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CUSTOMIZATION OF TAMOXIFEN THERAPY- NOT ONLY A DREAM

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“Our imagination is the only limit to what we can hope to have in the future”
Charles F Kettering

To my beloved and ever supportive family

ABSTRACT

Breast cancer (BC) is the most common form of cancer in western women. The grand majority of the afflicted women are eligible for endocrine treatment. Tamoxifen has been the golden standard treatment for more than three decades. Unfortunately, resistance towards this drug is a major concern in the clinic. The aim of this thesis is to increase our knowledge of the resistance mechanisms by merging information from cell model systems and human tumors.

One of the main inducers of angiogenesis, vascular endothelial growth factor (VEGF), is overexpressed in many types of cancers and has also been associated to poor outcome in estrogen receptor (ER) positive BC. When analyzing VEGF in 404 ER positive tumors from patients treated with tamoxifen as the sole adjuvant treatment, we found a significant negative effect of high VEGF on survival. Interestingly, the effect was overcome by a prolonged tamoxifen regimen. Moreover, studies on MCF7 cells and its 4-hydroxytamoxifen (4-OHT)-resistant counterpart, MCF/LCC2 (LCC2) revealed the existence of an autocrine signaling loop involving VEGF, its receptor VEGFR2 and p38 in LCC2 cells.

As the resistance mechanism likely involves alterations in multiple parts of the signaling machinery we used a high throughput methodology to study this further. We performed quantitative mass spectrometry (MS)-based proteomics on these cell lines to explore key proteomic differences between them and in response to 4-OHT treatment. Pathway analysis on significantly deregulated proteins revealed a connection to the retinoic acid receptor alpha (RARA), a receptor in close interaction with ER signaling. The 4-OHT-resistant cells exhibited attenuated anti-growth response in response to RARA activation in contrast to MCF7 cells. LCC2 cells were also dependent on RARA for maintenance of viability. Primary validation of the impact of RARA on survival, in the previously described cohort, revealed a correlation between high RARA expression and reduced relapse-free survival specifically in those treated with two years of tamoxifen. Moreover, VEGF was highly correlated to RARA expression. We conclude that RARA may be of potential prognostic or predictive value for patients eligible for tamoxifen therapy.

We used a similar MS-based proteomics approach in order to discover potential biomarkers predictive for tamoxifen response by comparing patients with early relapse (<2years) versus non-relapse (>7years). This pilot study of 24 patients yielded in the identification of 3101 proteins out of which 13 were classified as characteristic for tamoxifen-resistant tumors. CAPS and MX1 are part of this 13-protein signature and have shown to be overexpressed in patients with early relapses.

Altogether, our findings suggest that it is possible to apply information from cell lines to find patient-based associations with potential clinical utility. The response to tamoxifen may be dependent on the involvement of VEGF and RARA. The predictive value of CAPS and MX1 remains to be elucidated. The validation of these factors in larger patient populations is expected in the near future.

LIST OF PUBLICATIONS

- I. Aesoy R, **Sanchez BC**, Norum JH, Lewensohn R, Viktorsson K, Linderholm B. An autocrine VEGF/VEGFR2 and p38 signaling loop confers resistance to 4-Hydroxytamoxifen in MCF7 breast cancer cells. *Mol Cancer Res* Oct;6(10):1630-8, 2008
- II. **Sanchez BC**, Sundqvist M, Fohlin H, Spyrtos F, Nordenskjöld B, Stål O, Linderholm BK. Prolonged tamoxifen increases relapse-free survival for patients with primary breast cancer expressing high levels of VEGF. *Eur J Cancer* Jun;46(9):1580-7, 2010
- III. **Sanchez BC***, Johansson H*, Mundt F, Kovacs A, Máthé G, Yakhini Z, Hjerpe A, Forshed J, Krawiec K, Kanter L, Stål O, Linderholm BK, Lehtiö J. Deregulated retinoic acid receptor alpha signaling in tamoxifen resistance is of potential predictive value in steroid receptor positive breast cancer. *Manuscript*
- IV. Johansson H, **Sanchez BC**, Forshed J, Stål O, Fohlin H, Lewensohn R, Bergh J, Hall P, Lehtiö J, Linderholm BK. Proteomics-based characterization of potential biomarkers implicated in tamoxifen resistance in primary operable breast cancer. *Manuscript*

Additional publications

Koos B*, Paulsson J*, Jarvius M, **Sanchez BC**, Wrede B, Mertsch S, Jeibmann A, Kruse A, Peters O, Wolff JE, Galla HJ, Söderberg O, Paulus W, Ostman A, Hasselblatt M. Platelet-derived growth factor receptor expression and activation in choroid plexus tumors. *Am J Pathol* Oct;175(4):1631-7, 2009

De Petris L, Brandén E, Herrmann R, **Sanchez BC**, Koyi H, Linderholm B, Lewensohn R, Linder S, Lehtiö J. Diagnostic and prognostic role of plasma levels of cytokeratin 18 in patients with non-small cell lung cancer. *Eur J Cancer* Jan;47(1):131-7, 2011

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LIST OF ABBREVIATIONS

BC	Breast cancer
E2	Estrogen
NGS	Nottingham grading system
TNM	Tumor node metastasis
HER2	Human epidermal growth factor receptor 2
PR	Progesterone receptor
EGFR	Epidermal growth factor receptor
CMF	Cyclophosphamide Methotrexate 5-Fluorouracil
FEC	5-Fluorouracil Epirubicin Cyclophosphamide
FAC	5-Fluorouracil Adriamycin Cyclophosphamide
PI3K	Phosphoinositide 3 kinase
MAPK	Mitogen activating protein kinase
ERD	Estrogen receptor downregulator
SERM	Selective estrogen receptor modulators
LBD	Ligand binding domain
AF-2	Activating function 2
ERE	Estrogen responsive element
Rb	Retinoblastoma protein
RTK	Receptor tyrosine kinase
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
EMT	Epithelial mesenchymal transition
RA	Retinoid
RARA	Retinoic acid receptor alpha
RARE	Retinoid response element
MS	Mass spectrometry
iTRAQ	Isobaric tags for relative and absolute quantification
IPG	Immobilized pH gradient
IEF	Isoelectric focusing
MALDI	Matrix assisted laser desorption ionization
Q-TOF	Quadrupole Time of flight
LTQ	Linear trap quadrupole
IPA	Ingenuity pathway analysis
4-OHT	4-hydroxytamoxifen
TMA	Tissue micro array
IHC	Immunohistochemistry

1 INTRODUCTION

Despite all our efforts to combat cancer and improve its treatment, cancer mortality is still high causing approximately 7.6 million deaths around the world per year [1]. In the fight of prolonging a patient's life the quality of life is to some extent put aside. It is important not to forget that we are dealing with patients who desire a better quality of life above all. The dream of personalized medicine is to adjust therapy so that the right drug is given to the right patient at the right time. By doing so, we would also improve the effects of a specific drug and spare patients from ineffective treatments with severe side-effects. This work aims to show that although we are taking small steps towards this dream it is possible to achieve it.

1.1 BREAST CANCER

1.1.1 General overview

A prolonged period of time from the first menstruation to menopause thus prolonging the exposure to endogenous estrogen (E2) is strongly associated with breast cancer (BC) although contradictions have been reported [2]. What we also have to consider is that as our society evolves our way of living changes with all that comes with it such as an increased exposure and consumption to toxins, less physical activity and imbalanced nutrition etc. Despite this, medical advancement is allowing us to reach a high age but at what price? Some would probably argue that it is against nature itself to become as old as we are able to become nowadays, explaining why cancer would probably be nature's way of getting rid of us. High age is actually one of the main risk factors for BC and cancer in general [3]. These factors together with improved diagnosis are the main reasons to an increase in BC cases in developed countries. As less developed countries adapt to a similar lifestyle the incidence of BC is rapidly increasing. Moreover, economical limitation, lack of awareness and cultural beliefs in less developed countries contribute to the poor diagnosis of BC at an early stage thus resulting in higher BC-related deaths.

Worldwide, it is estimated that one million women are diagnosed with BC every year and approximately 40 % die from their disease [4]. In Sweden only, there were 7000 cases reported in 2007 constituting 30% of all cancer cases in women. Fortunately, early diagnosis, social awareness and proper treatment have made it possible to achieve a relative 10-year survival of 80% [3].

1.1.2 Clinical grading and staging

BC is a very heterogeneous disease comprising a wide range of subtypes which pathologists have had a tough time trying to classify. The most common histological type of BC is adenocarcinoma, that is, cancer originating in mammary epithelial cells. Within this group there are, according to the World Health Organization (WHO) classification, 18 different types out of which ductal invasive BC of no special type is the most common [5]. The classification according to histopathological features lacks prognostic information and does consequently not influence the choice of therapy [6].

Grading and staging describe the characteristics of the tumor more in detail as they determine its differentiation, proliferative activity and aggressiveness. Histological grading takes the differentiation level of cells into account dividing them into a low grade group, when they are well differentiated and slow growing, an intermediate group and high grade group, when they are poorly differentiated and rapidly growing (Bloom-Richardson). The Nottingham grading system (NGS) (Elston-Ellis modification of Bloom-Richardson) which is based on the degree of tubule or gland formation, nuclear pleomorphism and mitotic count, takes the evaluation of the morphology of cells to a more detailed level making it the most recommended system for BC worldwide [7].

	Stage	Relative Survival (5y)	TNM classification	
0	Tis N0 M0	93%	Tumor size	
			TX	Cannot be assessed
			T0	No evidence of primary tumor
I	T1 N0 M0	88%	Tis	Carcinoma <i>in situ</i> (lobular or ductal) or Paget's disease of the nipple with no associated tumor
			T1	≤2cm
			T2	≥2cm but ≤ 5cm
IIA	T1 N1 M0 T2 N0 M0	81%	T3	≥5cm
			T4	Tumor of any size with direct extension to chest wall or skin or both of the above.
			Lymph node	
IIB	T2 N1 M0 T3 N0 M0	74%	NX	Cannot be assessed
			N0	Cancer has not spread to nearby lymph nodes
			N1	Cancer has spread to the movable ipsilateral axillary lymph nodes
IIIA	T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1-2 M0	67%	N2	Cancer has spread to ipsilateral lymph nodes fixed to one another or to other structures under the arm
			N3	Cancer has spread to the ipsilateral mammary lymph node (s)
			Metastases	
IIIB	T4 any N M0 any T N3 M0	41%	MX	Cannot be assessed
			M0	No distant metastasis
IIIC	Any T N3 M0	49%	M1	Distant metastasis present (includes ipsilateral supraclavicular lymph nodes)
			IV	Any T any N M1

Table 1. Staging and survival of BC using guidelines from the Tumor Node Metastasis (TNM) classification system. (Adapted and modified from the National Cancer Institute and the National Cancer Data Base)

BC is staged by using the classical tumor node metastasis (TNM) classification based on four characteristics such as size, invasiveness, involvement of lymph nodes and presence of metastases and expressed as a number between 0 and 4 in roman letters

(Table 1). Although morphological evaluation is of importance, staging is one of the most important factors when selecting appropriate treatment for the patient. However, as technological advances feeds us with increasing knowledge in tumor biology it is of great importance to incorporate this information and update the present guidelines. That is why the latest edition of the American Joint Committee on Cancer (AJCC) cancer staging manual includes guidelines for standardization as well as implementation of available electronic analytical tools [8]. Moreover, the rules on TNM staging have been more clearly specified to reduce misinterpretations. Importantly, the MX category describing an unknown metastatic status has been removed and should be replaced by M0 [8].

The employment of high throughput technologies such as gene microarrays has enabled further characterization and classification of different subgroups of BC based on gene expression. The work of Perou et al enabled the classification of invasive carcinomas into five subtypes, two ER positive (luminal A and B), and three ER negative (HER2-enriched, normal breast-like and basal-like) [9]. There is so far no validated consensus in how to distinguish Luminal A, which is the most represented in BC, from Luminal B. Although both types are positive for progesterone receptor (PR) expression, Luminal A is the slower proliferating of the two [10]. In addition to high HER2 expression the HER2-enriched type is also defined by elevated expression of various genes close to the HER2 amplicon. The normal breast-like subtype exhibits high expression of genes characteristic of non-epithelial cell types and adipose tissue-related. The basal-like type, also called triple negative (ER-/PR-/HER2-) expresses keratins 5, 6 and 17 similarly to basal epithelial cells of the normal mammary gland, as well as laminin and fatty acid binding protein 7 [9]. Similarly, a subtype with the additional trait of low expression of cell-cell adhesion-related genes, hence the name, Claudin-low subtype was recently characterized. Moreover, it has been shown to be enriched with stem-like / mesenchymal characteristics and to be of poor prognosis. Although the basal-like and Claudin-low exhibit similar biology their difference in response to treatment remains to be elucidated [11].

1.1.3 Prognostic and predictive markers

A prognostic marker is used to acquire an insight into the natural progression of the disease without treatment by identifying patients with different risks of outcome. A predictive marker is an indicator of sensitivity or resistance to a specific treatment. In short, prognostic markers tell if a patient needs treatment whereas the predictive marker tells which treatment will be the most optimal. Needless to say, these types of markers are of great importance in the management of the disease.

At present, the established prognostic and predictive markers available in BC are ER, PR, HER2, age, tumor size, lymph node status and histological grade.

ER and HER2 are the best established markers available to oncologists as they decide if and which treatment would be the most appropriate for their patients. In fact, these two markers are the only BC markers so far having reached level of evidence I or II according to the American Society of Clinical Oncology's Tumor Marker Utility Grading System. ER and HER2 expression are evaluated routinely in every BC. Since approximately 80% of all BC tumors express the alpha isoform of ER, often simply referred as ER, it is a marker of great impact. ER is considered a key marker for endocrine therapy response although with a limited prognostic value. The extensive evaluation by the early BC trialists's collaborative group (EBCTCG) concluded that

endocrine therapy is of greater benefit for patients with ER positive BC tumors whereas no benefit was observed in ER negative BC. Interestingly, a small benefit was seen in patients with an ER negative and PR positive disease [12]. The degree of ER expression has also been shown to influence therapy response as higher levels of ER were related to improved outcome to endocrine therapy [13].

It has been shown that PR is strongly dependent to ER expression. In spite of this, the predictive significance of PR is poor. However, adjuvant trials comparing endocrine treatment with controls indicate a strong prognostic value of PR expression [14]. It is suggested that the β isoform of ER could be of potential prognostic and predictive value. Although ER β is structurally similar to ER α it is believed to mediate opposite effects, explaining its downregulation in BC. Surprisingly, ER β expression was found to be associated with poor prognosis and endocrine resistance. Due to various contradicting reports, ER β it has still a long way to go before it qualifies as a reliable marker [15].

HER2, a member of the epidermal growth factor receptor (EGFR) family, has been shown to be both a prognostic and predictive marker in BC. About 15 % of all BCs overexpress HER2 and thus likely to have a poor outcome. This group of patients is, however, expected to benefit from HER2 targeting therapy [16]. There have been some issues with accuracy and reproducibility of ER, PR and HER2 determination and recently an expert panel from the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) provided recommendations for the testing of these three markers for optimized methodology, interpretation and reporting of established assays [17]. The suggested definition of ER and PR positivity as a staining of at least 1% positive tumor nuclei will help avoid false negative evaluations [18]. To date, some countries including Sweden still use 10% as cut-off point.

BC is a disease of the elderly. Nevertheless, 1 in every 8 invasive BCs is found in women below 45 years of age and is often of poor prognosis. Tumor size and lymph node status are considered strong prognostic markers. They both correlate to each other suggesting that the larger the tumor the higher the risk of nodal involvement [19]. However, it is important to keep in mind that lymph node status does not reflect the behavior of a tumor entirely correctly [19, 20].

Although histological grading using the NGS alone identifies groups of different prognosis it has been shown to be limited since the intermediate group of grade II tumors can further be separated into a low and high grade by use of gene array analyses [21]. Studies have demonstrated that grade is an independent prognostic factor in specific subgroups of BC, including ER-positive and HER2 negative BC [22]. Importantly, the NGS has been incorporated in Adjuvant! Online and the St Gallen guidelines for use of adjuvant treatment [23]. However, the lack of reproducibility in general has been an issue of discussion.

The technological advancement in high-throughput microarray-based gene-expression methods has enabled the identification of various multi-gene signatures associated with prognosis. However, only a few have been appropriately validated. Oncotype Dx® has been shown to correlate with outcome, and also is also of predictive potential for endocrine therapy and chemotherapy [24]. Other successful gene signatures are the Mamaprint®, intended for the identification of patients with poor outcome [25] and the Rotterdam profile for the identification of patients likely to benefit from tamoxifen [26]. Actually, the value of Oncotype Dx® and Mamaprint® as superior prognostic tools is currently under investigation in large prospective randomized clinical trials

(TAILORx and MINDACT) [7]. In addition to all the factors discussed above there are some other factors of prognostic and predictive use which are not equally well established such as assessment of proliferation rate by Ki67, synthesis phase fraction (SPF), CyclinD1 and Cyclin E [15].

1.1.4 Treatment modalities

Treatment of BC is determined by the evaluation of prognostic and predictive markers. Furthermore, treatment approaches are classified according to how they act and at what phase of treatment they are given. The various therapy approaches are often used in combination. Generally patients are subjected to surgery and after that often eligible for additional treatment, i.e. adjuvant therapy, in order to avoid recurrence of disease. Neoadjuvant therapy, which previously was used to reduce tumor size if it was considered too large for surgery, is now being used at a higher extent since it gives direct feedback on the efficacy of treatment.

Surgery is still the most commonly used treatment strategy in BC patients. For a long time, BC was considered a local disease and therefore the only type of treatment offered to women with BC was the removal of the whole breast i.e., mastectomy. The original mastectomy advocated by William Hallstedt was a very aggressive surgery often resulting in severe side effects and metastatic recurrence [27]. BC surgical techniques have evolved since then into more breast conserving ones, including lumpectomies and quadrantectomies, in which only parts of the breast are taken away. Metaanalyses of various randomized trials showed that radiotherapy as supplementary treatment to surgery was superior to surgery alone in terms of recurrence risk and 15-year survival [28]. Moreover, improved radiotherapy methods involving more precise dose planning and delivery and have increased local control and reduced cardiac damage, a previously common adverse effect of BC radiotherapy [29, 30].

BC chemotherapy is often used as an adjuvant therapy with the purpose to eradicate possible micrometastases in any part of the body. Moreover, chemotherapy given to BC patients often includes drugs with different mechanisms of action. This multidrug-approach often results in synergistic effects of the drugs as well as reduced cytotoxicity as lower doses may be given. Following the pivotal CMF (Cyclophosphamide, Methotrexate and 5-Fluorouracil) several combinations are currently in clinical use, of which the most commonly used regimens include FEC or FAC (Epirubicin or Adriamycin) either followed by a taxane [12, 31].

The development of humanized antibodies inhibiting the growth factor receptor HER2, trastuzumab, was the turning point for targeted therapy. Since then it has been shown effective in reducing BC recurrence in a specific group of patients. Moreover, receiving 1 year of trastuzumab after completed primary treatment was shown to decrease the rate of recurrence by 50% [32]. As the impact on trastuzumab response through various downstream pathways, such as the phosphatidylinositol 3 kinase (PI3K) and mitogen activating protein kinase (MAPK), was uncovered, small molecular inhibitors have been considered alone or in combination with trastuzumab or chemotherapy. Several inhibitors targeting other BC-related proteins have been developed. However it is still too early to predict their impact on BC therapy [33].

Another therapy approach used adjuvantly includes endocrine therapy which aims to deprive BC cells from E2 as some of them need it for their survival. Suppression of E2 production is acquired by ovarian ablation (surgical or pharmacological) in

premenopausal patients [34] or by aromatase inhibitors (AIs) in post-menopausal women as most part of E2 is acquired from the conversion of androgens in peripheral tissues by the action of the aromatase enzyme [35]. The AIs in use are exemestane, anastrozole and letrozole and have all been proved to be of benefit for postmenopausal patients [36].

Another approach of inhibiting E2 signaling is to antagonize ER function by using either of two principal strategies, such as downregulation or selective modulation of ER signaling by ER downregulators (ERDs) and selective ER modulators (SERMs), respectively. With respect to ERDs, Fulvestrant is the only one in clinical use and classified as a second-line hormone-based treatment choice for post-menopausal women with advanced hormone-receptor-positive BC [37]. At present, there are three SERMs which have been tested for clinical use i.e., Tamoxifen, raloxifen, toremifen and of these tamoxifen is the best characterized one and also the only one currently in use in clinical practice of BC [38]. Tamoxifen has been in use for more than three decades having been successful in the treatment of both pre and postmenopausal women with BC and also in preventive purposes for women at elevated risk. In a survey conducted by EBCTCG, it was found that a 5-year use of tamoxifen in women with ER positive BC reduces the BC associated death rate by approximately 31%, regardless of age [12]. However, the ASCO guidelines from 2010 regarding endocrine therapy in post-menopausal patients recommend the use of AIs either upfront or after tamoxifen treatment for a maximum of five years for improve outcome [36]. Moreover, for premenopausal patients, the use of goserelin in addition to tamoxifen or exemestane is suggested to be more beneficial than tamoxifen alone and is currently under investigation in international randomized phase III trials [34].

1.2 TAMOXIFEN

Tamoxifen acts a competitive inhibitor of ER. Normally, E2 binds to the ligand binding domain (LBD) of ER thus activating the activation function 2 (AF-2) located nearby and promoting dimerization with another ER. The activation induces conformational changes of the LBD enabling dimerization and recruitment of co-activators e.g. SRC-3 and transcription factors e.g. AP-1 [39, 40]. This enables the binding of the complex to estrogen responsive elements (ERE) located on DNA ultimately inducing the transcription of target genes which transduce the proliferative signaling of E2. The binding of tamoxifen promotes conformational changes different to that of E2 enabling the recruitment of transcriptional repressors instead thus resulting in transcriptional inhibition [39]. Apart from its antiestrogenic characteristic tamoxifen has also been shown to interact with protein kinase C and affect calcium signaling by binding to calmodullin [41]. Moreover, tamoxifen is known to cause an arrest in the early phase of cell cycle, G1 [42].

1.2.1 Mechanisms of resistance

Although introducing tamoxifen into the treatment of BC significantly reduced BC-related deaths among women, resistance to tamoxifen is a major concern. Some BC patients exhibit tamoxifen resistance already in the beginning of treatment (intrinsic resistance) whereas others develop resistance during the course of the treatment (acquired resistance). Several parts of the cell signaling apparatus have been identified as potential contributors to the resistance.

1.2.1.1 Drug metabolism

Tamoxifen is metabolized into active compounds such as endoxifene and 4-hydroxytamoxifen in order to exert a more potent effect. There are a variety of enzymes in the cytochrome P450 system involved in these steps. CYP2D6 is the enzyme mediating the conversion of N-desmethyl-tamoxifen into endoxifene [43]. Variations of the CYP2D6 allele have been associated to different enzyme activity or expression and thus affecting the ability to metabolize the drug [44].

1.2.1.2 Altered Estrogen Receptor signaling

Being the main marker for eligibility to endocrine therapy it was believed that resistance could be caused by ER mutations and splice variants. However, despite much investigation their clinical role in outcome appears to be small [15]. About 20% of patients have been reported to lose ER expression over time, explaining the lack of therapy response in these patients [45, 46]. Since tamoxifen response is associated to co-repressor recruitment, the overexpression of co-activators such as SRC-3 also called AIB1[47] and p/CIP[48], as well as downregulation of NCoR[49] and SMRT[48] have been coupled to resistance. ER is also activated by growth factors acting through the PI3K/AKT and MAPK pathway and directly interacting with AF-1 in the N-terminal of ER [50, 51]. In addition, ER can also be activated through a variety of other proteins and post-translational modifications [52]. In addition to the nuclear role of ER its non-genomic effects are of interest as ER localized at the plasma membrane is suggested to interact with local proteins involved in signal transduction (Figure 1) [53].

1.2.1.3 Cell cycle

Various components of the cell cycle regulating system have been assigned a role in the resistance mechanism. Tamoxifen-resistant cells have been shown to manipulate the expression, activation and localization of positive regulators such as Cyclin D1 and Cyclin E1, and MYC [54, 55] and negative regulators such as p21, p27 as well as deactivation of the retinoblastoma protein (Rb) in order to circumvent the G1 arrest caused by tamoxifen [56, 57].

1.2.1.4 Growth factor crosstalk

Enhanced growth factor stimulation undermines the effectiveness of antiestrogen treatment. Regular growth factor signaling includes receptor tyrosine kinase (RTK) dimerization upon ligand-binding followed by autophosphorylation, resulting in the activation of several signal transductions cascades e.g. the PI3K/AKT as well as the MAPK pathway. Various components of these pathways have been shown to support BC cells circumvent the inhibitory effects of tamoxifen by opting for alternate proliferation and survival signaling through bidirectional crosstalk with ER signaling.

The best characterized mechanism involves the EGF family although insulin-like 1(IGF1)-, fibroblast (FGF)-, and vascular endothelial (VEGF) growth factor also have been implicated in the crosstalk [45, 58]. Various molecular alterations enabling the overexpression of ligand or receptor HER2 have been associated with tamoxifen resistance [47, 59, 60]. Moreover, the overexpression of SRC-3, constitutively activation of PI3K, loss of the tumor suppressor PTEN, increased activation of AKT, ERK, as well as p38, enhance signaling through HER2 and ER (Figure 1) [56, 60-63].

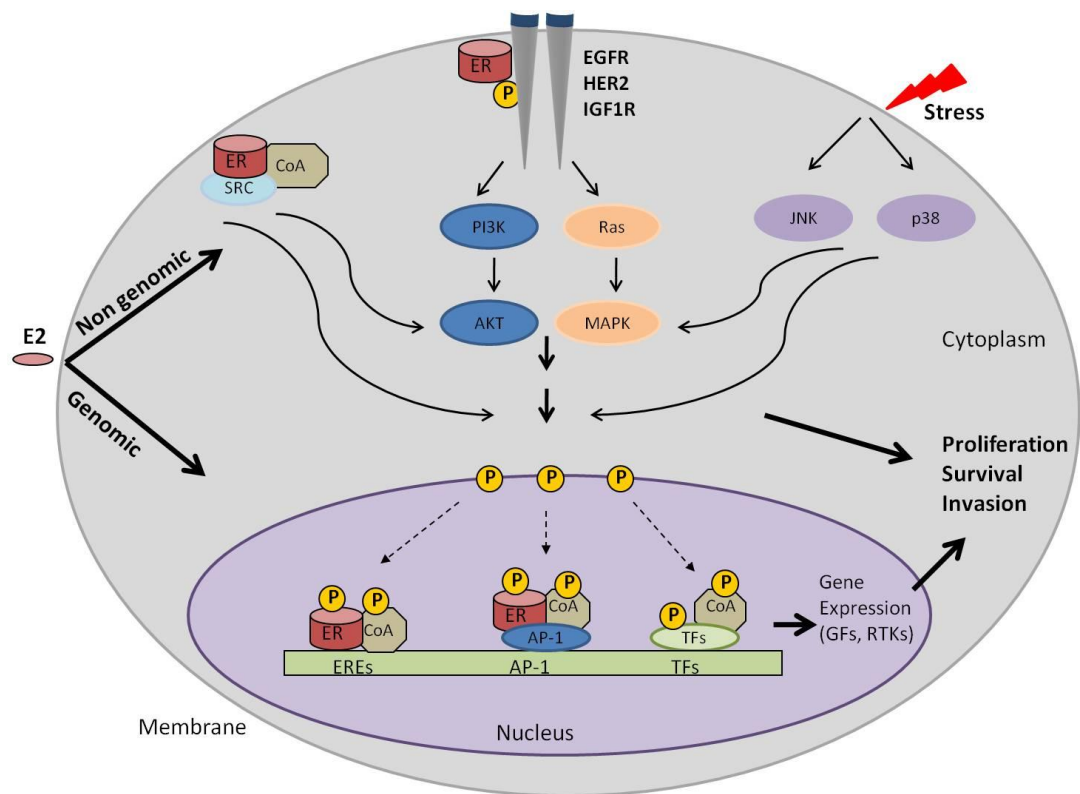


Figure 1. Simplified view of estrogen regulated pathways altered in tamoxifen resistance involving growth factor signaling. For details see text. Abbreviations: E2, estrogen; ER, estrogen receptor; EREs (estrogen responsive elements); CoA, co-activators; TFs, transcription factors; GFs, growth factors; RTKs, tyrosine kinase receptors.

Although VEGF and its receptor VEGFR2 expression on endothelial cells and their function as regulators of angiogenesis is important for tumor cell growth, several studies have shown that BC cells *per se* express and secrete VEGF to a higher extent than surrounding normal tissue [64, 65]. As a consequence, increased signaling through the action of VEGF/VEGFR2 has been found to induce downstream ERK and PI3K most probably in an auto- or paracrine mode [66]. Moreover, HER2 is able to induce VEGF protein synthesis (Figure 1) [67].

1.2.1.5 Other pathways

The extensive research performed in this area has implicated various others players downstream of RTK signaling ultimately altering regulation of apoptosis, microRNA etc. Various components of an anti-apoptotic (BCL-2, BCL-XL) nature have been seen upregulated while pro-apoptotic (Bak, Bik, Caspase 9) are suppressed in tamoxifen-resistant models [45]. Recently, microRNAs in conjunction with HER2 have also been assigned a role in the resistance mechanism as reduced expression of miR-221/222 and 342 as well as miR-451 were found to promote this state [68].

The importance of the surrounding tissue of a tumor has been recognized and thus not to be forgotten in this context. Various proteins from the extracellular matrix such as integrins and fibroblast of tumor have showed a direct association to tamoxifen resistance [69, 70]. Moreover, epithelial mesenchymal transition-like (EMT) behavior in BC cells is induced by tamoxifen resistance involving Pin1 as an inducing factor [71, 72].

1.3 VASCULAR ENDOTHELIAL GROWTH FACTOR IMPLICATIONS IN BREAST CANCER

VEGF exerts a major role in angiogenesis and plays a central role in local tumor growth and distant metastasis in BC [73, 74]. Increased VEGF expression and angiogenesis-related processes have been shown to be of adverse prognostic value in primary BC patients [75-77]. Moreover, increased VEGF expression correlated to poorer response in patients receiving adjuvant tamoxifen or chemotherapy [78, 79]. Interestingly, genetic polymorphisms in the VEGF gene have been associated to increased BC risk [80]. Having these facts in mind, the expectations were high for bevacizumab, a humanized antibody targeting VEGF, as it was implemented in the clinic. Although its use in combination with paclitaxel as first-line treatment of metastatic HER2 negative BC was approved by the Food and Drug administration (FDA) in 2008, the approval was revoked in 2010 due to poor benefit in relation to risks [81]. The obvious limitation is the lack of biomarkers predictive for antiangiogenic therapy. At present, various trials are investigating the potential of implementing bevacizumab to systemic adjuvant treatment. Even though other antiangiogenic agents are available none of them has been approved for clinical use so far [45].

1.4 RETINOIC ACID RECEPTOR ALPHA

Ever wondered why carrots are good for you? Well, carrots contain vitamin A which are metabolized into retinoids (mainly all-trans retinoid) (RA) generally known for being essential for the development of eyes but also skin, skeleton, and immunological function and stem cell differentiation [82, 83]. Once RA is transported to the nucleus it signals through the interaction of retinoid acid receptors (RARs) and retinoid X receptors (RXR), in a similar way as ER [84]. Interestingly, RAR and RXR are believed to be dimerized in the absence of the ligand remaining transcriptional inactive by lowering their affinity to transcriptional co-activators while increasing it for co-repressors such as N-CoR until ligand binding [85]. There are also reports suggesting the direction in which the receptor complex binds to the RA response element (RARE) on DNA is important for response to stimuli [86].

Retinoic Acid Receptor Alpha (RARA) is one of the three subtypes of RARs. There are many directly RARA-regulated genes involved in proliferation, apoptosis and differentiation. It is noteworthy that even genes lacking RAREs can be indirectly regulated by RA since many of its target genes include transcriptional regulators [83].

1.4.1 Signaling properties in cancer

RAs have been shown to act through various ways causing anti-proliferative, pro-apoptotic and pro-differentiation effects in cancer cells [83]. The G1 arrest caused by RAs in order to block cell cycle progression is achieved by increased degradation and lowered expression of cyclins, cyclin dependent kinases (CDKs), growth inducers such as c-myc as well as co-activators inducing transcription of proliferative target genes [83, 87]. In addition, the inhibition of Rb phosphorylation pushes Rb to act as a tumor suppressor and cause a cell cycle arrest by inhibiting further transcription of cyclins [88, 89]. By increasing expression and stability of CKIs such as p21 and p27, RAs are able to suppress the role of CDKs in inducing the cell cycle [90]. However, the inhibitory effect on proliferation is believed to occur mainly through the action of the RAR β isoform [91].

The apoptotic effects of RAs are mainly caused by induction of RARA and have been observed in acute promyelocytic leukemia (APL) blasts, T lymphoblastic and myeloid leukemia, medulloblastoma, and melanoma cells. There are reports implicating TGF β as an inducer of apoptosis in MCF7 cells [92]. However, apoptotic effects specifically implicating the RAR β have also been reported in BC cells [92].

Interestingly, the ability of RAs in inducing differentiation of various cancer cells into a less neoplastically transformed state has been of great interest in cancer research [83]. RA-induced differentiation is likely regulated by a complex transcription factor network involving FOXO3A and Hoxa 1 [93, 94]. However, RA signaling is often altered in cancer due to epigenetic alterations compromising expression of RARs or transcriptional regulators [92]. In addition, the PI3K/AKT pathway has also been shown to have an inhibitory effect on RAR β expression [95].

Recently, the interaction between RA and E2 signaling was revealed as it was shown that RARA and ER not only share a large number of DNA binding regions in MCF7 cells but also that the nature of the interplay is antagonistic [96]. The authors also showed that the transcription factor FoxA1 is required for RARA recruitment to specific target sites [96]. However, contradictory results presented evidence of a co-operative signaling between RARA and ER suggesting that ER is dependent on RARA and that RARA is able to interact with ER binding sites in an E2-dependent manner [97].

1.4.2 Retinoids in cancer therapy

Based on all the reported effects on cancer cells RAs were introduced to the treatment of various types of cancer. Although the effects are known, the exact mechanisms used by RAs to achieve these are still not fully elucidated. APL has been successfully treated with all-trans RA (ATRA) in combination with arsenic trioxide as it induces differentiation of cells and degrades the fusion protein PML/RARA, which is expressed by most part of APL patients [98]. RAs are also employed in the treatment of lymphoid malignancies, basal cell skin cancer [99, 100]. The use of RAs in solid tumors has been limited due to lack of significant effect. A recent metaanalysis including 248 studies evaluating RAs in lung cancer therapy and prevention concluded that bexarotene may be a promising agent. Notably, a large study presented indications of an increased risk of lung cancer in relation to bexarotene [101]. In BC, fenretinide has been suggested as a preventive agent for second primary BC in premenopausal women in combination with tamoxifen and is currently being studied further. However, preliminary results show no benefit of the combined treatment [102, 103].

1.5 PROTEOMICS

The word proteome was coined by Marc Wilkins in the 90's as a corresponding term for the genome, but as the name implies, referring to the entire set of proteins expressed by the genome in a certain cell at a specific time. Proteomics encompasses the studies of the proteome. Basically most peptide sequences of six amino acids or more are unique for a single gene product meaning that if their sequence is obtained the corresponding proteins can be identified. The development of technologies within proteomics has resulted in today's robust and reliable high throughput methods based on mass spectrometry (MS), capable of identifying and quantifying number of protein simultaneously, and protein array technologies as opposed to classic protein detection methods such as immunoblotting limited to studying only one protein at a time. MS has

also shown superiority in accuracy when measuring molecular weights compared to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [104]. Proteomics has developed tremendously fast and thanks to the integration and tuning of other tools besides MS, such as protein and peptide separation methods, it is receiving acknowledgement from the research community. It is exciting times seeing the field of proteomics evolve continuously aiming towards improved resolution, sensitivity and accuracy of protein identification and quantification.

1.5.1 Top-down versus bottom-up proteomics

In general, proteomics methods differ in the choice of studying either proteins themselves or peptides, the fragments of proteins. Top-down proteomics is an approach that studies proteins since identification and quantification occurs prior to digestion of proteins into peptides. In the present work we chose to do bottom-up proteomics which puts the digestion as the first step in the workflow. Although there are advantages and disadvantages with each approach we, in our group, focus on the optimization of bottom up proteomics. We consider this approach to be the most advantageous in identification efficacy, proteome coverage, and quantification accuracy.

The most commonly used protein separation method in top down proteomics is 2 dimensional (2D) SDS-PAGE. This technique separates proteins by their isoelectric point and size. As a result a snapshot of the most abundant proteins present in the sample is obtained. Staining of the gel enables the identification and quantification of the proteins of interest. These are consequently excised and digested prior to MS analysis [104, 105].

Although some researchers advocated the top-down approach, the bottom-up approach is the most widely used choice. The benefit of 2D gel separation is the visualization of proteins although not optimal for small, poorly soluble or low abundant proteins. There is also the issue of poor reproducibility. Another advantage of working with peptides as in bottom-up proteomics is that the complexity of samples can be reduced more easily. In addition, peptides are more suitable for MS analyses compared to proteins.

1.5.2 Methodological pipeline

The workflow in bottom-up proteomics integrates various components each with a specific task required for optimal results. It all starts with choosing the most optimal starting material and preparing the samples making them compatible to the analyzing purposes. The fractionation of a sample according to localization or characteristics of protein types is an approach allowing for a more targeted analysis and can also be of help with the interpretation of the final data. The main characteristic of the bottom-up approach is the upfront digestion of proteins in the samples into small peptides. This is usually done by using the enzyme trypsin which cleaves only after the amino acids arginine or lysine. Samples are labeled with isotope-containing tags called iTRAQ allowing quantification and thereafter fractionated. The samples are then inserted into the MS instrument where the proteins are identified through peptide sequencing and quantified by estimation of intensities of the reporter ions formed from iTRAQ. By applying advanced biostatistics and using appropriate software, the extensive list of identified proteins is processed narrowing the detected targets down to a manageable size as it is put into biological context (Figure 2).

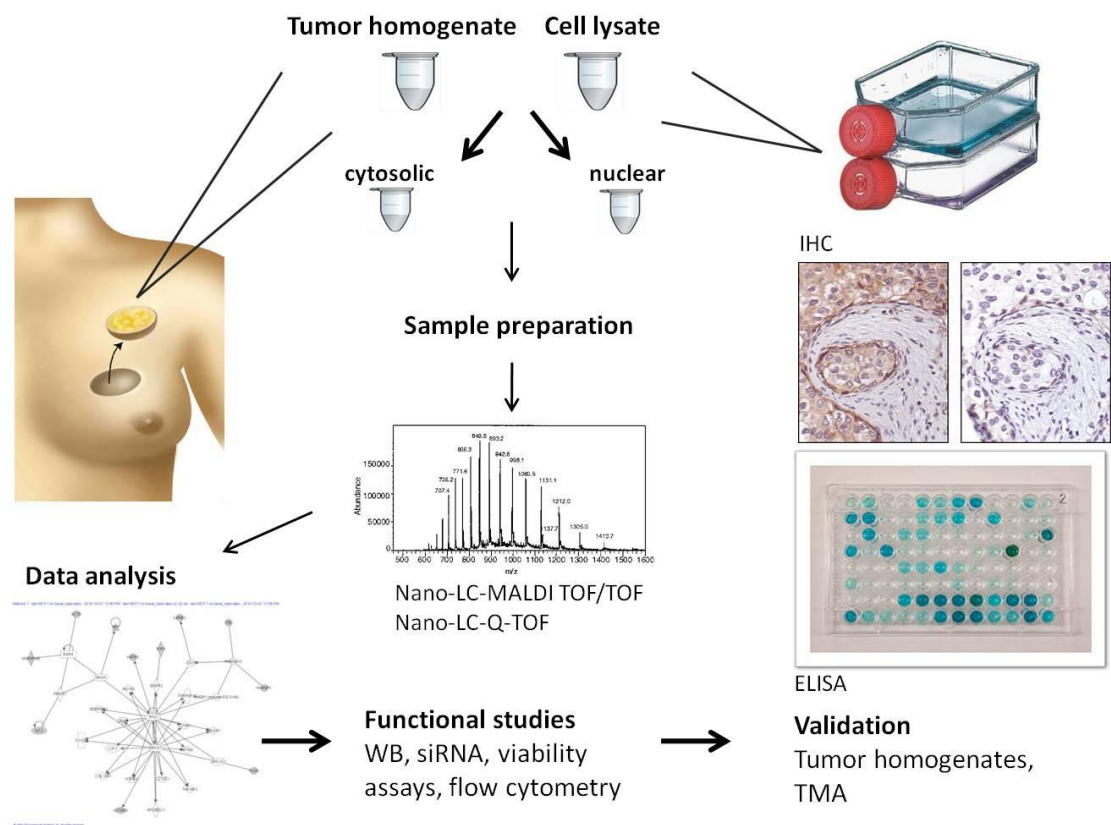


Figure 2. Overview of the experimental workflow followed by functional analysis and ultimately validation in patient samples. For details of the workflow see text. Abbreviations: WB, western blot; TMA, tissue micro array; IHC, immunohistochemistry.

1.5.2.1 iTRAQ labeling

Our group performs quantitative proteomic analyses in order to be able to compare proteome changes caused by perturbations such as treatment or biological differences. This is done by using up to eight stable isobaric tags for relative and absolute quantification, called iTRAQ, which consist of a charged reporter group, a peptide reactive group and a neutral balance portion balancing all eight tags in mass. Following protein digestion, the peptide mixture is labeled with these tags which bind covalently to primary amine groups, and all eight samples are consequently pooled. Each tag exhibits a different fragmentation pattern by MS/MS giving rise to unique reporter ions thus enabling the relative quantification of each labelled peptide [106].

1.5.2.2 Fractionation

One of the most problematic issues in proteomics has been to deal with the complexity of samples. Therefore the aim has been to optimize and simplify samples before the quantification and identification of peptides. It is believed that by reducing the influence of peptides from high abundant proteins the discovery of less abundant and perhaps more interesting proteins will be facilitated. Samples are therefore subjected to immobilized pH gradient-isoelectric focussing (IPG-IEF). This approach uses the difference in isoelectric point of peptides within a specific range of pH to separate them. Although the narrow range IPG-IEF applied from pH 3.5-4.5 only captures a subset of peptides they still exhibit high representation of all present proteins. This is justified by *in silico* data strongly suggesting that 96% of all proteins have at least a corresponding peptide within this range [107].

1.5.2.3 MS-based protein identification (tandem MS)

The next step in the workflow includes mass spectrometry, a method used for the identification of peptides. Before we get lost in translation a short description of the basic principle of MS is required. MS is used to measure the mass of peptides by first ionizing the molecules and then determining their mass to charge ratio. A sample has to travel through 3 key compartments in the mass spectrometer, the ion source, mass analyzer and detector. First, the sample is vaporized for ionization of molecules. Secondly, these ions are equally accelerated into a focused beam where the velocity of each ion depends on the mass to charge ratio. The ions are consequently guided to the compartment where mass separation and analysis is performed by using different methods depending on the MS-instrument of choice. The output is represented as a famous stick diagram or mass spectrum, showing ion intensity on the y-axis and the mass to charge ratio (m/z) on the x-axis [104].

In the current work we used two ionization methods called matrix assisted laser desorption ionization (MALDI) and electro spray ionization (ESI). As the name implies, the ionization in MALDI is performed by a laser in cooperation with the matrix which the sample was mixed onto [104]. ESI is based on the release of gas-phase ion-like molecules as the density charge on the surface of sample droplets becomes too much and tears the droplets open. Moreover, ESI is better at keeping molecule fragmentation at a minimum [108].

Next is a brief introduction to the mass analyzers, time of flight (TOF)-TOF, also known as MS/MS, quadrupole (Q)-TOF and linear ion trap quadrupole (LTQ)-Orbitrap, used in this work. When using TOF the determination of the mass to charge ratio is based on the time it takes for the accelerated ions to reach the detector in a vacuum free flight zone of the TOF analyzer as lighter ions travel at a higher speed and vice versa. The extra TOF is actually not a spelling mistake but the addition of such a component. It is responsible for analyzing the mass of fragments of peptides selected for fragmentation after the first mass analysis single MS-spectra. The merging of the precursor mass from MS and the fragmentation masses from MS/MS results in the determination of the amino acid sequence of a peptide by comparison of the experimental data to theoretical database. Q-TOF and ion trap both use a quadrupole as mass analyzer. The quadrupole consists of four metal rods arranged in parallel through which selected ionized molecules pass driven by the magnetic field within this rods. LTQ differs from the regular quadrupole as it analysis ions in a pulse mode as opposed to a continuous flow, by capturing them in an ion trap of extended volume by additional repulsive rods in the ends of the quadrupole rods [109]. The LTQ-Orbitrap is a mass analyzer representing the combination of two techniques which complement one another. The orbitrap uses an electrostatic field to separate the masses instead of a magnetic [28].

As a last step, a mass spectrum is obtained by the detector as it detects the masses and number of ions that reaches it. Once we have obtained the spectra with peptide fragments the job of identifying the corresponding protein begins. A database search algorithm (i.e. MASCOT, paragon) is used to match the experimental fragments to *in silico* generated ones in order to identify the peptide. Several commonly used existing sequence databases and various software are employed resulting in a very extensive list of identified proteins.

1.5.2.4 Tools for biological interpretation

The next step of putting all this data into context is complicated. Fortunately the quality of software developed to facilitate this analysis, is continuously improving. Despite this, reliability and coverage of databases are some of the issues that still have to be taken under account. The role of systems biology, which integrates computational, biological and medical input, is invaluable since it simplifies the handling of data severely [110]. Software, such as SIMCA, Ingenuity Pathway Analysis (IPA), Gorilla, Proteincenter (Proxeon) etc., were employed in paper III and IV. SIMCA is a program suitable for performing multivariate data analysis. The other mentioned programs use biological and medical knowledge to define protein characteristics and relationships between them.

2 AIMS OF THIS THESIS

The overall aim was to identify potential markers for tamoxifen resistance by merging knowledge from a cell model system and human tumors.

The specific aims of each paper were:

- I.** To investigate if VEGF/ VEGFR2 signaling implicating p38 MAPK is involved in the molecular mechanisms of tamoxifen resistance.
- II.** To validate the importance of VEGF expression on survival after adjuvant tamoxifen treatment and to explore a possible relation between VEGF levels and treatment duration.
- III.** To explore the proteomic profile of MCF7 BC cells and the 4-hydroxytamoxifen-resistant MCF7/LCC2 subline by using mass spectrometry-based proteomics in search of key differences involved with the resistance mechanisms.
- IV.** To discover potential predictive biomarkers for tamoxifen response by using mass spectrometry-based proteomics on tumor samples from primary BC patients.

3 MATERIAL

3.1 CELL LINES

The cell model of choice was MCF7 and its subline called MCF7/LCC2 (referred to as LCC2). The parental cell is the most commonly used in breast cancer research originating from a pleural effusion from a 69-year old BC female patient diagnosed with invasive ductal carcinoma [111]. LCC2 is a 4-OHT-resistant cell line derived from a series of sublines originating from MCF7. MCF7/MIII was the first cell line derived from MCF7 having obtained a hormone-independence for growth *in vitro* and *in vivo* as opposed to parental cells. Further selection of these resulted in the acquisition of MCF7/LCC1, a subline with a more malignant phenotype despite no apparent differences in genomic amplification compared to MCF7 [112]. Although MCF7/MIII and MCF7/LCC1 can form tumors in mice without supplemental E2 their growth is still induced by E2. LCC2 cells are the result of the exposure to stepwise increasing concentrations of 4-OHT, the main metabolite of tamoxifen, of MCF7/LCC1 cells until becoming resistant. They were defined resistant when 1 μ M of 4-OHT only caused a decrease in proliferation by 15%. An additional trait by the resistant cell line was the hormone-independence when growing both *in vitro* and *in vivo* while maintaining ER levels similar to that of MCF7 cells [113].

3.1.1.1 Reflecting on the choice of cell model

We opted for these cell lines since we considered them representative of a reliable model which could mirror the biological process of acquired tamoxifen resistance. The main characteristics that make LCC2 cells a useful model for studying resistance mechanisms is their ability to maintain similar levels of ER expression as the parental cells while sustaining tumorigenic ability to induce tumor growth without the need of hormone supplementation. Moreover, the proliferation of LCC2 cells is not induced by tamoxifen as opposed to several other tamoxifen resistant cell lines [114, 115].

Although cell lines may seem like the optimal tool due to their endless growth potential, we have to be aware of the limitations that come with them. Cells are relatively easy to take care of except that they may undergo phenotypic or genotypic changes during culturing. These changes can occur due to a variety of reasons such as altered temperature, carbon dioxide levels and oxygen, continuous exposure to trypsin and contamination with mycoplasma, viruses or even other cell types [111, 116]. However, it is important not to forget that cell models are great tools for research and that by taking the required precautions problems can be overcome to a certain degree. This is the reason till why we, in our lab, maintain all our cells under the same conditions using each stock of cells for up to a maximum of two months. In order to avoid contamination only mycoplasma-negative cells are allowed into the culture room and freezer storage.

3.2 PATIENTS

In general, patients from both cohorts received similar primary treatment according to standard guidelines. All patients underwent standardized breast conserving surgery followed by radiotherapy. Dissection of axillary lymph nodes was performed in eligible patients excluding those of high age or with concomitant diseases. It is important to mention that patients were randomized into two arms as part of a phase III clinical trial

studying two vs. five years of adjuvant tamoxifen until 1995 as the 5-year regimen became standard therapy. After primary treatment the patients were observed for a long period of time by annual clinical examinations and mammograms at their corresponding clinics. Survival times were calculated as the time from diagnosis to the date of first recurrence or death, and for relapse-free patients to last clinical examination. Recurrences were defined as the first documented evidence of new disease manifestations in the loco-regional area, contralateral breast, in distant sites or a combination of those. The REMARK criteria were taken into account during selection of both patient cohorts.

3.2.1 Cohort I

Data on a total of 711 frozen tumor homogenates with a known hormone receptor status from patients diagnosed with primary operable BC from 1991 to 1996 was made available to us from the Breast Cancer database at the Regional Oncologic Centre, Linköping University Hospital, Linköping, Sweden. This database contains information on all patients from the Southeast Sweden Health Care Region with primary BC. The clinical data obtained from this database included: Age, gender, tumor size, steroid receptor status, node status and S-phase fraction. For the purpose of studying the sole effects of tamoxifen we included 402 out of 449 eligible patients with ER positive BC of stages I–III having received tamoxifen as the only adjuvant therapy. Patients with locally advanced BC, displaying distant metastases at diagnosis, or having received neoadjuvant therapy, were excluded. A description of the patients' characteristics is listed in Table 2. Data on nodal status were limited to 96% of the patients. The median follow-up time in relapse-free patients was 9.8 years. The study design was approved by the research ethics board of Linköping University, Sweden. Apart from the analyses we performed on this patient material in the present work we had access to data on previous measurements done by our group on levels of p38, JNK and ERK.

Paper II

Four hundred and four patients were included in this study. 402 samples were included since 47 were unavailable due to sample limitation.

Paper III

Eight random matched samples from a total of the twenty four samples used in paper IV were used for the verification of MS data by western blot. 382 patients were included for the validation of functional studies by enzyme-linked immunosorbent assay. We were unable to analyze 67 samples from the original cohort due to limitations of tumor homogenate availability.

Paper IV

24 samples were included in this pilot study. Twelve patients relapsing within two years were matched to patients remaining relapse-free for more than seven years according to age, tumor size and nodal status.

Feature	Cohort 1 Nr of patients	Cohort 1 Nr of patients
<i>Patients enrolled</i>	402	45
<i>Tumor size, mm</i>		
Median (range)	20 (3-150)	15 (6-50)
<i>Stage</i>		
T1	222 (55)	32 (71)
T2-T3	180 (45)	13 (29)
<i>S-phase</i>		
<10%	267 (66)	NA
>10%	94 (23)	NA
Unknown	41 (11)	NA
<i>Lymph node status</i>		
Node-negative	200 (50)	32 (71)
Node-positive	184 (46)	10 (22)
Unknown	18 (4)	3 (7)
<i>Histopathological grade</i>		
I-II	NA	22 (49)
III	NA	19 (42)
Unknown	NA	4 (9)
<i>ER</i>		
Median	NA	1.93 (0.0 10.4)
Pos (≥ 0.05 fmol/ μ g DNA)	402 (100)	8 (18)
Neg (< 0.05 fmol/ μ g DNA)	0	37 (82)
<i>PR</i>		
Median (range)	3.0 (0-36.0)	1.45 (0-10.6)
Pos (≥ 0.05 fmol/ μ g DNA)	357 (89)	13 (29)
Neg (< 0.05 fmol/ μ g DNA)	45 (11)	31 (69)
Unknown		1 (2)
<i>HER2</i>		
Pos	NA	4 (9)
Neg	NA	41 (91)
<i>VEGF, pg/μg DNA</i>		
Median (range)	2.4 (0-85.3)	2.3 (0-320.9)
Low	201 (50)	21 (47)
high	201 (50)	24 (53)
<i>Tamoxifen therapy</i>		
2 years	149 (37)	27 (60)
5 years	253 (63)	18 (40)

Table 2. Summarizing table of tumor characteristics of the two cohorts included in this thesis.

3.2.2 Cohort II

The original cohort included 679 patients diagnosed with primary operable invasive BC at the Karolinska University Hospital and St Görans Hospital, Stockholm during January 1993 to December 1996. Clinical data for these patients included: Age, gender, tumor size, steroid receptor status, grade, node status and HER2 status (Table 2). 404 patients of these received adjuvant endocrine therapy up to five years out of which 295

had a BC positive for both ER and PR. Within the TMA used in paper III, there were 95 available tumor tissues of which 45 were evaluable by immunohistochemistry. The median follow-up time in recurrence free patients was 11.2 years. Detailed characteristics of patients included in the TMA are included in the third manuscript. The medical ethical committee of the Karolinska Institute, Stockholm, Sweden, approved the study design. Data on previous measurements of JNK and ERK on TMAs and VEGF, VEGFR2 and p38 on tumor homogenates from this cohort was available to us.

3.2.2.1 Reflecting on patient cohorts

When considering material for biomarker discovery or validation it is important that the handling and preparation of samples follows an approved standard protocol and is instantaneous from the moment it is collected from the patient [117]. This should be done in order to avoid decomposition or degradation of analytes of interest. Moreover, if all samples are prepared equally it sets an assumptive baseline for all samples. Therefore, the preparation of our samples followed a strict standardized operating procedure handled by experienced technicians as these samples were used for determination of ER and PR within clinical routine. As a matter of fact, both Linköping, as well as Karolinska University Hospital participated in the external quality assessment program arranged by the EORTC- Receptor and Biomarker Group at the Quality Assessment Laboratory, University Medical Centre Nijmegen, Netherlands.

Since a representative piece of the tumor was collected there is the issue of tumor heterogeneity to be considered. It is possible that the collected specimen not only consists of tumor cells but also stromal and other infiltrating cells making it difficult to decide the true origin of the analytes of interest. Considering the scarcity of tissue material it is noteworthy that tumor homogenates were adequate material for our purposes and were ample allowing measurements of various proteins. The strengths of our more extensive patient material (cohort I) are many including the homogeneity, the relatively long follow-up and the extensive and informative clinical data. One drawback could be that we did not limit ourselves to include randomized patients only in our studies. Moreover, we are aware that cohort II was too small to achieve any statistical power or significance. We included this cohort as a test material for another platform, the histological one, since it remains the standard platform for new biomarkers aiming to be included in post surgical routine analysis for primary BC. With these drawbacks in mind, we consider our data to be more of a hypothesis-generating character.

4 RESULTS AND DISCUSSION

4.1 PAPER I

An autocrine VEGF/VEGFR2 and p38 signaling loop confers resistance to 4-hydroxytamoxifen in MCF7 breast cancer cells

This study was initiated by our previous observations describing an association between high VEGF, VEGFR2 and positive p38 expression and early recurrences in ER positive patients during adjuvant tamoxifen treatment [118]. These results suggested involvement of these factors in the mechanism of tamoxifen resistance. In order to study this closer we turned to a cell model system consisting of two cell lines with different responsiveness to 4-hydroxytamoxifen (4-OHT), the active metabolite of tamoxifen, LCC2 and the parental sensitive MCF7 cell line [113]. We hypothesized that in 4-OHT-resistant LCC2 cells p38 could partly be activated as a consequence of increased VEGF/VEGFR2 signaling enabling them to proliferate even in the presence of 4-OHT.

Cells were exposed to 4-OHT to confirm their different responsiveness to the treatment by analyzing their viability. The most noticeable differences between cell lines were found six days post treatment defining the default time point for this study. Interestingly, in comparison to MCF7 cells, untreated LCC2 cells secreted higher levels of VEGF and although the secretion decreased in a dose-dependent manner, VEGF levels remained higher than in MCF7 cells. As for VEGFR2 expression, despite similar basal levels in both cell lines the proportion of activated receptor was higher in LCC2 cells. In response to increasing concentrations of 4-OHT, the expression of VEGFR2 in LCC2 cells remained intact while activated levels were somewhat lowered although still higher than in sensitive cells.

Moreover, basal p38 expression was also higher in LCC2 cells with an increasing tendency in response to 4-OHT. Interestingly, the treatment affected the phosphorylation pattern of p38 in these cell lines differently. The activity of p38 was affected in a dose-dependent way, increasing in MCF7 while decreasing in LCC2 cells arguing for potential difference in antibody specificity between the activated and total p38.

There are four different isoforms of p38 where α and γ have been mostly studied and found to be of pro-apoptotic and proliferative nature, respectively. Since they appear to regulate each other the dominating one will consequently influence which pathway becomes activated [119]. We speculate that the overexpressed and thus dominating isoform, inducing the activation of proliferative pathways in LCC2 cells, may be p38 γ by inhibiting the activation of p38 α and thereby also blocking its own degradation (Figure 3). Appropriately, recent studies assigned p38 γ a critical role in maintaining oncogenic properties and contributing to resistance to DNA damage in BC cells [120].

We also studied the role of p38 in viability by pre-exposing the cells to a pharmacological inhibitor called SB202190 followed by co-treatment with 4-OHT. The remarkable effect of the inhibitor per se led us to the conclusion that inhibition of p38 gave an additive effect to that of 4-OHT treatment on cell viability in both cell lines.

We proceeded by silencing VEGF or VEGFR2 expression in LCC2 cells with the intent to pinpoint if VEGF indeed was signaling through VEGFR2 and whether or not

p38 was involved in this pathway. VEGF silencing decreased the expression of both total and phosphorylated VEGFR2 and p38 although the latter did not decrease to the same extent. Moreover, when VEGFR2 expression was repressed a reduction of total and phosphorylated p38 was seen. However, VEGF was only slightly reduced.

In conclusion, our results from this paper suggest that the existence of an autocrine release and action of VEGF through VEGFR2 involving p38 is characteristic for 4-OHT-resistant BC cells.

Increased VEGF secretion has also been reported in TAMR-MCF7 cells, a similar type of 4-OHT-resistant MCF7 cells. Moreover, these cells form tumors with high angiogenic intensity regulated by Pin1 through activation of c-Jun [121]. Pin1 proved to be, implicated in the induction of an EMT-like state in TAMR-MCF7 cells, and regulated by the activation of E2F1/pRb, a key regulator for cell cycle progression, whose activation is in turn dependent on PI3K and p38 (Figure 3). Importantly, inhibition of p38 decreased VEGF secretion in the resistant cells [121, 122]. Additional functions assigned to p38 include both stabilization of VEGF mRNA and phosphorylation of ER (Figure 3) [123]. p38 is likely to have a wide influence in tamoxifen resistance thus explaining the significantly reduced viability of the LCC2 cells when inhibiting p38.

This data strengthens our theory about the autocrine loop active in tamoxifen-resistant cells involving VEGF/VEGFR2 and p38. However, although it is tempting to claim that VEGF/VEGFR2 signaling works as a collaborating pathway with HER2/PI3K signaling, as suggested by others [63, 67], MCF7 cells are HER2 negative and thus such statement cannot be applied in our model system [124].

Unfortunately, the effect of bevacizumab has not been consistently successful in BC therapy. There is, however, a possibility that the lack of effect is due to a diluted response as a consequence of not identifying the patients that would benefit the most. Considering our findings, perhaps a combination of Bevacizumab plus tamoxifen could increase response in patients exhibiting high levels of VEGF? Importantly, completed phase II trials investigating feasibility and effect of combined endocrine therapy plus bevacizumab have reported beneficial effects apart from recurrent side-effects [125, 126]. Similar randomized phase III trials are currently ongoing and thus the effect of bevacizumab in this setting remains to be seen.

4.2 PAPER II

Prolonged tamoxifen increases relapse-free survival for patients with primary breast cancer expressing high levels of VEGF

Earlier studies have shown that high VEGF levels in primary breast tumors correlate to shorter survival times for patients treated with adjuvant tamoxifen [127-130]. With this study we aimed to validate the potential adverse prognostic effect of VEGF. The expression of VEGF was determined, in the cytosolic fraction of tumor homogenates from 402 patients with an ER positive BC receiving tamoxifen as adjuvant therapy, by using enzyme linked immunoassays. As the majority of these patients (253/402) had received tamoxifen for five years while the rest were treated for two years, we also intended to evaluate the impact of treatment duration on survival taking expression of VEGF under consideration.

Statistical analyses were performed looking to validate the previously reported adverse prognostic value of VEGF in tamoxifen treatment. Moreover, associations between VEGF expression and survival were of interest to emphasize the importance of tamoxifen treatment duration.

Our results did indeed confirm the adverse prognostic value of VEGF expression in patients receiving tamoxifen. Moreover, when studying the cohort by treatment duration, no significant difference in outcome was observed between high vs. low VEGF-expressing patients in the 5-year regimen. There was, however, a significant decrease in survival for patients with high VEGF expression among those receiving tamoxifen for two years. In other words, high VEGF expression does not influence the outcome provided patients receive a prolonged tamoxifen treatment.

In addition, supporting evidence from a large randomized trial showed that the negative prognostic impact of peritumoral vascular invasion (PVI), which correlates with VEGF levels, was not seen in steroid receptor positive BC patients when receiving five years of tamoxifen [77].

When patients were separated and analyzed according to VEGF expression, the 5-year regimen proved to be more beneficial in the high VEGF-expressing patients. Interestingly, the positive effect of the prolonged tamoxifen regimen on survival was not seen in the subset of patients exhibiting low VEGF levels. These results imply that the duration of treatment does not influence the outcome of patients as long as they express VEGF at low levels.

The consensus on tamoxifen treatment duration for postmenopausal women has been of five years after presented evidence of a significant benefit in comparison to two years of tamoxifen [131]. Our results point at no benefit gain with the prolonged regimen if patients do not express VEGF at a higher extent. Interestingly, the latest recommendations regarding endocrine treatment in postmenopausal patients are that AIs should be used either upfront or after tamoxifen treatment for a maximum of five years or as an extension to five years of tamoxifen [36]. This includes often combining treatment in a sequential manner such as three years of tamoxifen plus two years of AI or the opposite starting with an AI upfront. Our results suggest that patients expressing high levels of VEGF would specifically benefit from an extended tamoxifen treatment followed by an AI rather than only three years of tamoxifen.

Although there are studies suggesting that treatment with AIs may be more beneficial than tamoxifen in patients expressing high VEGF levels, no such correlation has been observed [132]. Recently the combination of an AI and bevacizumab for the treatment of postmenopausal women with metastatic BC has shown promising effects and is still being evaluated [125, 126]. However, no trials evaluating tamoxifen or comparing both endocrine treatments in such setting have been performed.

It is important to keep in mind the possibility of an interaction between VEGF and HER2 considering the evidence presented by preclinical [63, 67] and clinical studies [133, 134] showing the existence of such a relationship.

In summary, high VEGF was significantly correlated to an impaired survival after adjuvant tamoxifen treatment in the whole patient population. However, an improved survival was observed in patients with high VEGF-expressing tumors provided they were given tamoxifen for a prolonged time period.

4.3 PAPER III

Deregulated retinoic acid receptor alpha signaling in tamoxifen resistance is of potential predictive value in steroid receptor positive breast cancer

Having concluded that 4-OHT-resistant LCC2 cells may have a VEGF/VEGFR2-driven autocrine loop involving p38 in paper I, we decided to continue exploring the characteristics of each of these two cell lines, however, this time with a high throughput methodology. Our aim was to do an unbiased discovery of potential key players in tamoxifen resistance by performing quantitative MS-based proteomics on MCF7 and LCC2 cells, prior and post exposure to 4-OHT.

We used a bottom-up proteomics approach for this purpose. The samples of origin were lysates from cells treated with 4-OHT for three days and corresponding untreated cells. In order to study the non-genomic and genomic effects of altered ER signaling associated with tamoxifen resistance, lysates were fractionated and analyzed separately as two subcellular fractions, a DNA-binding (nuclear) and a cytosolic.

In short, samples were consequently digested, labeled with iTRAQ and additionally fractionated by IPG-IEF. Next, they were analyzed by nLC-MALDI-TOF/TOF and nLC-Q-TOF where proteins were identified through peptide sequencing and quantified by estimation of intensities of the reporter ions formed from iTRAQ. By using in-house software, denoted PQPQ [135], quantitative accuracy was enhanced by removing outliers and thus quantification of biological replicates was enabled. We identified a total of 830 proteins of which 201 and 629 were found in the nuclear and cytosolic fraction, respectively.

The extensive list of identified proteins was processed using advanced biostatistics to include only significantly deregulated proteins. In general, the three top cellular functions in which the most significantly deregulated proteins were involved in were cell cycle, growth and cell to cell signaling when comparing untreated LCC2 to MCF7 cells. In response to 4-OHT, the most relevant functions of proteins included cellular assembly, morphology and proliferation in MCF7 cells while LCC2 cells appeared to circumvent the effect of 4-OHT by deregulating proteins involved in cell death, cellular compromise and cell cycle.

Consequently, pathway analyses revealed a connection between RARA and significantly deregulated proteins in the proteomics data set. RARA is normally involved in anti-proliferative signaling inducing apoptosis and differentiation. Recent work by Hua et al, showed an antagonistic interaction between ER and RARA signaling [96]. However, these two pathways were later reported to co-operate with each other [97]. When displaying proteins shown to be regulated by ER and RA [96, 136] separately within each fraction, the RA-regulated ones seemed to be the most abundant in our data set. This raised our interest in RARA and we proceeded by functionally exploring it in the cell model system.

RARA expression was similar at basal levels in both LCC2 and MCF7, increasing only in MCF7 after 4-OHT treatment. Moreover, exposure to the RARA agonist, AM580, resulted in G1 cell cycle arrest in MCF7 cells similarly to the effect of 4-OHT. In contrast, LCC2 cells remained more or less unaffected by either treatment. When analyzing viability, the antigrowth response caused by AM580 was decreased in LCC2

compared to MCF7 cells. Furthermore, depletion of RARA by siRNA increased viability in MCF7 while significantly decreasing viability in LCC2 cells.

RARA seems to act as a brake repressing the growth properties in MCF7 cells. In the absence of a brake the repressive growth regulation is decreased allowing cells to proliferate. However, RARA seems to exert a different function in LCC2 cells. RARA, supported by an altered environment seems to be of importance for sustaining viability of LCC2 cells.

Interestingly, RARA expression was shown to be upregulated in patients with early relapses (<2 years) compared to relapse-free patients (>7 years of relapse-free follow-up). We hypothesize that patients with early relapse may have acquired a switch in RARA signaling together with other co-regulators allowing increased levels of RARA to promote proliferation in the presence of tamoxifen.

A primary validation on tumor homogenates from 382 ER positive patients receiving adjuvant tamoxifen for two or five years showed a significant correlation between high RARA expression and shorter RFS in patients treated with the 2-year regimen (n=63) (p=0.0028). Analysis of RARA by IHC, in a second independent cohort of 45 patients, showed the same tendency in respect to RFS, although did not achieve statistical significance.

An issue to take under account is that RARA expression in the samples analyzed by IHC does not seem to correlate with HER2 status. The close proximity of the RARA gene to the HER2 amplicon [137] raises the question whether or not there is a relation between these two factors. Since HER2 status is unknown in the main validating cohort it is an issue to be pursued. Moreover, RA in combination with tamoxifen has been suggested to be of benefit in HER2-overexpressing, ER positive BT474 BC cells [138]. This connects HER2 with a regular anti-proliferative RARA signaling in contrast to RARA's role in high VEGF/VEGFR2-expressing cells, such as LCC2.

Since VEGF is a RA-regulated gene, we also analyzed the effect of RARA modulation on VEGF in both cell lines and found that VEGF was regulated differently between cell lines. Interestingly, VEGF secretion in LCC2 cells was increased by both induction and silencing of RARA suggesting that RARA is only partly involved in the overexpression of VEGF seen in the 4-OHT-resistant cells. Interestingly, a strong correlation was seen between RARA and VEGF expression (p<0.0001) in the main validating cohort.

RARA is controlled by multiple actors interacting with one another in a complex manner and thus resulting in the integration of various pathways of the molecular machinery. The transcriptional activity of RARA is modulated by phosphorylation of co-activators, such as SRC-3, and co-repressors. Apart from this, RARA is believed to exert non-genomic functions implicating the direct interaction with SRC-3 as a consequence of RA-induced activation of SRC-3 through p38 [139]. RARA can also be activated in a ligand-independent manner, for instance, in response to growth factors or through direct interaction with MAPKs. Speculating on possible associations to our findings in paper I, it is tempting to say that the possibility that RARA is part of the autocrine loop involving VEGF/VEGFR2 and p38 in LCC2 cells exists.

RARA has been also been shown to directly interact with Pin1, a protein shown to induce EMT-like behavior and tumor angiogenesis involving p38 and VEGF in BC cells [72, 121, 122]. However, Pin1 induces degradation of RARA unless a mutation on

a specific phosphorylation site on RARA is present [140, 141]. Therefore, inhibition of Pin1 results in increased RARA levels and as a consequence also improved response to RA treatment in acute myeloid leukemia (AML) [140]. In LCC2 cells, however, the role of RARA appears to be different, promoting survival of cells to a certain extent. Whether or not RARA, in LCC2 cells, contains the mutation that abolishes Pin1's ability to degrade RARA and instead is activated by their interaction, remains to be explored (Figure. 3).

In conclusion, we show that the function of RARA is deregulated in LCC2 cells in comparison to MCF7. LCC2 is dependent on the presence of RARA to sustain viability in contrast to MCF7 cells, suggesting that high expression of RARA in tumors from early relapse patients supports proliferation of these tumors. Also, RARA is suggested to be of potential prognostic and predictive value in ER positive BC patients.

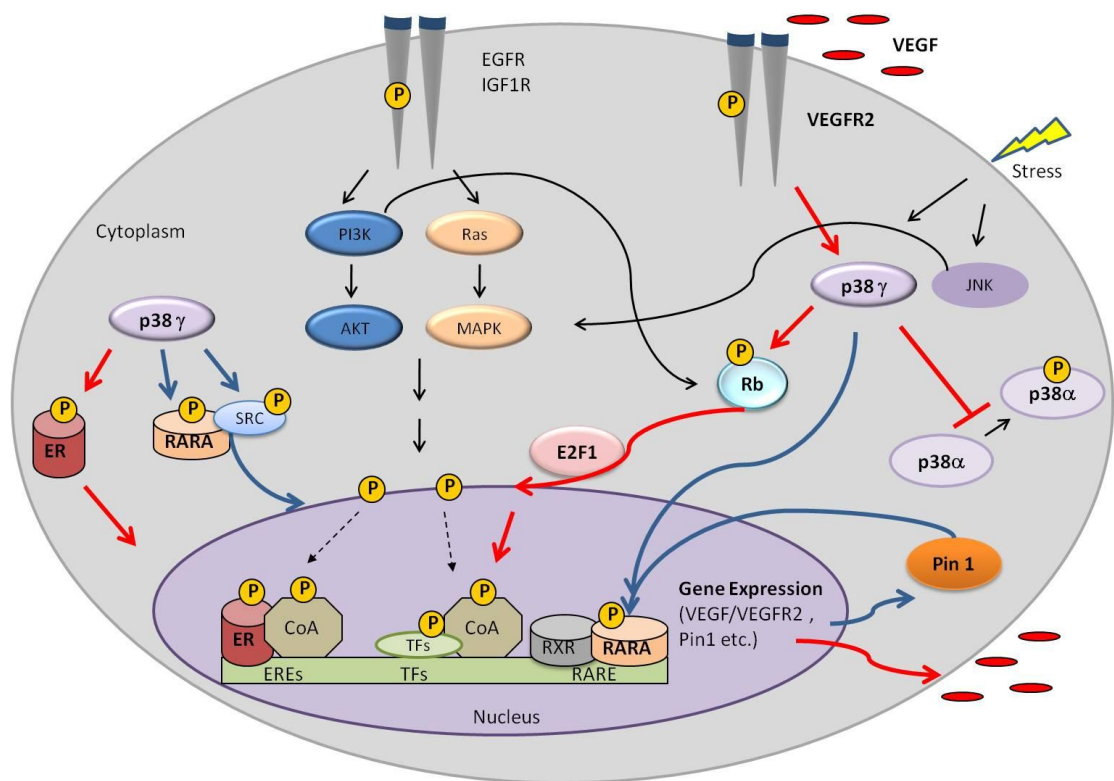


Figure 3. Illustration summarizing hypothetical implications of our findings based on related published observations. The potential role of p38 in the autocrine loop present in LCC2 cells (blue arrows). Potential implications of an altered RARA signaling in tamoxifen resistance (red arrows).

4.4 PAPER IV

Proteomics-based characterization of potential biomarkers implicated in tamoxifen resistance in primary operable breast cancer

For this study we used a similar MS-based proteomics approach as described in paper III with the aim to do an unbiased search for potential predictive markers of tamoxifen response on tumor samples from primary BC patients.

This pilot study included 24 out of the original cohort of 402 ER positive tumor samples from patients receiving tamoxifen as the sole adjuvant treatment. These samples were divided into two groups, 12 samples from patients exhibiting early relapses (<2 years), and 12 samples from relapse-free patients (>7 years). The groups, referred to as relapse and control, were matched to each other according to age, tumor size and node status in order to avoid the well established influence of these factors in outcome.

Samples from tumor homogenates were prepared to be compatible to the desired analysis. Moreover, the digestion, labeling with iTRAQ and fractionation with IPG-IEF was done similarly as in paper III. Next, samples analysis was performed by LTQ-Orbitrap Velos high resolution mass spectrometer yielding a total of 3101 identified proteins.

As the data generated was extensive and complex advanced biostatics tools such as orthogonal partial least square (OPLS) and principal component analysis (PCA) were requires as they include efficient and robust methods for analysis and interpretation of data [142]. Uni- and multivariate analyses by PCA and OPLS revealed a 13-protein signature, which separated relapse from control group (P-value 2.2e-005). Seven of them were upregulated and six downregulated in the relapse group compared to the control group.

These 13 proteins are involved in various processes such as Ca^{+2} signaling, metastasis, invasion, motility, EMT, and cellular metabolism [143-148]. We chose to proceed with the four most upregulated proteins, calcyphosine (CAPS), myxovirus resistance 1 (MX1), Ras-related protein Rab-21 (RAB21), and glutamine--fructose-6-phosphate transaminase 1 (GFPT1) based on relevant biological function and applicability. Interestingly, expression analysis by WB on eight random patient samples out of the 24 included in the study revealed an overexpression of CAPS and MX1 in the relapse group. GFPT1 was expressed at similar levels in both groups. We were not able to evaluate RAB21 expression due to antibody limitations.

CAPS has been associated to endometrial cancer, a disease believed to be induced by the agonistic characteristics of tamoxifen [144, 149, 150]. CAPS is a Ca^{2+} -binding protein whose synthesis and activation is induced by the cAMP cascade and implicated in cellular proliferation and differentiation [151]. This is of special interest since tamoxifen has been shown to bind to calmodulin, consequently inhibiting further functions of Ca^{2+} -binding proteins through reduction of cAMP [41]. The overexpression of CAPS seen in the relapse patients may be the result of tamoxifen failure in reducing cAMP or a salvation pathway. Appropriately, signaling through CAPS has been suggested to be an alternative pathway to calmodulin [151].

The role of MX1 in cancer has not been well explored. It is known that MX1 is a member of the dynamin superfamily of large GTPases which mediate vesicle trafficking [152]. Moreover, its expression is tightly regulated by interferons, signal transducer and activator of transcription (STAT) signaling [153, 154] and recently also, the PI3K/AKT pathway [155]. Importantly, connections between MX1 and resistance to tamoxifen and fulvestrant, an ERD also used in BC, have been previously reported [156, 157]. These observations suggest that the upregulation of MX1 seen in the relapse group may be a consequence of induction of growth signaling through various pathways.

Similarly, RAB21 can also interact with growth factor signaling although in an indirectly manner. RAB21, another GTPase, is known to regulate vesicular transport in cells and believed to enhance cell migration through integrin traffic modulation [158]. Integrins in turn, have been implicated in proliferation, survival, migration and invasion by conveying signals in two opposite directions, from the extracellular environment to the intracellular machinery and back. This is enabled by the interaction with growth factor signaling involving proteins such as, Ras, PI3K, and Src [159] and thus speaking for a potential involvement of cell motility to our findings in paper I. It is therefore of importance to pursue with the search of a specific antibody which will reveal the certainty in this statement.

GFPT1 is involved in the entry of glucose into the hexosamine signaling pathway acting as generator of UDP N-acetylglucosamine (UDP-GlcNac). These molecules are recognition tags enabling the O-linked GlcNac modification which influences transcriptional regulation and is believed to crosstalk with phosphorylation [160]. Interestingly, this modification is also able to prevent ER degradation and induce STAT signaling [160] in turn increasing MX1 expression. Despite its theoretical potential the verification analysis by WB did not show variations of GFPT1 expression between groups. The discrepancy between quantitative MS-data and WB results could be explained by the detection of different protein variants by these two methods and is aim for further studies.

In conclusion, a 13-signature characteristic of tamoxifen resistance was obtained when comparing patients with early relapses vs. relapse-free ones. Moreover, the two most upregulated proteins in the quantitative MS-data were confirmed to be overexpressed in patients with relapse by WB. The possible value of CAPS and MX1 as predictive markers for tamoxifen response will be further explored by validation in the original cohort and two independent ones.

5 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The work included in this thesis shows the importance and necessity to combine the use of a robust and reliable high throughput method and other classical protein detection methods which study a few proteins at a time. The implementation of both types of analyzing methods is useful for the generation of hypotheses and for adding detailed knowledge to the big picture when trying to dissect disease mechanisms.

An important drawback with many preclinical findings of tumor markers is the lack of consequent validation revealing their clinical usefulness. However, it is important to follow guidelines for tumor marker studies and to consult statisticians when doing so, as poor study design and inappropriate statistical analyses can lead to unreliable results despite having access to a large patient material.

The studies presented in this thesis have increased our knowledge in molecular implications of tamoxifen resistance. These findings are hypothesis-generating and entail possible clinical implications. To explore this further, VEGF, RARA, CAPS, MX1 and possibly RAB21 will be validated in two independent patient populations of similar characteristics to the main cohort used in this thesis. These cohorts are expected to include 800 and 1071 consecutive ER positive patients, randomized to two years of tamoxifen vs. non adjuvant treatment and having received a 5-year tamoxifen regimen, respectively. The optimal study design for studying the true predictive value of our potential markers would require patients randomized between the standard 5-year regimen and no adjuvant treatment and thus will not be performed. However, the randomized cohort will enable the exploration of a true prognostic and predictive significance of RARA in intrinsic tamoxifen resistance.

An important issue throughout the studies included in this thesis has been the lack of HER2 status in our most extensive cohort. In order to clarify the potential involvement of HER2 expression in our analyses the determination of HER2 is paramount.

Another important point conveyed with this work is the utility of cell lines when studying tumor biology. We showed that findings from cell lines can be applied in patients tumors when looking for additional clues.

High throughput proteomics has evolved tremendously and is continuously improving its reliability as tool for the discovery of new biomarkers. The implementation of techniques such as multiple reaction monitoring (MRM), which enables highly accurate targeted analysis of multiple proteins simultaneously, in the validation of our findings is set as a future endeavor.

6 CONCLUSIONS IN SUMMARY

Paper I

An autocrine loop through VEGF/VEGFR2 involving p38 is characteristic of 4-OHT-resistant BC cells.

Paper II

High VEGF expression is significantly correlated to impaired survival in ER positive primary BC patients. High VEGF-expressing tumors benefit more than low-expressing ones from prolonged tamoxifen treatment.

Paper III

High expression of RARA is significantly associated to reduced survival in patients treated with two years of tamoxifen. RARA is suggested to be a potential predictive marker for tamoxifen resistance.

Paper IV

CAPS and MX1 are part of a 13-protein signature characteristic of tamoxifen resistance in primary operable BC patients. CAPS and MX1 are potential predictive markers of tamoxifen response.

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