with early breast cancer, while adjuvant tamoxifen significantly reduced the risk of recurrence and death in ER-positive

patients, approximately 30% relapse by 15 years (Duffy, et al., 2011). In an effort to improve the positive predictive accuracy of ER, PR was investigated as a biomarker and, again, evidence supporting and refuting its predictive value in hormone therapy has been reported (Bardou, Arpino, Elledge, Osborne, & Clark, 2003; Dowsett et al., 2006).

Because of those groups that concluded that PR status was a good predictive marker, it is used to better define the likelihood of endocrine responsiveness in the clinic. When compared to ER+/PR- tumours, ER+/PR+ tumours – particularly if they are strongly PR-positive – are associated with a higher rate of response to endocrine therapy in patients with metastatic disease and possibly longer survival in the adjuvant setting (Elledge, 2010).

ER and PR expression are also independent prognostic factors in breast cancer. Patients with ER and/or PR-positive tumours have a better survival than hormone receptor negative tumours, with a 5-year OS of 83% in the ER+/PR+ group versus 69% in the double negatives. Hormone receptor status can change during the course of disease and may differ across lesions: the ER status of metastatic disease is different from the primary tumour in about 20% of cases and PR expression is lost in 40% of previously positive tumours when they metastasize (Oldenhuis, et al., 2008). Therefore, ASCO guidelines recommend measurement

of both ER and PR in metastatic lesions if these results might influence treatment planning (Hammond, et al., 2010).

The value of ER status as an independent prognostic variable is diminished by its association with other established indicators of favourable prognosis, including older age, low-grade histology, a favourable nuclear grade, a normal complement of DNA (diploid), and a low proliferative index. In addition, ER-positive patients receive and benefit from either adjuvant or palliative hormone therapy so regularly that it is difficult to evaluate the prognosis apart from the influence of therapy (Esteva & Hortobagyi, 2004).

ER and PR status supply important information to patients and health professionals on treatment options based on tumour biology and the expected progression of the disease. Clearly, however, there are limitations to their value. These limitations are especially important when making patient care decisions. In a quest to better elucidate the benefits or drawbacks of tamoxifen treatment, numerous other biological factors have been explored in vivo and in vitro.

2.5 Putative Biomarkers

Several biomarkers supplementing ER and PR show promise in breast cancer cell lines and tumour samples from small tamoxifen clinical trials. To reach a level of evidence that would spur trials randomized for these biomarkers and/or

clinical use, these biomarkers must be retrospectively analyzed in prospective placebo-controlled randomized trials (Simon, Paik, & Hayes, 2009).

2.5.1 CCND1

Amplification of the gene encoding cyclin D1, CCND1, has been shown to be correlated with an adverse tamoxifen effect in breast cancer patients (Lundgren, Holm, Nordenskjold, Borg, & Landberg, 2008). This protein is a cell cycle regulatory protein that binds to and activates cyclin-dependent kinases 4/6 (CDK4/6) to drive progression through the G_1 phase of the cell cycle (Kirkegaard et al., 2008).

G_1 is the phase of the cell cycle during which the cell reaches the restriction (R) point, where it must make the commitment to advance through the remainder of the cell cycle through M phase to two daughter cells, to remain in G_1, or to retreat from the active cell cycle into G_0 senescence. After the R-point transition, cells are growth factor-independent and will continue to mitosis lest issues arise during the steps leading up to division that would prevent viable daughter cells (Weinberg, 2007). Cyclins are proteins which act as key controlling elements of the eukaryotic cell cycle. In mammalian cells, cyclins bind to cyclin-dependent kinases and form complexes that are involved in regulating different cell cycle transitions, depicted in Figure 3: cyclin-D-CDK4/6 complex for G_1 progression, cyclin-E-CDK2 for the G_1-S transition, cyclin-A-CDK2 for S-phase progression,

and cyclin A/B-CDK1/CDC2 for entry into M-phase (Stamatakos et al., 2010). The levels and availability of cyclins during the phases of the cell cycle change in order to impose control on the specific steps, while the levels of most CDKs vary only minimally. CDK4 is nonetheless regulated via the INK4 CDK inhibitors (CdkIs) $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$ (Weinberg, 2007).

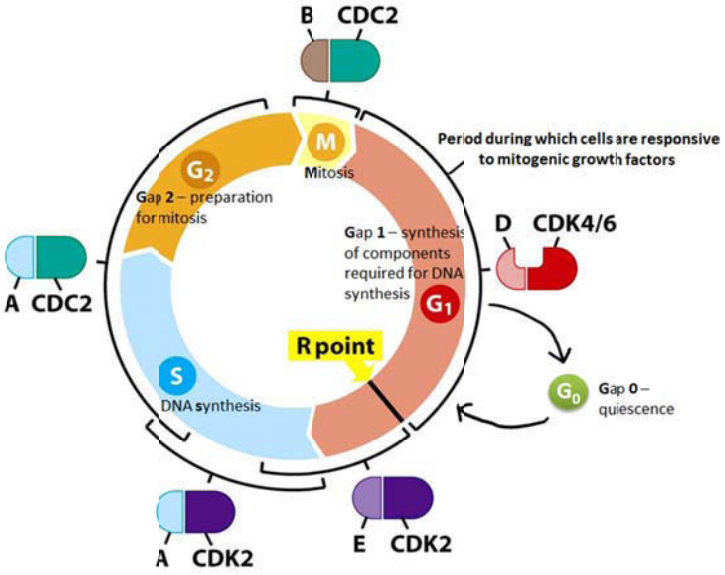

Figure 3: Cyclin-CDK pairing and responsiveness to extracellular signals. Each type of cyclin pairs with a specific CDK or set of CDKs. The brackets indicate the periods during the cell cycle when these various cyclin-CDK complexes are active. Cells respond to extracellular mitogens and inhibitory factors (like TGF-β) only in a discrete window of time that begins at the onset of G_1. The end of this time window is designated as the R point (adapted from Weinberg, 2007).

The D-type cyclins (D1, D2, and D3) do not share the cyclical fluctuations in protein levels of the other cyclins: their levels are controlled largely by mitogenic growth factors, where cyclin D1 is stimulated via growth factor activation of tyrosine kinase receptors (RTKs) (Figure 4). Thus, cyclin D1 levels will drop with growth factor removal. The three D-type cyclins continuously inform the cell cycle clock of current condition in the extracellular environment. While cyclin D1 is present in other cell cycle phases, following the G_1/S transition, it is exported from the nucleus into the cytoplasm, where it can no longer influence cell cycle progression (Weinberg, 2007). Cyclin D1 gene expression is also induced by many oncogenic signalling pathways, including Ras, Src, Her2/neu, β-catenin, and members of the signal transducer and activator of transcription (STAT) family (Sherr, 2004).

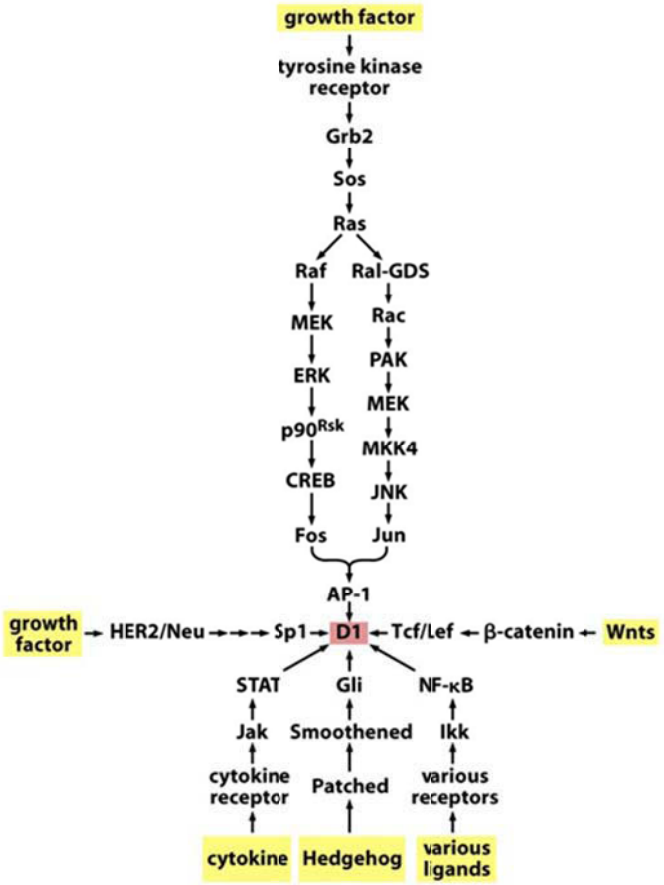

Figure 4: Control of cyclin D1 levels.

The control of cyclin D1 levels can be explained in part by a signal transduction cascade that leads from RTKs to the AP-1 TF. A number of signalling cascades with other factors modulating transcription converge on the promoter of this gene, CCND1: the RANK receptor acts through the NF-κB pathway; the prolactin receptor acts through the Jak/STAT pathway; focal adhesion kinase (FAK) acts through E2F and Sp1 TFs; and ER may act through AP-1 TFs (Weinberg, 2007).

Cyclin D1 is the regulatory subunit of the holoenzymes that phosphorylate and, together with sequential phosphorylation by cyclin E-CDK2, inactivate the cell cycle-inhibiting function of the retinoblastoma protein (Rb). Rb is the tumour suppressor that serves as a gatekeeper of the G_1 phase and is thought to silence specific genes that are active during S phase through active repression of E2F transcriptional activity, which is then reversed by cyclin D1 (Fu, Wang, Li, Sakamaki, & Pestell, 2004).

Rb begins as essentially unphosphorylated in G_0 and becomes hypophosphorylated by cyclin D-CDK4/6 on a small number of serine and threonine residues after entrance into G_1, maintaining its growth-inhibitory function. In concert with the advance of cells through the R point, it becomes hyperphosphorylated on a much larger number of residues by cyclin E-CDK2 holoenzymes and loses its growth-inhibitory powers, having been functionally inactivated by this phosphorylation. Rb remains hyperphosphorylated (pRb) throughout the remainder of the cell cycle until exit from mitosis, when protein phosphatase 1 (PP1) removes the phosphate groups, setting the stage for the next cell cycle and a new cycle of Rb phosphorylation. Should the cell experience serious physiologic stress while in S phase or G_2, Rb phosphorylation can be reversed, returning Rb to its inhibitory state. Without strong and continuous mitogenic signalling throughout early and mid-G_1 up to the R point, cyclin D1

levels drop and Rb loses its phosphate group, remaining inhibitory and a poor substrate for further phosphorylation by cyclin E-CDK2 (Weinberg, 2007).

In its active state, Rb (or associated pocket proteins p107 or p130) occludes the transactivation domains of E2F TFs, acting as a transcriptional repressor. E2F is a class of heterodimeric proteins, the subtypes of which can bind to DP1 or 2 and attract histone acetylases (HACs) or histone deacetylases (HDACs) and chromatin remodelling proteins (Fu, et al., 2004), depending on the subtype. Levels of E2F4/5 are high during G_0: indeed, they associate with p107/p130 and attract HDAC to repress transcription. E2F1/2/3 are expressed largely in proliferating cells, and attract HAC to activate transcription, prominently of cyclin E, which goes on to hyperphosphorylate pRb and phosphorylate p27 to liberate additional cyclin E-CDK2 complexes. When they are repressed by active or hypophosphorylated pRb, HDAC is recruited to repress transcription (Weinberg, 2007).

Cyclin D-CDK4/6 complexes also act to sequester $p21^{Cip1}$ and $p27^{Kip1}$ CDK inhibitors. These proteins are important stimulators of the formation of D-CDK4/6 complexes in early- and mid-G_1 as well as inhibitors of cyclin-CDK complexes active in later phases, including the immediately subsequent E-CDK2 complex. In G_0, p27 is bound to any E-CDK2 that may be present in the cell. As D-CDK4

complexes form, they sequester p27 and p21 and E-CDK2 can begin to trigger passage through the R-point (Weinberg, 2007).

Cyclin D1 additionally has CDK-independent functions, such as regulating nuclear hormone receptors: cyclin D1 is a positive regulator of ER-mediated transcription of ERE-responsive genes. Via physical interactions, cyclin D1 can regulate the transactivation of these receptors, both positively and negatively. Associating with the p300/CBP-associated factor (P/CAF), cyclin D1 can activate ER independent of estrogen stimulation. In addition to being an upstream regulator of ER activity, cyclin D1 also appears to be a downstream target of the ER, as transcription of the CCND1 locus is increased following ER activation. Activation of ER also induces the activation of other cellular pathways, including the Src/Ras/MAPK pathway, upstream of cyclin D1 (Fu, et al., 2004). Because tamoxifen acts as a competitive inhibitor of ligands activating ER, theoretically cyclin D1 overexpression should render this therapy ineffective through its ability to activate the receptor in a ligand-independent manner (Bostner, et al., 2007). Increased cyclin D1 levels shorten the G_1 phase, rescuing growth factor-deprived and antiestrogen-arrested cells and enabling them to complete the cell cycle (Elsheikh et al., 2008; Kirkegaard, et al., 2008). *In vitro* studies have linked tamoxifen resistance to the expression of cyclin D1 in cell lines (Elsheikh, et al., 2008; Hui et al., 2002).

Overexpression of cyclin D1 is observed in about 50% of breast cancer specimens (Bostner, et al., 2007; Kirkegaard, et al., 2008). In several clinical studies, early relapse and shorter survival were observed in women with cyclin D1–positive breast cancer who received tamoxifen (Ahnstrom, Nordenskjold, Rutqvist, Skoog, & Stal, 2005; Bostner, et al., 2007; Rudas et al., 2008; Stendahl et al., 2004) but the opposite has also been shown. Cyclin D1 expression has been shown to be an independent poor prognostic factor but the data on this aspect of cyclin D1's clinical value are conflicting (Reis-Filho et al., 2006; Roy & Thompson, 2006; Rudas, et al., 2008).

CCND1 amplification has also been shown to have independent prognostic value (Bostner, et al., 2007; Elsheikh, et al., 2008). Found at locus 11q13, CCND1 amplification is found in about 15% of breast tumours (Bostner, et al., 2007). It is generally believed that CCND1 is the most important driver gene of the 11q13 amplicon because the CCND1 locus is usually included in the amplicon (Ormandy, Musgrove, Hui, Daly, & Sutherland, 2003), cyclin D1 protein expression is upregulated in 11q13-amplified cancers (Bieche, Olivi, Nogues, Vidaud, & Lidereau, 2002; Elsheikh, et al., 2008; Reis-Filho, et al., 2006), as is the gene's cell cycle regulatory function (Dancau et al., 2010). CCND1 amplification is associated with decreased time to recurrence in ER+ breast cancer on tamoxifen (Bieche, et al., 2002; Bostner, et al., 2007; Jirstrom, et al.,

2005; Roy & Thompson, 2006). Jirstrom reported a highly significant interaction between tamoxifen treatment and CCND1 amplification with regards to recurrence-free survival in ER+ breast cancer (Jirstrom, et al., 2005).

The predictive value of CCND1 amplification has not been validated in a placebo-controlled randomized trial using standard of care treatment parameters to reach a level of significance that would permit this information to be of value when treating patients: Bostner was the only group that looked at CCND1 amplification in patients receiving tamoxifen or placebo, but treatment was administered for only two years and at 40 mg/day (Bostner, et al., 2007), whereas the standard of care is currently 20mg/day for five years (Decensi et al., 2003). Jirstrom also had a placebo group but the treatment group was divided into 20 mg/day and 40 mg/day and were again treated for two years (Jirstrom, et al., 2005). We hypothesize that CCND1 will predict for decreased RFS in patients treated with tamoxifen.

2.5.2 RSF1

11q13 is amplified in 15% of breast cancers, and while CCND1 is considered the driving force behind this amplification, amplicons in cancer are usually large, spanning up to several megabases in size, and contain multiple genes, several of which might contribute to the process of malignant transformation. The driver genes are not always easily identifiable from the bystanders, and the degree of

cooperation between the genes located at the same amplicon is essentially unknown (Dancau, et al., 2010). Likewise, 11q13 delineates approximately 2–10 megabases (Gibcus et al., 2007). It has been suggested that some of these genes may be important for breast cancer development and progression besides CCND1 (Dancau, et al., 2010): several distinct core regions within the 11q13 amplicon have been shown to be amplified independently of one another (Bostner, et al., 2007), and up to eight cores have been suggested for this region (Albertson, 2006).

I. PAK1

For example, p21-activated kinases (PAKs) are serine/threonine protein kinases that serve as important mediators of Rac and Cdc42 GTPase function as well as pathways required for Ras-driven tumorigenesis. The Pak1 protein has been implicated in signalling by growth factor receptors and morphogenetic processes that control cell polarity, invasion, and actin cytoskeleton organization (Ong et al., 2011). Overexpression of the protein has been suggested to influence mammary hyperplasia, malignancy, anchorage-independent growth, invasiveness and cell survival. Pak1 can activate the ER via phosphorylation of the ligand-dependent domain of the receptor leading to transcription of ER-responsive genes, such as, interestingly, CCND1 (Wang, Mazumdar, Vadlamudi, & Kumar, 2002). The pak1 pathway, starting at the cell surface by growth factor activation of receptors,

could also influence cyclin D1 oncogenic functions in breast cancer through an NF-κ-B-dependent pathway (Balasenthil et al., 2004).

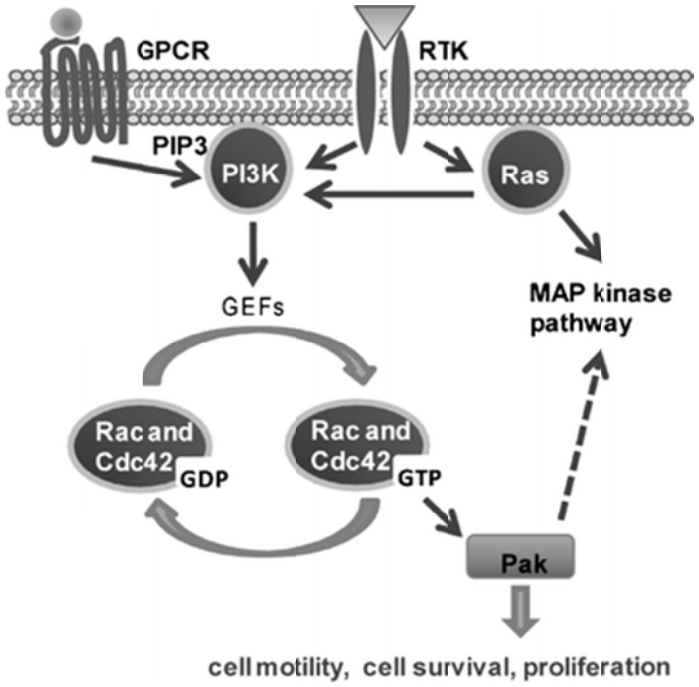

Figure 5: Pak activation by the small GTPases Rac and Cdc42.

Signals from receptor tyrosine kinases, (e.g. insulin, EGF, PDGF, and VEGF receptors) and G protein-coupled receptors lead to activation of Pak via GTP-bound Rac and Cdc42. Activated Pak in turn initiates signalling cascades that culminate in the cellular response. In addition, activated Pak potentiates activation of the MAP kinase pathway. Of note, while activation of Pak via Rac and Cdc42 is well characterized, a number of GTPase-independent mechanisms for Pak activation have also been identified. GPCR, G protein-coupled receptors; RTK, receptor tyrosine kinase; PI3 K, phosphatidylinositol-3 kinase; PIP3,

phosphatidylinositol (3, 4, 5) trisphosphate (Dummler, Ohshiro, Kumar, & Field, 2009).

Pak1 has been shown to phosphorylate histone H3.3a, thereby controlling gene expression, and it may also influence mitotic events. Cytoplasmic and nuclear overexpression of Pak1 has been associated with high expression of the proliferation marker Ki-67 (Holm et al., 2006).

Patients with overexpressed Pak1 have been shown to have decreased response to tamoxifen (Bostner, et al., 2007; Holm, et al., 2006). 11q13 is often amplified with 11q14, where the PAK1 gene is located (Bocanegra et al., 2010). ER+ patients with PAK1 amplification have also been shown to have decreased benefit from the drug (Bostner, et al., 2007). aCGH performed in our lab on 90 breast tumours, however, revealed a tumour with a very clear 11q14 amplification in which PAK1 was not involved (Figure 6). Comparing the genes involved in the amplicon with expression data from the sample, RSF1 became a candidate driver of this amplification.

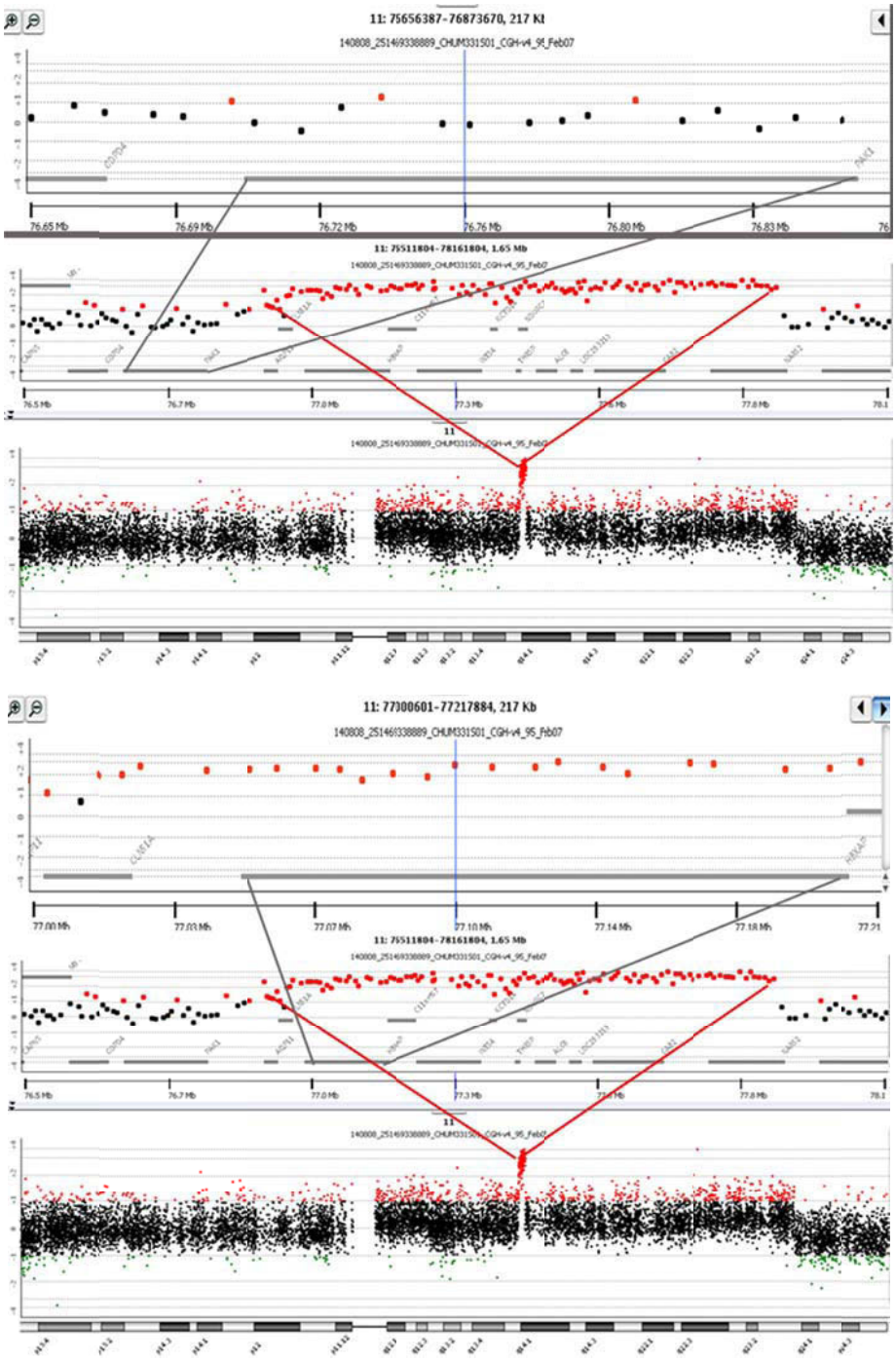

Figure 6: 11q14 amplification as shown by aCGH.

PAK1 is not involved in a clear 11q14 amplification (top) whereas RSF1 is amplified (bottom).

II. Rsf1 and RSF1 amplification

In eukaryotic cells, DNA is wrapped around histone octamers to form nucleosomes, the primary unit of chromatin structure. Nucleosomes compact the genome but also restrict the access of TFs, so there is a balance to strike between effective genome packaging and accessibility. Cells therefore tailor the way that chromatin is packaged to help regulate gene expression, involving dynamic competition between nucleosomes and TFs for important cis-regulatory sequences in gene promoters. This competition is influenced by enzymes that covalently modify nucleosomes, termed chromatin modifiers, and enzymes that reposition, reconfigure or eject nucleosomes, termed 'chromatin remodellers'. Together, these factors help create promoter architectures — the density, composition and positioning of nucleosomes relative to important cis-regulatory sites. These factors also collaborate to alter promoter architecture to expose regulatory sites and allow activation under the appropriate conditions (Cairns, 2009). Rsf1 acts as part of the chromatin remodelling complex RSF (remodelling and spacing factor). RSF is an imitation switch (ISWI) complex, part of a family of chromatin remodelling ATPases that assembles chromatin and slides and spaces nucleosomes, making the chromatin template fluid and allowing appropriate regulation of events such as transcription, DNA replication, recombination and repair (Mellor, 2006).

To form this imitation switch (ISWI) complex, Rsf1 interacts with SNF2H. The former functions as a histone chaperone, which, in general, associates with histones to facilitate their interaction with other molecules without being a component of the final reaction product (Loyola & Almouzni, 2004). The latter possesses nucleosome-dependent ATPase activity (Sheu et al., 2010). Together, the ISWI complex formed moves nucleosomes ATP-dependently along DNA without major disruption to allow access to DNA of TFs, enhancers, repressors, and enzymes (Varga-Weisz, 2010). Growth signals and environmental cues cause RSF to participate in nucleosome assembly and chromatin remodelling, and RSF has been shown to interact with centromere protein A, suggesting a role in chromosome segregation (Sheu, et al., 2010).

Experiments in non-transformed cells suggest that increased Rsf-1 expression and excessive RSF activity, which occurs in tumours harboring Rsf-1 amplification, can induce chromosomal instability, likely through induction of the DNA damage. Acute Rsf-1 expression resulted in the induction of DNA damage as evidenced by DNA strand breaks, nuclear γH2AX foci, and activation of the ATM-CHK2-p53-p21 pathway, leading to growth arrest and apoptosis. Chronic induction of Rsf-1 expression, on the other hand, results in chromosomal aberrations. Formation of a functional RSF complex with SNF2H has been

shown to be required for Rsf-1 to trigger this DNA damage response. (Sheu, et al., 2010).

Both overexpression of Rsf1 and amplification of the RSF1 (also known as HBXAP) gene have been implicated as having important roles in cancer, most studied in ovarian cancer (Sheu, et al., 2010), where it is amplified in about 10-15% of ovarian carcinomas (Brown, et al., 2008; Shih et al., 2005). They are also significantly correlated with high-grade ovarian serous carcinoma (Mao et al., 2006; Shih, et al., 2005), the most aggressive ovarian cancer (Mao, et al., 2006), and significantly worse outcome in ovarian cancer patients (Brown, et al., 2008; Shih, et al., 2005).

With regards to therapeutic interventions, Rsf-1 has been implicated in resistance to paclitaxel, which stabilizes the microtubule polymer and protects it from disassembly, leading to an inability of the chromosomes to achieve a metaphase spindle configuration and the subsequent triggering of apoptosis (G. M. Chin & Herbst, 2006). Rsf-1 knockdown sensitizes ovarian cancer cells to paclitaxel and Rsf-1 is found upregulated in paclitaxel-resistant ovarian cancer cell lines. As well, disruption of the interaction between SNF2H and Rsf-1 enhances paclitaxel sensitivity in tumour cells with Rsf-1 upregulation (Choi et al., 2009).

Shih found that RSF1 was the only gene that demonstrated consistent overexpression of its protein in all ovarian tumours with an 11q13.5 amplification

(Shih, et al., 2005). In breast cancer, only Rsf-1 protein expression has been explored. It is expected that RSF1 amplification will predict for a worse RFS in patients treated with tamoxifen, considering its observed correlation with Rsf-1 protein expression and its clear importance in other cancers.

2.5.3 PAX2

Pax (paired box) genes comprise a small but developmentally crucial gene family that encode a set of TFs. Nine members of the family have been described in mammals, subclassified into four groups (PAX1/PAX9, PAX 2/PAX5/PAX8, PAX4/PAX6, and PAX 3/PAX7) based on genomic structure, sequence similarities, and expression patterns in developing tissues and organs. Each PAX gene encodes a protein that is hypothesized to modify downstream gene transcription by binding to enhancer DNA sequences. These TFs regulate tissue development and aid with cell-lineage specification, proliferation, migration, and survival. PAX genes influence embryogenesis, and expression levels usually attenuate during terminal differentiation of most organ systems (Tung et al., 2009). However, in a few tissues, Pax gene expression either persists into adult life or is re-expressed (Robson, He, & Eccles, 2006): functions in adult tissues include direction of organ-specific regenerative events (Seale et al., 2000) and protection against stress-induced cell death (Cai et al., 2005).

Pax proteins are involved in stem-cell self-renewal, both during fetal development and in adult life. This feature is important in morphogenesis, regeneration and repair of tissues, and is particularly relevant in the context of possible pathways by which cancer cells undergo self-renewal and division to generate a tumour.

Embryonic development requires absolutely precise regulation to ensure correct spatial and temporal generation of diverse precursor cell types. Populations of these cells must be tightly controlled to prevent inappropriate proliferative, migrational or transdifferentiation events. The key developmental processes that are regulated by Pax genes — proliferation, stem-cell self-renewal, resistance to apoptosis, and cell migration and invasion (Robson, et al., 2006) — make up four of the characteristics suggested by Hanahan and Weinberg dictating malignant growth (Hanahan & Weinberg, 2000). Pax subgroups II (of which PAX2 is a member) and III in particular seem to be highly susceptible to deregulated expression and, consequently, may contribute these characteristics in an uncontrolled fashion to cancers (Robson, et al., 2006).

PAX2 specifically encodes a TF crucial to the organogenesis and development of the central nervous system, eyes, ears, mammary glands, and urogenital tract. Mutations in PAX2 have been associated with renal hypoplasia, and increased PAX2 expression has been found in various cancers, including ovarian, renal,

prostate, and breast cancers, Wilms tumor, and Kaposi sarcoma (Tung, et al., 2009).

It is therefore curious that Hurtado found a role for PAX2 as a repressor in breast cancer (Hurtado, et al., 2008) as it had been shown to be a tamoxifen-regulated gene that can induce endometrial cancer (Wu et al., 2005). PAX2 is expressed in 40-60% of breast carcinomas (Hurtado, et al., 2008; Silberstein, et al., 2002) and is required for the survival and morphogenesis of tissues in the developing kidney, eye, ear and mammary gland (Robson, et al., 2006).

Tamoxifen-resistant breast tumours may be characterized by elevated ERBB2 levels, and ER-positive cell line models overexpressing ERBB2 acquire resistance to tamoxifen. Crosstalk between the ER and ERBB2/HER-2 pathways has long been implicated in breast cancer aetiology and drug response, and using genome-wide ER chromatin immunoprecipitation (ChIP)-on-chip analyses in ER-positive MCF-7 cells, Hurtado found an ER-binding site within the intron of the HER-2 genomic region. Estrogen–ER and tamoxifen–ER complexes were found to directly repress ERBB2 transcription by means of a cis-regulatory element within the ERBB2 gene in human cell lines: PAX2 and the ER co-activator AIB-1/SRC-3 compete for binding and regulation of ERBB2 transcription, the outcome of which determines tamoxifen response in breast cancer cells (Hurtado, et al., 2008), depicted below.

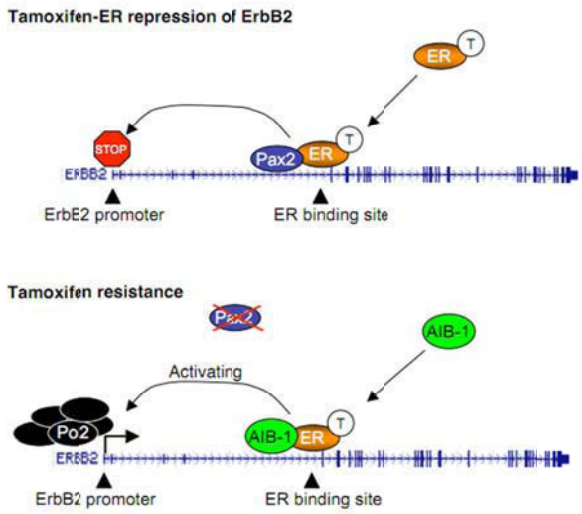

Figure 7: Model of tamoxifen-ER transcriptional regulation of ERBB2.

In ER+ breast epithelial cells, treatment with tamoxifen results in recruitment of ER and the putative repressor Pax2 to an ER binding site within the intron of the ERBB2 genomic region. The presence of Pax2 results in active repression of ErbB2 transcription. Tamoxifen resistance can be potentially achieved by a number of mechanisms, including a decrease in Pax2 levels (which results in the opportunity for increased binding of the co-activator AIB-1), an increase in AIB-1 levels (which can outcompete Pax2 for binding) or by amplification of the ErbB2 genomic locus, all of which increase ErbB2 levels (Hurtado, et al., 2008).

Looking at patients treated with tamoxifen, it was found that PAX2-positive patients had a significantly increased RFS compared to PAX2-negative patients. Tumours that were PAX2 positive and AIB-1 negative had the best prognosis of all, with a recurrence rate of only 5.8%, and PAX2-positive, AIB-1-negative

tumours had the lowest percentage of ERBB2-positive staining (Hurtado, et al., 2008). Given that tamoxifen has anti-proliferative effects in the breast but possesses agonist properties in the endometrium (Fisher, et al., 2005), it is possible that PAX2 may have tissue-specific effects and may be one of the primary determinants for SERM action in female reproductive tissue (Hurtado, et al., 2008). Moving to a cohort of patients randomized to tamoxifen or placebo, it is expected that the PAX2-positive patients will also have a significantly increased RFS compared to those who are negative.

2.5.4 TC21

Ras proteins serve as signalling nodes activated in response to diverse extracellular stimuli. Activated Ras interacts with multiple, catalytically distinct downstream effectors, which regulate cytoplasmic signalling networks that control gene expression and regulation of cell proliferation, differentiation, and survival (Wennerberg, Rossman, & Der, 2005). TC21/R-Ras 2 is a member of the Ras superfamily of small GTP-binding proteins that, like Ras, has been implicated in the regulation of growth-stimulating pathways. It is the first Ras-related protein shown to have potent transforming activity and its aberrant function has been proposed to play an important role in human carcinogenesis (Graham et al., 1994) .

Non-genomic or membrane-initiated ER signalling activities and crosstalk with growth factor signal transduction pathways has been implicated in tamoxifen resistance. Activation of ER outside the nucleus leads to the activation of surface tyrosine kinase receptors (e.g., EGFR and HER2) as well as interaction with cellular kinases and adaptor molecules (e.g., Src, Shc, PI3K), which in turn lead to the activation of MAPK and AKT pathways, orchestrating cell proliferation and survival (Schiff et al., 2004). These signalling pathways in turn can activate ER itself or its coactivators and corepressors, thereby increasing the potential of genomic/nuclear ER activity. Among the key components of growth factor signalling are the Ras proteins for which an involvement in tamoxifen resistance has been suggested (Rokavec, et al., 2008).

TC21's role in tumorigenesis and cell growth regulation is well-studied, especially in oral cancer (Sharma, Sud, Chattopadhyay, & Ralhan, 2005). TC21 expression has been found to be elevated in transformed cell lines compared to non-transformed. Ectopic overexpression of TC21, but not Ras, has been shown to cause transformation of MCF-10A cells (Clark, et al., 1996). TC21 correlates with poor prognosis in oral squamous cell carcinoma (Macha et al., 2010) and it is suggested that TC21 is associated with esophageal progression (Sharma, et al., 2005). While mutations of TC21 are uncommon in breast cancer (Barker & Crompton, 1998), Rokavec found high cytoplasmic TC21 expression in 44% of

breast tumours. It was also found that high TC21 protein and mRNA expression predicted for decreased RFS compared to no/low expression in tamoxifen-treated patients. In patients receiving placebo, this difference was no longer seen. TC21 expression was shown to be repressed by ER, and the presence of tamoxifen metabolites increased TC21 expression, promoting acquired resistance in cells (Rokavec, et al., 2008).

The cohort used by Rokavec was not randomized to treatment: the study was performed on a set of archival material (Werner Schroth et al., 2007). We hypothesize that, using specimens from a placebo-controlled randomized trial, the predictive value of TC21 with regards to tamoxifen treatment will persist: patients, when treated with tamoxifen, will show decreased RFS when expressing high levels of TC21 compared to those with no/low expression, and this correlation will disappear in patients in the placebo group.

3. Methods and materials

3.1 National Cancer Institute of Canada Mammary (MA).12 study

MA.12 was an RCT studying tamoxifen in ER+ and ER- high-risk premenopausal women with early breast cancer who had received adjuvant chemotherapy. When the trial started in 1992, the benefit of tamoxifen in women who were ER- was unresolved. As well, the value of sequential use of adjuvant tamoxifen with adjuvant chemotherapy was unclear. The patients of the MA.12 were therefore randomized to tamoxifen or placebo for five years after standard polychemotherapy treatment (Bramwell et al., 2010).

MA.12 recruited 672 premenopausal women with histologically-confirmed breast cancer who had undergone complete or segmental mastectomy plus axillary node dissection between 1993 and 2000. The median age of the sample was 46 years, ranging from 29 to 58 years. With a median follow-up time of 9.7 years, patients were excluded if they had distant metastases, residual disease in the breast or axilla, other serious medical illnesses, or a previous cancer. Women considering pregnancy or using hormones were excluded. Brief exposure to tamoxifen (≤2 weeks) was permitted. Initially, entry was restricted to women with node-positive disease but, from February 1995, women with high-risk node-negative tumours (tumour ≥1 cm and high histological grade or lymphovascular

invasion or both) were included. Complete inclusion and exclusion criteria can be found in Appendix 4.

Patients were given the standard schedules of one of three chemotherapy regimens: six cycles of cyclophosphamide/methotrexate/5-fluorouracil (CMF) or cyclophosphamide/ epirubicin/5-fluorouracil(CEF) or four cycles of doxorubicin (adriamycin)/cyclophosphamide (AC). After surgery to remove the tumour, patients were stratified by type of chemotherapy (CMF versus CEF versus AC), hormone receptor status (ER- and/or PR-positive versus ER- and PR-negative), and nodal status (0 versus 1–3 versus 4–9 versus 10+), and randomized to 20 mg/day tamoxifen tablets (338 women) or placebo tablets (334 women) (Bramwell, et al., 2010). Figure 8 depicts the study's design.

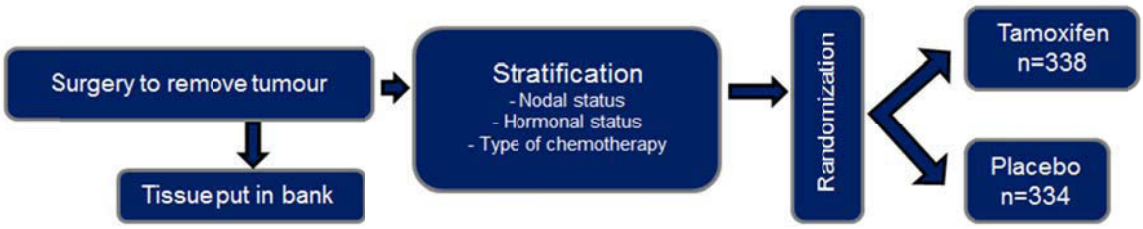

Figure 8: MA.12 patient flow.

Tumours removed at surgery were later used for TMA construction. Patients were stratified before randomization to tamoxifen or placebo (Bramwell, et al., 2010).

MA.12 has found that adjuvant treatment with tamoxifen significantly improves recurrence free survival at five years compared to placebo (78.2% vs. 71.3%; HR 0.77; p = 0.056) (Bramwell et al., 2010). A trend toward improved OS was also observed with tamoxifen after five years vs. placebo (86.6% vs. 82.1%; HR 0.78; p = 0.12). Interestingly, there was no evidence of greater benefit for the hormone receptor-positive or ER+ subgroups, with p = 0.71 and .14, respectively. The authors admit that this has no biological basis and has not been observed in similar trials. Patient and survival data from this study can be found in Appendix 5, Tables 1-4 and Appendix 5, Figure 1.

One of the drawbacks of MA.12 was the slow accrual that led to the closing of patient recruitment before the projected sample size was attained. This was attributed to an increasing conviction among physicians and patients that tamoxifen was beneficial in this setting, despite limited data to that effect at the time. Another concern was the influence of compliance on the validity of the results, with the authors discussing a likely dilution of the beneficial effects on tamoxifen. Of women starting treatment, 103 (31%) on tamoxifen and 70 (21%) on placebo stopped early because of toxicity, refusal, protocol violation, and other causes (Bramwell, et al., 2010).

3.2 Tissue Microarrays (TMAs)

Patient tumour samples were harvested at the time of tumour-removal surgery, fixed in formalin, and then embedded in paraffin. A TMA was built using a Beecher Instruments MTA II tissue micro-arrayer, wherein 5-micron cores were removed from the samples and injected in duplicate into a paraffin block. Sections of the four resulting paraffin blocks were cut by the NCIC immediately before being provided for the current project and placed on slides. Three kidney tumour cores were included as quality controls.

Four slides of each block were sent for the current project by the NCIC, one for each biomarker to be tested. Figure 9 is a depiction of the TMA construction process.

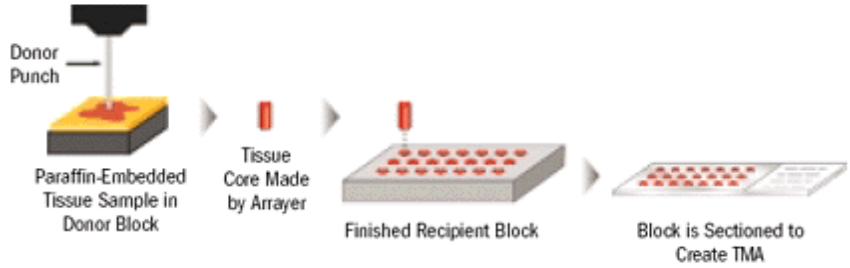

Figure 9: TMA construction process.

A needle is inserted into the paraffin-embedded tissue sample to remove a donor punch. This “core” is inserted into a paraffin block, which contains punches from multiple patients. This block is sectioned to provide slides for analysis. Adapted from (Gabrenya, 2004).

3.3 FISH

FISH was performed to visualize CCND1 and RSF1 gene statuses. A BAC probe, nick translated to be orange, was hybridized to these genes, and a green centromeric probe was used to evaluate whether a change in gene copy number was related to a whole-chromosome loss or gain or a target of amplification or deletion.

BAC clones specific to the two genes of interest were ordered from BACPAC Resources Centre (Oakland, CA) (CCND1 RP11-300I6, RSF1 RP11-1081L7 andRP11-1107J12). Two clones were ordered for RSF1 on suggestion from the authors of a previous paper exploring RSF1 by FISH (Brown, et al., 2008) who required two probes to visualize a signal bright enough to read. After testing, use of both sequences was deemed unnecessary on the NCIC TMAs and only probes from J12 were used.

BACs were amplified in 12.5 ug/mL chloramphenicol, incubated overnight in a 37ºC shaker. Because of the large molecular weight of the DNA desired for hybridization, Qiagen's Large Construct Kit (Cat. No. 12462, Qiagen, Valencia, CA) was used to purify the probe sequences.

Purified DNA was then nick translated using a commercially-available nick translation kit (07J00-001, Abbott Molecular, Mississauga, ON) to incorporate a fluorochrome conjugated to dUTP (Bayani & Squire, 2001). The use of orange

dUTP (02N33-050; Inter Medico, Markham, ON) to tag the probes has become a popular colour and was used for both CCND1 and RSF1. The nick translation method is based on the ability of DNAse I to introduce randomly distributed nicks into DNA at low enzyme concentrations in the presence of Mg2+. E. coli DNA polymerase I synthesizes DNA complementary to the intact strand in a 5' → 3' direction using the 3'-OH termini of the nick as a primer. The 5' → 3' exonucleolytic activity of DNA polymerase I simultaneously removes nucleotides in the direction of synthesis. The polymerase activity sequentially replaces the removed nucleotides with hapten-labelled deoxyribonucleoside triphosphates. At low temperatures (15°C), the unlabelled DNA in the reaction is thus replaced by newly synthesized labelled DNA (Science, 2009).

Before precipitating the probes from the nick translation mixture, human COT-1 DNA (Cat. No. 15279-011; Invitrogen, Burlington, ON) and salmon sperm DNA (Cat. No. 15632-011; Invitrogen, Burlington, ON) were added to compete for repetitive sequences in the tissue genome, eliminating background staining (Trifonov, Vorobieva, & Rens, 2009). 100% ethanol and 3M sodium acetate were used to precipitate the combination of probes and competitor DNA. The mixture was first frozen at -80°C for at least 20 minutes and then centrifuged at 4°C for 10 minutes at 10 000 RPM. The supernatant was removed and the DNA pellet was air-dried in the dark. It was then resuspended with a SpectrumGreen

chromosome-11 centromere enumeration probe (CEP11) (Order # 32-112011; Abbott Molecular, Mississauga, ON) and hybridization buffer (Order # 06J67-001; Abbott Molecular, Mississauga, ON). Immediately before application to the slide during the FISH protocol, this combination was denatured by heating in a 73°C water bath for 5 minutes.

The FISH protocol was optimized on test TMAs built by the Research Pathology Facility at the McGill Centre for Experimental Therapeutics in Cancer, Jewish General Hospital, Montreal. The method was then checked for validity by performing FISH on tissue samples (provided by the Département de Pathologie, Hôtel-Dieu de Montréal, Montreal) having amplifications, deletions, and normal copy numbers. Amplification statuses were known thanks to aCGH previously performed on these tissues in the Basik lab.

Slides were first deparaffinised for ten minutes in each of three jars of fresh xylenes and rehydrated with a 100%-95%-70% ethanol series. 0.2*N* hydrochloric acid was used to permeabilize the tissues for 30 minutes. It is presumed that the high-pH fixation reaction is reversed with the introduction of a low-pH acid. It is thought also that this acid deproteination increases probe penetration, decreasing the amount of time required for proteolysis, thus causing less tissue damage (Watters & Bartlett, 2002). Pre-treatment included 1.5 hours in citric acid pre-treatment solution (PT-0001-1000; Zytovision, Bremerhaven, Germany). It is

believed that heating cells in an acidic environment may remove chromatin proteins, allowing DNA exposure (Tojo et al., 2010).

As previously stated, the tissues were fixed in formalin, an aldehyde that crosslinks proteins and nucleic acids. A tissue digestion step to unmask nucleic acids was therefore required for proper probe attachment (Watters & Bartlett, 2002). Digestion occurred with 0.5g of the protease pepsin in 6 Coplin jars of 0.2*N* HCl warmed to 37°C for 15 minutes in each jar. The amount of time required for digestion and the optimal concentration of the solution was determined on test TMAs and the extent of digestion this standard conferred on the experimental tissue was verified with DAPI II counterstain (Order # 06J50-001; Abbott Molecular, Mississauga, ON). If digestion was poor, slides were reimmersed in protease until DAPI II revealed optimal digestion, with the nuclei showing a bright blue outline with a light blue interior.

The tissue was stabilized in 4% paraformaldehyde in PBS and then denatured in formamide denaturation buffer at 73°C for 6 minutes. After dehydration in a 75%-90%-100% ethanol series, the probe mixture was applied. A coverslip sealed with rubber cement kept the probes from evaporating during hybridization, which took place in a dark, humidified Hybridizer (Code S2450; Dako, Burlington, ON) hybridizing chamber at 37°C for 18 hours.

The rubber cement was removed after hybridization and the coverslips were allowed to slide off in an IGEPAL (Product # I7771-100ML; Sigma-Aldrich, Oakville, ON) wash buffer in the dark. Excess probe was removed in three Coplin jars of super pure formamide (Catalog # BP228-100; Fisher Scientific, Ottawa, ON) wash solution heated to 43°C for 10 minutes in each. The slides were then dried in the dark and counterstained with DAPI II. A coverslip was applied and the slides were stored at -20°C protected from light.

3.3.1 FISH Scoring

I. Metafer Counting

The Metafer (MetaSytems Group Inc., Waltham, MA) slide scanning system was used to automatically count the probe ratios in the test tissue. The system was validated on tissues – and TMAs of those tissues – known to be amplified and normal by visual analysis and aCGH. The machine is also in clinical use for HER2 analysis in the Department of Pathology, Jewish General Hospital, Montreal.

The Metafer system pre-scans the slide at low power magnification to create a map of the TMA with the position of each core. The machine uses the map to direct its analysis of the TMA at high power magnification. A tile sampling algorithm is used to choose nine areas of each core to analyze and count, and each colour channel is automatically focused for each tile to give the best picture

of the signals. The Metacyte software analyzes the pictures taken and reports the amplification states of each core based on the average of the ratios of orange to green signals within each tile.

II. Manual Counting

If DAPI II staining was suboptimal for the Metafer system, the map would consider that core as missing and skip over the sample during its read. There were also cores where Metacyte considered the probe colouring to be suboptimal, in which case pictures were taken but no amplification status was reported. In many cases, the cores could be read manually.

If no picture was taken because of poor DAPI II staining, pictures were taken using a Jenco Epi-fluorescent Microscope (Model No. EPI-F223, Jenco International Inc., Portland, OR) in the Department of Pathology, Jewish General Hospital, Montreal. Similar to the automated system, a picture was taken through each colour channel and then merged for counting. At least two areas from each core were used to count the orange to green ratios of 15 nuclei.

If Metafer had taken the pictures of the nine tiles, these were adjusted for optimal clarity using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA). At least two pictures were used to count the orange to green ratios of 15 nuclei.

The ratios of the 15 nuclei were averaged for each core. This number was considered the amplification state score for the core.

III. Determining amplification state

Based on a literature review of clinical trials, international studies and protocols, expert consensus, and US Food and Drug Administration Panel findings, the American Society of Clinical Oncologists-College of American Pathologists (ASCO/CAP) expert panel published guidelines the clinical use of HER2 amplification testing via FISH. It is recommended that a ratio of probe to centromere of >2.2 be considered amplified, that 1.8-2.2 be considered equivocal, and that <1.8 be considered a negative result (Wolff et al., 2007). Because of the very small proportion of tumours with a ratio of >2.2, samples with ratios over 1.8 were considered amplified.

3.4 Immunohistochemistry

Indirect immunohistochemistry was performed using the labelled streptavidin biotin (LSAB) method. LSAB has been shown to increase sensitivity eight-fold compared to the avidin-biotin complex (ABC) method (Giorno, 1984), possibly due to streptavidin being uncharged relative to tissue so that electrostatic binding is eliminated and that streptavidin lacks carbohydrate groups that can bind tissue lectins (Shi, Itzkowitz, & Kim, 1988).

Slides were first deparaffinised for ten minutes in each of three Coplin jars of xylenes and rehydrated in a 100%-95%-70% ethanol series. The tissue was then steamed in low-pH antigen-unmasking solution (H-3300; Vector Laboratories,

Burlington, ON), which breaks protein crosslinks formed by formalin fixation (Hayat, 2002), for 30 minutes.

Horseradish-peroxidase (HRP) is used in this method to create a brown staining to localize the protein of interest. To avoid background staining from endogenous peroxidase activity, tissue was blocked in 3% hydrogen peroxide (H_2O_2).

Background staining can also be caused by non-specific binding of the secondary antibody to reactive sites. Normal serum, generated in the same animal as the secondary antibody, carries antibodies that bind these sites and thus prevents the associated background staining. As both the primary antibodies against our proteins of interest were generated in rabbit, goat-anti-rabbit secondary antibodies were used, and 5% goat serum in phosphate-buffered saline with 0.1% Tween-20 (PBS-T) was used as a blocking buffer for 30 minutes at room temperature.

The primary antibodies used were the same as the ones used by the groups exploring TC21 (Rokavec, et al., 2008) and PAX2 (Hurtado, et al., 2008) in tamoxifen-treated patients: a rabbit polyclonal anti-TC21 (sc-883; Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit polyclonal anti-PAX2 (ab38738; Abcam, Cambridge, MA). Slides were incubated with the desired antibody at a dilution of 1:100 in the blocking buffer overnight at 4°C in a humidity chamber. Biotinylated goat-anti-rabbit secondary antibody (111-065-003; Jackson

Immunoresearch Laboratories, West Grove, PA) was applied at a dilution of 1:300 in the blocking buffer for one hour at room temperature in a humidity chamber. Horseradish peroxidase-conjugated streptavidin (016-030-084; Jackson Immunoresearch Laboratories, West Grove, PA) was then applied at a dilution of 1:2500 in the blocking buffer for one hour at room temperature in a humidity chamber.

To visualize protein of interest, a solution of 3,3' diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide is applied to the slide. In the presence of the HRP enzyme, these are converted to a brown precipitate. This brown staining thus represents an area in which the protein of interest was found. The DAB reaction (SK-4100; Vector Laboratories, Burlingame, CA) was stopped in PBS and then the slide was counterstained in Harris-modified haematoxylin solution (HHS16; Sigma-Aldrich, Oakville, ON). This allows the protein staining to be localized as haematoxylin stains the nuclei purple. The slides were then dehydrated in a 70%-95%-100% ethanol series for three minutes each and immersed twice in xylenes for seven minutes each. Curemount II (Cat# 475233; Instrumedics, Inc., Richmond, IL) was used as the mounting medium to stabilize the staining.

3.4.1 Immunohistochemistry Scoring

The slides were scored in duplicate by Dr. Mark Basik (MB) and Dr. Olga Aleynikova (OA). A four-point scale was used to score intensity of the staining: 0 for no staining, 1+ for low staining, 2+ for medium staining, and 3+ for high staining. PAX2 was only scored for nuclear staining, while TC21 was scored for both nuclear (MB only) and cytoplasmic staining. The percentage of the cells in the core staining at that intensity was also reported by MB.

These scores were compiled using several approaches to take into consideration the multiple values collected for each patient. Table 1 describes the parameters analyzed.

Protein	Cellular localization	Analysis method	Description
TC21	Cytoplasm	Average combined cytoplasmic intensity (ACCI)	The average of the intensities of the two cores of one sample was taken. These two averages, one from each reader, were themselves averaged to give the ACCI.
		Average combined maximum cytoplasmic intensity (ACMCI)	The maximum score of the two cores was taken. These scores, one from each reader, were averaged to give the ACMCI.
		Average combined cytoplasmic product score (ACCPS)	For MB readings, the intensity of each core was multiplied by the percentage of cells staining at that intensity ('percentage'). For OA readings, the average of the percentages reported by MB was multiplied by each core's intensity. These numbers were averaged to give the ACCPS.
	Nucleus	Average nuclear intensity (ANI)	The average of the intensities of the two cores was taken to give the ANI.
		Maximum nuclear intensity (MNI)	The maximum score of the two cores was taken to give the MNI.
		Average nuclear product score (ANPS)	The intensity of each core was multiplied by its percentage. These numbers were averaged to give the ANPS.
PAX2	Nucleus	Average combined nuclear intensity (ACNI)	The average of the intensities of the two cores of one sample was taken. These two averages, one from each reader, were themselves averaged to give the ACNI.

Protein	Cellular localization	Analysis method	Description
		Average combined maximum nuclear intensity (ACMNI)	The maximum score of the two cores was taken. These scores, one from each reader, were averaged to give the ACMNI.
		Average combined nuclear product score (ACNPS)	For MB readings, the intensity of each core was multiplied by its percentage. For OA readings, the average of the percentages reported by MB was multiplied by each core's intensity. These numbers were averaged to give the ACNPS.

Table 1: IHC analysis methods.

TC21 expression was measured in the cytoplasm (average combined cytoplasmic intensity, average combined maximum cytoplasmic intensity, and average combined cytoplasmic product score) and in the nucleus (average nuclear intensity, maximum nuclear intensity, average nuclear product score). PAX2 expression was measured in the nucleus (average combined nuclear intensity, average combined maximum nuclear intensity, and average combined nuclear product score).

4. Results

4.1 Validation of Methodology

4.1.1 FISH

To ensure that the FISH protocol was sensitive and specific, FISH was performed on 12 tissue sections from 6 tumours on which aCGH had been previously performed in our lab, therefore permitting knowledge of the amplification status of the samples. Two tumours had both CCND1 and RSF1 amplified, three tumours were amplified at only CCND1, and three tumours had only RSF1 amplifications. All samples were tested for both CCND1 and RSF1. All experiments resulted in Metafer reporting ratios within the appropriate ratio ranges according to the status that had been reported by aCGH. Figure 10 shows the FISH results.

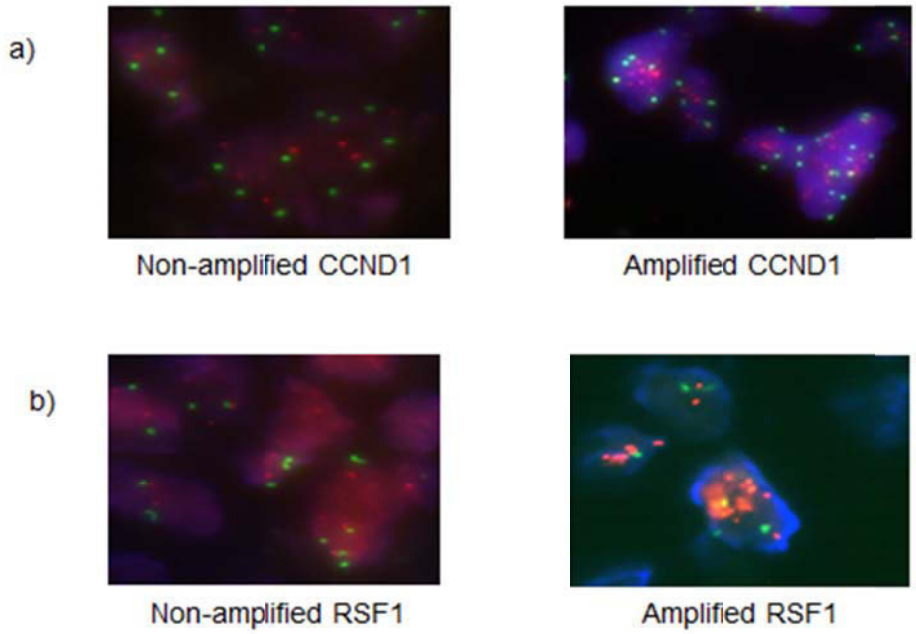

Figure 10: FISH performed on tumours amplified and non-amplified for CCND1 (a) and RSF1 (b).
Orange probe: CCND1/RSF1; green probe: CEP11; blue: DAPI counterstain

4.1.2 IHC

To ensure that the IHC protocol was sensitive and specific, a microarray of cores containing samples of cell lines was obtained from Dr. Sylvie Mader at the Institut de Recherce en immunologie et cancérologie, Université de Montréal, and stained for PAX2 and TC21. Among the cell lines, the expression of MDA-MB-361, MDA-MB-453, MDA-MB-231, BT-20 was considered aberrant according to expression data. The expression of the cells by IHC matched the expression reported in the expression data.

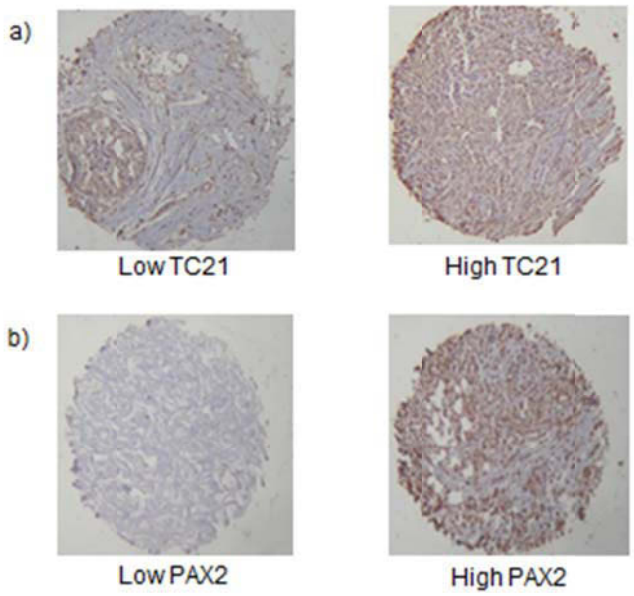

Figure 11: IHC performed on cell lines with known TC21 (a) and PAX2 (b) expression.
Brown: DAB staining; purple: haematoxylin counterstain

4.2 CCND1

4.2.1 Patient distribution and baseline characteristics

CCND1 status was measured in 448 patients. 410 patients had a FISH ratio of <1.8, 12 patients had a FISH ratio of 1.8-2.2, and 26 patients had a FISH ratio of >2.2. 38 tumours (8.7%) were considered amplified with ratios ≥1.8. Figure 12 shows this distribution of patients.

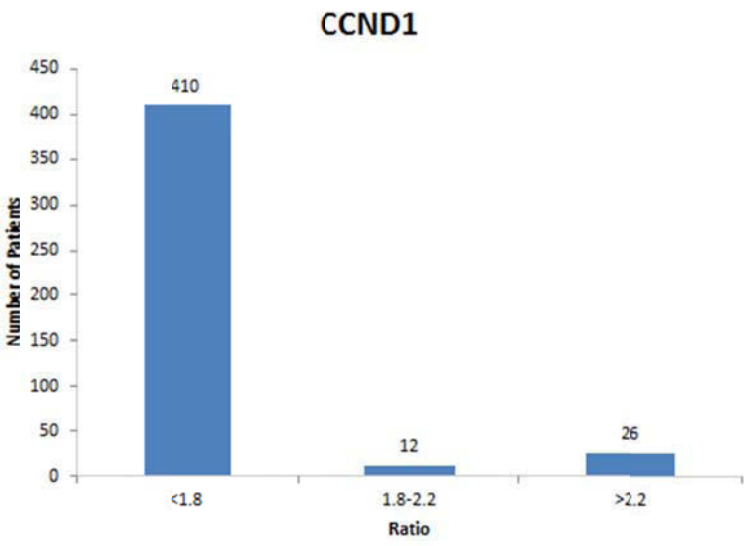

Figure 12: Patient distribution according to CCND1 status.

Baseline characteristics were calculated for amplified and non-amplified CCND1 respectively and are presented in Appendix 6. The p-value for variable age was calculated by Wilcoxon two sample test, while for others were calculated by Chi-square test. Patients with amplified CCND1 were significant more likely to be ER+ (p = 0.01) and to have higher ECOG status (measuring the physical restrictions a disease puts on a patient) (p = 0.04). There was no significant difference between groups in nodal status, adjuvant chemotherapy choice, age, stage, or treatment group.

4.2.2 Predictive analysis

5-year RFS and OS were measured comparing tamoxifen and placebo arms for patients with respectively amplified and non-amplified CCND1. Hazard ratios (HRs) and p-values were adjusted for treatment (when appropriate), age,

performance status, time from diagnosis to randomization, nodal status, stage, receptor status, and type of chemotherapy treatment. All the HRs were compared to the placebo arm.

i. Overall survival

CCND1 status was not predictive of OS between treatment groups (interaction p = 0.41). However, there may be a trend in OS in patients with non-amplified CCND1 between those treated with tamoxifen vs. placebo [adjusted HR 0.69 (95% CI 0.44-1.08); p = 0.10].

CCND1 Status and Treatment		# of patients	5-Year OS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
CCND1 non-amplified	T	198	0.85 (0.79, 0.90)	0.28	0.69 (0.44, 1.08) [0.10]	0.41
	P	203	0.84 (0.78, 0.88)			
CCND1 amplified	T	21	0.95 (0.71, 0.99)	0.96	0.58 (0.11, 3.21) [0.54]	
	P	17	0.81 (0.52, 0.94)			

Table 2: CCND1 status as a predictive marker of 5-year OS.
T: tamoxifen; P: placebo

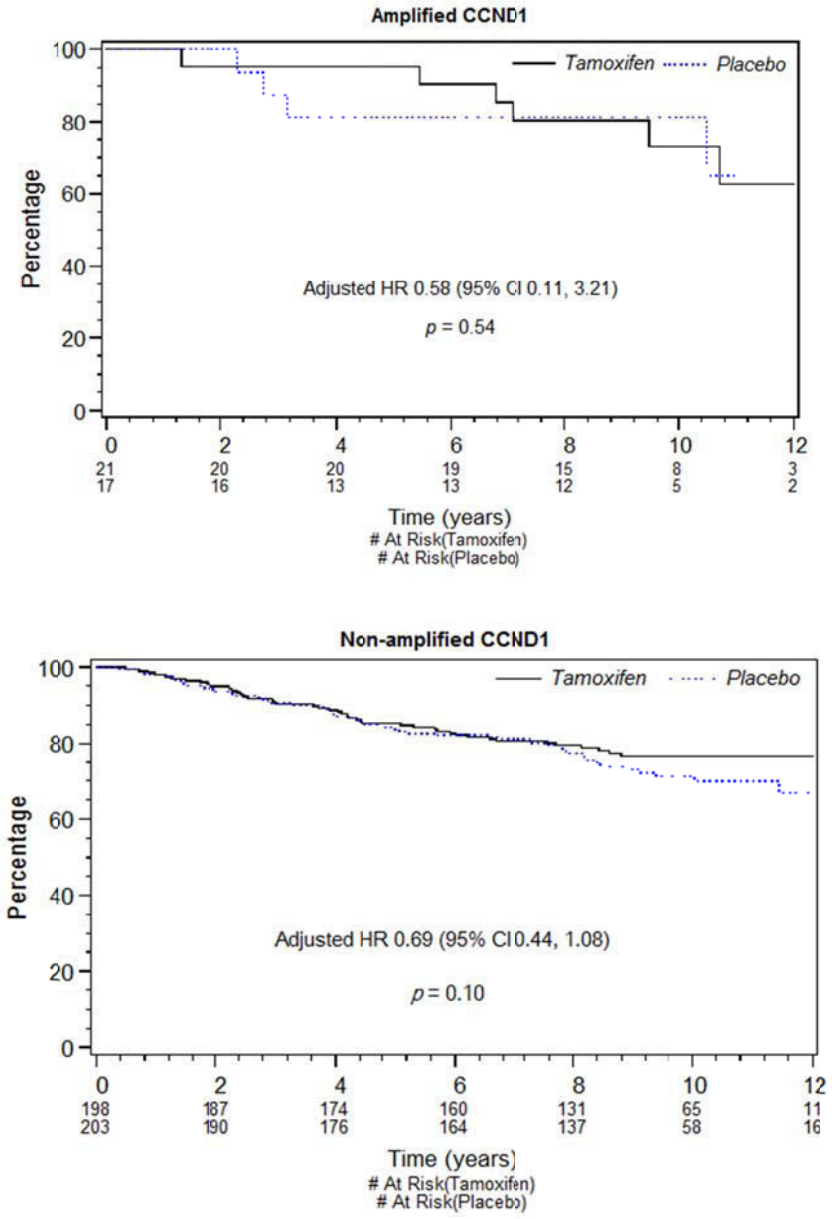

Figure 13: OS for patients by CCND1 amplification status.

ii. Recurrence-free survival

In patients with non-amplified CCND1 status, the 5-year RFS in patients receiving tamoxifen (0.78, 95% CI 0.72-0.83) was significantly higher than those receiving placebo (0.72, 95% CI 0.65-0.77), with an adjusted HR of 0.62 (95% CI

042-0.91, p = 0.01). CCND1 status was not found, however, to be a predictive biomarker of recurrence-free survival between treatment groups (interaction p = 0.90).

CCND1 Status and Treatment		# of patients	5-Year RFS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
CCND1 non-amplified	T	197	0.78 (0.72, 0.83)	0.06	0.62 (0.42, 0.91) [0.01]	0.90
	P	203	0.72 (0.65, 0.77)			
CCND1 amplified	T	21	0.81 (0.57, 0.92)	0.26	0.42 (0.12, 1.46) [0.17]	
	P	17	0.59 (0.33, 0.78)			

Table 3: CCND1 status as a predictive marker of 5-year RFS.

T: tamoxifen; P: placebo

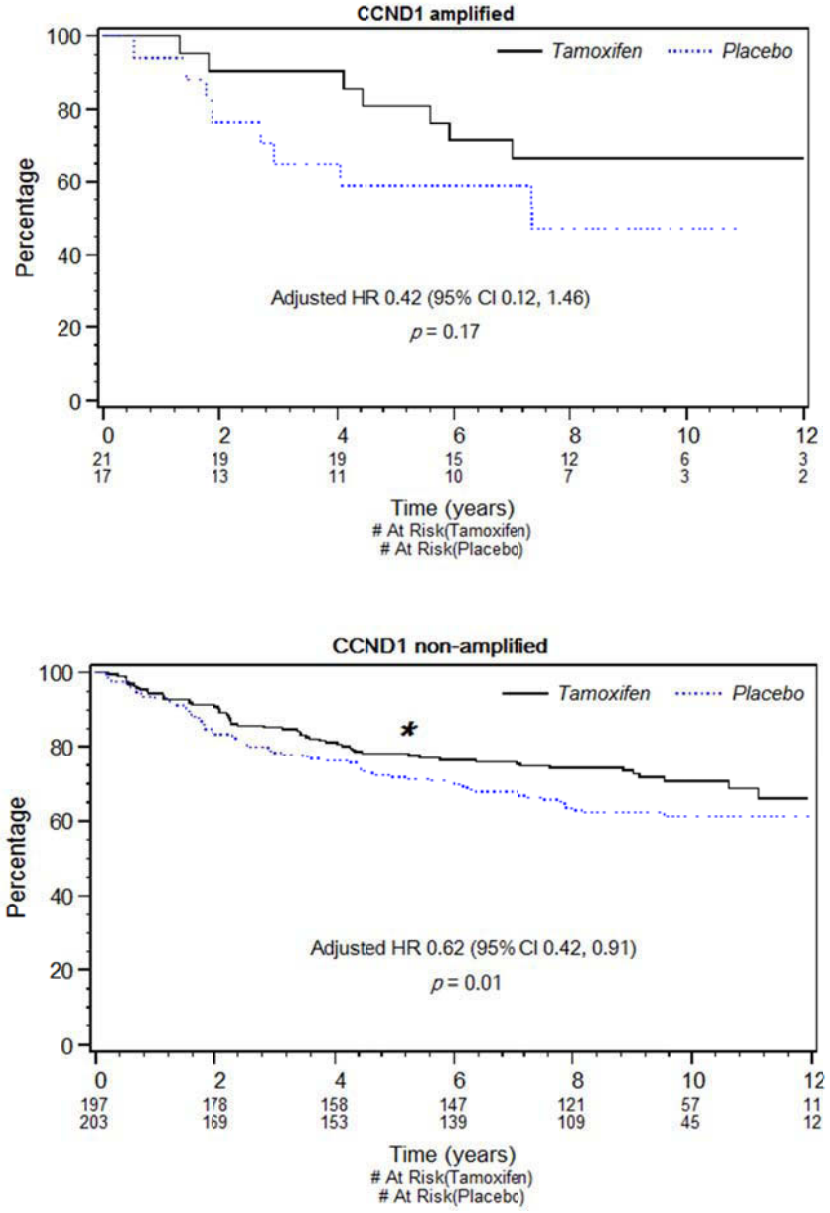

Figure 14: RFS for patients by CCND1 amplification status.

* denotes $p < 0.05$

4.3 RSF1

4.3.1 Patient distribution and baseline characteristics

RSF1 status was measured in 410 patients. 382 patients (93.2%) had a FISH ratio of <1.8, 11 patients (2.7%) had a FISH ratio of 1.8-2.2, and 17 patients (4.1%) had a FISH ratio of >2.2. 28 tumours (6.8%) were considered amplified with ratios ≥1.8. Figure 15 depicts this distribution of patients.

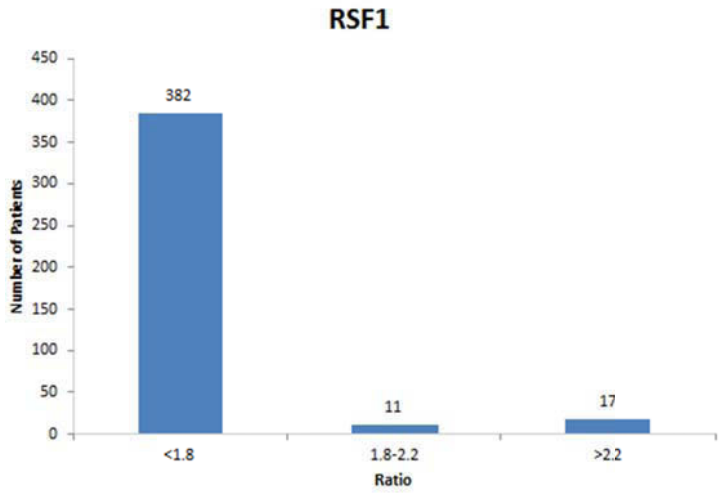

Figure 15: Patient distribution according to RSF1 status.

Baseline characteristics were calculated for patients with respectively amplified and non-amplified RSF1 and are presented in Appendix 7. Again, the p-value for age was calculated by Wilcoxon two sample test, while for all others the Chi-square test was used. Patients with amplified RSF1 were more likely to be ER+ (p = 0.05), There was no significant difference between groups in nodal status, adjuvant chemotherapy choice, age, stage, ECOG status, or treatment group.

4.3.2 Predictive analysis

5-year RFS and OS were measured comparing tamoxifen and placebo arms for patients with respectively amplified and non-amplified RSF1. Hazard ratios (HRs) and p-values were adjusted for treatment (when appropriate), age, performance status, time from diagnosis to randomization, nodal status, stage, receptor status, and type of chemotherapy treatment. All the HRs were compared to the placebo arm.

I. Overall survival

RSF1 status was not predictive of OS between treatment groups (interaction p = 0.30). However, in patients with non-amplified RSF1, the 5-year OS in patients receiving tamoxifen [5-year OS 0.85 (95% CI 0.79-0.89)] was significantly higher than those receiving placebo [5-year OS 0.83 (95% CI 0.77-0.88)], with an adjusted HR of 0.62 (95% CI 0.38-0.99; p = 0.04).

RSF1 Status And Treatment		# of patients	5-Year OS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
RSF1 non-amplified	T	188	0.85 (0.79, 0.89)	0.21	0.62 (0.38, 0.99) [0.04]	0.30
	P	194	0.83 (0.77, 0.88)			
RSF1 amplified	T	12	0.92 (0.54, 0.99)	0.52	0.69 (0.07, 6.47) [0.75]	
	P	16	0.80 (0.50, 0.93)			

Table 4: RSF1 status as a predictive marker of 5-year OS.
T: tamoxifen; P: placebo

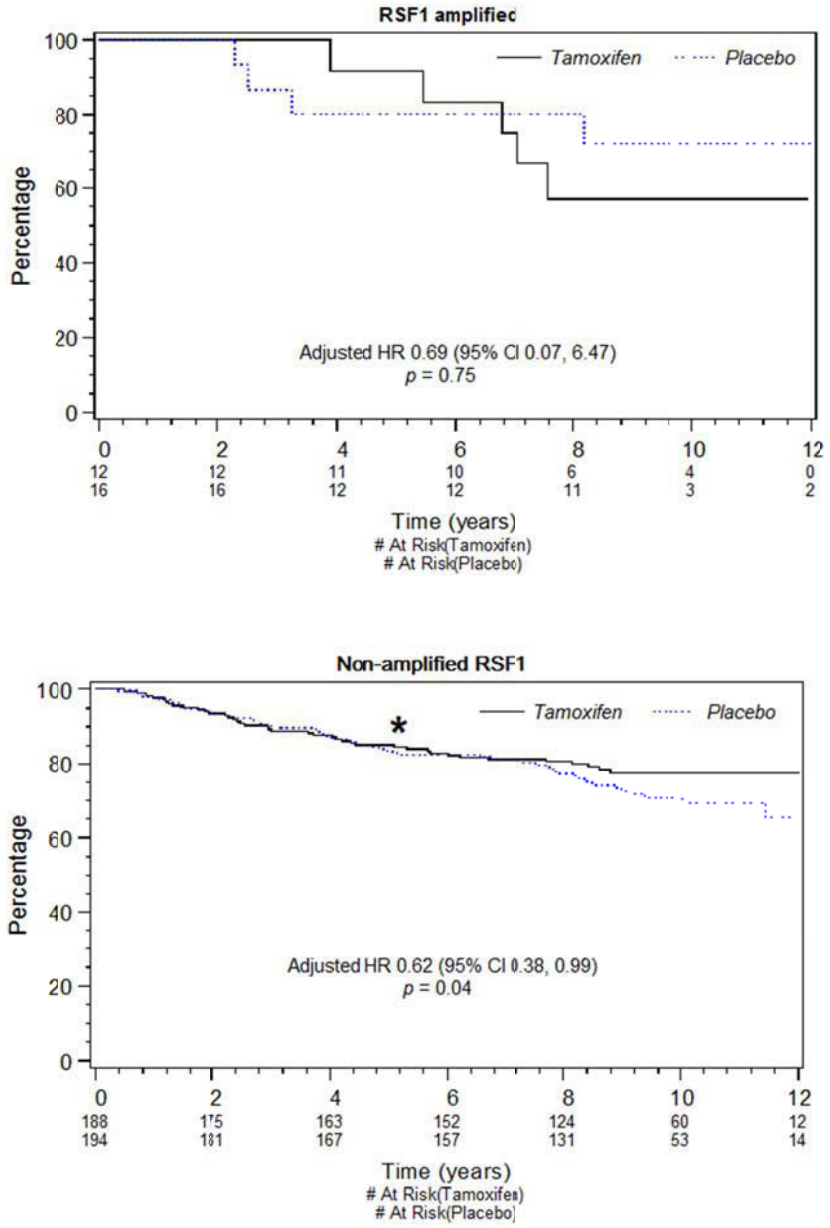

Figure 16: OS for patients by RSF1 amplification status.

* denotes $p < 0.05$

II. Recurrence-free survival

In patients with non-amplified RSF1 status, the 5-year RFS in patients receiving tamoxifen (5-year RFS 0.79, 95% CI 0.72-0.84) was significantly higher than those receiving placebo (5-year RFS 0.70, 95% CI 0.63-0.76). While the p-value for the interaction between treatment group and RSF1 status did not reach 0.05, there was a trend toward significance (interaction $p = 0.09$).

RSF1 Status And Treatment		# of patients	5-Year RFS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
RSF1 non-amplified	T	187	0.79 (0.72, 0.84)	0.02	0.51 (0.34, 0.77) [0.001]	0.09
	P	194	0.70 (0.63, 0.76)			
RSFI amplified	T	12	0.67 (0.34, 0.86)	0.63	1.11 (0.24, 5.15) [0.89]	
	P	16	0.69 (0.40, 0.86)			

Table 5: RSF1 status as a predictive marker of 5-year RFS.

T: tamoxifen; P: placebo

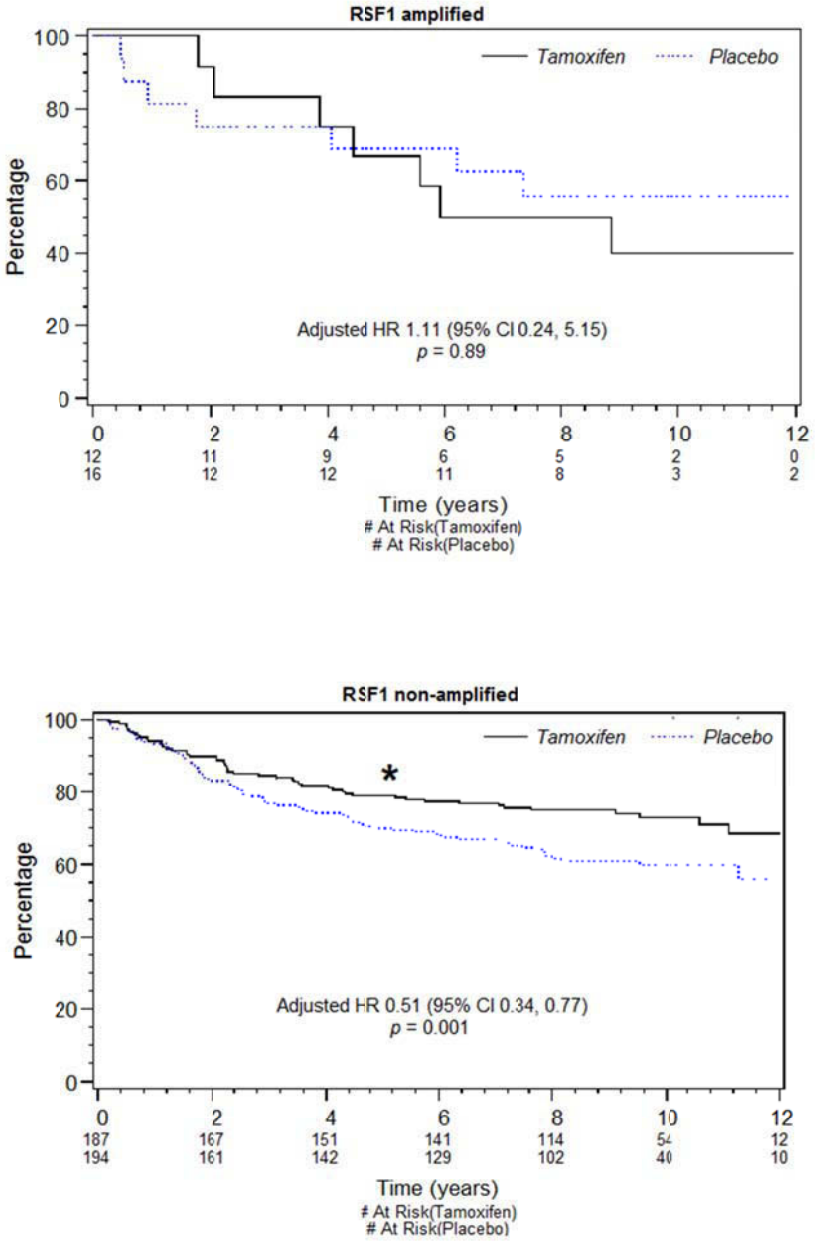

Figure 17: RFS for patients by RSF1 amplification status.

* denotes $p < 0.05$

4.4 PAX2

4.4.1 Patient distribution and baseline characteristics

PAX2 was measured in 452 patients. Measured using ACNI, 259 (57%) of patients expressed PAX2 (nuclear staining score of 1+, 2+, or 3+) and 193 (43%) showed no expression (nuclear staining score of 0). Baseline characteristics were measured for patients with respectively no and high ACNI. The p-value for age was calculated by Wilcoxon two-sample test and all others were calculated by the Chi-square test. There was no apparent difference in any of these characteristics between the two groups presented in Appendix 8.

4.4.2 Predictive analysis

5-year RFS and OS were measured comparing tamoxifen and placebo arms for patients respectively PAX2-positive or PAX2-negative. Hazard ratios (HRs) and p-values were adjusted for treatment (when appropriate), age, performance status, time from diagnosis to randomization, nodal status, stage, receptor status, and type of chemotherapy treatment. All the HRs were compared to the placebo arm.

I. Overall survival

PAX2 expression was not predictive of OS between treatment groups (interaction $p = 0.86$).

ACNI Status and Treatment		# of patients	5-Year OS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
ACNI-negative	T	92	0.86 (0.77, 0.91)	0.34	0.72 (0.38, 1.40) [0.33]	0.86
	P	101	0.84 (0.75, 0.90)			
ACNI-positive	T	134	0.86 (0.79, 0.91)	0.64	0.65 (0.37, 1.14) [0.13]	
	P	125	0.83 (0.75, 0.89)			

Table 6: PAX2 as a predictive marker of 5-year OS.

T: tamoxifen; P: placeo

II. Recurrence-free survival

In patients with PAX2 expression, the 5-year RFS in patients receiving tamoxifen (5-year RFS 0.78, 95% CI 0.69-0.84) was significantly higher than those receiving placebo (5-year RFS 0.72, 95% CI 0.63-0.79), with an adjusted HR of 0.52 (95% CI 0.32-0.85, p = 0.008). However, the adjusted HR for patients with no PAX2 expression was of borderline significance (p = 0.08). Accordingly, the p-value for interaction was 0.60.

ACNI Status and Treatment		# of patients	5-Year RFS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
ACNI-negative	T	91	0.80 (0.70, 0.87)	0.15	0.61 (0.35, 1.06) [0.08]	0.60
	P	101	0.69 (0.59, 0.77)			
ACNI-positive	T	134	0.78 (0.69, 0.84)	0.16	0.52 (0.32, 0.84) [0.008]	
	P	125	0.72 (0.63, 0.79)			

Table 7: PAX2 as a predictive marker of 5-year RFS.

T: tamoxifen; P: placebo

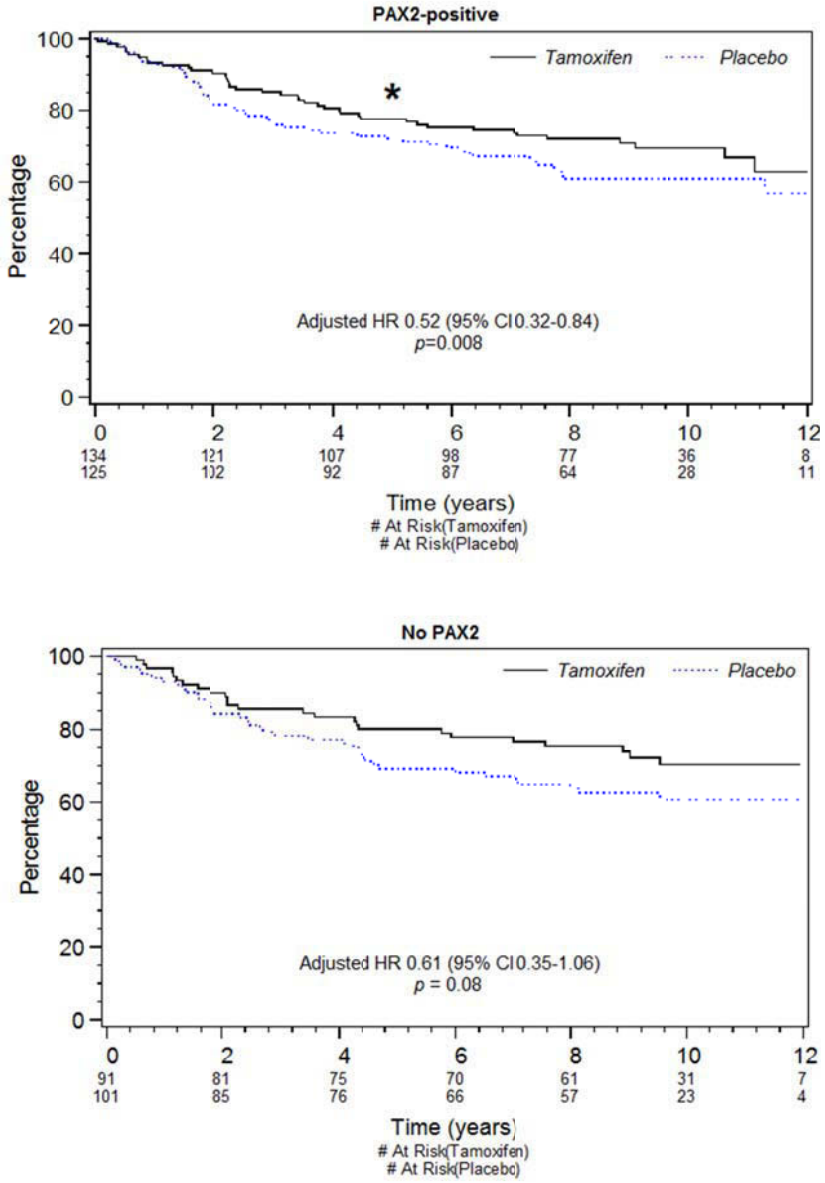

Figure 18: RFS for patients by PAX2 expression.

* denotes $p < 0.05$

4.5 TC21

4.5.1 Patient distribution and baseline characteristics

TC21 was measured in 450 patients. Measured using ACMCI, 167 (37%) of patients expressed low levels of TC21 (cytoplasmic staining 0 or 1+) and 283 (63%) expressed high levels of TC21 (cytoplasmic staining 2+ or 3+). Baseline characteristics were measured for patients with respectively low and high ACMCI, presented in Appendix 9. The p-value for age was calculated by Wilcoxon two-sample test and all others were calculated by the Chi-square test. With high ACMCI, patient were more likely to be HR+ ($p = 0.03$), ER+ ($p = 0.05$), be older [median age in high TC21 group 45.7 years (range 27.1-57.8) vs. median age in low TC21 group 45.4 years (range 29.3-55.2)] ($p = 0.05$), and have high ECOG status ($p = 0.04$). There was no difference between the expression groups for PR status, nodal status, adjuvant chemotherapy choice, stage, or treatment group.

4.5.2 Predictive analysis

5-year RFS and OS were measured comparing tamoxifen and placebo arms for patients with high and low TC21 expression. Hazard ratios (HRs) and p-values were adjusted for treatment (when appropriate), age, performance status, time from diagnosis to randomization, nodal status, stage, receptor status, and type of chemotherapy treatment. All the HRs were compared to the placebo arm.

I. Overall survival

TC21 expression was not predictive of OS between treatment groups (interaction $p = 0.30$). There was, however, a trend toward a significantly increased 5-year OS in patients with low TC21 expression having received tamoxifen vs. placebo [adjusted HR 0.51 (95% CI 0.24-1.09), $p = 0.08$].

ACMCI status And Treatment		# of patients	5-Year OS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
ACMCI Low	T	77	0.84 (0.74, 0.91)	0.20	0.51 (0.24, 1.09) [0.08]	0.30
	P	90	0.83 (0.74, 0.90)			
ACMCI High	T	146	0.86 (0.79, 0.91)	0.89	0.81 (0.48, 1.37) [0.44]	
	P	137	0.84 (0.76, 0.89)			

Table 8: TC21 as a predictive marker of 5-year RFS.
T: tamoxifen; P: placebo

II. Recurrence-free survival

II-i. All patients

In patients with low TC21 expression, those treated with tamoxifen had a significantly increased 5-year RFS (5-year RFS 0.82, 95% CI 0.71-0.89) compared to those treated with placebo (5-year RFS 0.72, 95% CI 0.62-0.80), with an adjusted HR of 0.39 (95% CI 0.20-0.78, $p = 0.007$). The interaction

between TC21 expression and treatment in all patients was found to be insignificant ($p = 0.15$).

ACMCI Status and Treatment		# of patients	5-Year RFS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
ACMCI Low	T	77	0.82 (0.71, 0.89)	0.06	0.39 (0.20, 0.78) [0.007]	0.15
	P	90	0.72 (0.62, 0.80)			
ACMCI High	T	145	0.76 (0.68, 0.82)	0.43	0.77 (0.50, 1.20) [0.25]	
	P	137	0.70 (0.62, 0.77)			

Table 9: TC21 as a predictive marker of 5-year RFS.

T: tamoxifen; P: placebo

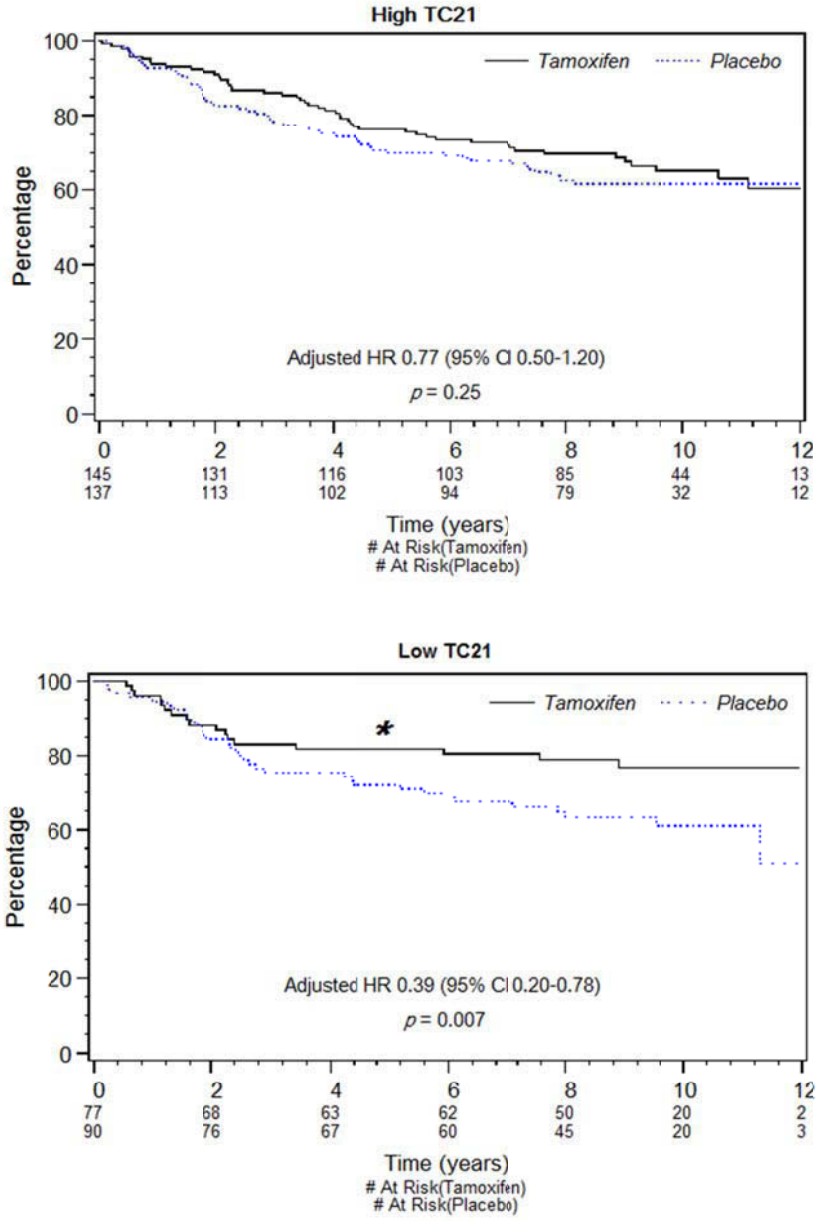

Figure 19: RFS for patients by TC21 expression.

* denotes $p < 0.05$

II-ii. ER+ subgroup

Because of the trend toward significance in predicting recurrence-free survival between treatment groups and the clear utility of tamoxifen in patients with low TC21 expression, it was hypothesized that the inclusion of ER- patients in the trial may account for the non-significant p-value for interaction. A subgroup analysis of ER+ patients was performed.

In this subgroup analysis, the adjusted HR in patients with low TC21 expression maintained its significance at $p = 0.04$ and patients with high TC21 expression continued to receive no benefit from tamoxifen treatment with an adjusted HR of 1.02 ($p = 0.93$). The p-value for interaction improved to borderline significance with $p = 0.08$.

ACMCI Status and Treatment		# of patients	5-Year RFS (95% CI)	Log-rank p-value	Adjusted HR (95% CI) [p-value]	P-value for Interaction
ACMCI Low	T	46	0.85 (0.71, 0.92)	0.03	0.44 (0.20, 0.95) [0.04]	0.08
	P	53	0.72 (0.58, 0.82)			
ACMCI High	T	94	0.77 (0.67, 0.85)	0.73	1.02 (0.63, 1.66) [0.93]	
	P	99	0.77 (0.67, 0.84)			

Table 10: TC21 as a predictive marker of 5-year RFS in ER+ patients.
T: tamoxifen; P: placebo

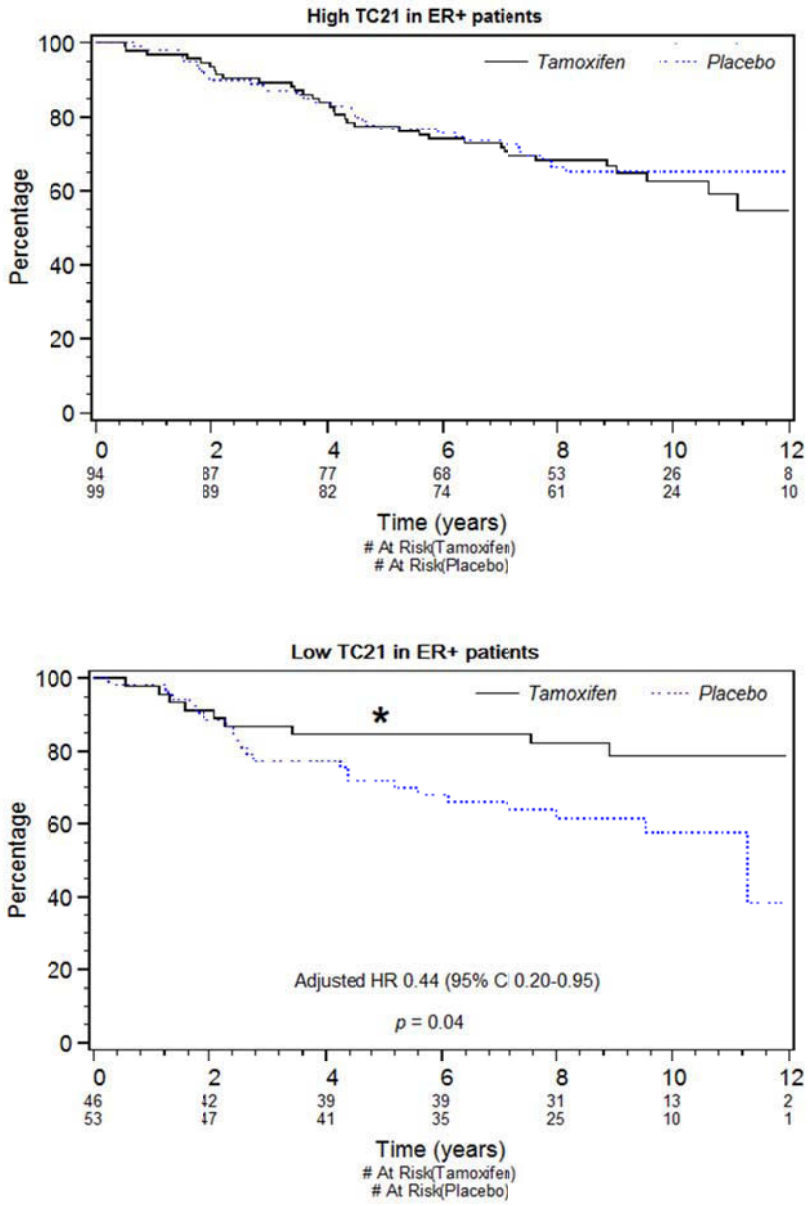

Figure 20: RFS for ER+ patients by TC21 expression.

* denotes $p < 0.05$

5. Discussion

Though tamoxifen is an extremely effective anti-hormonal therapy, resistance – both intrinsic and acquired – thereto is a major concern. Better treatment choices lead to better outcomes, and women can be given more years cancer-free or even be counted among cancer survivors with the ability to decide appropriately whether tamoxifen should be considered a therapeutic option.

Tamoxifen therapy is currently based on hormone receptor status. Current understanding of breast cancer subgroups is focused on gene expression profiles, wherein patient prognosis can be correlated with the expression of groups of genes (Sørlie et al., 2001). It could thus be expected that hormone receptor status alone may be an imperfect standard by which to treat, and could be improved as a predictive assay by including the analysis of other changes in the tumour.

Changes that are easily measured in the clinic are those of gene amplification and protein expression. Indeed, testing patients for ERBB2 amplification is currently used to determine whether to treat with Herceptin, which was shown to be more effective in predicting response to the drug than Her2 expression (Dybdal et al., 2005).

In an effort to enhance the ability to predict response to tamoxifen, we aimed to validate four biomarkers that have shown promise in preliminary studies but were

previously explored in studies that could not reach a level of significance necessary to have these candidates brought to the clinic. For CCND1, most groups exploring its ability to predict for tamoxifen response used a consecutive cohort or simply available tumours from a bank rather than a trial (Bieche, et al., 2002; Dancau, et al., 2010; Elsheikh, et al., 2008; Kirkegaard, et al., 2008; Reis-Filho, et al., 2006). One trial had only a tamoxifen arm and tested the addition of a second treatment to tamoxifen (Rudas, et al., 2008) and another tested two years of tamoxifen at two different doses vs. placebo (Jirstrom, et al., 2005). CCND1 was also studied in another randomized trial comparing tamoxifen to placebo, but patients were also randomized to chemotherapy or radiation treatment and treatment lasted two years at 40 mg/day (Ahnstrom, et al., 2005; Bostner, et al., 2007). Possibly part of the 11q13-14 amplicon, RSF1 amplification has not been evaluated in breast cancer. TC21 expression as it relates to tamoxifen response has only been assessed by one group (Rokavec, et al., 2008), which retrospectively analyzed a set of archived tissues. PAX2 has been studied in breast cancer in relation to tamoxifen response by, again, one group (Hurtado, et al., 2008) that looked at a set of tamoxifen-treated tumours. The literature suggested much promise for these biomarkers and their validation in an RCT was necessary for defining their possible clinical use.

Our data found that 8.7% of patients were amplified for CCND1, using a much less conservative cut-off defining amplification. This is compared with the reported amplification rate of 15% (Bostner, et al., 2007; Jirstrom, et al., 2005; Reis-Filho, et al., 2006). One group found CCND1 amplification to be an independent prognostic factor in ER+ breast cancer (Elsheikh, et al., 2007) while another found that CCND1 was not an independent prognostic factor for RFS/OS (Reis-Filho, et al., 2006). Our analysis did not uncover a role for CCND1 as a prognostic factor (Appendix 10a). While the p-value for interaction in prediction for OS was not significant, there was a trend toward better OS in patients with non-amplified CCND1. Kirkegaard (2008) found that CCND1 amplification predicted for decreased OS in ER+ patients. In non-amplified tumours, there was a significant difference in RFS between those treated with tamoxifen vs. placebo. Compared to other studies (Bieche, et al., 2002; Bostner, et al., 2007; Jirstrom, et al., 2005), however, CCND1 amplification could not presently be categorized as a predictive marker. Jirstrom (2005) found a significant interaction between tamoxifen and CCND1 amplification for RFS in ER+ breast cancers: tumours amplified for CCND1 in MA.12 were more likely to be ER+, and this may be a worthwhile subgroup to analyze. This finding is not unexpected, as cyclin D1 is both an upstream regulator and downstream target of ER (Fu, et al., 2004). Indeed, because of the large body of evidence pointing to CCND1 being clinically

useful, we plan to move to a larger trial to explore its utility. It could be that, in MA.12, the influence of CCND1 could not overcome the dilution of tamoxifen effects suggested by the authors as a result of low compliance rates.

RSF1 was found to be amplified in 6.8% of patients. No evidence exists as to the rate of RSF1 amplification in breast cancer, but it was found that expression of the Rsf1 protein was low in this cancer (Mao, et al., 2006). RSF1 was, along with CCND1, significantly more frequently amplified in ER+ tumours than in ER- tumours (p = 0.01). This could be an effect of co-amplification with 11q13: 9/28 tumours amplified for RSF1 also harboured a CCND1 amplification. When examining the functional role of Rsf-1, Sheu et al. (2010) found that Rsf-1's overexpression causes double-stranded breaks in the DNA, though the RSF complex is known to be involved in DNA damage repair. The group hypothesized that extra Rsf-1 may sequester available levels of its RSF pair, SNF2H, and compromise the actions of other complexes of which SNF2H is a part. This biological explanation does not seem to be the case in the MA.12 cohort, as RSF1 amplification was not found to be a prognostic factor for OS or RFS (Appendix 10b). RSF1 was found to be a borderline-significant predictor of 5-year RFS with tamoxifen (p for interaction 0.09). Patients in this trial with normal RSF1 copy numbers could also expect to have a significantly longer OS and RFS when treated with tamoxifen compared to placebo. In ovarian cancer, two groups have

found that RSF1 amplification leads to significantly worse outcomes (Brown, et al., 2008; Shih, et al., 2005). RSF1 certainly deserves further study as a biomarker of tamoxifen response, and moving to a larger cohort of patients will no doubt yield more patients amplified for RSF1 for further validation.

PAX2 was found to be expressed in 57% of patients, within the reported range of 40-60% in breast cancer (Silberstein, 2002; Hurtado, et al., 2008). In our study, PAX2 was not found to be a predictive or a prognostic (Appendix 10c) marker. While PAX2-positive patients showed a significantly increased RFS with tamoxifen vs. placebo, patients with no PAX2 expression also had a borderline significantly increased RFS with treatment – patients showed benefit from tamoxifen regardless of their PAX2 expression. Hurtado (2008) explored the predictive value of this protein in a cohort of patients with metastatic breast cancer that were all ER+ and all treated with tamoxifen and found that positive PAX2 staining corresponded to a significantly improved RFS compared to PAX2-negative tumours ($p < 0.0001$). It is unexpected that a subgroup analysis of our data in ER+ patients treated with tamoxifen would result in this suggestive result: in prognostic analysis of the association of ACNI status and outcomes for tamoxifen-treated patients, the adjusted OS HR was 1.14 ($p = 0.67$) and the adjusted RFS HR was 1.04 ($p = 0.88$) (Appendix 10d). PAX2 does not seem to be a predictive marker of tamoxifen response in breast cancer in our study. It

may be interesting, however, to analyze PAX2 in the ER+ subgroup of MA.12 and to correlate it with expression of AIB1, its competitive partner in regulating tamoxifen's interaction with the promoter of the ERBB2 gene.

Elevated TC21 expression was found in 63% of patients, compared to the 44% of patients with high TC21 expression found by Rokavec (2008). While our group used ACMCI to define TC21 expression levels, the Rokavec group used a modified semiquantitative scale that included the percentage of stained cells in its score (Rokavec, et al., 2008; Tlaczala et al., 2008). Patients with high TC21 levels were more likely to be hormone receptor- and ER-positive. Rokavec (2008) concluded that TC21 expression is regulated by ER by finding that ER transfection into ER-negative breast cancer cells decreased TC21 mRNA expression. However, the group also found that a genetic polymorphism within the TC21 gene was associated with increased TC21 expression in ER+ breast cancer. Further, because of the non-genomic ER signalling activities and their dependence on Ras signalling, a correlation between ER positivity and TC21 expression may be expected. Patients with high TC21 expression were also found to be older, though the clinical use of this statistically significant information, considering the median ages, is negligible. TC21 expression was not found to be a prognostic marker in our study (Appendix 10e), while TC21 was found to correlate with poor prognosis in oral squamous cell carcinoma (Macha,

et al., 2010). In all MA.12 patients, low TC21 was found to predict for a significantly increased 5-year RFS for patients treated with tamoxifen compared to placebo, and the p-value for the interaction between treatment and TC21 expression suggests a trend. To delineate this possible correlation, the ER+ subgroup was analyzed. The prediction for an increased 5-year RFS with tamoxifen treatment persisted, and a borderline significant p-value for interaction was found. The p-value for interaction in the prediction of OS by treatment arm and TC21 expression also decreased from $p = 0.30$ to 0.15 (Appendix 11). Again, this result may be expected considering the growing evidence of the contribution of cross-talk between ER and growth factor signalling to tamoxifen resistance (Rokavec, et al., 2008). Further validation of RSF1 will be performed in a larger placebo-controlled tamoxifen RCT of ER+ women, the receptor status for which tamoxifen treatment is recommended. The women in this larger study are also node-negative, allowing analysis in a less restrictive patient population. Additionally, this study has found equivalent compliance rates between control and treatment groups (Hadji, 2010).

6. Conclusion

This study measured four potential predictive biomarkers for outcomes in the NCIC CTG MA.12 trial of tamoxifen after adjuvant chemotherapy in premenopausal women with early breast cancer. Although none of the candidates were completely validated as predictive biomarkers for tamoxifen, the predictive effect of TC21 expression and RSF1 amplification evidently deserve further study. Because of the large body of evidence pointing to the clinical utility of CCND1, it may also be worthwhile to re-explore this amplification in a trial with fewer limitations on the interpretability of the results, caused in MA.12 by issues with treatment compliance and inadequate numbers of patients.

The consequences of breast cancer on a woman, her family, and society are far-reaching and severe. Women now have more choices than ever in treating their breast cancer, and expanding the body of knowledge that can be applied to making a suitable treatment choice can save years of suffering and inappropriate health care spending. We show here that women with high-risk early breast cnacer with no RSF1 copy number gains should receive tamoxifen and that women with low TC21 expression also benefit from this therapy. We hope that this information, validated in a larger RCT, will inform women and their caregivers on how to make their care as personalized as possible.

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Appendix

1. Breast Cancer Staging

TABLE 7: TNM staging system for breast cancer

Primary tumor (T)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	DCIS
Tis	LCIS
Tis	Paget's disease of the nipple with no tumor
T1	Tumor ≤ 2 cm in greatest dimension
T1mic	Microinvasion ≤ 0.1 cm in greatest dimension
T1a	Tumor > 0.1 but not > 0.5 cm in greatest dimension
T1b	Tumor > 0.5 cm but not >1 cm in greatest dimension
T1c	Tumor > 1 cm but not > 2 cm in greatest dimension
T2	Tumor > 2 cm but not > 5 cm in greatest dimension
T3	Tumor > 5 cm in greatest dimension
T4	Tumor of any size, with direct extension to (a) the chest wall or (b) skin only, as described below
T4a	Extension to the chest wall, not including the pectoralis muscle
T4b	Edema (including peau d'orange) or ulceration of the skin of the breast or satellite skin nodules confined to the same breast
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Note: Paget's disease associated with a tumor is classified according to the size of the tumor.	
Regional lymph nodes (N)	
Nx	Regional lymph nodes cannot be assessed (eg, previously removed)
N0	No regional lymph node metastasis
N1	Metastasis in movable ipsilateral axillary lymph node(s); pathologic involvement of 1-3 axillary lymph nodes
N2	Metastasis in ipsilateral axillary lymph node(s) fixed or matted, or in clinically apparent* ipsilateral internal mammary nodes in the **absence** of clinically evident axillary lymph node metastasis; pathologic involvement of 4-9 axillary lymph nodes
N2a	Metastasis in ipsilateral axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastasis only in clinically apparent* ipsilateral internal mammary nodes and in the **absence** of clinically evident axillary lymph node metastasis
N3	Metastasis in ipsilateral infraclavicular lymph node(s) with or without axillary lymph node involvement, or in clinically apparent* ipsilateral internal mammary lymph node(s) and in the presence of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement; pathologic involvement of ≥ 10 axillary lymph nodes
N3a	Metastasis in ipsilateral infraclavicular lymph node(s) and axillary lymph node(s)
N3b	Metastasis in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastasis in ipsilateral supraclavicular lymph node(s)

DCIS = ductal carcinoma in situ; LCIS = lobular carcinoma in situ
*"Clinically apparent" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination or grossly visible pathologically.

Pathologic classification (pN)	
pNx	Regional lymph nodes cannot be assessed (eg, previously removed)
pN0	No regional lymph node metastasis
pN0(i-)	No regional lymph node metastasis histologically; negative IHC
pN0(i+)	No regional lymph node metastasis histologically; positive IHC; no IHC cluster > 0.2 mm
pN0(mol-)	No regional lymph node metastasis histologically; negative molecular findings (RT-PCR)
pN0(mol+)	No regional lymph node metastasis histologically; positive molecular findings (RT-PCR)
pN1	Metastasis in 1-3 axillary lymph nodes, and/or internal mammary nodes with microscopic disease detected by SLN dissection but not clinically apparent
pN1mi	Micrometastasis (> 0.2 mm but ≤ 2.0 mm)
pN1a	Metastasis in 1-3 axillary lymph nodes
pN1b	Metastasis in internal mammary nodes with microscopic disease detected by SLN dissection but not clinically apparent
pN1c	Metastasis in 1-3 axillary lymph nodes and in internal mammary lymph nodes with microscopic disease detected by SLN dissection but not clinically apparent
pN2	Metastasis in 4-9 axillary lymph nodes, or in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastasis to ipsilateral axillary lymph node(s) fixed to each other or other structures
pN2a	Metastasis in 4-9 axillary lymph nodes (≥ 1 tumor deposit > 2.0 mm)
pN2b	Metastasis in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastasis
pN3	Metastasis in ≥ 10 axillary lymph nodes, or in infraclavicular lymph nodes, or in clinically apparent ipsilateral mammary lymph nodes(s) in the presence of ≥ 1 positive axillary lymph node(s); or, in > 3 axillary lymph nodes with clinically negative microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastasis in ≥ 10 axillary lymph nodes (at least 1 tumor deposit > 2.0 mm); or, metastasis to the infraclavicular lymph nodes
pN3b	Metastasis in clinically apparent (ipsilateral internal mammary lymph nodes in the presence of ≥ 1 positive axillary lymph node(s) in > 3 axillary lymph nodes and in internal mammary lymph nodes with microscopic disease detected by SLN dissection but not clinically apparent
pN3c	Metastasis in ipsilateral supraclavicular lymph nodes
Distant metastasis (M)	
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
cM0(i+)	No clinical or radiographic evidence of distant metastases; deposits of molecularly or microscopically detected tumor cells in blood, bone marrow, or other nonregional nodal tissue
M1	Distant metastasis

TABLE 7: TNM stagging system for breast cancer

Stage grouping			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mic	M0
	T1*	N1mic	M0
Stage IIA	T0–1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0–3	N2	M0
	T3	N1–2	M0
Stage IIIB	T4	N0–2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

*T1 includes T1mic
From: Edge SB, Byrd DR, Compton CC, et al (eds): AJCC Cancer Staging Manual, 7th ed. New York, Springer-Verlag, 2009.

(Edge, et al., 2010)

2. Histologic Grade

Each of the three following features is assigned a score ranging from 1 to 3, with 1 indicating slower cell growth and 3 indicating faster cell growth (Imaginis, 2011b).

Tubule Formation (% of Carcinoma Composed of Tubular Structures)	Score
> 75%	1
10-75%	2
less than 10%	3
Nuclear Pleomorphism (Change in Cells)	
Small, uniform cells	1
Moderate increase in size and variation	2
Marked variation	3
Mitosis Count (Cell Division)	
Up to 7	1
8 to 14	2
15 or more	3

Adapted from Dutra, Azevedo, Schmitt, & Cassali, 2008

The scores of each of the cells' features are then added together for a final sum that will range between 3 and 9. A tumor with a final sum of 3, 4, or 5 is considered a Grade 1 tumor (well-differentiated). A sum of 6 or 7 is considered a Grade 2 tumor (moderately differentiated), and a sum of 8 or 9 is a Grade 3 tumor (poorly differentiated) (Imaginis, 2011b).

Grade	Description	Score
GX (undetermined grade)	Grade cannot be assessed	
G1 (low grade)	Well-differentiated breast cells; cells generally appear normal and are not growing rapidly; cancer arranged in small tubules.	3,4,5
G2 (intermediate grade)	Moderately-differentiated breast cells; have characteristics between Grade 1 and Grade 3 tumors.	6,7
G3 (high grade)	Poorly differentiated breast cells; cells do not appear normal and tend to grow and spread more aggressively.	8,9

Adapted from Institute, 2004 and Society, 2010

3. Gail Score

Question1: Does the woman have a medical history of any breast cancer or of ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS)?

Explanation: A medical history of ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) increases the risk of developing invasive breast cancer. The method used by the Breast Cancer Risk Assessment Tool to calculate the risk of invasive breast cancer is not accurate for women with a history of DCIS or LCIS. In addition, the tool cannot accurately predict the risk of another breast cancer for women who have a medical history of breast cancer.

Question 2: What is the woman's age?

Explanation: The risk of developing breast cancer increases with age. The great majority of breast cancer cases occur in women older than age 50. Most cancers develop slowly over time. For this reason, breast cancer is more common among older women.

Note: This tool only calculates risk for women 35 years of age or older.

Question 3: What was the woman's age at time of her first menstrual period?

Explanation: Women who had their first menstrual period before age 12 have a slightly increased risk of breast cancer. The levels of the female hormone estrogen change with the menstrual cycle. Women who start menstruating at a

very young age have a slight increase in breast cancer risk that may be linked to their longer lifetime exposure to estrogen.

Question 4: What was the woman's age at her first live birth of a child?

Explanation: Risk depends on many factors, including age at first live birth and family history of breast cancer. The relationship of these two factors is shown in the following table of relative risks.

Relative Risk of Developing Breast Cancer

Age at first live birth	# of affected relatives		
	0	1	2 or more
20 or younger	1	2.6	6.8
20-24	1.2	2.7	5.8
25-29 or no child	1.5	2.8	4.9
30 or older	1.9	2.8	4.2

For women with 0 or 1 affected relative, risks increase with age at first live birth. For women with 2 or more first degree relatives, risks decrease with age at first live birth.

Question 5: How many of the woman's first-degree relatives (mother, sisters, daughters) have had breast cancer?

Explanation

Having one or more first-degree relatives (mother, sisters, daughters) who have had breast cancer increases a woman's chances of developing this disease.

Question 6: Has the woman ever had a breast biopsy?

6a: How many previous breast biopsies (positive or negative) has the woman had?

6b: Has the woman had at least one breast biopsy with atypical hyperplasia?

Explanation: Women who have had breast biopsies have an increased risk of breast cancer, especially if their biopsy specimens showed atypical hyperplasia. Women who have a history of breast biopsies are at increased risk because of whatever breast changes prompted the biopsies. Breast biopsies themselves do not cause cancer.

Question 7: If known, please indicate the woman's race/ethnicity.

Explanation: While race/ethnicity is included in the calculation, it does not influence breast cancer risk as much as other factors. The model for African American women was derived from the Women's Contraceptive and Reproductive Experiences (CARE) Study (see reference 5) and NCI's SEER Program. For Hispanic women, part of the model is derived from white women who participated in the Breast Cancer Detection Demonstration Project and from SEER data. The risk estimates for Hispanic women are therefore subject to

greater uncertainty than those for white women. Calculations for American Indian, Alaskan Native, Asian, and Pacific Islander women are based entirely on data for white women and may not be accurate. Researchers are conducting additional studies, including studies with minority populations, to gather more data and to increase the accuracy of the tool for women in these populations.

Note: If the woman's race/ethnicity is unknown, the tool will use data for white females to estimate the predicted risk.

Adapted from Institute, 2008 (Institute, 2008)

4. Inclusion criteria for NCIC MA.12

DISEASE CHARACTERISTICS: Adenocarcinoma of the breast with 1 or more histologically proven positive axillary nodes OR Adenocarcinoma of the breast with negative axillary nodes or adverse prognostic factors such that the patient is at high risk for recurrence and node negative lesion is characterized by the following features: Tumor at least 1 cm Poorly differentiated, SBR grade III, or MSBR grade V and/or lymphatic/vascular invasion Pathologic review by experienced breast pathologist recommended if grade is unspecified and lymphatic/vascular invasion is absent Disease considered potentially curable and treated by 1 of the following: Complete surgical removal of the breast plus axillary node dissection Partial surgical removal of the breast plus axillary node

dissection, with the intention of giving breast irradiation following completion of an adjuvant chemotherapy regimen Regional nodal or chest wall irradiation not prohibited but strongly discouraged No evidence of residual tumor in the axilla following dissection No microscopic evidence of residual tumor at the resection margins following total mastectomy Further excision highly recommended if there is microscopic residual disease present at partial mastectomy margins If further excision is not undertaken, a radiotherapy boost to the tumor bed is required in addition to breast irradiation given following protocol chemotherapy Disease clinically staged prior to surgery as T1-T3a, N0-2, M0 No clinical T4 disease, i.e.: No extension to the chest wall No edema (including peau d'orange) No skin ulceration No satellite skin nodules confined to the same breast No inflammatory carcinoma Disease pathologically staged following surgery as TNM stage I, II, or III (T0-4; N0-2; M0) T4 allowed only with dermal involvement on pathology assessment No evidence of metastatic disease beyond the homolateral axillary nodes on pre-chemotherapy chest x-ray, bone scan (with radiographs of suspicious areas), and abdominal ultrasound (required only if bilirubin, alkaline phosphatase, or AST/ALT are elevated) Simultaneous bilateral breast carcinoma allowed Complete tumor resection on both breasts required Axillary dissection on both sides must meet criteria as above if both sides are clinically node-positive Axillary dissection on the second side optional if the axilla is clinically negative at

the time of surgery and the other side is node-positive Adjuvant chemotherapy must begin within 14 weeks of initial pathologic diagnosis Hormone receptor status: Any receptor level allowed (values must be available if biochemical method used; immunocytochemical assay permitted)

PATIENT CHARACTERISTICS: Age: Not specified Sex: Female Menopausal status: Pre- or perimenopausal, i.e., meeting at least 1 of the following criteria: Normal menstruation Amenorrhea for less than 1 year (up to 3 years in patients under age 52) Biochemical evidence of ovarian function Hysterectomy without bilateral oophorectomy in patients under age 56 Premenopausal women no greater than age 50 who were started on replacement hormone therapy before amenorrhea are eligible Performance status: ECOG 0-2 prior to chemotherapy Hematopoietic: WBC at least 3,000/mm3 Polymorphs and bands at least 1,500/mm3 Platelet count at least 100,000/mm3 Hepatic: (unless abdominal ultrasound indicates liver metastasis) Alkaline phosphatase no greater than 2 times normal AST and/or ALT no greater than 2 times normal Renal: Not specified Other: No history of serious underlying medical illness or psychiatric or addictive disorder No second malignancy within 5 years except: Curatively treated nonmelanomatous skin cancer Curatively treated endometrium, colon, or thyroid cancer or carcinoma in situ of the cervix No plan for pregnancy during the

5-year study period Fertile women must use effective contraception (other than oral contraception) Accessible for treatment and follow-up

PRIOR CONCURRENT THERAPY: Biologic therapy: Colony-stimulating factors allowed (use must be documented) Chemotherapy: No prior chemotherapy No concurrent other cytotoxic therapy Endocrine therapy: Adjuvant tamoxifen (20 mg po daily) allowed up to 2 weeks before or during adjuvant chemotherapy provided drug is discontinued at randomization No long-term prednisone or other hormones.

Adapted from ClinicalTrials.gov, 2011 (ClinicalTrials.gov, 2011)

5. MA.12 Patient Data and Survival Curves

Table 1.
Patient characteristics according to treatment arms

Characteristic	Tamoxifen		Placebo		Total	
	n	%	*n*	%	*N*	%
Receptor status						
ER and/or PgR positive	252	74	253	76	505	75
ER and PgR negative	50	15	26	8	76	11
ER negative and PgR unknown	36	11	55	16	91	14
ER positive	223	66	231	69	454	68
ER negative	115	34	103	31	218	32
PgR positive	132	39	119	36	251	37
PgR negative	60	18	41	12	101	15
PgR unknown	146	43	174	52	320	48
Nodal status						
Node negative	84	25	83	25	167	25
1–3 nodes	190	56	187	56	377	56
4–9 nodes	55	16	53	16	108	16
10+ nodes	9	3	11	3	20	3
Adjuvant chemotherapy						
CEF	73	22	73	22	146	22
CMF	153	45	151	45	304	45
AC	112	33	110	33	222	33
Age						
≤29	1	0	2	0	3	0
30–39	64	19	66	20	130	19
40–49	210	62	210	63	420	63
≥50	63	19	56	17	119	18
Stage (pathological)						
I	33	10	37	11	70	10
II	284	84	278	83	562	84
III	21	6	19	6	40	6
Pathological T stage						
1	140	41	149	45	289	43
2	179	53	165	49	344	51
3/4	19	6	20	6	39	6
ECOG status						
0	234	69	206	62	440	65
1	100	30	125	37	225	34
2	4	1	3	1	7	1
Menstrual status (at randomization)						
Regular menses	85	25	93	28	178	26
Irregular menses	36	11	39	12	75	11
No menses >3 months	129	38	121	36	250	37
Previous hysterectomy	17	5	20	6	37	6
No data	71	21	61	18	132	20

ER, estrogen receptor; PgR, progesterone receptor; CEF, cyclophosphamide/epirubicin/fluorouracil; CMF, cyclophosphamide/methotrexate/5-fluorouracil; AC, doxorubicin(adriamycin)/cyclophosphamide; ECOG, Eastern Cooperative Oncology Group.

Table 2.
Outcome events—deaths and recurrences by treatment arm

	Tamoxifen		Placebo	
	n	%	*n*	%
Patients	338		334	
OS—total deaths	77	23	92	28
Breast cancer	72		86	
Other malignancy	2		4	
Other[a]	3		2	
DFS—total events	105	31	121	36
Recurrences	101	30	116	35
Locoregional only	17	5	14	4
Breast	11		10	
Chest wall	2		1	
Regional nodes	4		3	
Distant only	51	15	57	17
Multiple sites	33	10	45	13
Bone[b]	59		69	
Liver	45		54	
Lung	40		53	
Brain	24		27	
Ascites/effusions	38		47	
Other	30		34	
Death without recurrence	4	1	5	1

[a] Tamoxifen: nonprotocol treatment complication (1), intracranial bleed (1), unknown, but extensive metastases (1); placebo: motor vehicle accident (1), suspected suicide (1).

[b] Single patient can have multiple sites of recurrence.

OS, overall survival; DFS, disease-free survival.

Table 3.
Effect of tamoxifen on OS and DFS according to hormone receptor subgroups

	Tamoxifen		Placebo		
	Total patients	% Surviving 5 years	Total patients	% Surviving 5 years	HR (95% CI)
OS					
Receptor status					
ER and/or PgR positive	252	90	253	85	0.83 (0.58–1.18)
ER and PgR negative	50	76	26	73	0.78 (0.34–1.81)
ER negative, PgR unknown	36	78	55	72	0.67 (0.29–1.56)
ER status					
Positive	223	90	231	86	0.94 (0.65–1.38)
Negative	115	81	103	72	0.61 (0.37–1.01)
DFS					
Receptor status					
ER and/or PgR positive	251	80	253	73	0.82 (0.61–1.11)
ER and PgR negative	50	70	26	69	0.83 (0.38–1.82)
ER negative, PgR unknown	36	78	55	65	0.65 (0.30–1.43)
ER status					
Positive	222	79	231	75	0.90 (0.66–1.24)
Negative	115	77	103	64	0.65 (0.41–1.03)

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PgR, progesterone receptor.

Table 4.
Compliance with study medication

	Tamoxifen		Placebo	
	n	%	*n*	%
Total off treatment	338	100	334	100
Reasons				
Treatment completed	175	52	174	52
Recurrence/progression	50	15	78	23
Death	1	0	1	0
Intercurrent illnessa	9	3	11	3
Toxicity treatment	29	8	15	5
Refusal	44	13	35	11
Protocol violation	13	4	12	4
Otherb	17	5	8	2
Number treated patients	328	100	327	100
Duration of study medication (months)				
0–12	64	19	45	14
13–24	33	10	41	13
25–36	19	6	31	9
37–48	23	7	21	6
49–60	189	58	189	58
Other hormones before progression and during protocol treatmentc	7	2	2	1
Tamoxifen	0		2	
Letrozole	1		0	
Progestogen	6		0	
Other hormones before progression and after protocol treatmentc	34	10	30	9
Not completed 5 years ofblinded treatment				
Tamoxifen	17		20	
Letrozole	12		1	
Anastrozole	1		0	
Exemestane	0		3	
Progestogen	4		2	
Completed 5 years of blinded treatment				
Tamoxifen	0		5	
Letrozole	11		2	
Exemestane	1		0	

[a] Included development of contralateral breast cancer.

[b] Ineligible, noncompliant, stopped early/unblinded in error, physician advice, desire for pregnancy, left the country.

[c] A patient may have received more than one hormonal agent.

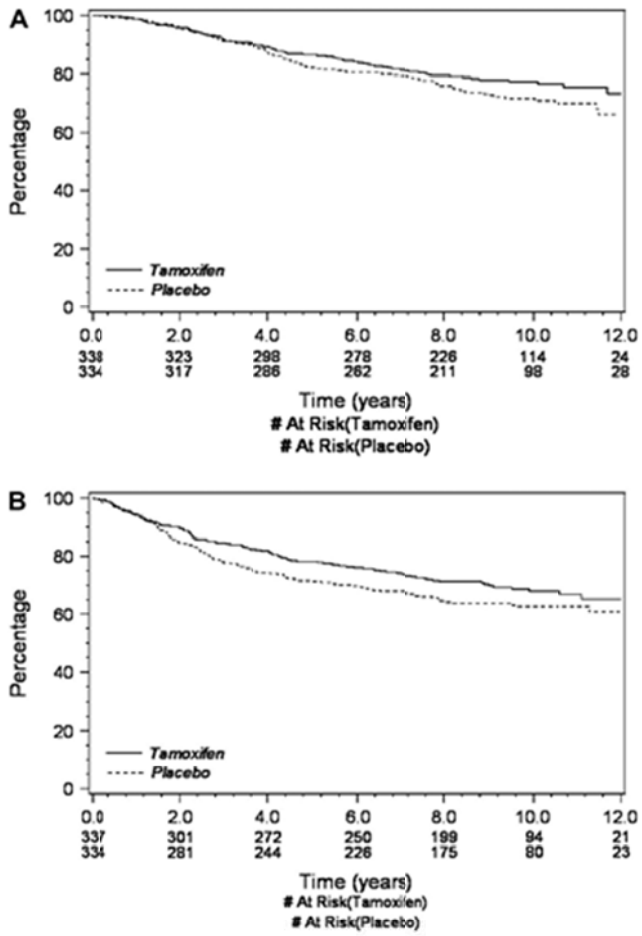

Figure 1.
(**A**) Kaplan–Meier curves for overall survival, tamoxifen versus placebo; (**B**) Kaplan–Meier curves for disease-free survival, tamoxifen versus placebo.

Adapted from Bramwell, et al., 2010 (Bramwell, et al., 2010)

6. Comparison of baseline characteristics between patients with amplified and non-amplified CCND1

Characteristic	Non-amplified CCND1		Amplified CCND1	
	No.	%	No.	%
Receptor status (p=0.14 for receptor status; p=0.01 for ER status; p=0.35 for PR status)				
ER and/or PR positive	294	73	33	87

Characteristic	Non-amplified CCND1		Amplified CCND1	
	No.	%	No.	%
ER and PR negative	48	12	1	3
ER negative and PR unknown	59	15	4	10
ER positive	254	63	32	84
ER negative	147	37	6	16
PR positive	165	41	12	32
PR negative	63	16	5	13
PR unknown	173	43	21	55
Nodal status (p=0.15)				
Node negative	105	26	4	10
1-3 nodes	217	54	27	71
4-9 nodes	69	17	6	16
10+ nodes	10	3	1	3
Adjuvant chemotherapy (p=0.29)				
CEF	98	24	5	13
CMF	175	44	19	50
AC	128	32	14	37
Age (years) (p=0.64)				
Median	45.7		45.0	
Range	29.3-57.8		27.1-54.1	
Stage (pathological) (p=0.39)				
I	39	10	2	5
II	336	84	35	92
III	26	6	1	3

Characteristic	Non-amplified CCND1		Amplified CCND1	
	No.	%	No.	%
Pathological T stage (p=0.67)				
1	167	42	15	39
2	204	51	22	58
3/4	30	7	1	3
ECOG status (p=0.04)				
0	268	67	23	61
1	130	32	13	34
2	3	1	2	5
Treatment (p=0.49)				
Tamoxifen	198	49	21	55
Placebo	203	51	17	45

7. Comparison of baseline characteristics between patients with amplified and non-amplified RSF1

Characteristic	Non-amplified RSF1		Amplified RSF1	
	No.	%	No.	%
Receptor status (p=0.13 for receptor status; p=0.05 for ER status; p=0.75 for PR status)				
ER and/or PR positive	277	73	25	89
ER and PR negative	46	12	2	7
ER negative and PR unknown	59	15	1	4
ER positive	243	64	23	82
ER negative	139	36	5	18

Characteristic	Non-amplified RSF1		Amplified RSF1	
	No.	%	No.	%
PR positive	144	38	10	36
PR negative	61	16	6	21
PR unknown	177	46	12	43
Nodal status (p=0.31)				
Node negative	97	25	3	11
1-3 nodes	211	55	17	61
4-9 nodes	64	17	7	25
10+ nodes	10	3	1	3
Adjuvant chemotherapy (p=0.80)				
CEF	89	23	5	18
CMF	168	44	13	46
AC	125	33	10	36
Age (years) (p=0.95)				
Median	45.5		45.5	
Range	29.3-56.6		27.1-57.8	
Stage (pathological) (p=0.47)				
I	36	10	1	3
II	322	84	26	94
III	24	6	1	3

Characteristic	Non-amplified RSF1		Amplified RSF1	
	No.	%	No.	%
Pathological T stage (p=0.10)				
1	157	41	11	39
2	199	52	15	54
3/4	26	7	2	7
ECOG status (p=0.08)				
0	264	69	14	50
1	114	30	14	50
2	4	1	0	0
Treatment (p=0.52)				
Tamoxifen	188	49	12	43
Placebo	194	51	16	57

8. Comparison of baseline characteristics between PAX2-positive and PAX2-negative patients

Characteristic	PAX2-negative		PAX2-postiive	
	No.	%	No.	%
Receptor status (p=0.22 for receptor status; p=0.31 for ER status; p=0.60 for PR status)				
ER and/or PR positive	150	78	185	71
ER and PR negative	21	11	30	12
ER negative and PR unknown	22	11	44	17
ER positive	131	68	164	63

Characteristic	PAX2-negative		PAX2-postiive	
	No.	%	No.	%
ER negative	62	32	95	37
PR positive	82	42	98	38
PR negative	29	16	41	16
PR unknown	82	42	120	46
Nodal status (p=0.63)				
Node negative	45	23	70	27
1-3 nodes	106	55	141	54
4-9 nodes	38	20	41	16
10+ nodes	4	2	7	3
Adjuvant chemotherapy (p=0.48)				
CEF	49	25	57	22
CMF	86	40	111	43
AC	58	30	91	35
Age (years) (p=0.94)				
Median	45.6		45.5	
Range	29.3-57.8		27.1-55.0	
Stage (pathological) (p=0.08)				
I	17	9	28	11
II	159	82	221	85
III	17	9	10	4

Characteristic	PAX2-negative		PAX2-postiive	
	No.	%	No.	%
Pathological T stage (p=0.18)				
1	84	43	104	40
2	92	48	141	54
3/4	17	9	14	6
ECOG status (p=0.29)				
0	133	69	166	64
1	59	30	88	34
2	1	1	5	2
Treatment (p=0.39)				
Tamoxifen	92	48	134	52
Placebo	101	52	125	48

9. Comparison of baseline characteristics between patients with low and high TC21 expression

Characteristic	Low ACMCI		High ACMCI	
	No.	%	No.	%
Receptor status (p=0.03 for receptor status; p=0.05 for ER status; p=0.10 for PR				
ER and/or PR positive	111	67	221	78
ER and PR negative	24	14	26	9
ER negative and PR unknown	32	19	36	13
ER positive	99	59	194	69
ER negative	68	41	89	31
PR positive	55	33	122	43

Characteristic	Low ACMCI		High ACMCI	
	No.	%	No.	%
PR negative	29	17	40	14
PR unknown	83	50	121	43
Nodal status (p=0.29)				
Node negative	51	31	65	23
1-3 nodes	89	53	159	56
4-9 nodes	24	14	51	18
10+ nodes	3	2	8	3
Adjuvant chemotherapy (p=0.56)				
CEF	39	23	65	23
CMF	78	47	120	42
AC	50	30	98	35
Age (years) (p=0.05)				
Median	45.4		45.7	
Range	29.3-55.2		27.1-57.8	
Stage (pathological) (p=0.86)				
I	18	11	26	9
II	139	83	240	85
III	10	6	17	6
Pathological T stage (p=0.39)				
1	75	45	111	39
2	78	47	155	55
3/4	14	8	17	6
ECOG status (p=0.04)				
0	123	74	175	62
1	42	25	104	37
2	2	1	4	1

Characteristic	Low ACMCI		High ACMCI	
	No.	%	No.	%
Treatment (p=0.26)				
Tamoxifen	77	46	146	52
Placebo	90	54	137	48

10. Prognostic analyses

The following tables summarize 5-year RFS and OS for patients with respectively amplified/non-amplified CCND1/RSF1, PAX2-positivity or negativity, or low/high TC21. HRs adjusted for treatment (when appropriate), age, performance status, time from diagnosis to randomization, nodal status, stage, receptor status, and type of chemotherapy treatment. Associated 95% confidence interval and p-values are also included for the comparisons between patients in the two groups. All the p-values in the tables refer to the adjusted ones. All HRs are compared to the non-amplified/negative/low group.

a) Association of CCND1 status and outcomes for all patients

CCND1	# of patients	5-Year OS (95% CI)	OS HR (95% CI) [P-value]	5-Year RFS (95% CI)	RFS HR (95% CI) [P-value]
Non-amplified	401	0.84 (0.80, 0.88)	1.08 (0.54, 2.19) [0.82]	0.75 (0.70, 0.79)	1.47 (0.85, 2.57) [0.17]
Amplifiied	38	0.89 (0.74, 0.96)		0.71 (0.54, 0.83)	

b) Association of RSF1 status and outcomes for all patients

RSF1	# of patients	5-Year OS (95% CI)	OS HR (95% CI) [P-value]	5-Year RFS (95% CI)	RFS HR (95% CI) [P-value]
Non-amplified	382*	0.84 (0.80, 0.88)	1.46 (0.72, 2.98) [0.29]	0.74 (0.70, 0.78)	1.48 (0.82, 2.68) [0.19]
Amplified	28	0.85 (0.65, 0.94)		0.68 (0.47, 0.82)	

c) Association of PAX2 status and outcomes for all patients

ACNI	# of patients	5-Year OS (95% CI)	OS HR (95% CI) [P-value]	5-Year RFS (95% CI)	RFS HR (95% CI) [P-value]
negative	193*	0.85 (0.79, 0.89)	1.13 (0.75, 1.70) [0.56]	0.74 (0.67, 0.80)	1.10 (0.78, 1.56) [0.59]
positive	259	0.84 (0.79, 0.88)		0.75 (0.69, 0.80)	

d) Assocation of PAX2 status and outcomes for tamoxifen-treated patients

ACNI	# of patients	5-Year OS (95% CI)	OS HR (95% CI) [P-value]	5-Year RFS (95% CI)	RFS HR (95% CI) [P-value]
negative	92*	0.86 (0.77, 0.91)	1.14 (0.61, 2.14) [0.67]	0.80 (0.70, 0.87)	1.04 (0.61, 1.80) [0.88]
positive	134	0.86 (0.79, 0.90)		0.78 (0.69, 0.84)	

e) Association of TC21 status and outcomes for all patients

ACMCI	# of patients	5-Year OS (95% CI)	OS HR (95% CI) [P-value]	5-Year RFS (95% CI)	RFS HR (95% CI) [P-value]
Low	167	0.84 (0.77, 0.89)	0.99 (0.64, 1.53) [0.95]	0.77 (0.69, 0.82)	1.07 (0.74, 1.55) [0.73]
High	283*	0.85 (0.80, 0.89)		0.73 (0.68, 0.78)	

11. Predictive analysis of OS by treatment arm and TC21 status in ER+ patients

ACMCI Status and Treatment		# of patients	5-Year OS (95% CI)	Log-rank p-value	Adjusted Hazard Ratio (95% CI) [p-value]	P-value for Interaction
ACMCI Low	TAM	46	0.89 (0.76, 0.95)	0.15	0.58 (0.25, 0.39) [0.22]	0.15
	No TAM	53	0.83 (0.70, 0.91)			
ACMCI High	TAM	95	0.90 (0.82, 0.95)	0.32	1.27 (0.69, 1.32) [0.44]	
	No TAM	99	0.92 (0.84, 0.96)			