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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Metastatic Breast Cancer: Biomolecular Characterization and Targeted Therapy

Siker Sokintam Juvita Kimbung



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DOCTORAL DISSERTATION

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Faculty opponent

Associate Professor Therese Sørлие

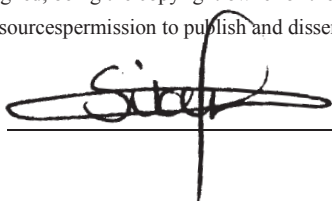
Department of Genetics, Institute for Cancer Research

OUS Radiumhospitalet, Oslo, Norway

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<p>Abstract</p> <p>Metastasis is a complex process that remains a major challenge in the clinical management of cancer, because most cancer-related deaths are attributed to disseminated disease rather than the primary tumor. Despite the significant advances in the prediction of prognosis, and therapeutic management of primary breast cancers, coupled with the substantial improvement in our understanding of the molecular determinants of metastasis, breast cancer relapse and death rates remain unacceptably high.</p> <p>The aim of the research presented in this thesis was to characterize the biomolecular heterogeneity of breast cancer across tumor progression stages and to identify novel biomarkers and therapeutic strategies which may improve prognostication and personalization of therapy for women diagnosed with metastatic breast cancer.</p> <p>By analysis of tumor biopsies collected at different stages of disease progression, we showed that, in general, the phenotype of the primary tumor is typically conserved during tumor progression. However, in a clinically relevant number of cases, a phenotypic drift in biomarkers and tumor molecular subtypes occurs longitudinally with disease progression, with a change to a more aggressive phenotype being associated with an inferior clinical outcome. We also uncovered that breast cancer liver metastases are transcriptionally different from metastases in other anatomical sites and identified candidate liver metastasis-selective genes with the potential to specifically predict liver metastatic relapse and more generally, the time to any recurrence in early stage breast cancer. Furthermore, we demonstrated that, co-targeting of PARP1 and PI3K may represent an improved and specific treatment strategy for BRCA1 deficient breast cancers.</p> <p>The results we present continue to emphasize the clinical significance of breast cancer heterogeneity and highlight possible ways to improve the accuracy of predicting prognosis and effectively treating patients with metastatic disease, a step towards achieving the promise of personalized cancer management and overcoming the clinical burden of metastatic breast cancer.</p>		
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To Mum and Dad
because you believed...

Table of contents

TABLE OF CONTENTS	7
LIST OF ORIGINAL PAPERS	9
ABBREVIATIONS	10
SUMMARY	11
BACKGROUND	13
BRIEF INTRODUCTION TO CANCER.....	13
BREAST CANCER EPIDEMIOLOGY	13
<i>Risk factors</i>	14
DIAGNOSIS.....	15
SUBTYPES	16
<i>Histological subtypes</i>	16
<i>Immuno-pathological subtypes</i>	17
<i>Molecular subtypes</i>	17
RISK ASSESSMENT: PROGNOSTIC AND TREATMENT PREDICTIVE FACTORS	19
<i>Established factors</i>	19
<i>Emerging biomarkers</i>	21
<i>Prognostic factors specific to metastatic breast cancer</i>	24
<i>Emerging prognostic factors in MBC</i>	27
TREATMENT.....	27
<i>Treatment of early stage breast cancer</i>	28
<i>Systemic treatment</i>	28
<i>Treatment of MBC</i>	29
THE BIOLOGY OF BREAST CANCER METASTASIS.....	33
<i>Tumor progression models</i>	33
<i>Genes mediating breast cancer metastasis</i>	34
<i>Organ specific metastasis</i>	35
AIMS OF THE THESIS	39
MATERIALS	41
EXPERIMENTAL AND METHODOLOGICAL CONSIDERATIONS	43
<i>Sample selection</i>	43
<i>Immunological assays</i>	44

<i>Gene expression microarrays</i>	45
<i>In vitro 2D experimental models</i>	46
RESULTS AND DISCUSSION	49
TRENDS IN THE SURVIVAL OF MBC PATIENTS TREATED WITHIN THE TEX CLINICAL TRIAL (PAPERS I-III)	49
LONGITUDINAL HETEROGENEITY OF PATHOLOGICAL BIOMARKERS DURING TUMOR PROGRESSION (PAPER I).....	50
<i>ER, PR, HER2</i>	50
<i>Molecular subtype</i>	51
PROGNOSTIC SIGNIFICANCE OF METASTASIS-SPECIFIC BIOMARKERS AND MOLECULAR SUBTYPE (PAPER I)	52
TRANSCRIPTIONAL LANDSCAPES OF BREAST CANCER METASTASES (PAPER II)	53
SITE SPECIFIC METASTASIS (PAPERS II & III).....	55
<i>Predicting metastatic potential in early breast cancer</i>	55
<i>Claudin-2: a potential prognostic factor for predicting breast cancer liver relapse</i>	55
<i>Transcriptional biology of breast cancer liver metastases</i>	57
<i>The 17-gene liver metastasis selective signature: a potential luminal A subtype specific prognostic marker</i>	58
TARGETED THERAPY FOR <i>BRCA1</i> DEPENDENT MBC: COMBINATION OF PARP1 AND PI3K INHIBITORS (PAPER IV).....	59
CONCLUSIONS	61
FUTURE PERSPECTIVES	63
POPULAR SCIENCE SUMMARY	65
ACKNOWLEDGEMENTS	69
REFERENCES	71

List of original papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

- I. Significance of biomarker expression and molecular subtype at different stages of tumor progression for the prognosis of metastatic breast cancer.

Kimbung S, Kovács A, Danielsson A, Bendahl P, Lövgren K, Stolt MF, Tobin NP, Lindström L, Bergh J, Einbeigi E, Fernö M, Hatschek T, and Hedenfalk I, in collaboration with the TEX Trialists Group.

Submitted.

- II. Transcriptional profiling of breast cancer metastases identifies liver metastasis-selective genes associated with adverse outcome in luminal A primary breast cancer.

Kimbung S, Johansson I, Danielsson A, Veerla S, Egyhazy S, Bergh J, Einbeigi Z, Linderholm B, Lidbrink E, Loman N, Malmström P, Söderberg M, Walz T, Fernö M, Hatschek T and Hedenfalk I, in collaboration with the TEX study group.

Manuscript.

- III. Claudin-2 is an independent negative prognostic factor in breast cancer and specifically predicts early liver recurrences.

Kimbung S, Kovács A, Bendahl P, Malmström P, Fernö F, Hatschek H, and Hedenfalk I.

Molecular Oncology 8 (2014) 119-128.

- IV. Co-targeting of the PI3K pathway improves the response of BRCA1 deficient breast cancer cells to PARP1 inhibition.

Kimbung S, Biskup E, Johansson I, Aaltonen K, Ottosson-Wadlund A, Gruvberger-Saal S, Cunliffe H, Fadeel B, Loman N, Berglund P, Hedenfalk I.

Cancer Letters 319 (2012) 232-241.

Abbreviations

BRCA1	breast cancer 1, early onset
CI	Confidence interval
CTC	Circulating tumor cells
DSB	Double strand break
ER	Estrogen receptor
FFPE	Formalin-fixed paraffin embedded
FNA	Fine needle aspirate
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
IHC	Immunohistochemistry
ISH	<i>In situ</i> hybridization
MBC	Metastatic breast cancer
mTOR	Mammalian target of rapamycin
PARP1	Poly (ADP-ribose) polymerase 1
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
SAM	Significance analysis of microarrays
TMA	Tissue microarray
TN	Triple negative
ASCO	American Society of Clinical Oncology
NCCN	National Cancer Center Network
ESMO	European Society for Medical Oncology

Summary

Metastasis is a complex process that remains a major challenge in the clinical management of cancer, because most cancer-related deaths are attributed to disseminated disease rather than the primary tumor. Despite the significant advances in the prediction of prognosis, and therapeutic management of primary breast cancers, coupled with the substantial improvement in our understanding of the molecular determinants of metastasis, breast cancer relapse and death rates remain unacceptably high.

The aim of the research presented in this thesis was to characterize the biomolecular heterogeneity of breast cancer across tumor progression stages and to identify novel biomarkers and therapeutic strategies which may improve prognostication and personalization of therapy for women diagnosed with metastatic breast cancer.

By analysis of tumor biopsies collected at different stages of disease progression, we showed that, in general, the phenotype of the primary tumor is typically conserved during tumor progression. However, in a clinically relevant number of cases, a phenotypic drift in biomarkers and tumor molecular subtypes occurs longitudinally with disease progression, with a change to a more aggressive phenotype being associated with an inferior clinical outcome. We also uncovered that breast cancer liver metastases are transcriptionally different from metastases in other anatomical sites and identified candidate liver metastasis-selective genes with the potential to specifically predict liver metastatic relapse and more generally, the time to any recurrence in early stage breast cancer. Furthermore, we demonstrated that co-targeting of PARP1 and PI3K may represent an improved and specific treatment strategy for BRCA1 deficient breast cancers.

The results we present continue to emphasize the clinical significance of breast cancer heterogeneity and highlight possible ways to improve the accuracy of predicting prognosis and effectively treating patients with metastatic disease, a step towards achieving the promise of personalized cancer management and overcoming the clinical burden of metastatic breast cancer.

Background

Brief introduction to cancer

Cancer describes a group of genetic diseases affecting different cell types and organs, and together are a leading cause of mortality globally. Despite their variation in cell or organ of origin and clinical manifestation, all cancers arise as a result of chronological acquisition of genetic and epigenetic alterations which endows a normal cell with the ability to divide irrespective of the homeostatic constraints that limit growth of normal tissues. The leading cause of cancer related death is metastasis [1-3]. Metastasis is the process through which malignant cells spread from the primary tumor site to colonize other distant vital organs. Generally, the path of transformation of a normal cell, from a benign state into a malignant phenotype capable of progressing into the lethal metastatic tumor, is today well-characterized. In 2000, Hanahan and Weinberg proposed six key transformation steps necessary for tumor initiation to metastasis development which they called the “Hallmarks of Cancer” [4]. Ten years later, these same authors, after considering the remarkable advances in cancer research over the decade, published a revised and updated lists of eight cancer hallmarks [5]. These hallmarks of cancer include: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, resistance to cell death cues, replicative immortality, induction of angiogenesis, activation of tissue invasion and metastasis, deregulation of cellular energetics and avoidance of immune destruction. The research described in this thesis has attempted to characterize the molecular heterogeneity of breast cancer across tumor progression stages into metastasis, in view of identifying biomarkers to improve prognostication and personalization of therapy for women diagnosed with metastatic breast cancer (MBC).

Breast cancer epidemiology

The 2012 global report on cancer incidence and mortality compiled by the International Agency for Research on Cancer (IARC) [6] classified breast cancer as the second most common cancer form worldwide and the most prevalent female malignancy. In 2012, about 1.67 million new cases were diagnosed worldwide,

while an estimated 521,000 deaths were attributed to this disease [6]. That same year in Sweden, approximately 8,490 women were diagnosed with breast cancer and about 1,450 breast cancer-related deaths were registered [7]. These data clearly highlight the clinical burden of this devastating disease. Of interest, the incidence of breast cancer is on the rise in many countries, but this high incidence rate is being tempered by a decline in mortality. Independent studies have principally attributed these opposite trends between breast cancer incidence and mortality to recent advances in disease detection and adjuvant therapeutic management, respectively [8, 9]. Metastasis is a very important clinical and socio-economic problem since it accounts for more than 90% of all cancer related deaths [3]. While most patients with breast cancer are diagnosed with localized disease, 5-10% of newly diagnosed breast cancer patients present with cancer that has metastasized to other body parts [1], clinically known as stage IV breast cancer. Nonetheless, the risk of subsequently developing metastatic disease in patients with localized primary disease is relatively high. Depending on a combination of different prognostic factors, approximately 30-50% of patients receiving chemotherapy for early stage disease will develop metastatic disease [1, 10]. These figures suggest that the prevalence of MBC is high, but because most national cancer registries do not capture relapses, it is difficult to give an accurate estimate of the number of women living with MBC worldwide [1]. Nevertheless, the high numbers of annual breast cancer related deaths demonstrate the severity of the clinical and socio-economic burden of MBC compared to early stage disease [11], underscoring the need for solutions to improve metastatic disease prevention and clinical management of affected patients.

Risk factors

Although a complete understanding of the etiology of primary breast cancer is still actively being researched, several factors have been linked with the risk of developing breast cancer. These include: the female sex (99% of breast cancers are diagnosed in women), age (the risk increases with age until menopause [12]), race (risk increases from Caucasian to African American, to Hispanic and Asian [13]) lifestyle and dietary choices (including high alcohol and coffee consumption, oral contraception use, lack of regular physical activity and obesity [14-16]), and exposure to ionizing radiation to the chest area at a young age. However, reproductive factors and hormonal imbalances seem to be among the strongest predisposing factors, with women with early menarche and late full-term pregnancies having higher risks due to the longer exposure to estrogens [17]. Interestingly and primarily important to this thesis, family history of breast cancer also increases a woman's risk of developing breast cancer. 10% of breast cancers cluster within families [18]. Mutations in two highly penetrant tumor suppressor genes [breast cancer 1, early onset (*BRCA1*) and breast cancer 2, early onset

(*BRCA2*)] have been associated with 15-20% of familial breast cancers and 2-3% of all breast cancer cases. Germ line mutations in any of the *BRCA* genes confer up to 80% increased lifetime risks of developing breast cancer by the age of 70 years [18]. *BRCA1* mutated breast cancers are typically diagnosed early (between the ages of 40-50), are of high histological grade, often hormone receptor negative and of the basal molecular subtype [19, 20]. *BRCA2* mutated tumors are also frequently diagnosed in younger women, but on the other hand, they are often hormone receptor positive and are frequently of the luminal B subtype [21, 22]. Once diagnosed with breast cancer, the prognosis for patients with tumors harboring a *BRCA* mutation is considered to be relatively poor since they cluster within two molecular subtypes associated with inferior outcome. However, it is not clear if the prognosis is different between matched mutation and non-mutation carriers. In a very recent study including only young Polish women (≤ 50 years) with early-stage breast cancer diagnosed between 1996 to 2006 and for whom genetic testing for three *BRCA1* founder mutations was offered, no difference in 10-year survival between mutation and non-mutation carriers was observed, with all groups displaying an overall survival of above 80% [23]. These high survival rates may be attributed to increased disease awareness and advances in post-diagnosis chemotherapeutic management, but the generalization of these results to all women with *BRCA1* mutations is questionable. Hence, until more data become available, the general consensus still considers *BRCA1* mutated tumors to be of a relatively poor prognosis, especially in the metastatic setting where treatment options are limited. In paper IV of this thesis, we have investigated one approach of improving treatment efficacy and ultimately prolonging the survival of MBC patients with tumors harboring mutations in the *BRCA1* gene.

All the risk factors mentioned above contribute to an individual's risk of developing breast cancer, which is the root of MBC. Specifically, the risk of developing MBC subsequent to primary breast cancer diagnosis is influenced by a combination of patient and tumor pathological and biological factors which will be discussed under subsequent sections of this thesis.

Diagnosis

The improving survival rates observed amongst patients diagnosed with primary breast cancer are largely attributed to early detection [8, 9]. Screening mammography is directed towards the detection of clinically occult disease. In Sweden, generalized mammography screening was introduced between 1986 and 1997 and is recommended for women between 40-74 years, which has led to about 50% reduction in breast cancer deaths amongst women who participate in screening programs [24]. However, in a more recent report from the United

Kingdom [9], the relative risk reduction in breast cancer mortality was reported to be approximately 20%. Women with suspicious breast masses detected upon screening are further subjected to a “triple diagnostics” workup which includes physical examination of the breast and regional lymph nodes, followed by imaging tests and finally with a histological examination of a fine or core needle biopsy to make a definitive breast cancer diagnosis.

Routine screening for distant metastases is not recommended by many guidelines (e.g. ASCO, NCCN, ESMO) for management of patients with early breast cancer (reviewed in [25]), so symptoms are key to the diagnosis of recurrent disease and may vary from person to person, with some patients even asymptomatic. Symptoms of breast cancer recurrence may include a new lump or mass in the breast, skin-peeling, flaking, redness and unexplained weight loss. However, some symptoms are more specific to the anatomical location of the metastasis; bone pain and fractures for bone metastases, shortness of breath for pulmonary spread, seizures, unsteadiness and headaches for brain metastases and abdominal swelling and jaundice for hepatic recurrences [3]. Unlike in early stage breast cancer where clinical biopsies are mandatory, routine histological verification of metastatic disease was only previously recommended to resolve cases of ambiguity, where a biopsy is taken to confirm the presence of metastatic disease [26]. Based on results from a series of studies presenting evidence of alteration of disease biomarkers at time of recurrence (Table 3, reviewed in [27]), this paradigm is now gradually changing and biopsying metastases for reassessment of biological markers is now routinely performed wherever possible, in compliance with recently revised guidelines for MBC management [1, 28, 29]. In addition, hematological tests and imaging of frequent metastatic sites including the bone, liver and lungs is performed [28]. Radiologic assessment of the central nervous system and the brain may be considered for patients with HER2 positive or triple negative tumors [26, 28] due to the high risk of brain relapse [30, 31], but this is only recommended for symptomatic patients.

Subtypes

Today, breast cancer is considered to be a heterogeneous disease that can be classified using histology, immuno-pathological and molecular criteria into distinct subtypes displaying diverse biology and clinical outcome.

Histological subtypes

The morphological features of breast cancer are complex and heterogeneous between patients and have been used for decades by pathologists as a classification

tool. The dominant histological subtypes of breast cancer are invasive ductal carcinoma (about 75% of cases) and invasive lobular carcinoma (10%). The remainder are comprised of the medullary, tubular, neuroendocrine, apocrine, metaplastic, mucinous, inflammatory, comedo, adenoidcystic and micropillary subtypes [32]. Ten-year survival rates are relatively different between the histological subtypes, with invasive ductal, invasive lobular, apocrine and medullary carcinomas displaying relatively similar outcomes, but somewhat inferior compared to the other subtypes [32]. Nevertheless, the rarity of many histological subtypes has limited the utility of this classification system for clinical decision making and very little is known about the contribution of the histological subtypes to tumor heterogeneity and other factors relating to tumor progression and response to therapy.

Immuno-pathological subtypes

Historically, in breast cancer, specific biomarkers have been identified and used to stratify patients into subgroups with distinct biology, therapy response and prognosis. The cardinal markers analyzed are the hormone receptors [estrogen receptor α (ER) and progesterone receptor (PR)] and the human epidermal growth factor receptor 2 (HER2). While ER and PR are exclusively analyzed by immunohistochemistry (IHC) nowadays, the HER2 receptor is quantified by both IHC and *in situ* hybridization techniques. Based on these markers, breast cancer can be broadly classified into three main subtypes: hormone receptor positive (ER+ or PR+ and HER2-), HER2 positive (HER2+ regardless of ER or PR status) and triple negative (TN) (ER- and PR- and HER2-) subclasses. Most (70-80%) invasive primary breast cancers are ER positive, 15-20% overexpress the HER2 oncogene [33-35] while 12-17% are of the TN subtype [36].

Molecular subtypes

The advent of high-throughput analytical techniques has revolutionized our understanding of breast cancer development and biology. Through whole genome transcriptional profiling of primary tumors performed in several independent cohorts, at least four stable molecular subtypes of breast cancer have been identified [19, 37-39]. These molecular subtypes are characterized by distinct transcriptional portraits, which largely recapitulate the immuno-pathologically defined subtypes and in addition provide extra biological and prognostic information. The molecular subtype classifiers identify two luminal subtypes (luminal A and luminal B), which are principally ER+ and can be distinguished from the other subtypes by deregulation of genes involved in the ER signaling pathway [19, 37-39]. Luminal A tumors tend to express higher levels of *ESR1* and

other ER regulated genes [38], display decreased proliferation [37, 40-42] and generally have a better outcome compared with luminal B tumors [19, 37-39]. The other main subtypes are enriched for ER- tumors: the HER2 enriched subtype which displays significant, albeit imperfect, overlap with the IHC defined HER2 positive subtype and the basal-like subtype which is enriched (80% overlap) for IHC TN tumors [43].

Other rare, but clinically relevant molecular subtypes of breast cancer include:

- a) the normal-like subtype which displays a gene expression profile similar to that of normal breast epithelial cells [19, 37, 39]
- b) the claudin-low subtype which is also enriched for ER- tumors, shows decreased expression of claudins (3, 4 and 7) and displays similarities to the stem cell and epithelial-to-mesenchymal transition (EMT) gene signatures [44, 45]
- c) the molecular apocrine subtype which is enriched for ER-/HER2+ tumors which display high androgen receptor signaling [46, 47].

The classification of breast cancer into molecular subtypes as discussed here-in is however not exhaustive. A recent study including a very large collection of primary tumors integrated data from global DNA copy number aberrations and gene expression and identified 10 subgroups with distinct prognoses [48]. In addition, a few studies including only tumors of one specific subtype have further sub-divided these into groups with different clinical behavior, e.g. the HER2 enriched [49] and TN subtypes [50] have been further stratified into distinct and reproducible subgroups, which continues to highlight the marked heterogeneity of breast cancer.

The routine application of genomics based assays in clinical practice is limited by the complicated nature of the technologies and high expertise and costs necessary to run these assays. The St Gallen expert's consensus [51, 52], recognizing the importance of the molecular taxonomy of breast cancer for optimal management of early breast cancer, has approved a surrogate IHC based criterion for determination of molecular subtypes, which is directed for use in situations where technological and financial constraints limit the conduction of genomic tests. This surrogate classification considers different combinations of four biomarkers (ER, PR, HER2 and Ki67) to assign molecular subtypes to breast tumors as follows:

- luminal A-like (ER+, PR+, HER2-, Ki67 low)
- luminal B-like (ER+, HER2- and at least one of PR- and Ki67 high; or alternatively ER+, HER2+, any PR, any Ki67)
- HER2 positive (ER-, PR-, HER2+, any Ki67)
- triple negative (ER-, PR-, HER2-, any Ki67).

The concordance or agreement between the transcriptionally derived and IHC based subtypes is relatively high for the luminal A and B and the basal/TN subtypes, but only moderate for the HER2 positive subtype [39, 53, 54]. However, efforts to improve the concordance rates are in progress. By including a panel of basal markers (epidermal growth factor receptor type 1 (EGFR) and cytokeratin 5/6) to ER, PR and HER2, Nielsen et al. [55] and Cheang et al. [56] using IHC, extended the classification of TN tumors into two groups (basal-like and non-basal), which also differed significantly in prognosis [56]. Furthermore, Keam et al. [57] were able to classify a cohort of 105 TN breast cancers using IHC into two groups with distinct clinical outcomes by using only the proliferation marker Ki67.

Risk assessment: prognostic and treatment predictive factors

The main goal of oncological therapy is to minimize morbidity and maximize efficacy. To achieve this goal, oncologists make use of a collection of patient and tumor pathological and biological (molecular) factors to predict a patient's outcome if left untreated and also to calibrate treatment with the extent of disease aggressiveness. While prognostic biomarkers are intended to identify patients at sufficiently low risk to safely omit systemic treatment, treatment predictive factors help to inform on sensitivity to specific treatments in high risk patients. Whereas some biomarkers are widely acknowledged and implemented in routine clinical management of breast cancer patients in many countries globally, other promising markers are still being validated with the hope of future mass inclusion into clinical practice.

Established factors

Age

The incidence of breast cancer increases with age and as a result, the majority of breast cancers are diagnosed in older women. However, women under 40 years diagnosed with breast cancer tend to present with tumors which are hormone receptor negative and of high histological grade; all features associated with an inferior prognosis [58, 59]. Notably, in some studies including only patients with MBC, a positive association between older age (>50 years) at primary diagnosis and an inferior outcome was reported [2, 60], probably due to other age related comorbidities and decreased tolerance to therapy.

TNM classification

The clinical gold standard for prognostication is the TNM staging system which combines anatomic and pathologic factors to gauge the extent of disease progression and determine a clinical course of action. TNM associates tumor size and degree of local invasion (T), the number, size and location of lymph nodes (N) and presence or absence of distant metastases (M) [61] to determine the clinical stage of the disease and how aggressive a therapeutic strategy should be administered. Both tumor size and lymph node involvement are very strong and independent prognostic factors for early recurrence and breast cancer related mortality [62-64]. These factors are however not routinely used for decision making in the metastatic setting [1, 26, 28] even though they have been shown to affect survival after recurrence [2, 65]. The presence of distant metastasis at time of primary diagnosis is indicative of incurable disease, although prolonged survival may be achieved with systemic therapy.

Histological grade

The histological grade of the tumor reflects the degree of differentiation and the proliferative rate of the cancer cells compared to normal breast epithelial cells. A poorly differentiated tumor suggests deviation from normal breast function and is associated with an aggressive phenotype and overall poor prognosis. A very common method of evaluating the histological grade of a tumor in the clinic is the Nottingham histological grading system described by Elston and Ellis [66], which considers tubule formation, nuclear pleomorphism and mitotic count to stratify tumors semi-quantitatively into three categories associated with outcome. Grade 1 tumors are well-differentiated, grade 2 tumors moderately differentiated, while grade 3 tumors are poorly differentiated.

Hormone receptors (ER and PR)

Estrogen receptor alpha (ER α) and progesterone receptor (PR) are established prognostic biomarkers in early breast cancer as well as in MBC. The expression of ER and PR is associated with favorable tumor pathological characteristics and prognosis. In addition, ER expression predicts the response to systemic therapeutics targeting estrogen signaling and ER positivity is the principal qualifier for endorsing endocrine therapy administration [67]. The sensitivity of ER positive tumors to endocrine therapy is however variable and studies have associated degree of sensitivity to the percentage of cells staining positive for ER by IHC [68, 69]. On the other hand, ER expression has been associated with poor response to chemotherapy. While ER negative tumors show no appreciable benefit from endocrine therapy [70], this phenotype has been linked to superior response to chemotherapy [71-73].

Greater than 50% of ER positive tumors concordantly express PR and the prognostic relevance of PR expression has been shown within the ER positive

group. Low/absent PR expression is associated with higher proliferative rates, lower sensitivity to endocrine treatment and an inferior outcome [74-76]. Importantly, PR expression adds prognostic information within the IHC based luminal A subtype by improving the identification of patients within this “good” prognosis group who may benefit from additional treatment [54]. As a result of this recent finding, the St Gallen consensus guidelines for molecular subtyping using IHC markers were recently updated and now recommend that an ER positive tumor devoid of PR and HER2 expression be classified as luminal B [51].

Human epidermal growth factor receptor-2 (HER2)

HER2 is also an important prognostic factor in both early and metastatic breast cancer. About 15-20% of primary breast cancers display amplification/over-expression of the HER2 oncogene [34, 35]. HER2 amplification correlates with poor prognosis in the absence of targeted intervention. In addition, HER2 amplification is the main predictive biomarker for response to targeted anti-HER2 therapy with the humanized monoclonal antibody trastuzumab [77] and small molecule inhibitors of the tyrosine kinase family of receptors such as lapatinib [78]. Conversely, HER2 amplification is associated with poor endocrine responsiveness [74, 76] but displays higher response rates to chemotherapy with anthracyclines [79, 80] and taxanes [81, 82].

To assist clinicians in making informed decisions that balance the baseline risks and the potential associated toxicities due to adjuvant treatment in early breast cancer, prognostic indices (constituting a combination of the factors mentioned above) have been developed which provide a numeric index that can be readily correlated with risk levels and survival. The oldest is the Nottingham Prognostic Index [83, 84], which uses histological features of the tumor (TNM and histological grade) to generate a numeric score that can be correlated with prognosis. The most common prognostic index with extensive clinical utility is Adjuvant! Online (<http://www.adjuvantonline.com>), which can predict 10-year breast cancer survival and can be used to calculate potential benefits from adjuvant systemic treatment [85, 86]. Factors included in calculating survival estimates with Adjuvant! Online include TNM, ER status, age, performance status, and proposed adjuvant therapies.

Emerging biomarkers

The marked heterogeneity within the clinical groups defined by the aforementioned prognostic and treatment predictive factors limits their specificity and sensitivity, and as a result, many patients are still currently provided unnecessary adjuvant treatment. This warrants the identification of novel biomarkers for optimal personalization of breast cancer therapy. To address this

need, several new biomarkers are emerging, and are being incorporated into national and international guidelines for management of early stage breast cancer. Importantly, the successful application of these biomarkers mandates robust inter and intra-laboratory technical and analytical consistency, which has been the principal impediment for the widespread recognition of these markers despite their proven clinical significance [87]. Clinical trials to validate their prognostic and/or predictive importance as well as analytical validity and utility are underway, and this will hopefully lead to their unanimous endorsement for standard clinical routine wherever feasible.

Ki67

Uncontrolled proliferation is one of the hallmarks of cancer [5], and proliferation plays significant roles in determining the efficacy of cancer chemotherapeutics and radiotherapy. Ki67 is a nuclear protein whose expression has been shown to correlate with the proliferative rate of tumor cells and this biomarker has shown independent prognostic utility in primary breast cancer, especially amongst patients with ER positive tumors [40, 42, 88]. Ki67 expression can separate hormone receptor positive tumors into two subclasses; a low proliferative group (luminal A) which is associated with a favorable prognosis, and a high proliferative group (luminal B) which is associated with a less favorable prognosis. Some studies have also reported a predictive role of Ki67 in determining response to chemotherapy [89-91]. This biomarker is endorsed for clinical management of early stage disease by some national (e.g. Swedish National guidelines) and international (e.g. St Gallen guidelines), guidelines but variations in analytical methods across laboratories hamper its widespread application. Rigorous efforts to standardize the assessment of this biomarker are ongoing [92].

Multi-gene signatures

Through the interrogation of multiple genes or even complete cancer transcriptomes in one experiment (qPCR or microarrays), multi-gene signatures capturing key tumor biological factors affecting prognosis and treatment sensitivity have been identified. A few of these multi-gene signatures are listed in Table 1 (Adapted from [93]). Studies are ongoing to prospectively validate some of these signatures for introduction into routine clinical protocols. Signatures validated for prognostic and treatment predictive purposes in early stage, node-negative, and tamoxifen treated breast cancer include the Oncotype Dx™ 21-gene risk of recurrence score ([94, 95]), and the MammaPrint® 70-gene signature [96]. These signatures can identify high risk patients who may derive significant benefit from the addition of chemotherapy to complement hormonal treatment, as well as a low risk group of patients for whom chemotherapy can be safely withheld.

Table 1. Characteristics of some selected multi-gene tests available for clinical decision making in early breast cancer (Adapted from [93])

Generic name	uPA/PAI-1	2-gene ratio HOXB13:IL17R and molecular grade index	ER, PR, HER2, KI67	70-gene signature	21-gene signature	PAM50	11-gene assay	97-gene genomic grade	Rotterdam signature
Marketed test	Femtole®	Breast cancer Index SM	IHC4	Mammaprint®	Oncotype DX™	Prosigna®	Endopredict®	MapQuant Dx®	76-gene assay
Company	Sekisui Diagnostics	Biotheranostics	n.a.	Agendia	Genomic Health	NanoString	Sivdon	Ipsogen	Veridex
Period of first commercialization	~1990ies			~2006	~2007	~2013	~2011	~2008	~2005
Approval	CE marking		n.a.	CE marking	CE marking	CE marking	CE marking	CE marking	n.a.
Detection method	-	qRT-PCR	-	FDA 2007	-	FDA 2013	-	-	-
	ELISA		IHC,	Microarray	qRT-PCR	qRT-PCR	qRT-PCR	Microarray	Microarray
			FISH-CISH			nCounter			
Tissue	Fresh/Frozen	FFPE	FFPE	Fresh/Frozen, FFPE (2011)	FFPE	FFPE	FFPE	Fresh/Frozen, FFPE	Fresh/frozen
Prognostic Index	uPA/PAI-1 combination	BCI score	IHC4 score	Mamma- Printindex	RS	ROR	EP/EPclin	GGI	-
Indication	Prognostic	Prognostic	Prognostic	Prognostic	Prognostic	Prognostic	Prognostic	Prognostic	Prognostic
	Predictive	Predictive	Predictive	Predictive	Predictive	sub-type classifier		Genomic grade	
Population studied	N0 (-)	N0-1, ER+	N0-1, ER+	N0-1	N0-1, ER+	N0-1, ER+	N0-1, ER+	N0-1, ER+	N0, ER+
		Post- menopausal	Post- menopausal	< 62 years old		Post- menopausal	HER2-		

CE marking, European Community marking; FFPE, formalin-fixed paraffin-embedded; BCI, Breast Cancer Index; EP, EndoPredict score; Epclin, EndoPredict score combined with nodal status and tumor size; GGI, Genomic Grade Index; qRT-PCR, quantitative RT-PCR; ROR, Risk Of Recurrence; RS, Recurrence Score; n.a., not available

The performance of these signatures in identifying low risk patients amongst patients presenting with node-positive, hormone receptor positive disease is however only moderate [97].

The PAM50 breast cancer intrinsic subtype classifier (and PAM50-ROR) is the only breast cancer molecular subtype classifier that has been validated and approved for prognostication purposes in early breast cancer. However, based on current evidence, it is not approved for guiding decisions regarding prescription of chemotherapy [51].

The validity of these prognostic signatures for predicting late distant recurrences has been questioned. ER+/HER2- tumors display lower annual recurrence rates in the first years following diagnosis compared to ER- and HER+ tumors. However, the annual recurrence rates for ER+/HER2- tumors persist after the first 5 years [98]. Quite recently, the EndoPredict score [99], PAM50-ROR score [100], BCI [101] and MammaPrint® [102] were shown to provide additional prognostic information for the identification of late distant recurrences.

Prognostic factors specific to metastatic breast cancer

Although several primary tumor pathological and biological characteristics affect the prognosis of MBC, other distinct factors, clinically relevant for determining therapy choice and prognosis after disease recurrence are summarized in Table 2.

Table 2. Conventional MBC prognostic factors (Adapted from [1, 26])

Prognostic factor	Favorable	Unfavorable
Performance status	Good	Poor
Site of relapse	Loco-regional, bone,	Lung, liver and brain
Number of sites	Oligo	Multiple
Metastasis-free interval	> 2 years	≤ 2 years
Hormone receptor status	Positive	Negative
HER2 status	Negative	Positive
Age at primary diagnosis	≤ 50 years	> 50 years
Adjuvant therapy	No	Yes
Prior treatment for MBC	No	Yes

One of the most important of these factors is tumor burden, which considers the number of metastatic lesions and the specific anatomical location of the metastases. Patients presenting with solitary (oligo) lesions survive longer than patients with multiple lesions [2]. In addition, a significantly superior prognosis has been observed amongst patients with loco-regional and bone metastases compared to patients with metastases in visceral organs [2, 60, 103, 104].

The importance of reassessing biomarkers at time of recurrence has only been recently acknowledged; hence previously, prognosis and treatment decisions in MBC were mainly guided by primary tumor characteristics. Consistent with their role in early breast cancer, ER, PR and HER2 status of the primary tumor have shown similar prognostic significance for survival after MBC diagnosis [2, 103]. Remarkably, many studies have reported that significant phenotypic drifts in the expression of standard biomarkers occur across tumor progression stages (summarized in Table 3, [27]). A recent meta-analysis reported that the discordance rates of ER, PR and HER2 are estimated to be 20%, 33% and 8% respectively, and loss of biomarker expression is frequently observed at recurrence [105].

A change in biomarker status may reflect tumor heterogeneity or alterations in tumor biology following selective pressures of adjuvant treatment, which may also necessitate a change in therapeutic management to improve clinical outcome. On the other hand, biomarker discordance may reflect less than perfect accuracy and reproducibility of analytical techniques [29, 106-108]. Two prospective studies utilizing the same technique to analyze and compare the expression of pathological markers between paired primary tumors and metastases confirmed that hormone receptors were more unstable than HER2 throughout tumor progression [109, 110], supporting a biological consequence of this change. Nevertheless, data revealing the significance of metastasis specific biomarker expression in determining prognosis of MBC are very limited. Such studies have largely been restricted by the scarcity of clinical metastasis biopsies. Today, biopsies of metastases are routinely collected whenever possible as part of the diagnostic workup for MBC. As this scarce resource becomes more readily available, our understanding of the importance of metastasis specific biomarkers will be improved. In paper I of this thesis, we have specifically investigated the significance of ER and molecular subtypes assessed at time of recurrence for the prognosis of MBC.

Table 3: Summary of some studies on discordance of single biomarkers (ER, PR and HER2) and molecular subtype status between primary tumors and MBC (Adapted from [27])

Reference	ER discordance (%)	PR discordance (%)	HER2 discordance (%)	Molecular subtype discordance (%)
Lindstrom et al. 2012	32	41	15	n.a.
Niikura et al. 2012	n.a.	n.a.	43	n.a.
Curigliano et al. 2011	15	49	14	n.a.
Gong et al. 2011	7	n.a.	n.a.	n.a.
Amir et al. 2012	13	31	6	n.a.
Bogina et al. 2011	6	21	1	n.a.
Wilking et al. 2011	n.a.	n.a.	10	n.a.
Aitken et al. 2010	28	23	9	n.a.
Amir et al. 2012	16	40	10	n.a.
Idirisinghe et al. 2010	16	38	5	n.a.
Simmons et al. 2009	40	40	8	n.a.
Liedtke et al. 2009	18	40	14	n.a.
Lower et al. 2009	n.a.	n.a.	33	n.a.
Broom et al. 2009	18	37	6	n.a.
Santinelli et al. 2008	n.a.	n.a.	19	n.a.
Tapia et al. 2007	n.a.	n.a.	8	n.a.
Lower et al. 2005	30	39	n.a.	n.a.
Carlsson et al. 2004	n.a.	n.a.	0	n.a.
Edgerton et al. 2003	n.a.	n.a.	15	n.a.
Gancberg et al. 2002	n.a.	n.a.	9	n.a.
Simon et al. 2001	n.a.	n.a.	3	n.a.
Mobbs et al. 1987	14	28	n.a.	n.a.
Guarneri et al. 2008	22	36	16	n.a.
Hoefnagel et al. 2010	10	30	5	n.a.
Thompson et al. 2010	10	25	3	n.a.
Brogi et al. 2011	16	21	5	n.a.
Chang et al. 2011	25	n.a.	13	n.a.
Falck et al. 2013	13	33	27	46
Kimbung et al. Paper I	17	39	2	55

n.a., not available

Emerging prognostic factors in MBC

Clinical tumor markers

The evaluation of tumor derived carbohydrate and glycoprotein markers has proven useful in monitoring tumor burden, which is known to influence MBC prognosis. Elevated levels of the CA15.3 and CEA antigens are correlated with the risk of metastasis and death in early stage disease [111, 112]. Expression of these markers is elevated at first relapse and serial measurements during the course of treatment can inform on disease progression [113]. The ASCO guidelines recommend the use of CA15.3 to monitor advanced disease that is not amenable to conventional follow-up [114].

Circulating and disseminated tumor cells

In early stage breast cancer, baseline circulating tumor cell (CTC) numbers have been associated with tumor growth in the lymph nodes and with progression free survival [115], but CTCs are primarily gaining recognition in MBC disease monitoring and prognosis. A reduction in CTC numbers from baseline levels after treatment has been associated with improved progression-free survival and overall survival [116, 117]. Bidard and colleagues recently performed a pooled analysis of CTC data from 1,944 individuals with MBC assessed at baseline and after commencing treatment, confirming that high baseline CTC levels were associated with decreased progression-free survival and overall survival [118]. In addition, they found that an increase in CTC levels 3-8 weeks after start of treatment was associated with poor outcome. These data confirm that CTCs are a potentially important marker for determining prognosis and assessing treatment response in MBC. Efforts are underway to test if circulating tumor DNA can also serve as a biomarker for monitoring treatment response in MBC [119]. However, the requirement of expensive machinery and complicated analytical techniques may limit the clinical utility of these circulating biomarkers.

Treatment

By considering the prognostic and treatment predictive biomarkers described in the previous section, patients are stratified into low or high risk groups and treated accordingly.

Treatment of early stage breast cancer

Surgery and radiotherapy

Loco-regional control through surgical excision of the tumor mass is the primary therapeutic intervention for stage I–III primary breast cancer. Breast conserving surgery and complete mastectomy are surgical procedures offered based on prognostic factors. This is usually followed with postoperative radiotherapy to minimize loco-regional recurrences and to improve survival [51, 67]. Furthermore, because the axillary lymph nodes are the primary site of breast cancer spread, with lymph node involvement being a strong predictor of loco-regional and distant recurrence, the sentinel nodes are surgically examined by a technique which utilizes blue dye and radio-isotopes to assist in the determination of lymph node status. This sentinel node biopsy has been proven to be a sufficient, safe and effective method for the assessment of lymph node status [120].

Systemic treatment

Systemic treatment which is aimed at eliminating any circulating or disseminated tumor cells follows local treatment with the hope of preventing their outgrowth as overt metastases in distant vital organs. Adjuvant systemic therapies include:

Hormonal therapy

More than 70% of breast cancers are positive for the ER and may therefore be sensitive to therapies targeting estrogen dependent signaling as previously mentioned. ER signaling can be abrogated by directly blocking the estrogen receptor with drugs like tamoxifen or by blocking estrogen synthesis with drugs which inhibit the enzyme aromatase, e.g anastrozole, letrozole or exemestane. In addition, estrogen levels can be reduced by ovarian ablation (achieved clinically by oophorectomy, irradiation of ovaries or by using a luteinizing hormone-releasing hormone (LHRH) agonist), and this has been shown to decrease breast cancer recurrence and mortality [121]. In premenopausal women, the ovaries are the principal source of circulating estrogen [17]; hence ovarian ablation and adjuvant systemic treatment with the ER antagonist tamoxifen is the treatment of choice [51]. On the other hand, estrogen is produced via aromatization of ovarian and adrenal androgens in the liver, muscle and fatty tissue in postmenopausal women [17] and although some benefit may be derived from tamoxifen treatment, aromatase inhibitors are the preferable and recommended line of therapy for high-risk postmenopausal women with ER positive primary breast cancer [51].

HER2 targeted therapy

Patients with tumors showing overexpression or amplification of the HER2 oncogene are treated with drugs specifically targeting and disrupting HER2

dependent signaling, culminating in decreased proliferation and death of tumor cells. Agents in clinical use include recombinant monoclonal antibodies like trastuzumab and small molecule tyrosine kinase inhibitors like lapatinib. Since their introduction, HER2 targeted agents have revolutionized the management of patients with tumors of this biological subtype, reversing the poor prognosis and improving recurrence-free and overall survival of patients receiving treatment [122, 123].

Cytotoxic chemotherapy

Depending on the level of risk following assessment of prognostic factors, high-risk patients with hormone receptor positive and HER2 positive disease are prescribed cytotoxic chemotherapeutic treatment to complement endocrine and/or HER2 targeted treatment. However, chemotherapy is the treatment of choice for patients presenting with TN primary breast cancers. Current standard chemotherapy regimens include anthracyclines and taxanes [51]. Poly-chemotherapy seems to be a more efficient way of treatment compared with single-agent regimens, and has been shown to reduce breast cancer mortality by approximately 30% [10].

Treatment of MBC

Once metastases are diagnosed, the goal of therapy is to improve the patient's quality of life, prevent and palliate symptoms and prolong survival. This is because MBC is considered to be incurable by current medical interventions. However, there exists a small but clinically relevant subset of MBC patients who can benefit from more radical treatment with a curative intent. No global consensus exists for treating MBC. Recently, the European School of Oncology - MBC Task Force (ESO-MBC Task Force; [25, 124]), the European Society for Medical Oncologists (ESMO guidelines working group; [1]) and the 1st International consensus for advanced breast cancer (ABC 1; [28]) have published guidelines for clinical management of patients with MBC.

Local treatment

The role of local treatment by surgical removal of metastases is controversial. For the 5-10% of early breast cancer patients diagnosed with *de novo* metastatic disease [1] some argue that surgical removal of the primary tumor is beneficial. The primary tumor is a source of further metastatic spread and debulking the tumor burden may decrease the percentage of chemoresistant cells and improve response to systemic therapy [125-127]. In addition, immunologic competence of the host may be restored by removing the tumor [126, 128]. In contrast, others are of the opinion that surgery may modify the growth kinetics of metastases by inducing an angiogenic surge, since the primary tumor is thought to be a source of

anti-angiogenic factors [129, 130]. Furthermore, surgery may result in the release of growth factors in response to wound healing, and these growth factors may enhance the proliferation of metastases [131]. In addition, the immunosuppressive effects of anesthesia and surgery are potentially risky for accelerated relapses [132]. Notably, several studies evaluating the effect of surgical removal of the primary tumor in early stage IV disease have consistently reported a survival advantage in patients undergoing surgery compared to those who do not receive surgical intervention (reviewed in [124]). Importantly, in these studies, a high percentage (37-61%) of patients underwent surgery, reflecting that although not generally approved by treatment guidelines, surgery is still widely provided. Interestingly, all recently published MBC guidelines suggest that surgical removal of localized primary tumors be considered for well selected patients [1, 25, 28].

On the other hand, surgery for distant recurrent MBC is not a recommended practice. Resection of pulmonary and hepatic metastases in MBC patients has been shown to improve survival [133-136], but most of these studies were small and patients were highly selected making it difficult to generalize the results. Consequently, the value of surgery in recurrent MBC needs to be prospectively assessed since it is clear that there exists a small but clinically significant group of patients with oligo distant metastases for whom surgical excision of the isolated metastatic lesion may culminate in complete remission and prolonged survival [124].

Systemic treatment

Similar to early stage disease, the key therapeutic agents in MBC consist of endocrine treatment and HER2 targeted therapy, and these are often complemented with chemotherapy. The preferred first-line treatment for postmenopausal women presenting with endocrine sensitive and HER2 negative MBC is aromatase inhibitors with or without chemotherapy, but tamoxifen remains a viable option [26, 137]. Tamoxifen, ovarian function suppression or a combination thereof is recommended for premenopausal women. However, treatment response is often not durable and the median survival of patients with MBC is estimated to range between 2-3 years only [2, 28]. The fact that a patient develops recurrent disease may reflect that tumor cells have acquired resistance to the treatment regimen provided in the adjuvant setting.

Despite having consistently hormone receptor positive tumors at recurrence, up to 50% of patients with ER positive metastases do not respond to first-line endocrine treatment (*de novo* resistance), and the remainder will eventually relapse despite an initial response (acquired resistance) [138]. This therefore warrants the introduction of novel drugs and treatment regimens to re-activate therapy sensitivity. Resistance to hormonal therapy has been linked to cross-talk between signal transduction pathways, particularly the PI3K/AKT/mTOR pathway [139]. This pathway regulates key cellular processes like proliferation, metabolism,

angiogenesis, motility and survival [140-142]. The combination of hormonal therapy with mTOR inhibitors has been compared with hormonal therapy alone in various phases of clinical trials, consistently showing significant improvements in time to progression and overall survival in postmenopausal women with aromatase inhibitor resistant MBC [143-146]. However, significant adverse effects were also recorded in some patients.

Anti-HER2 targeted therapy is recommended as first-line treatment for all patients with HER2 positive MBC. Hormonal therapy is combined with anti-HER2 therapy for patients with ER+/HER2+ disease, and this may be followed by chemotherapy depending on other prognostic factors [1, 28]. In the case of disease progression after trastuzumab, combination of trastuzumab and lapatinib has been shown to prolong survival [147].

MBC patients presenting with TN disease generally have the worst outcome and the scarcity of well validated therapeutic targets for this biological subgroup further compounds the treatment challenge. Chemotherapy remains the standard of care for patients with tumors of this phenotype. Combinations of anthracyclines and taxanes in the metastatic setting have been associated with higher response rates and longer progression-free intervals, but minimal effects on overall survival [28]. Poly-chemotherapy treatment is more often provided because of high frequency of visceral metastases in patients of this subgroup, and also because the TN phenotype is in itself suggestive of an aggressive disease. Importantly, because the response to chemotherapy is not durable, patients succumb faster to their disease [36, 148]. Huge efforts are being made to find novel, effective and durable treatment strategies for this group of patients. This vision has been another specific aim of the present thesis (paper IV).

Patients with TN MBC harboring inherited or sporadic mutations in the *BRCA* genes have shown remarkable sensitivity to poly (ADP-ribose) polymerase 1 (PARP1) targeting [149-151], and this line of targeted therapy is currently being researched in many preclinical and a few clinical trials and has also been specifically addressed in paper IV of this thesis. PARP1 is an enzyme which is crucial for the repair of both single and double strand breaks in the DNA [152], and therefore very important in maintaining genomic stability. The *BRCA1* and *BRCA2* genes are also important for the repair of DNA double strand breaks (DSBs), which are potentially lethal lesions if left unrepaired [153, 154]. The proposed mechanism of action of PARP inhibitors in a *BRCA* deficient background is that inhibition of PARP1 will result in impaired DNA single strand break repair, leading to their accumulation in the cell and subsequent conversion into DSBs when the DNA is replicated during the cell cycle S-phase. Effective DSB repair is achieved through the error-free homologous recombination process which requires functional *BRCA* genes [153, 154]. Because of the inactivating mutations in these genes, the cells are unable to repair these DSBs and as a result undergo cell cycle arrest and ultimately cell death [155, 156]. This phenomenon is

called “synthetic lethality” and has been proven in several early phase trials to be an effective therapeutic option for *BRCA* mutated MBC [149-151]. Unfortunately, *de novo* or acquired resistance occurs in a significant number of tumors. Against the background that the PI3K/AKT/mTOR pathway is linked to the acquisition of resistance to oncologic therapy, and mindful of the data showing that mutations in the phosphatase and tensin homolog (*PTEN*) tumor suppressor gene, which is a principal regulator of PI3K/AKT signaling, is frequently observed in breast cancers harboring *BRCA1* mutations [157], and that *PTEN* mutations result in defective homologous recombination [158, 159], we have investigated the potential of dual targeted inhibition of PARP1 and PI3K as a therapeutic approach to overcome resistance to PARP inhibition in *BRCA1* deficient cells (paper IV).

Studies have indicated that TN breast cancer cell lines and sporadic tumors share similar DNA double strand break defects as *BRCA* mutant cells [160-162]. The hope of extending PARP inhibition targeted therapy to include *BRCA* proficient TN tumors was raised by an early phase II study reporting benefit from the drug iniparib [163], but these results were not confirmed in phase III [164]. The drug iniparib was later shown to possess no specific PARP inhibition potential *in vitro* [165]. The significance of PARP inhibition in TN breast cancer still needs to be further explored and other biomarkers to predict sensitivity are currently being investigated.

Other emerging treatments

Other agents that are proving to be of importance in MBC management include:

- Anti-angiogenic therapy with the Vascular Endothelial Growth Factor (VEGF) targeting antibody bevacizumab which has shown better efficacy in TN than in endocrine sensitive MBC [166, 167].
- MBC patients with bone metastases may also be treated with bone modifying agents like zoledronic acid, the human monoclonal RANK ligand antibody denosumab and bisphosphonates, which have been shown to either reduce the frequency or delay the time to bone metastasis specific death as well as improve the quality of life by modulating bone pain [1].

The biology of breast cancer metastasis

Tumor progression models

Tumor progression from an early neoplastic lesion to the development of a metastasis in a distant organ is viewed conventionally as an evolutionary process, involving multiple genetic alterations, and culminating in the selection and propagation of aggressive metastatic clones. An implication of evolution is intra-tumor heterogeneity, i.e. the co-existence of multiple, molecularly distinct cell populations within a tumor [168]. By performing either comparative genomic hybridization analysis, exome sequencing, chromosome aberration analysis, or ploidy profiling on multiple spatially separated samples obtained from primary tumors or metastases, multiple studies have confirmed that solid tumors display significant intra-tumor heterogeneity [169, 170]. It is however unclear when the selection of the metastatic phenotype occurs during tumor progression [171, 172].

Several models have been proposed to explain the complexity of the metastatic cascade. The unconventional model is a parallel progression model, which postulates that micrometastases may arise early in the course of tumor progression (when the primary tumor is still very small or even undetectable) and undertake an independent genetic course towards eventually becoming lethal metastatic lesions in distant organs. This theory is backed by the observation of significant genetic disparity between paired samples of primary tumors and metastases [169, 173, 174]. Likewise, the detection of tumor cells in the circulation or other distant organs in experimental animal models [175] and women with MBC [176, 177] when the primary tumors are still very small further supports parallel progression, although it is not known if these early disseminated cells can grow into overt metastases. However, most clinical metastases are detected several years and even decades following the diagnosis and treatment of the primary tumor, lending support to the conventional step-wise, linear (sequential) progression model. This model of metastatic progression suggests that as the primary tumor grows, only a sub-clone of cells acquires additional genetic and epigenetic changes endowing them with the potential to dislodge from the primary tumor mass, extravasate, invade and successfully colonize distant organs [178]. Experimental evidence advocating that a sequential selection process is in force is provided by several studies [179-181] demonstrating that cells with variable metastatic potential can be isolated from within the same cell population. Moreover, only a very small number of circulating or disseminated tumor cells are potentially able to successfully grow as overt metastases, often after a long period of dormancy [182]. This implies that some of the genetic changes necessary for successful outgrowth of metastases are acquired very late in the metastatic cascade and may not be present or detectable in the primary tumor. Navin and colleagues [183], by

using DNA copy number analysis and whole genome sequencing techniques, investigated the clonal composition of primary breast cancer lesions and compared this to the associated liver metastases. They could infer a clonal progression pattern between the tumor pairs, and concluded that a single clonal expansion occurred in the primary tumor, giving rise to the metastasis [183].

Interestingly, global gene expression studies based on microarray technology have revealed that the transcriptional landscape of paired primary tumors and metastases from the same individual are very similar [184-186] and gene signatures that predict, with high accuracy, the ability of a tumor to metastasize are already available and can be identified by analyzing the bulk primary tumors [96, 187, 188]. This has been interpreted by some investigators to imply that from the very onset, primary tumors are destined to be either metastatic or non-metastatic, requiring no further selection before metastasis formation [171], challenging the conventional longitudinal clonal selection progression model. Nevertheless, no studies asserting a functional role of the genes in these poor prognosis signatures in mediating metastasis are available, putting into question the biological relevance of these poor prognosis signatures for facilitating tumor invasion and metastasis [178].

Taken together, irrespective of tumor progression model, the path to successful metastatic colonization is heterogeneous, differing from patient to patient, and may depend on the aggressiveness of each independent tumor and/or host related factors.

Genes mediating breast cancer metastasis

Genes mediating primary tumor progression towards metastasis can be classified into three principal categories [61, 178]:

- *Metastasis initiation genes* which confer tumor cells with the ability to alter cell-cell adhesion and adopt a motile phenotype, paving the way for local tissue invasion and extravasation into the circulation. Genes associated with epithelial to mesenchymal transition (e.g. cadherins and *TWIST*), extracellular matrix degradation (e.g. *MMPs*), evasion of cell death (e.g. *Caspase 8*) and angiogenesis (e.g. *VEGF*) are included in this category.
- *Metastasis progression genes* are necessary for both primary tumorigenesis and successful colonization of specific distant metastatic niches. Genes involved in vascular remodeling, immune evasion and extravasation are included in this category. Metastasis progression genes may confer specific advantages in specific target organs, making it possible to mechanistically couple primary tumor progression with tissue

specific tropisms. Examples of metastasis progression genes include *EREG*, *COX2* and *MMP1*, which regulate extravasation of circulating tumor cells and are involved in colonization of the lung.

- *Metastasis virulence genes* provide a selective survival advantage to disseminated tumor cells when colonizing and growing into overt metastases in specific secondary microenvironments. These genes are exclusively involved in the colonization of distant organs and might not be differentially expressed between primary tumors since they may not be important for primary tumor growth. An example of a metastasis virulence gene is the chemokine receptor *CXCR4*, which together with its ligand, the chemokine stromal-cell-derived factor 1 (*SDF-1*) has been shown to facilitate the colonization and outgrowth of breast cancer cells in the bone, lung and brain microenvironments [61]. Also, based on data presented by Tabaries et al. [189, 190], *CLDN2*, a mediator of cell adhesion, may be considered to be a liver metastatic virulence gene.

Apart from genes promoting tumor invasion and metastasis, a group of genes have been characterized with the ability to repress tumor dissemination, often referred to as “metastasis suppressor genes”. These genes do not have any effect on primary tumor growth. Examples of metastasis suppressor genes include: *NM23*, which encodes a histidine kinase and loss of heterozygosity of this gene has been associated with metastatic progression in colorectal cancer [191], and *KISS1*, which when deleted or downregulated in several tumor types, is inversely correlated with tumor progression, metastasis and overall survival [192].

Organ specific metastasis

Regardless of the tumor progression model favored, it is widely accepted that the site of metastasis is not randomly selected since different cancers display specific metastatic site preferences [193]. As early as 1889, the English surgeon Stephen Paget, after reviewing autopsy records from over 700 women who died from breast cancer, noticed that there was a discrepancy between the blood supply and the incidence of metastases in specific organs [194]. He concluded that metastases (seeds) could only grow in congenial organs (soils), a hypothesis which was later on confirmed by Fidler and co-workers [195-197]. Paget’s and Fidler’s theory is however contradictory to the beliefs of Virchow and Ewing, who propose that metastasis is merely associated with the arrest of tumor cells in the vasculature and that the circulatory patterns between primary tumors and secondary sites is primarily responsible for site specificity of metastases [198, 199]. The preferential distant sites of breast cancer recurrence are the bone, lung, liver and brain [200].

The risk of breast cancer metastatic recurrence in specific tissues may be inferred with marginal specificity from some pathological features of the primary tumors

([30, 31] and paper III in this thesis). ER positive (luminal A and B) tumors frequently metastasize to the bone and liver, while ER negative tumors preferentially colonize visceral organs (lung, liver and brain). HER2 positive tumors on the other hand have been associated with a predilection to spread to the central nervous system, including the brain. As previously mentioned, the site of relapse is one of the strongest prognostic factors for survival after the diagnosis of metastatic disease, with the survival time decreasing in magnitude from bone, to lung, to liver and to brain [2, 60, 103, 104]. Markers for predicting the future metastatic site of a primary breast cancer are very scarce and tissue specific metastasis progression and virulence genes are not well characterized.

Efforts to identify metastasis progression and virulence genes have mainly relied on *in vivo* animal models and human tissue based approaches, since the prevailing micro-environmental conditions in metastatic target organs cannot be fully mimicked using *in vitro* systems. Over the past decade, Massagué and colleagues have pioneered this research field towards deciphering the molecular basis of breast cancer organ specific metastatic tropism. They have identified factors intrinsic and extrinsic to breast tumor cells, which mediate their selective colonization of the bone, lung and brain, respectively [179, 201, 202]. By using the heterogeneous breast cancer cell line (MDA-MB-231) derived from a plural effusion of a patient with generalized MBC, these researchers isolated sub-populations of cells exhibiting distinct preferences for colonizing the bone, lung and brain, respectively, when inoculated into immune-compromised mice. They went on to compare the global gene expression profiles of these site specific variants with the parental line and by so doing, identified putative site specific metastasis progression and virulence genes. Importantly, by integrating bioinformatics analyses of human primary tumor transcriptional data with survival data, they were able to identify genes within their experimental signatures predictive of lung, bone and brain relapses while only analyzing primary tumors. Finally, functional validation experiments were carried out both *in vitro* and *in vivo* to confirm the role of the identified genes in mediating organ specific metastases. A similar approach was recently replicated by Peter Siegel's laboratory to identify candidate liver metastasis genes [189]. Figure 1 provides a summary of some important genes mediating organ specific metastases in breast cancer. In general, the conclusion from all these studies is that overt colonization critically depends on the capacity of disseminated cells to benefit from specific stromal components in different organs [203].

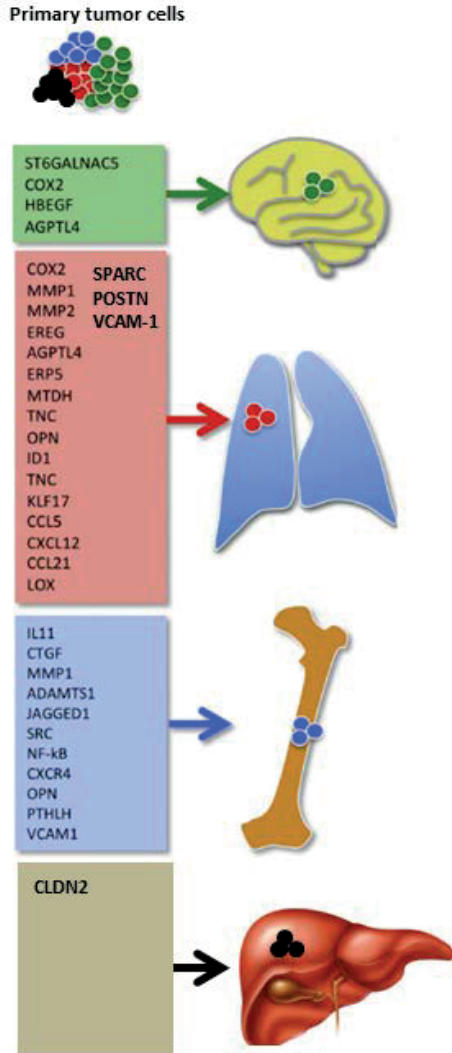


Figure 1. Examples of breast cancer organ-specific metastasis progression and virulence genes (Adapted from [204]).

Aims of the thesis

The overall aim of the research described in this thesis was to characterize the biomolecular heterogeneity of breast cancer across tumor progression stages and identify novel biomarkers and therapeutic strategies to improve prognostication and personalization of therapy for women diagnosed with MBC. The specific objectives of the respective papers were:

- To evaluate the stability of conventional breast cancer pathological biomarkers and molecular subtypes across tumor progression stages, and investigate how metastasis specific biomarker status and molecular subtype influences MBC prognosis (paper I).
- To describe the transcriptional landscape of breast cancer metastases, identify liver metastasis selective genes and investigate their potential in predicting prognosis in early stage breast cancer (paper II).
- To specifically study the expression of the breast cancer liver metastasis selective gene *CLDN2* across different stages of breast cancer progression and evaluate its potential for predicting liver metastatic propensity after primary tumor diagnosis (paper III).
- To investigate if the sensitivity of *BRCA1* deficient breast cancer cells to PARP1 inhibition can be enhanced by co-targeting the PI3K signaling pathway (paper IV).

Materials

To realize the study goals of papers I-III, we retrospectively analyzed tumor material collected within a randomized phase III trial (the TEX trial) conducted between 2002 and 2007 across different treatment centers in Sweden. The TEX trial enrolled 304 women with documented locally advanced (in-operable) or MBC for whom first line chemotherapy treatment for metastatic disease was indicated [205]. The trial was designed to specifically compare the efficacy of the combination of epirubicin and paclitaxel (ET) versus epirubicin, paclitaxel and capecitabine (TEX). Conditions for exemption from the trial included brain metastases, indication for HER2 targeted therapy, or other malignancies diagnosed within five years of trial commencement. A well annotated database was constructed and included information about previous clinical history (primary tumor pathological information and adjuvant treatment, metastasis-free interval), and prospectively collected data with regards to the metastatic disease (tumor burden) and survival for each patient. Before the commencement of chemotherapy treatment for advanced or metastatic disease, fine needle aspiration biopsies (FNAs) of at least one metastatic lesion were collected whenever possible for whole genome transcriptional profiling. In addition, we collected archival formalin-fixed paraffin embedded (FFPE) tumor blocks of primary tumors and synchronously diagnosed lymph node metastases wherever feasible. A board certified breast pathologist performed central re-assessment of the histological grade of the primary tumors. Tissue microarrays (TMAs) were constructed for re-evaluation of conventional markers and subsequent characterization of any potentially interesting novel prognostic biomarkers. Figure 2 represents a flowchart of the subset of tumors (patients) included in the different studies. The regional ethics committees at all participating centers approved these studies.

Paper IV of this thesis was a proof-of-concept study performed *in vitro* using a panel of five breast cancer cell lines (MDA-MB-436, SUM149, HCC1397, L56Br-C1 and MCF7) with defective *BRCA1* and/or PI3K signaling as experimental models. A detailed description of each cell line has been provided in paper IV.

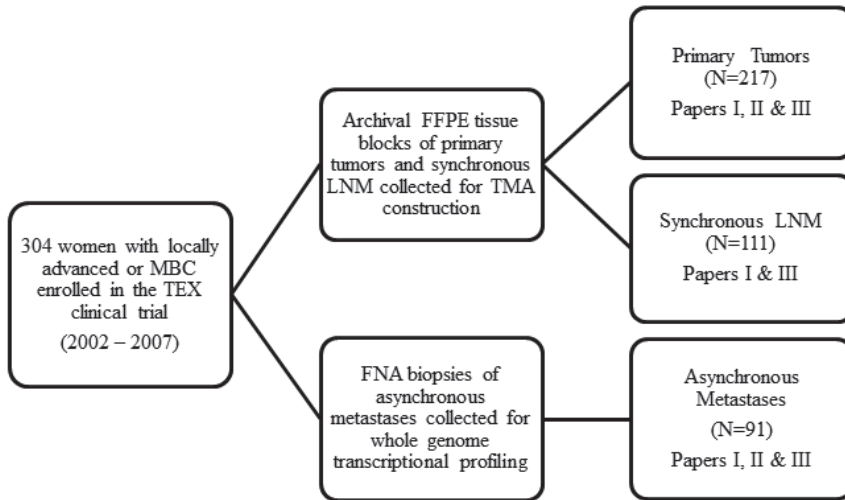


Figure 2. Flow chart showing the selection of patients included in Papers I-III of this thesis. Cases were excluded due to a) missing clinical data; b) unavailable tumor blocks; c) missing TMA data due to core loss or <10% tumor cells; or d) failed quality control for transcriptional profiling.

Experimental and methodological considerations

Listed below are the main methods used in papers I-IV of this thesis. For detailed description of experimental procedures, refer to the “Materials and Methods” sections in the corresponding paper(s).

- Immunohistochemistry (papers I-III)
- Gene expression microarrays (papers I-IV)
- *In vitro* cytotoxicity assays (paper IV)
- Western blotting (paper IV)
- Immunofluorescence assay (paper IV)
- siRNA interference (paper IV)
- Statistical analyses (papers I-IV)

Sample selection

Careful experimental design and implementation of analytical techniques are crucial for obtaining meaningful results. An important feature of experimental design is choosing a relevant study population and a representative sample to reach statistically significant results and make meaningful and if possible generalizable conclusions about the population under study. Primarily, we aimed to investigate tumor heterogeneity across breast cancer progression stages and its consequence on outcome after the diagnosis of metastasis. Such a study requires a large collection of tumor material from metastatic lesions, which is a very scarce resource. The 304 patients with advanced breast cancer examined in papers I-III in this thesis were prospectively recruited and monitored within a phase III trial for first line chemotherapy treatment for metastatic disease. This cohort therefore contains a well-selected group of patients for whom reliable and complete clinical data on many variables was available, making it possible to adjust for the effects of multiple important factors in statistical analyses. However, the cohort may not completely mirror the heterogeneous nature of MBC. For example, HER2 amplification or overexpression and the presence of brain metastases were among

the exclusion criteria for the clinical trial, which resulted in an under-representation of HER2 positive tumors in our study.

Fresh biopsies from metastatic lesions were collected wherever possible for re-assessment of standard pathological biomarkers and for transcriptional profiling. In addition, a central re-assessment of standard pathological biomarkers for primary tumors and synchronous lymph node metastases was performed on TMAs. Central assessment of biomarkers greatly reduces the confounding effects of technical and analytical variability. The gene expression dataset generated in this study is to our knowledge one of the largest breast cancer metastasis datasets currently available. In papers II and III, we aimed to identify liver metastasis selective genes. Although we were able to include a fairly significant number of liver metastases, the difficulties in obtaining biopsies from some specific sites (lung and bone) and the exclusion of patients with brain recurrences from the trial, also limits the generalization of our findings. In addition, the retrieval of intra-individual tumor samples representing the different tumor progression stages was only moderate and thus reduced the power of these statistical analyses.

Immunological assays

The assessment of protein (biomarkers) expression using antibodies has been widely used in this thesis. These antibody based techniques rely on the principle that an antibody can specifically recognize and bind to an epitope on an antigen. The antigen-antibody complex can be visualized using a secondary antibody conjugated to an enzyme that catalyzes a reaction resulting in the production of a colored substrate. This makes it possible to separate and quantify the expression of the specific target antigen from a complex protein mixture. In papers I-III, IHC was performed on TMAs to quantify the expression of pathological biomarkers (ER, PR, HER2, and Ki67) and claudin-2. Performing IHC on TMAs enables parallel analyses of hundreds of tumors, which significantly improves the throughput of biomarker studies. Kononen and colleagues [206] developed the TMA technique currently used in many laboratories. Although IHC is a relatively cheap and simple technique, the outcome is very sensitive to technical variability associated with the quality of reagents (sensitivity and specificity of antibodies), sample handling (age of FFPE tissue blocks, length of fixation, antigen retrieval and staining method) and the method of evaluating the staining (image analysis software or manual evaluation) amongst other factors. The quantification of a new biomarker by this technique therefore warrants robust analytical validation of all steps in the protocol. Furthermore, to allow for generalization of results, independent validation studies within and between laboratories are important. One drawback of using TMAs for evaluating biomarkers is that, the small size of the tumor cores may not be representative of a heterogeneous tumor.

Other immunological assays used in this thesis such as Western blotting and immunofluorescence also rely on antibody specificity for accurate identification and quantification of target proteins. In addition, careful implementation of all procedures prior to the antibody application is mandatory for obtaining meaningful results.

Gene expression microarrays

The microarray technology, which allows for the simultaneous analysis of thousands of gene probes, has revolutionized molecular biological research. Gene expression microarrays were used in papers I-IV of this thesis to simultaneously measure the expression of all the mRNA transcripts in a sample and compare global transcriptional patterns between specified groups of samples. Specifically, only single-channel microarray platforms were used in this thesis. For single-channel microarrays, the test sample is hybridized to the microarray, and the intensity of each probe represents its relative abundance compared to other samples processed in the same experiment. This is different from dual-channel microarrays, which typically co-hybridize two independent samples to the same microarray. The samples are often labeled with different fluorescent dyes, and the intensity of each dye at each spot is combined to give an intensity ratio for each probe, reflecting the difference between the two samples.

Gene expression microarray technology is thought to be a reliable technique, with high reproducibility both between and within platforms observed by independent research groups when experiments are carefully designed and executed [207, 208]. Intra- and inter tumor heterogeneity affects global gene expression. Tumor biopsies consist of a collection of tumor cells with different genetic aberrations, embedded in the tumor stroma which is also comprised of a variable collection of cell types including infiltrating lymphocytes, endothelial cells and epithelial cells from the normal tissue in which the tumor is growing. The global gene expression signature is therefore a representation of signals from all these cell types and high percentages of stromal infiltrates will “dilute” and limit the detection of tumor intrinsic changes. Micro-dissection of tumor tissue to enrich for tumor cells is an approach to enrich for tumor cells, but the disadvantage of this approach is that it completely discounts the contribution of the stromal cells, which are also key mediators of tumor metastasis. In papers I-III, we analyzed FNA biopsies of a relatively large collection of breast cancer metastases. FNA biopsies have been reported to provide transcriptional profiles that are a purer representation of tumor cell populations [209] and can reliably identify routine molecular markers and global molecular differences between breast cancer subtypes [210, 211]. Of the 91 FNA samples included in our analyses, 86 (95%) had an estimated tumor cellularity greater than 75%. However, because of the relatively small size of an FNA biopsy, the possibility that it may only capture a fraction of the molecular

complexity in a typically heterogeneous tumor cannot be ruled out. In paper IV, gene expression profiling was performed on RNA extracted from cell lines; hence the problem of heterogeneity in tumor cell composition is not critical in such a system.

Very high quality mRNA is necessary for the generation of reliable microarray data. All RNA samples were therefore run on the 2100 BioAnalyzer for quality control, and only samples with RNA integrity (RIN) values above 6 were hybridized to the gene expression arrays.

Another major source of systemic technical bias common to microarray studies is hybridization batch effects. To minimize technical biases in our tissue based studies, mRNA amplification and labeling were performed in parallel in 96-well plates and all hybridizations were performed within a period of 48 hrs. For cell lines, all samples were processed in a single batch.

Normalization of microarray data is a very important process aimed at adjusting for and minimizing variation arising from technical rather than biological differences. Depending on the specific normalization method used, it is possible that some biologically relevant information may be lost, but this trade-off is generally widely acceptable in microarray studies. Following data normalization, extraction of the relevant biological information from thousands of related measurements is not a trivial task. Several powerful bioinformatics and statistical methods for analyzing multi-dimensional “omics” data are currently available.

In this thesis, we focused on identifying genes which were significantly differentially expressed between defined groups of samples and pinpointed molecular functions, biological processes and pathways significantly enriched amongst these differentially expressed genes. A serious statistical problem encountered in such multi-dimensional analyses is the risk of obtaining high numbers of potentially false positive results due to multiple testing (as a consequence of the huge discrepancy between the numbers of mRNA transcripts measured compared to the number of samples). This problem is generally dealt with by making some adjustment to the p -value. In this thesis, multiple testing was addressed by performing False Discovery Rate (FDR) adjustments of p -values as proposed by Benjamini and Hochberg [212], and the q -value (described by Storey [213]) as implemented in the Significance Analysis of Microarray (SAM) algorithm [214].

***In vitro* 2D experimental models**

Significant gains in our current understanding of the molecular basis of breast cancer over several decades have been achieved through the use of established breast cancer cell lines as *in vitro* experimental models. Most cell lines used in

cancer research are derived from plural effusions or metastases [215, 216]. *In vitro* experimental models using cancer cell lines are a cheap and reproducible way for rapid screening and characterization of anti-cancer drugs, and for exploring functional and mechanistic characteristics of biomolecules. It has been shown that cell lines maintain, to a large extent, the genetic and phenotypic complexity of the original tumors from which they are derived [217, 218]. Thus, specific oncogenic aberrations in cells lines can be therapeutically targeted and the response rapidly evaluated in cytotoxicity assays to determine resistance or sensitivity, and to identify biomarkers of response. Nonetheless, the use of such experimental models also mandates caution.

In paper IV of this thesis, we used a panel of established and well characterized human breast cancer cell lines (MDA-MB-436, SUM149, HCC1397, L56Br-C1 and MCF7) with genetic defects in either *BRCA1* and/or the PI3K signaling pathway to investigate the cytotoxicity and other molecular effects of combination treatment with PARP1 and PI3K inhibitors. To avoid the risk of “false” cell lines, we obtained all cell lines directly from authentic manufacturers or vendors. In addition, because cell lines are prone to genotypic and phenotypic drift when cultured for extensive periods [215, 216], we only used early passages of cells (under 40) in all experiments. However, considering the complex transformations necessary for primary tumor cells to complete the metastatic cascade, and the protocols for establishing cell lines, these models may only represent a clone from an otherwise heterogeneous tumor mass. Also, cancer cells are genetically unstable, hence culturing conditions may induce specific genetic alterations, which may in turn influence their behavior and response to external stimuli. In addition, the effects of the tumor microenvironment and host immune responses are fundamentally ignored when using this two dimensional (2D) experimental model system. Hence, interpretation of results from these 2D *in vitro* experiments should make allowances for these limitations.

Results and Discussion

Trends in the survival of MBC patients treated within the TEX clinical trial (papers I-III)

All patients recruited into the TEX trial were diagnosed with locally advanced or metastatic breast cancer between 1993 and 2007. Survival data were last updated in 2013. The median survival of all patients from time of metastasis diagnosis to last database update was approximately 38 months (95% CI=32-43 months). This estimate is consistent with results from other larger studies in which the median survival for patients diagnosed with advanced breast cancer was reported to range between 15-34 months [2, 65, 219-223]. Whether these results represent an improvement in the survival of patients with metastatic disease remains controversial. While it is clear that the introduction of targeted treatment with trastuzumab has led to significant improvements in the survival of patients with HER2 positive MBC, two recent studies by Tevaarwerk et al. [65] and Ufen et al. [223] after stratifying patients according to time period of MBC diagnosis, reported that no significant improvement in the survival of patients with MBC has been achieved in the last decade.

Importantly, we confirmed the inferior prognosis associated with liver relapse [2, 103, 104, 224, 225] and in particular demonstrated in paper II that this relatively poor outcome for patients with liver relapses may be specific to individuals with liver metastases concomitantly diagnosed with metastases in other anatomical sites (Figure 3). Liver metastases are seldom diagnosed as solitary lesions but when this happens, a number of small studies have confirmed the benefits of surgical excision of the metastasis, which prolongs overall survival [135, 136]. Our results show that even without the provision of surgery for the metastatic disease, patients with solitary liver metastatic lesions experience a relatively longer survival, which may probably be further extended if treated more radically.

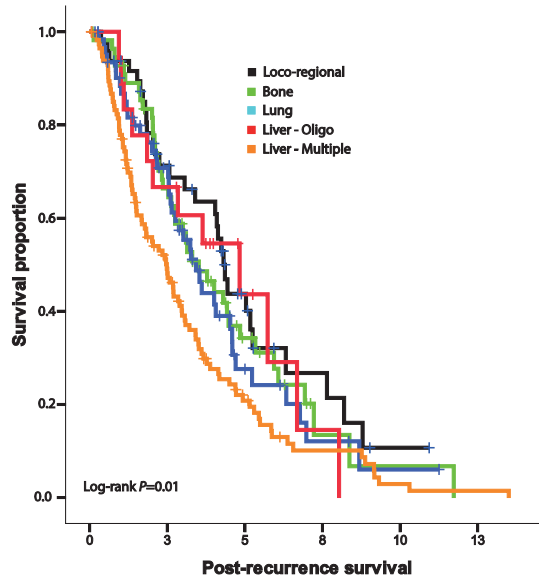


Figure 3. Post-recurrence survival according to metastatic category. Patients were categorized according to the most advanced metastatic site (loco-regional, locally advanced or regional metastases in the lymph nodes or skin; bone, skeletal metastases with or without loco-regional metastases; lung, plural metastases with or without skeletal and loco-regional metastases; liver, hepatic metastases with or without plural, skeletal or loco-regional metastases). In addition, patients with liver recurrences were further stratified into two groups based on the number of sites involved (oligo, $n=1$ and multiple, $n>1$). A significantly inferior survival was observed for patients with liver metastases occurring parallel with metastatic deposits in other organs.

Longitudinal heterogeneity of pathological biomarkers during tumor progression (paper I)

ER, PR, HER2

Tumor progression is considered to be an evolutionary process and several studies have reported that standard breast cancer pathological biomarkers including ER, PR and HER2, which are pivotal for optimal therapeutic management of breast cancer patients, are unstable across tumor progression. In paper I, we investigated the degree of concordance in the expression of ER, PR and HER2 across different tumor progression stages. We observed ER discordance rates of approximately 14% and 17% for the comparison between paired primary tumors and synchronous lymph node metastases, and primary tumors and asynchronous metastases, respectively. PR was more unstable, with discordance rates of 21% and 39% observed between primary tumors and synchronous lymph node metastases, and

primary tumors and asynchronous metastases, respectively. HER2 status, on the other hand, was more frequently preserved, with discordance rates ranging only between 2-8% for corresponding comparisons between the different progression stages. These results are representative of many previous studies (summarized in Table 3), indicating that the discordance rates of ER, PR and HER2 between primary tumors and asynchronous metastases range between 7-40%, 21-41%, and 1-43%, respectively. Of interest, by using McNemar's test, we observed a statistically significant pattern in the direction of biomarker conversion, with loss of expression of ER ($P=0.007$) and PR ($P<0.001$) more frequently observed in the asynchronous metastases relative to the primary tumors. Furthermore, a significantly inferior survival was observed for patients who presented with an ER negative metastasis after adjuvant treatment for an ER positive primary tumor (HR=2.6, CI=1.5-4.7, $P=0.001$).

Molecular subtype

In a similar analysis, we compared molecular subtypes between paired primary tumors and asynchronous metastases. Primary tumors were classified into molecular subtypes based on the 2013 St Gallen criteria for assigning surrogate molecular subtypes to breast cancers by using a panel of four (ER, PR, HER2 and Ki67) IHC based markers [51]. Asynchronous metastases were subjected to whole genome transcriptional profiling and subtyped using the PAM50 genetic classifier [39]. We found that the TN (basal) subtype was remarkably stable, while the luminal primary tumors were relatively unstable (McNemar-Bowker's test $P=0.001$). Luminal A primary tumors preferentially changed to the luminal B subtype at recurrence, while luminal B tumors frequently changed to the HER2 enriched subtype. Of note, the majority of the luminal tumors still displayed concordant ER expression between the different tumor progression stages as measured by IHC. Interestingly, a change from a luminal-like to a non-luminal subtype showed a trend towards an inferior post-recurrence survival (HR=1.8, CI=0.82-3.9, $P=0.14$). These results, in concordance with previous studies (Table 3), continue to highlight that phenotypic shifts in biological features between primary and secondary tumors can occur and may have clinical significant implications.

A novel and potentially important finding in this work was the remarkable instability of the luminal subtypes, which occurred in the absence of hormone receptor conversion. Specifically, patients with metastases displaying a conversion from a luminal-like to a HER2 enriched subtype may derive benefit from treatment with anti-HER2 agents. Only one study [226], to our knowledge, has previously compared the molecular subtypes between primary tumors and asynchronous metastases, and analogous to our results, they found a similar instability trend amongst luminal A tumors, which frequently converted to luminal

B/HER2+ (5/9) at recurrence. However, their analyses were underpowered (n=24) and resulted in statistically non-significant comparisons. The likelihood that some of the discordances we observed in our study may be attributed to differences in the analytical methods used for assignment of subtypes for the primary tumors and metastases respectively cannot be overlooked. In this context, however, the agreement between the IHC based classification and the PAM50 gene expression classification, especially for luminal tumors, has been shown to be relatively high [39, 53, 54]. Hence, our results may reflect a true change in tumor biology, since we observed a correlation between subtype conversion and poor outcome, albeit statistically non-significant, probably due to the small number of cases included in the analysis. Our results have serious implications for the management of metastatic disease. Decisions regarding prognosis and treatment of MBC patients are conventionally guided by reviewing the biomarker status of the primary tumors. Some retrospective [227-229] and two prospective studies [109, 110] have reported changes in choice of treatment based on analyses of metastases, and therapy change was often effected when a gain in the expression of a biomarker was observed. However, the impact of biopsy driven treatment decisions on survival is currently unknown and needs to be prospectively investigated within the framework of a clinical trial. Changes in biomarker status may indicate an alteration in tumor biology, or may be due to technical variation or even reflect tumor heterogeneity, since only a small portion of the tumor is often analyzed. Huge efforts are continuously being made by the clinical and research communities to standardize and improve the analytical assessment of biomarkers within and between laboratories. To avoid withholding potentially beneficial treatment or providing unnecessary treatment with potential toxicity to patients, more studies are needed to affirm the significance of our results.

Prognostic significance of metastasis-specific biomarkers and molecular subtype (paper I)

Re-testing biomarker status after the diagnosis of metastatic disease has only been recently introduced in the routine clinical diagnostic work-up for the management of MBC in many treatment centers. Consequently, data demonstrating the prognostic relevance of conventional biomarkers (e.g. ER and HER2) or molecular subtypes, assessed directly in metastases, are very limited. In separate Cox-proportional multivariable models adjusting for age at primary diagnosis, metastasis-free interval, tumor burden, nodal status and adjuvant treatment, we found that the ER status ($P<0.001$) and molecular subtype ($P<0.018$) of metastases are both significant independent prognostic factors for survival after recurrence. Our analyses were not adjusted for the patients' performance status, which is an important prognostic factor in MBC. The fact that all patients were recruited

subjects in a clinical trial would however suggest that the general performance status of the majority of the patients most likely exceeded the average threshold expected for a patient with MBC. Also, because the results of the clinical trial did not show any significant differences in survival based on the different treatment arms [205], we did not adjust our prognostic models for the treatment of metastatic disease. Of note, the multivariable models including metastasis specific biomarker status seemed to predict higher relative risks for mortality when compared with models including biomarker status from primary tumors. However, the number of cases included in the different models (biomarker status from primary tumor or metastases) was dissimilar; hence caution must be exercised when interpreting these results. It is worth emphasizing that using the biomarker status of the metastasis will account for any effects associated with discordant expression of biomarkers between primary tumors and metastases, which would be overlooked if only the primary tumor biomarker status was considered. Taken together, our results confirm the validity of using metastasis specific biomarkers for determining prognosis after breast cancer recurrence. However, whether more prognostic information is gained by using metastasis specific biomarker status compared with primary tumor biomarker status remains unclear and needs to be further investigated.

Transcriptional landscapes of breast cancer metastases (paper II)

The transcriptional landscapes of breast cancer metastases have generally been inferred from primary tumors due to scarcity of clinical biopsies from metastases to perform independent studies. Principal component analyses and unsupervised hierarchical clustering analyses of our 91 breast cancer metastases dataset consistently revealed that, similar to the global gene expression landscapes of primary breast cancers, the transcriptional fingerprints of metastases were principally associated to ER expression and the intrinsic molecular subtypes. In addition, we observed that the site of metastasis was also an important discriminator in the transcriptional space (Figure 4). Remarkably, liver metastases displayed a distinct transcriptional pattern relative to metastases from other sites. Also important was the observation that metastatic biopsies from the same patient (biological replicates) clustered together pair-wise and adjacent to each other, confirming that intra-individual tumors are highly similar [185, 186]. However, the higher similarity between matched tumor pairs compared to unmatched pairs may simply reflect the background genetic polymorphisms that exists between different individuals, which have been shown to influence global gene expression patterns [230].

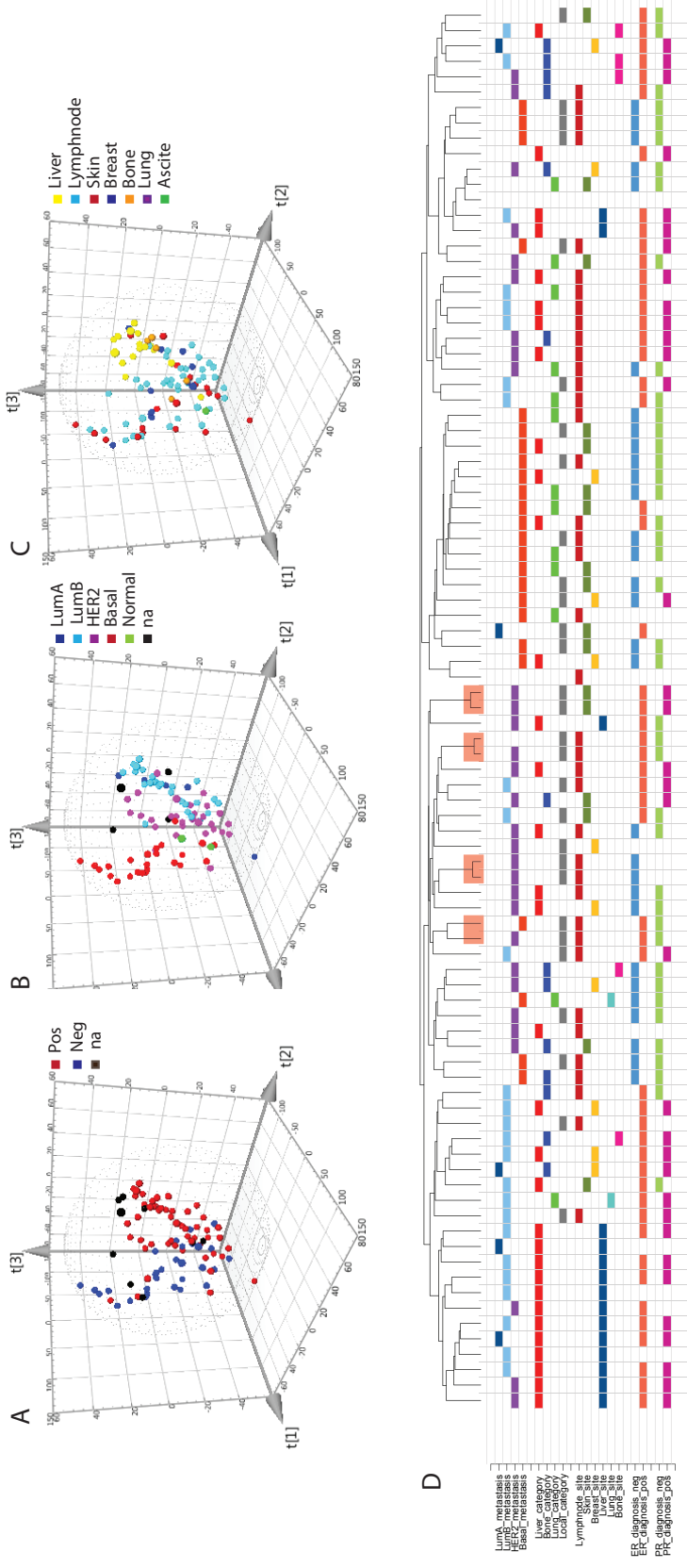


Figure 4. Unsupervised analyses of transcriptional similarities and differences between breast cancer site-specific metastases. PCA analyses showing differences in global transcriptional profiles. Each circle represents the global gene expression profile of a sample and samples with similar profiles cluster close to each other in the three-dimensional space. Samples in the PCA score plots are colored according to A) ER status of the primary tumor, B) Intrinsic subtype of the metastasis and C) specific site of the metastatic biopsy profiled. D) Dendrogram showing HCL of samples using the top 3,000 most variable probes. Highlighted samples in the tree represent biological replicates.

Site specific metastasis (papers II & III)

Predicting metastatic potential in early breast cancer

As mentioned previously, the pattern of breast cancer recurrences is not random, and the preferential organs of breast cancer relapse are the bone, lung, liver and brain. The risk of breast cancer recurrence is influenced by the stage at initial presentation (e.g. nodal status and tumor size) and the underlying tumor biology (e.g. grade, ER and HER2). However, factors predisposing selectivity of metastatic sites are yet to be comprehensively described. The molecular subtypes of breast cancer have been linked with different metastatic site preferences. While ER positive tumors show a predilection to metastasize to the bone, ER negative tumors prefer to colonize the lung and brain. Preference for the liver is however more heterogeneous, but a significant association between liver recurrences and the HER2 enriched and luminal/HER2 (luminal B) subtype has been reported [30, 31]. Similar to these studies, we found a significant positive association between liver relapses and ER positivity, as well as the luminal B subtype. However, a high prevalence of liver metastases was noted within all pathological and molecular subgroups. There is therefore a need for more specific biomarkers to improve prediction of the future metastatic site(s) of a primary breast cancer.

Claudin-2: a potential prognostic factor for predicting breast cancer liver relapse

Although liver metastases are associated with a relatively poor outcome, they have been relatively less characterized at the molecular level compared with bone, lung and brain metastases. In papers II and III, using SAM analyses, we identified a set of genes that were significantly differentially expressed in liver metastases compared to metastases from other anatomical sites. Gene ontology analyses revealed that down regulation of genes involved in cell adhesion, extra-cellular matrix remodeling, skeletal system development and blood vessel development were characteristic of liver metastases. Remarkably, we observed in contrast that *CLDN2*, a mediator of cell adhesion, was overexpressed in liver metastases. Similarly, overexpression of *CLDN2* was also recently reported in an experimental mouse model of breast cancer liver metastases, with supporting data functionally validating claudin-2 as a liver metastasis virulence gene [189, 190]. These results prompted us to investigate the potential of claudin-2 as a biomarker for predicting the liver metastatic propensity in early breast cancer. For the first time, our results revealed a connection between high expression of claudin-2 protein in the primary breast tumor and subsequent relapse in the liver ($P=0.02$). In addition, high claudin-2 protein expression was found to be a significant and independent

negative prognostic factor for shorter relapse-free survival and liver metastasis-free survival, respectively (Table 4). Specifically, only claudin-2 and tumor size were significant independent prognostic factors for early liver recurrence in multivariable analyses.

Table 4. Prognostic factors for liver metastasis-free survival in the TEX cohort

Factor	Univariable			Multivariable		
	HR	95% CI	P	HR	95% CI	P
CLDN2 (High versus Low)	2.3	1.3 – 3.9	0.003	2.0	1.1 – 3.8	0.03
Age (>50 years versus ≤50 years)	1.6	1.0 – 2.5	0.04	1.4	0.81 – 2.3	0.23
ER status (Neg versus Pos)	1.2	0.58 – 2.2	0.68	1.3	0.58 – 3.1	0.49
Histological grade (3 versus 1&2)	1.1	0.7 – 1.7	0.66	1.3	0.79 – 2.2	0.29
Nodal status (N+ versus N0)	1.5	0.94 – 2.3	0.09	1.2	0.69 – 2.0	0.54
Tumor size (>2 cm versus ≤ 2 cm)	1.4	0.92 – 2.2	0.12	1.7	1.0 – 2.9	0.04

Importantly, we performed an independent validation of our results in an external cohort of 237 pre-menopausal women with early stage, lymph node negative breast cancer, thereby confirming the negative prognostic value of claudin-2 for predicting time to relapse. Due to scarcity of liver recurrences in this external cohort, we were unable to validate the prognostic relevance of claudin-2 for predicting liver metastasis-free survival specifically. Our results are clinically important because claudin-2 may serve as a biomarker to select patients who may benefit from disease monitoring for early detection of liver metastases. Intensive surveillance of early breast cancer survivors for early detection of secondary tumors is discouraged by many guidelines (ASCO, NCCN, ESMO) due to imbalances in the costs to benefits from early distant metastasis diagnosis, as demonstrated in two randomized trials [231-233] which were conducted in unselected patient cohorts and in an era when very few treatment options were available for MBC [25]. However, costs may be reduced and more clinically relevant results may be obtained if specific biomarkers like claudin-2 are used to select high risk patients and inform on the specific site to focus surveillance. It is important to mention that improvement in overall survival through early detection may be confounded by a lead-time bias [222]. Nonetheless, the emerging recognition that the survival of patients diagnosed with oligo-metastatic disease may be prolonged by a more radical treatment, with even a curative intent [124, 133-136], coupled with the advances in imaging techniques and discovery of novel site specific biomarkers will enable the design of better studies to re-address the importance of monitoring of high risk early stage breast cancer patients following adjuvant treatment.

Transcriptional biology of breast cancer liver metastases

In paper II, we attempted to provide a detailed description of the main biological processes that may be specifically activated in breast cancer liver metastases by using a set of eight gene modules previously described to represent key biological processes specifically shaping the transcriptional landscape of breast cancer [234]. Four modules were found to be significantly differentially active between the metastases after grouping them according to the anatomical location of the lesions. One striking observation was the significantly lower expression of the “stroma” module in the liver metastases. In addition, through the integration of gene expression and clinical data from metastases and primary tumors from several independent cohorts as outlined in the Methods and Results sections in paper II, we identified a set of 17 genes to be significantly overexpressed in ER positive primary tumors with a propensity to metastasize to the liver. However and rather surprisingly, the majority of these genes (14/17) were down regulated in liver metastases compared to metastases from other anatomical sites. The inverse correlation of the pattern of expression of the majority of the genes between the liver metastases and primary tumors with liver metastatic propensity is still unclear. Of note, patients in the external primary tumor cohort were frequently diagnosed with metastatic lesions at multiple sites, which may limit the liver selectivity of the identified genes. Notwithstanding, the 17 genes were validated in a large independent cohort of primary breast tumors (n>1,800) as a significant prognostic marker for outcome after primary breast cancer diagnosis.

Gene ontology analyses revealed that the 17-gene signature was enriched for stromal genes involved in cell adhesion and skeletal development, and the signature was significantly correlated to the “stroma” module reported by Fredlund and colleagues [234]. These findings suggest that the stroma may be an important mediator of site specific metastases. This hypothesis is supported by results from a very recent study by Wolf and colleagues [235], showing a significant association of an extracellular (stroma) matrix-rich gene module with the site of breast cancer metastases, although liver metastases were not considered in this study. Furthermore, there is evidence that stromal signals resembling those of the bone play very important roles in primary breast tumors to prime them for colonization of the bone [236]. Due to scarcity of annotations for the specific site of relapse in publicly available datasets, we were however unable to test the potential of our signature to predict liver metastatic propensity after primary tumor diagnosis.

The 17-gene liver metastasis selective signature: a potential luminal A subtype specific prognostic marker

A very interesting and clinically relevant finding in this thesis was the observation that the 17-gene liver metastasis signature was prognostic for outcome in ER positive early breast cancer. Specifically, low expression of the signature was prognostic of early recurrence ($P < 0.001$) and shorter overall survival ($P = 0.026$) in multivariable analyses. More importantly, in separate analyses including only patients with luminal A tumors (Figure 5), the signature was independently prognostic for relapse-free survival ($P = 0.004$) but not overall survival ($P = 0.29$). This result is particularly interesting because the luminal A subtype, although considered to be of generally good prognosis, still accounts for a small but clinically relevant proportion of patients diagnosed with MBC. The prediction of the prognosis of ER positive tumors at the transcriptional level is mainly restricted to proliferation genes. However, proliferation alone cannot satisfactorily account for all the recurrences recorded amongst ER positive breast cancers, especially within the luminal A subtype which are often of a low proliferative potential and recurrence frequently occurs late. Bergamaschi et al. [237] have previously suggested that extracellular matrix genes may provide additional prognostic information within the ER positive subset of breast cancers; a recommendation which is supported by our results. Metastases remain the root of breast cancer related deaths. Given the importance of these findings for optimal personalization of breast cancer management, further studies are needed to validate and extend these results.

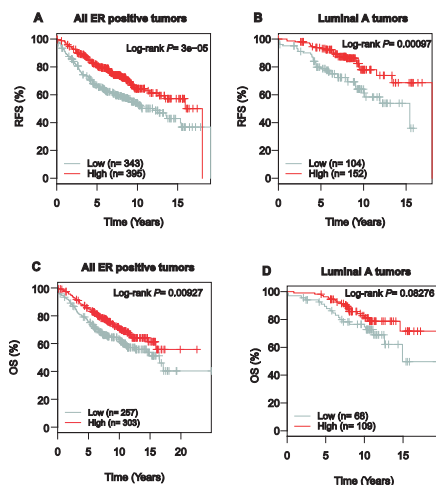


Figure 5. Prognostic impact of the 17 liver metastasis-selective genes in ER positive primary breast cancer. Relapse-free survival (RFS) and overall survival (OS) are shown. A) RFS for all ER positive tumors, B) RFS for luminal A (PAM50) tumors only, C) OS for all ER positive tumors, and D) OS for luminal A tumors only.

Targeted therapy for *BRCA1* dependent MBC: combination of PARP1 and PI3K inhibitors (paper IV)

Paper IV was a proof-of-concept study, performed to investigate if co-targeting of PI3K could potentiate the sensitivity of *BRCA1* mutated cells to PARP1 inhibition. Earlier studies showing remarkable sensitivity and specificity of PARP inhibitors in killing *BRCA* mutated breast cancer cells [155, 156] had led to the rapid development of PARP inhibitors and their prompt entry into clinical trials. Even though very encouraging responses were recorded in tumors harboring mutations in either *BRCA1* or *BRCA2* in these trials [149-151], a considerable number of tumors displayed *de novo* or acquired resistance to single agent PARP inhibition therapy, warranting the search for methods to improve sensitivity and durability of treatment without compromising the specificity, which is a main attraction to this therapeutic option for patients with *BRCA* mutated tumors. The PI3K pathway has been implicated in resistance to both endocrine therapy [139] and HER2 targeted therapy [138]. Furthermore, gross mutations in the *PTEN* tumor suppressor gene are a specific and very frequent oncogenic event occurring in *BRCA1* mutated tumors [157]. This suggest that the PI3K pathway may be constitutively activated in these tumors and may drive resistance to treatment through its involvement in the repair of breaks in the DNA, in addition to its anti-apoptotic and pro-survival effects [238].

We found that sequential combination of the PARP1 and PI3K inhibitors interacted in synergy and was significantly more cytotoxic compared with treatment with either drug as a single agent in *BRCA1* mutant cells (Figure 6). In addition, we uncovered that the mechanism of action involved the induction of cell cycle block (G2/M arrest) due to accumulation of DNA double strand breaks, which culminated in a mild induction of apoptotic cell death. Taken together, our results confirmed the hypothesis that the combination treatment may be a better therapeutic option for specific targeting of tumors with defective *BRCA1* signaling. However, the clinical relevance of this finding can only be tested in more complex *in vivo* studies such as experimental animal models or directly in patients within randomized clinical trials. Two independent studies validating and extending our results by using both *in vitro* and experimental animal models have since been published. In the first study, by Juvekar et al. [239], the combination of PI3K and PARP1 inhibition in an *in vivo BRCA1* deficient mouse model resulted in the delay of tumor doubling time by up to 70 days, confirming the efficacy of this therapeutic approach. The second study, by Ibrahim and colleagues [240], reported that PI3K inhibition sensitized *BRCA* proficient TN breast cancer cells to PARP1 inhibition through impairment of the homologous recombination pathway via down regulation of *BRCA1* expression. The effects of the combination treatment in TN breast cancer cells were further validated recently by De and

colleagues [241]. The possibility of expanding this therapeutic approach to include TN *BRCA* proficient tumors is particularly interesting, since less than 5% [18] of all breast cancers harbor mutations in the *BRCA1/2* genes. However, to avoid repeating past mistakes, biomarkers predicting sensitivity are needed to aid in the proper selection of patients. The search for biomarkers to identify the “BRCAness” phenotype within TN breast cancers and predictors of sensitivity to drugs completely targeting the PI3K signaling pathway or specific components of the pathway like mTOR or AKT continues to be an important research priority.

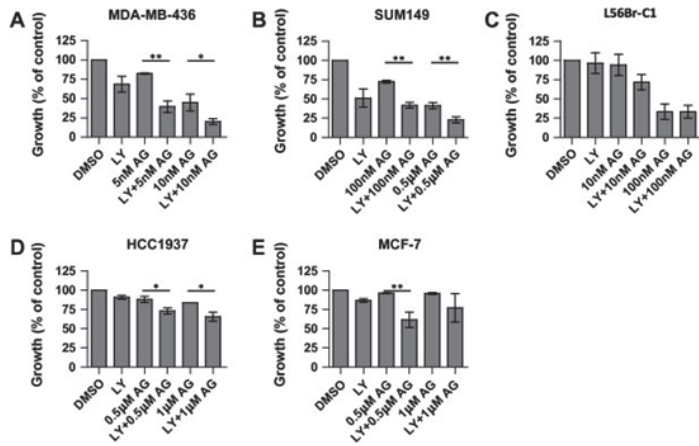


Figure 6. Cytotoxic effects after a two cycle sequential combination of the PARP1 inhibitor AG014699 (AG) with the PI3K inhibitor LY294002 (LY) in breast cancer cell lines.

Conclusions

The main conclusions drawn from the research presented in this thesis are:

- MBC patients with oligo liver metastatic disease experience relatively longer post-recurrence survival compared with patients presenting with liver metastases diagnosed in parallel with metastases in other organs. The survival of patients with oligo liver metastases may be further prolonged with more radical treatment such as surgical excision of the metastatic lesion. This needs to be investigated further in prospective clinical trials.
- The standard pathological markers ER, PR and HER2 and the tumor molecular subtype may change between primary tumors and metastases, with important consequences for post-recurrence survival if patients are not properly therapeutically managed.
- Tumor pathological markers (e.g. ER) and molecular subtypes assessed at time of recurrence are significant and independent prognostic factors for post-recurrence survival, and may be better prognostic markers in MBC compared to primary tumor derived markers.
- The transcriptional landscape of primary tumors is generally maintained in metastases. However, the site of metastasis is a small but significant discriminating factor in the global gene expression space.
- Liver metastatic relapse is associated with high grade tumors, ER positivity and the luminal B subtype. However, the prevalence of liver metastases among most pathological and molecular subtypes of early breast cancer is remarkably high.
- Claudin-2 is a potential prognostic marker of early liver relapse in breast cancer.
- The stroma may be an important mediator of site specific metastasis in breast cancer.
- Integrating stroma (extracellular matrix) related factors to proliferation may improve prognostication in ER positive breast cancer, especially within the luminal A subtype.
- Co-targeting PARP1 and PI3K signaling may be a more effective therapeutic strategy for *BRCA* deficient breast cancers.

Future perspectives

The data presented in papers I-III of this thesis corroborate previous studies and extends our understanding of the complexity and clinical significance of tumor heterogeneity across tumor progression stages. In addition, we have shed some light onto the biology of breast cancer liver metastasis and have identified a candidate liver metastasis marker gene. However, some of our analyses were statistically underpowered due to limited number of cases. This is particularly true for analyses including asynchronous metastases and also for analyses requiring annotations regarding the specific sites of metastases. A larger and well annotated sample collection of paired intra-individual tumors is needed to validate our results. Also, the exclusion of patients with HER2 positive disease, as well as patients presenting with brain metastases from the TEX clinical trial resulted in a potential bias in the distribution of molecular subtypes and type (site of relapse) of metastatic lesions studied. Hence, further studies are needed to assess if our findings hold true in a more balanced MBC cohort. Furthermore, comparison of biomarker status across tumor progression stages should preferably be done using the same analytical methods. In addition, the true clinical significance of biomarker conversion needs to be prospectively studied in clinical trials. With the advent of routine biopsying of metastatic disease in many treatment centers, the scarcity of clinical samples from metastases will be overcome in the near future, paving the way for better studies to address the limitations of our studies and validate our results.

Our observation of a cytotoxic superiority of the combination of PARP and PI3K inhibitors compared to the single agents has already been validated by more than two independent studies using *in vitro* and *in vivo* xenograft models, and clinical trials to test this combination are warranted. However, the sensitivity of tumors to these targeted agents is quite variable and biomarkers to predict sensitivity are in need. For example PTEN loss may result in preferential activation of the PI3K β subunit, suggesting that PI3K β -specific inhibitors could be an effective treatment option.

Popular science summary

Every year globally, an estimated 1.7 million women are diagnosed with breast cancer, while over half a million are killed by this disease. The principal cause of death is metastasis, which is the spreading of the cancer cells from the breast to other vital organs like the brain, liver or lungs, resulting in organ failure and subsequently death. Even though the past decades have seen significant progress in the management of patients with primary breast cancer, when the tumor is still localized in the breast, the prevention and treatment of metastases is however lagging behind. Many women still get disseminated disease, sometimes decades after successful surgical removal and treatment of the primary tumor. Unfortunately, a metastasis diagnosis is conceived to be a “death sentence” by many people since metastatic disease is incurable by current medical interventions.

Huge efforts have been made by researchers and oncologists to characterize the properties of a breast cancer that makes it spread and grow in particular vital organs and identify factors that make the cancer cells sensitive or resistant to treatment. The research described in this thesis has primarily aimed to unravel the molecular similarities and differences of breast cancer tumors as they progress from the localized tumors in the breast to deadly metastatic tumors in distant vital organs. In addition, we have investigated a novel treatment option for eradicating a subset of breast cancers presenting with mutations in the *BRCA1* gene, which are associated with an inferior outcome and are currently difficult to treat despite the poor prognosis.

In paper I, we investigated the stability of conventional biomarkers (ER, PR and HER2) and molecular subtypes of tumors across tumor progression stages. These biomarkers are used by clinicians to make decisions regarding prognosis and therapeutic management of patients with metastatic breast cancer. This research question is very important because, when a metastasis is diagnosed nowadays, only the biomarker status of the primary tumor is used for decision making, ignoring the possibility that a drift in biomarker expression may have occurred in the metastasis which may modify response to treatment and ultimately, affect the length of survival. We found that, on average, biomarker expression was often conserved between primary tumors and metastases from the same patient. However, more frequently than expected by chance, and in a clinically relevant number of cases, a change in biomarker status occurred and this conversion was shown to affect the length of survival, probably due to lack of response to the

treatment received since these biomarkers are also predictors of response to treatments administered. More interestingly, and a novel finding, we observed that the molecular subtype, which is a more accurate estimation of the biological phenotype of a breast cancer compared to only using the single biomarkers like ER, PR and HER2, was more unstable across tumor progression stages. Tumors expressing the single biomarker ER can be stratified into two molecular subtypes; luminal A and luminal B. Luminal B tumors are more aggressive tumors requiring a more intensive and radical treatment approach because of their poor prognosis characteristic. Since all the luminal subtypes express ER, a change from a luminal A to B phenotype will be undetected by analyzing only the biomarker ER. We detected a molecular subtype change which may require a modification of treatment in a significant number of patients. Our results emphasize a need to re-test biomarker expression in metastases to enable better management of patients with metastatic breast cancer.

In paper II, we studied and compared patterns in the global expression of all genes in clinical metastases biopsies collected from different organs. Specifically, we searched for liver metastasis selective genes which could be used as markers for predicting if and when a primary breast cancer will spread. We observed that the global gene expression pattern in metastases was similar to what has been previously described in primary tumors. In addition, by using a combination of different statistical and bioinformatics analyses, we identified a set of 17 liver metastasis selective genes which showed significant potential in predicting time to recurrence after primary tumor diagnosis and treatment. Importantly, this signature could identify patients within the luminal A molecular subtype at risk of developing metastatic disease after shorter intervals following primary tumor diagnosis and treatment. This result is clinically significant because patients with luminal A tumors are often considered to have a good prognosis, yet, they still account for a small but clinically relevant number of patients diagnosed with metastatic disease. Metastasis remains the root cause of cancer related deaths, hence accurate identification of all patients at risk of developing disseminated disease is important.

The goal of paper III was to test the ability of the liver metastasis selective gene, *CLDN2*, to predict the potential of a primary tumor to spread specifically to the liver. With the exception of the brain, liver metastases are the most lethal type of breast cancer metastases. We showed that high *CLDN2* expression in the primary tumor was significantly associated and prognostic of an early liver relapse. This result is important because *CLDN2* may serve as a marker to guide surveillance (where to focus screening) of patients after primary tumor diagnosis. It has been reported that early detection of an isolated liver metastasis could lead to more radical therapeutic interventions, which may improve the quality of life and prolong the survival for patients diagnosed with liver metastases, which is relatively short compared to patients with metastases in the bone or lungs.

Finally, in paper IV, our aim was to test the novel combination of two compounds specifically targeting distinct genetic defects found in *BRCAl* mutated breast cancer cells. These compounds inhibit two key enzymes; PARP1, which is important for fixing breaks in the DNA, and PI3K, which is important for maintaining cell growth and survival. Our results indicated a superior growth inhibitory and killing effect of the combination compared to each of the single agents. If validated in clinical studies, this therapeutic strategy may greatly benefit patients diagnosed with this deleterious subset of breast cancer.

In summary, the results we present in this thesis shed more light onto the complexity of the process of breast cancer progression and we propose methods of improving prediction of outcome, disease monitoring and treatment, which will facilitate the personalization of therapy for women diagnosed with breast cancer.

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Paper I

Significance of biomarker expression and molecular subtype at different stages of tumor progression for the prognosis of metastatic breast cancer

Siker Kimbung^{1,2}, Anikó Kovács³, Anna Danielsson⁴, Pär-Ola Bendahl¹, Kristina Lövgren¹, Marianne Frostvik Stolt⁵, Nicholas P. Tobin⁵, Linda Lindström^{6,7}, Jonas Bergh⁵, Zakaria Einbeigi⁴, Mårten Fernö¹, Thomas Hatschek⁵, and Ingrid Hedenfalk^{1,2}, in collaboration with the TEX Trialists Group

¹Division of Oncology, Department of Clinical Sciences, Lund, Lund University, Sweden;

²CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden;

³Department of Clinical Pathology and Cytology, Sahlgrenska University Hospital, Gothenburg, Sweden;

⁴Department of Oncology, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden;

⁵Department of Oncology and Pathology, Karolinska Institutet and Karolinska University Hospital, Sweden;

⁶University of California at San Francisco (UCSF), Department of Surgery, USA;

⁷Department of Biosciences and Nutrition, Karolinska Institutet and University Hospital, Sweden.

Corresponding author: Associate Professor Ingrid Hedenfalk, Division of Oncology, Department of Clinical Sciences, Lund, Lund University Cancer Center/Medicon Village, SE-22381 Lund, Sweden. Phone: +46-46-2220652. Fax: +46-46-147327.

E-mail: Ingrid.Hedenfalk@med.lu.se

Abstract

Discordant expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) between a primary tumor and metastasis may significantly alter tumor biology and clinical outcome. However, modification of tumor biology and prognosis as captured by the intrinsic molecular subtypes may occur even when ER, PR and/or HER2 are concordantly expressed. This study aimed to evaluate the concordance and prognostic relevance of individual tumor biomarkers and molecular subtypes measured at different tumor progression stages. A cohort of 304 women with locally advanced and metastatic breast cancer enrolled in a clinical trial was studied. Tissue microarrays of primary tumors ($N=217$) and synchronous lymph node metastases (LNM, $N=111$) were constructed. ER, PR, HER2 and Ki67 were quantified by immunohistochemistry and *in situ* hybridization and molecular subtypes were determined following the 2013 St Gallen guidelines. In addition, fine-needle aspirates of asynchronous metastases ($N=85$) were transcriptionally profiled and molecular subtypes determined using the PAM50 classifier. Discordant expression of all biomarkers and molecular subtype was observed across different tumor progression stages. Specifically, loss of ER ($P=0.007$) and PR ($P<0.001$) expression were significantly observed in the asynchronous metastases. The luminal subtypes displayed significant instability, frequently changing to a more aggressive subtype at recurrence ($P=0.001$). Interestingly, ER and molecular subtype at time of primary diagnosis and notably, at recurrence, were independent prognostic factors for post-recurrence breast cancer mortality ($P<0.05$). Furthermore, concordant ER expression resulted in a favorable outcome ($P=0.001$), and the prognosis of patients with luminal primary tumors which changed subtype at recurrence correlated with the direction of subtype conversion. These results confirm that pathological biomarkers may change and that molecular subtype conversion occurs during tumor progression, and unravel important consequences on outcome. In addition, the independent prognostic significance of ER and molecular subtype at recurrence is demonstrated.

Keywords: metastatic breast cancer, estrogen receptor, molecular subtype, biomarker conversion, prognosis

Introduction

Breast cancer mortality is mainly associated to metastases [1, 2]. The introduction of targeted therapies and optimized combinations of chemotherapeutics, coupled with better surveillance, have increased survival, especially for patients with estrogen receptor (ER) and progesterone receptor (PR) or human epidermal growth factor receptor 2 (HER2) positive primary breast cancer; however the significance of these therapeutic and technological advances for metastatic breast cancer (MBC) survival remains controversial [2-5]. Conventionally, risk assessment and treatment decision-making for MBC is based on both clinical (length of metastasis-free interval, adjuvant systemic therapy, tumor burden, age at diagnosis, and the patient's general performance status) and primary tumor pathological factors (*i.e.* ER and HER2) [1, 2]. Typically, distant metastases are not systematically biopsied, rationalizing the personalization of therapy based on primary tumor biomarker status. However, the notion that the hormone receptor and HER2 phenotype of metastases may significantly change from that of the primary tumor has been validated [6-10] and biomarker conversion may affect the choice of systemic therapy [11]. Furthermore, loss of ER expression at relapse has been linked with an inferior survival [8, 9]. Thus, many national and international guidelines for MBC management now recommend re-testing of at least one metastatic biopsy for better individualization of therapy [12-15]. Studies evaluating the prognostic significance of biomarker expression in metastases are of importance, especially in an era when re-testing of metastases is being mandated.

The molecular taxonomy of breast cancer is complex and currently at least four stable intrinsic subtypes with distinct biology and clinical outcome have been defined by transcriptional profiling [16-19]. However, translation of the intrinsic subtypes into daily clinical practice is limited by the requirement for fresh frozen specimens, technological complexity and high costs, and has led to the adoption of an immunohistochemistry-based surrogate definition for approximation of the intrinsic subtypes [20, 21]. The prognostic importance of the subtypes is well established in early stage disease, and they are now included in several clinical guidelines (*e.g.* St Gallen and ASCO). In contrast, the importance of the molecular subtypes is poorly defined in MBC. Hormone receptor positive tumors are comprised of two distinct subtypes; luminal A and luminal B, which differ in terms of proliferation, response to therapy and outcome. Even when ER is concordantly expressed during disease progression, a biological change in for instance proliferation, as captured by the subtypes, may occur in a metastasis, thereby altering the therapeutic management and prognosis.

This study aimed to evaluate the agreement in expression of individual tumor biomarkers and breast cancer molecular subtypes measured at time of primary tumor diagnosis, including synchronous LNMs, and at

recurrence, and to investigate the significance of ER expression and molecular subtype of metastases for the prognosis of MBC.

Materials and Methods

Patients

A cohort of 304 women with locally advanced and MBC who were enrolled in a randomized phase III trial (TEX) conducted between 2002-2007 in Sweden was studied. Patients with brain metastases, indicated for HER2-targeted therapy, or with other malignancies diagnosed within five years were exempted from the trial. Detailed information on the trial design and outcome has been reported [22]. As first-line treatment for MBC, patients received a combination of an anthracycline (epirubicin) and a taxane (paclitaxel) with or without the addition of capecitabine; no significant difference in overall survival was observed between the treatment arms.

Data collection

This sub-study was approved by the regional ethics committees at all participating centers. The flow chart in Supplementary Figure S1 depicts patient/sample selection at the respective stages. Pathological data, adjuvant systemic treatment and outcome data were collected from the central clinical trial database. Archival formalin-fixed paraffin-embedded primary tumor and LNM blocks were collected for tissue microarray (TMA) construction and central assessment of biomarkers. Furthermore, fine-needle aspirates (FNAs) from metastatic lesions were collected for transcriptional profiling whenever possible; these data can be accessed from the Gene Expression Omnibus database (GSE46141).

Biomarker evaluation

Standard pathological markers (ER, PR, HER2 and Ki67) were centrally evaluated on the TMAs. Information describing the experimental procedures and evaluation of these markers has been published [23]. All scorings were performed independently by a pathologist, blinded to outcome information. ER, PR and Ki67 were analyzed by IHC and HER2 status was analyzed by both IHC and chromogenic *in situ* hybridization (CISH). Furthermore, IHC data for ER, PR and HER2 (performed on core or fine-needle biopsies) for asynchronous metastases were extracted from the clinical trial database. Cut-offs used to indicate positive staining were $\geq 10\%$ for ER and PR, IHC 3+ or positive CISH for HER2 and $\geq 20\%$ for Ki67.

Molecular subtyping

The St Gallen International experts' consensus on primary therapy for early breast cancer recommends the inclusion of a surrogate tumor molecular subtype classification based on ER, PR, HER2 and Ki67 [20, 21]. Following the guidelines from the 2013 experts'

consensus [20], tumors were classified into the following molecular subgroups: luminal A-like (ER+, PR+, HER2-, Ki67 low), luminal B-like (ER+, HER2- and at least one of PR- and Ki67 high; or alternatively ER+, HER2+, any PR, any Ki67), HER2 positive (ER-, PR-, HER2+, any Ki67) and triple negative (ER-, PR-, HER2-, any Ki67). In addition, the PAM50 genetic classifier was used for classifying metastases into intrinsic subtypes (luminal A, luminal B, HER2-enriched, basal-like and normal-like) as previously described [18]. For accurate determination of the intrinsic subtype of a tumor, the sample must be centered against an appropriately large and representative sample set. HER2 amplification was a basis for exclusion from the TEX trial, hence the distribution of the subtypes in the present cohort may be different from that of the original dataset used by Parker and colleagues [18], and thus violates the specifications for accurate assignment of subtypes using the PAM50 classifier. To circumvent this bias, the present dataset was merged with an external primary breast cancer dataset which was profiled using the same microarray platform (Affymetrix HuRSTA-2a520709 gene chips). The data were normalized and classified into the intrinsic subtypes as previously described [22].

Statistical analyses

Paired dichotomized data were compared using the McNemar test. McNemar-Bowker's test of symmetry was used when comparing molecular subtypes between tumor progression stages. The primary endpoint for survival analyses was post-recurrence breast cancer mortality (BCM) and the follow-up was restricted to five years. Cumulative incidence curves were used to visualize differences in BCM between groups and the Log-rank test to evaluate statistical significance. Univariable and multivariable Cox-proportional hazards models were used to evaluate the independent prognostic importance of biomarkers and molecular subtype. Proportional hazards assumptions were checked by graphical methods. All *P*-values correspond to two-sided statistical tests and values <0.05 were considered significant. The statistical software packages IBM SPSS Statistics 19 (IBM Corporation, NY) and STATA version 12 (StataCorp, College Station, TX) were used.

Results

Table 1 and Supplementary Table S1 represent the distribution of baseline clinico-pathological factors in the entire cohort and the subgroups of

patients included on the different TMAs and representing each progression stage. Most clinico-pathological characteristics were similarly distributed between the subsets of patients included at the different stages compared to the entire study cohort. PR was however lost more frequently in the metastases. Likewise, the surrogate molecular subtypes were similarly distributed between the primary tumors and the LNMs, but an increase in the proportions of basal-like and HER2-enriched cases was observed among asynchronous metastases processed by transcriptional profiling (Table 1).

Concordance/discordance of individual biomarkers across tumor progression stages: ER, PR, HER2 and Ki67

The expression of each biomarker was compared across different progression stages (Table 2). A total of 126 cases had paired data for ER expression between primary tumors and asynchronous metastases and a discordance rate of 17% was observed. This discordance was significantly skewed (McNemar's $P=0.007$) as the majority (17/21, 81%) changed from positive status in the primary tumor to negative in the metastasis. Similarly, an ER discordance rate of 14% was observed between the paired primary tumors and synchronous LNMs, with the majority (10/13, 77%) losing expression in the LNM (McNemar's $P=0.09$). Conversely, 12/52 (23%) paired cases had discordant ER expression between LNMs and asynchronous metastases, but no skewness towards any direction was observed as exactly half (6/12) of the discordant cases lost ER and the other half gained ER (McNemar's $P=1.0$).

PR expression was more unstable between the different tumor progression stages. Discordance rates of 21% and 39% were observed between primary tumors and LNMs, and primary tumors and asynchronous metastases, respectively (Table 2). A significant skewness from positive to negative status (16/19, 84%) was observed for primary vs. LNM (McNemar's $P=0.004$) and similarly, 32/41 (78%) discordant cases lost PR expression when primary tumors were compared with paired asynchronous metastases (McNemar's $P<0.001$). Conversely, no statistically significant bias in the direction of change was noted for LNMs compared with asynchronous metastases. Although 18/44 (41%) paired cases were discordant, only 10/18 (56%) lost PR expression (McNemar's $P=0.82$).

Table 1. Distribution of tumor biomarkers and molecular subtypes at different tumor progression stages

	Tumor progression stage		
	Primary Tumors* (N=217) ^a N (%)	Synchronous LNM* (N=111) ^a N (%)	Asynchronous Mets** (N=304) ^b N (%)
ER status			
Positive	158 (81%)	75 (73%)	100 (71%)
Negative	36 (19%)	28 (27%)	41 (29%)
Missing/unknown	23	8	163
PR status			
Positive	110 (58%)	39 (38%)	53 (41%)
Negative	81 (42%)	64 (62%)	77 (49%)
Missing/unknown	26	8	174
HER status			
Amplified	17 (9%)	13 (14%)	7 (7%)
Normal	180 (91%)	77(86%)	95 (93%)
Missing/unknown	20	21	202
Ki67 status			
High	65 (36%)	33 (33%)	n.a.
Low	122 (64%)	66 (67%)	n.a.
Missing/unknown	30	12	n.a.
Molecular subtype			
Luminal A-like ^a /Luminal A ^b	65 (36%)	25 (29%)	5 (6%)
Luminal B-like ^a /Luminal B ^b	81 (45%)	44 (50%)	26 (31%)
HER2 positive ^a /HER2-enriched ^b	9 (5%)	5 (6%)	27 (32%)
Triple negative ^a /Basal-like ^b	24 (14%)	13 (15%)	24 (29%)
Normal-like ^b	n.a.	n.a.	2 (2%)
Unclassified/missing	38	24	210

*Analyzed centrally on TMAs; **from clinical records and transcriptional profiling; a, St Gallen subtype; b, PAM50 subtype; n.a., not applicable

Table 2. Biomarker discordance at different stages of tumor progression

Biomarker	N	Loss	Gain	Discordance (%)	P
Primary tumor vs. synchronous LNM					
ER	94	10	3	14	0.09
PR	92	16	3	21	0.004
HER2	83	2	5	8	0.45
Ki67	90	11	15	29	0.56
Primary tumor vs. asynchronous metastasis					
ER	126	17	4	17	0.007
PR	105	32	9	39	<0.001
HER2	64	1	0	2	1.0
Synchronous LNM vs. asynchronous metastasis					
ER	52	6	6	23	1.0
PR	44	10	8	41	0.82
HER2	30	3	0	10	0.25

Abbreviations: N, number of cases with paired data; P, P-value (McNemar's test)

Discordance rates for HER2 and Ki67 expression between primary tumors and LNMs were 8% and 29% for HER2 and Ki67, respectively (Table 2). Five of seven (71%) discordant cases gained HER2 expression while 15/26 (58%) of cases with discordant Ki67 displayed high proliferation in the LNM. However, no skewness was observed for these biomarkers (McNemar's $P>0.05$).

Molecular subtypes across tumor progression stages

Surrogate molecular subtypes could be assigned to 179 primary tumors and 87 LNMs respectively, of which 74 cases had paired data. A subtype conversion was observed in 13/33 (39%) patients with luminal A-like primary tumors, 92% (12/13) of which changed to the luminal B-like subtype. A luminal B-like subtype in the primary tumor changed to the less aggressive luminal A-like subtype in 5/28 (18%) patients, and to the more aggressive triple negative subtype in 2/28 (7%) patients. Three of nine (33%) triple negative primary tumors also changed subtype. The McNemar-Bowker's test of symmetry was used to test the null hypothesis that the shift in the distribution of subtypes was balanced, resulting in no significant deviation from the null hypothesis (Table 3; McNemar-Bowker's $P=0.42$).

Next, we compared the molecular subtypes between primary tumors and asynchronous metastases. To simplify the analyses, the triple negative and basal-like subgroups were considered as the same entity since the basal-like subtype is known to constitute a majority of the triple negative group [19, 24, 25]. Furthermore, because the surrogate IHC classification does not include the normal-like subgroup, the two metastases assigned to this subgroup by PAM50 were excluded, resulting in 49 cases for inclusion in the final analyses (Table 3). Eleven out of 13 (85%) cases changed subtype from luminal A-like in the primary tumor to a more aggressive subtype in the metastasis [luminal B (8/11, 73%) and HER2-enriched (3/11, 27%)]. In addition, of the 14 cases that transitioned from the luminal B-like subtype, 11 (79%) switched to the HER2-enriched subtype. Of note, the majority (60%) of the HER2 amplified primary tumors were of the HER2-enriched subtype at recurrence and all (100%) triple negative primary tumors displayed a basal-like subtype at recurrence. Overall, the symmetry of subtype distribution between the primary tumors and asynchronous metastases was significantly altered, particularly for the luminal tumors favoring a shift to a more aggressive subtype at recurrence (11/11 for luminal A-like and 13/14 for luminal B-like (Table 3; McNemar-Bowker's $P=0.001$).

Table 3. Breast cancer molecular subtype concordance/discordance at different stages of tumor progression

		LNM (St Gallen subtype)				
Primary tumor (St Gallen subtype)		Luminal A-like	Luminal B-like	HER2-Positive	Triple negative	P^*
Luminal A-like		20	12	0	1	0.42
Luminal B-like		5	21	0	2	
HER2-positive		0	0	3	1	
Triple negative		0	2	1	6	
		Asynchronous metastasis (PAM50 subtype)				
Primary tumor (St Gallen subtype)		Luminal A	Luminal B	HER2-enriched	Basal-like	P^*
Luminal A-like		2	8	3	0	0.001
Luminal B-like		1	7	11	2	
HER2-positive		0	1	3	1	
Triple negative		0	0	0	10	

* P -value from McNemar-Bowker's test

Post-recurrence breast cancer mortality in relation to ER and molecular subtype status at different progression stages

The median breast cancer specific survival for patients alive at last follow-up was approximately 45 months (range 9 -135 months). Negative ER status at primary diagnosis was associated with inferior survival in both univariable (Fig 1a; HR=1.7, CI=1.3-2.4, $P=0.002$) and multivariable analyses (Table 4; HR=2.2, CI=1.5-3.3, $P<0.001$).

Interestingly, the mortality in the ER negative group increased when ER at recurrence was considered [univariable (Fig 1b; HR=2.3, CI=1.5-3.6, $P=0.0001$) and multivariable analyses (Table 4; HR=3.0, CI=1.8-5.0, $P<0.001$)].

Next, the prognostic significance of the molecular subtypes on outcome of MBC was investigated. Five-year mortality was significantly different based on molecular subtype of the primary tumors (Figure 2a; Log-rank $P=0.01$), and subtype

remained an independent factor in multivariable analysis (Table 4; $P=0.003$). Importantly, a significant difference in post-recurrence breast cancer mortality was observed when the PAM50

intrinsic subtypes of the metastases were considered [univariable (Figure 2b; Log-rank $P=0.04$) and multivariable (Table 4; $P=0.02$) analyses].

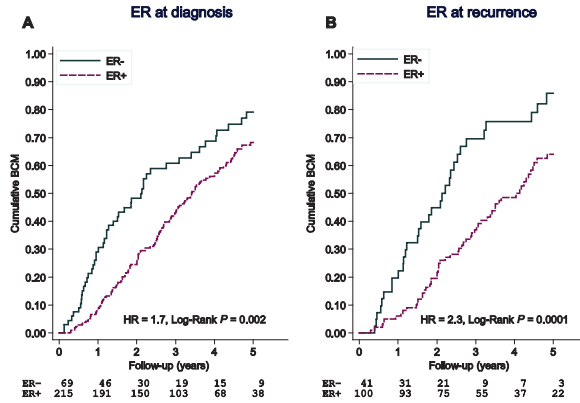


Figure 1. Cumulative breast cancer mortality (BCM) following metastasis diagnosis according to A) ER status at primary diagnosis and B) ER status at recurrence. Abbreviations: ER+, estrogen receptor positive; ER-, estrogen receptor negative

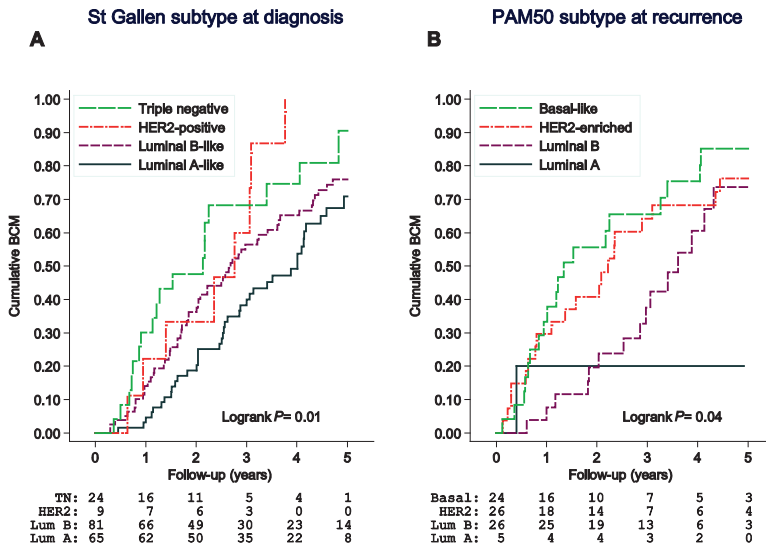


Figure 2. Cumulative breast cancer mortality (BCM) following metastasis diagnosis according to A) St Gallen molecular subtype at primary diagnosis and B) PAM50 molecular subtype at recurrence. Abbreviations: LumA, Luminal A; LumB, Luminal B; HER2, HER2-enriched; TN, Triple negative.

Finally, the effect of losing ER expression or changing from a luminal-like subtype to a non-luminal subtype between primary tumors and asynchronous metastases was investigated in sub-

analyses. 17/101 (17%) evaluable cases lost ER expression at recurrence, resulting in a significantly increased mortality (HR=2.6, CI=1.5-4.7, $P=0.001$). Similarly, 16/34 cases

changed from a luminal-like to a non-luminal subtype at recurrence and this correlated with an inferior outcome (HR=1.8, CI=0.82-3.9, $P=0.14$). Interestingly, for eligible cases with IHC scores for ER at recurrence, 70% (7/10) of the tumors that converted to a non-luminal subtype had concordant ER positive status.

Discussion

This study confirms that conversion of biomarkers occurs between the stages of tumor progression, with loss of hormone receptors (ER and PR) most commonly observed at recurrence, and is significantly associated with an unfavorable outcome. Importantly, we show that tumor biological properties captured by the molecular subtypes may also change, even in the absence of hormone receptor conversion. The implication of these results for the management of MBC is important given the poor prognosis associated with an MBC diagnosis. ER is an acknowledged independent prognostic and treatment predictive factor in primary breast cancer and the intrinsic subtypes have also been validated for prognostication purposes [26]. However, the role of the subtypes is not well defined in MBC. We observed a 17% discordance rate for ER expression between primary tumors and asynchronous

metastases, which is consistent with the 7-31% discordance rates previously reported [6, 9, 13]. The triple negative and HER2 amplified IHC/CISH subtypes were often conserved as basal-like and HER2-enriched subtypes respectively in the asynchronous metastases. Remarkably, primary luminal tumors were significantly unstable, with luminal A-like to luminal B, and luminal B-like to HER2-enriched conversions predominantly observed, which suggests a more aggressive and proliferative phenotype of the metastases. Importantly, the fact that most of the cases with discordant subtypes still had concordant ER expression implies that these cases will be missed in analyses considering only single biomarkers. Analogous to our findings, Falck *et al.* by using the IHC-based classification as outlined in the 12th St Gallen consensus guidelines [21], reported more frequent, albeit statistically non-significant, conversion from luminal A to luminal B subtype at recurrence [27]. Despite the fact that the compatibility between the current IHC-based subtype classification and the gene expression-based PAM50 classification is imperfect [18, 25, 28], the expected discordance rate associated with analytical bias is between 15-19% [25, 28] for the luminal A subtype, which is significantly lower than the 85% discordance observed in this study.

Table 4. Multivariable Cox proportional hazards analyses for 5-year post-recurrence breast cancer mortality (BCM)

	No. of cases	Relative hazard	95% CI	<i>P</i>
Biomarker status of primary tumors				
Estrogen Receptor	275			
Positive (reference)	210	1.0		
Negative vs. positive	65	2.2	1.5 - 3.3	<0.001
St Gallen subtype	175			0.003
Luminal A-like (reference)	64	1.0		
Luminal B-like vs. Luminal A-like	81	1.3	0.82 - 1.9	0.31
HER2 positive vs. Luminal A-like	8	2.5	1.0 - 5.8	0.04
Triple negative vs. Luminal A-like	23	3.1	1.7 - 5.9	<0.001
Biomarker status of metastases				
Estrogen Receptor	135			
Positive (reference)	95	1.0		
Negative vs. positive	40	3.0	1.8 - 5.0	<0.001
PAM50 subtype	78			0.018
Luminal A (reference)	5	1.0		
Luminal B vs. Luminal A	26	4.4	0.51 - 36.8	0.18
HER2-enriched vs. Luminal A	24	8.1	0.93 - 70.1	0.06
Basal-like vs. Luminal A	23	17.3	1.7 - 176.7	0.016

The analyses were adjusted for age at primary diagnosis (>50 years or ≤50 years), metastasis-free interval (≤2 years or >2 years), number of metastatic sites (multiple or single), site of metastasis (loco-regional vs. bone or lung or liver), nodal status (N+ or N0), adjuvant endocrine therapy (yes or no), and adjuvant chemotherapy (yes or no)

In addition, one would expect more frequent misclassification of luminal B to luminal A (35-52%) and not to the HER2-enriched subtype as observed herein. Taken together, a true subtype conversion may have occurred in a significant proportion of the discordant cases and the inferior outcome observed for these patients further emphasizes the clinical relevance of these results. The concordance between the clinical HER2-positive and the HER2-enriched subtype is moderate, while the triple negative and basal subtypes show the highest concordance [18, 25]; for the evaluable cases in the present study, we observed better preservation of primary tumor properties for these subtypes. Nonetheless, given the small number of cases included in our study and that of Falck and colleagues [27], further investigations are warranted to validate and extend these interesting results.

Currently, prognosis of MBC is established based on primary tumor characteristics, including ER and HER2 status. Although the independent prognostic power of these factors has been repeatedly validated [1], such analyses do not account for conversion of biomarkers and its consequences. In separate Cox-proportional multivariable models, ER and molecular subtype at time of primary diagnosis and at recurrence were both independent prognostic factors of breast cancer mortality; but interestingly, mortality risk was higher when biomarkers assessed at time of recurrence were modelled. However, because of unequal numbers of cases included in the different models, statistical tests to evaluate the best performing model were not performed. Notwithstanding, our data confirm the validity of ER and molecular subtypes both at time of primary diagnosis and at recurrence as independent prognostic biomarkers for clinical outcome in MBC.

Re-testing of hormone receptors and HER2 at recurrence is now included in many local and international guidelines to guide treatment decisions, but the consequences of receptor conversion on treatment decisions, especially in patients with previous receptor positive disease, remain controversial. We show retrospectively that ER loss or conversion from a luminal to a non-luminal subtype at recurrence is associated with an inferior prognosis, a finding corroborated by previous reports for ER [8, 9] and underscoring the importance of re-assessment of biomarkers in the event of breast cancer recurrence. Also, classifying primary tumors into molecular subtypes provides information beyond the standard pathological markers [18], but the clinical utility of currently available molecular subtype classifiers is being questioned [29]. Receptor discordance may be attributed to changes in disease biology, clonal selection following adjuvant systemic treatment, tumor heterogeneity, and less-than-perfect accuracy and reproducibility of analytical techniques [8, 12, 13, 30] and specific to this study, differences in the methods used for assigning subtypes to the primary tumors (IHC) and the asynchronous metastases (mRNA

profiles). In lieu of improving the agreement between the IHC and PAM50 subtype classifications, the revised St Gallen consensus guidelines [20], based on a recent report [28], recommends that a luminal A-like tumor must express both ER and PR, and the threshold for PR positivity was increased to 20%. In this study, 10% PR expression was considered as positive staining but almost all cases classified as positive using this threshold showed >20% positive staining. Among the individual biomarkers, we observed the highest discordance rates for PR (39% between primary tumor and asynchronous metastasis) with significant loss of expression at relapse (41% between synchronous LNM and asynchronous metastasis). This could in part explain the high rate of conversion from luminal A-like to luminal B at recurrence, with important implications of such a conversion on outcome as uncovered herein. Clearly, given the importance of translating the intrinsic subtypes into clinically useful groups, there is an urgent need for prospective data to strengthen the clinical validity and utility of individual biomarkers, especially cut-offs for Ki67 and PR, to perfect the surrogate identification of molecular subtypes [29].

In summary, receptor conversion and change in molecular subtype at recurrence can potentially affect tumor biology and prognosis of MBC as evidenced by data presented here. However, given the limitations in the size and design of this study, further prospective studies making provisions to minimize these biases are warranted to better understand the significance of these interesting results and also to improve the personalization of therapy for women diagnosed with MBC.

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Author contributions

SK, MF, and IH designed the study. JB, ZE, MF, and TH collected material and data. SK, AK, AD, KL, MFS, NPT, LL and IH carried out experiments or analyzed data. SK, PB, MF, and IH interpreted the results. SK and IH wrote the manuscript. All authors read and approved the submitted version of the manuscript.

Supplementary information

Supplementary Figure S1. Flow chart showing the selection of patients included at different tumor progression stages. Cases were excluded due to missing clinical data, unavailable tumor blocks, missing TMA data due to core loss or <10% tumor cells, or failed quality control for transcriptional profiling, respectively.

Supplementary Table S1. Baseline clinical and pathological characteristics of patients and primary tumors for the entire study cohort and the subsets of patients included on the two different tissue microarrays (TMAs).

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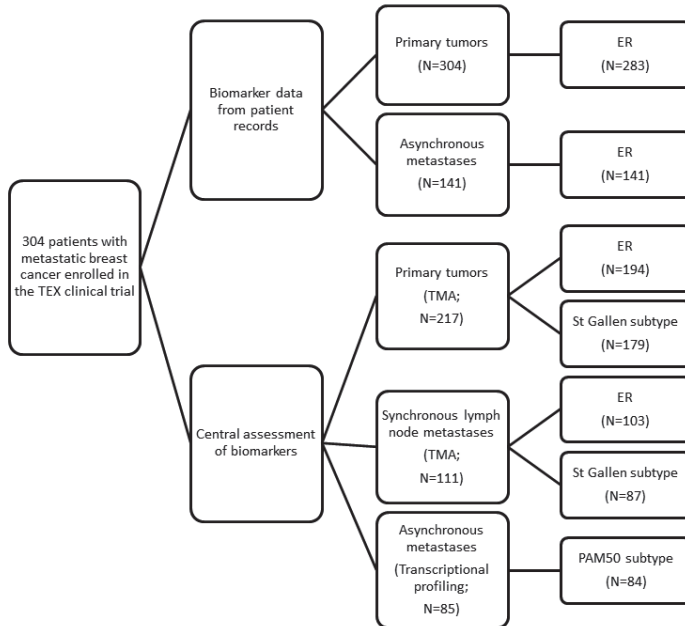
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Supplementary Table 1. Baseline clinical and pathological characteristics of all patients with metastatic breast cancer included in the TEX clinical trial and the subset of patients included on the 3 different tissue microarrays (TMAs).

	Tissue Microarrays (TMAs)		
	All Patients (N=304)	Primary Tumors (N=217)	Synchronous LNMs (N=111)
	N (%)	N (%)	N (%)
Age at diagnosis			
Median (Range)	50 (27-71)	51 (27-71)	51 (27-71)
< 50 years	154 (51%)	97 (45%)	52 (42%)
≥ 50 years	149 (49%)	118(55%)	72 (58%)
Missing/unknown	1	2	0
ER status			
Positive	215 (76%)	158 (81%)	75 (73%)
Negative	68 (24%)	36 (19%)	28 (27%)
Missing/unknown	21	23	8
PR status			
Positive	151 (57%)	110 (58%)	39 (38%)
Negative	114 (43%)	81 (42%)	64 (62%)
Missing/unknown	39	26	8
HER status			
Positive	n.a	17 (9%)	13 (14%)
Negative	n.a	180 (91%)	77(86%)
Missing/unknown	n.a	20	21
Ki67 status			
Positive	n.a	65 (36%)	33 (33%)
Negative	n.a	122 (64%)	66 (67%)
Missing/unknown	n.a	30	12
Histological grade (Pri tumour)			
1/2	121 (49%)	80 (43%)	39 (43%)
3	125 (51%)	105 (57%)	52 (57%)
Missing/unknown	58	32	20
Tumor size			
≤ 20 mm	119 (40%)	84 (40%)	31 (28%)
> 20 mm	180 (60%)	128 (60%)	79 (72%)
Missing/unknown	5	5	1
Nodal status			
N0	92 (31%)	68 (33%)	0 (0%)
N+	203 (69%)	141 (67%)	111 (100%)
Missing/unknown	9	8	0
Adjuvant chemotherapy			
Yes	150 (49%)	101 (47%)	72 (65%)
No	152 (50%)	114 (53%)	39(35%)

n.a, not available



Supplementary figure S1

Paper II

Transcriptional profiling of breast cancer metastases identifies liver metastasis-selective genes associated with adverse outcome in luminal A primary breast cancer

Siker Kimbung^{1,2}, Ida Johansson^{1,2}, Anna Danielsson³, Srinivas Veerla^{1,2}, Susanne Egyhazy⁴, Jonas Bergh⁴, Zakaria Einbeigi³, Barbro Linderholm³, Elisabet Lidbrink⁴, Niklas Loman⁵, Per-Olof Malmström⁵, Martin Söderberg⁵, Thomas Walz⁴, Märten Fernö¹, Thomas Hatschek⁴ and Ingrid Hedenfalk^{1,2} in collaboration with the TEX study group.

¹Division of Oncology and Pathology, Department of Clinical Sciences, Lund, Lund University, Sweden;

²CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden;

³Department of Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden;

⁴Department of Oncology and Pathology, Karolinska Institutet and Karolinska University Hospital, Sweden;

⁵Department of Oncology, Skåne University Hospital, Lund/Malmö, Sweden.

Corresponding author: Associate Professor Ingrid Hedenfalk, Division of Oncology, Department of Clinical Sciences, Lund, Lund University, Medicon Village, SE-22381 Lund, Sweden. Phone: +46-46-2220652. Fax: +46-46-147327. E-mail: Ingrid.Hedenfalk@med.lu.se

Abstract

The site of relapse is associated with the prognosis of metastatic breast cancer, but our understanding of the molecular determinants of organ-specificity of metastasis is incomplete. This study aimed to provide additional insight into the biology of breast cancer liver metastases and to identify liver metastasis-selective genes associated with outcome in primary breast cancer. Whole genome transcriptional profiling of clinical breast cancer metastasis biopsies from different anatomical sites revealed that the major variation in the transcriptional landscape of breast cancer metastases was associated with tumor pathological characteristics and molecular subtype. However, liver metastases displayed unique transcriptional fingerprints, characterized by down-regulation of extracellular matrix (*i.e.* stromal) genes involved in adhesion and skeletal development. Importantly, we identified a set of 17 liver metastasis-selective genes which were frequently down-regulated in estrogen receptor (ER) positive tumors with high histological grade and of the luminal B subtype. Furthermore, the 17-gene signature was significantly and independently prognostic of shorter relapse-free ($P = 3 \times 10^{-5}$) and overall ($P = 0.000927$) survival among patients with ER positive primary tumors. Remarkably, the 17-gene signature remained an independent predictor of shorter relapse-free survival ($P = 0.00097$) among patients with luminal A primary tumors. Taken together, these results highlight a possible role of stroma-related genes in liver metastasis biology and validate the prognostic relevance of extracellular matrix/stromal genes in hormone receptor positive primary breast cancer.

Keywords: breast cancer metastasis, transcriptional profiling, liver metastasis-selective genes stroma, luminal A, prognosis

Introduction

Metastasis is a very important clinical and socio-economic problem, accounting for over 90% of cancer-related deaths [1]. After the diagnosis of advanced or recurrent breast cancer, the site of metastasis is one of the most significant and independent prognostic factors for post-relapse survival, with liver colonization being associated with the poorest survival relative to loco-regional, bone and lung colonization [2-7]. Although only 5-20% of metastatic breast cancer (MBC) patients present with liver metastases as the first metastatic site, subsequently, about 50% of MBC patients will develop liver metastases through the clinical course of the disease [5, 8-11]. Noteworthy, diagnosis of liver metastases among MBC patients is on the rise [8, 12], which may suggest that available adjuvant therapies have limited efficacy for preventing the development of liver metastases, or may alternatively reflect technological improvements in the detection of liver metastases. Consequently, given the large number of patients potentially at risk of developing overt liver metastases, new biomarkers for predicting future site/s of relapse of a primary tumor are necessary to guide surveillance and improve personalization of therapy.

Metastatic colonization in breast cancer is not a random process. Once disseminated, circulating tumor cells exhibit tissue specific tropisms beyond what can be explained by normal circulatory patterns. This tissue specificity of metastases has been associated with pathological and molecular characteristics of the primary tumor [13, 14], but the marked heterogeneity within groups defined by these factors limits their ability to accurately predict the site/s of recurrence. For example, the intrinsic breast cancer subtypes display divergent metastatic site preferences, with luminal tumors frequently colonizing the bone and liver, while the non-luminal subtypes show preferences for the lungs, liver and brain. Conventionally, at time of primary diagnosis, the prognosis for a favorable outcome and decision for exemption from toxic chemotherapy treatment is based on a combination of factors including estrogen receptor (ER) positivity, negative nodal status, small tumor size and low histological grade (1 and 2) [4]. These favorable prognostic factors are also significantly enriched within the luminal A intrinsic subtype. However, intrinsic or acquired resistance to therapy and disease recurrence to distant sites including the liver may eventually occur, albeit late and in a

limited, but clinically significant, number of patients with luminal A breast cancers, underlining the heterogeneity even within this favorable subtype. Metastases remain the main cause of breast cancer-related mortality and these patients eventually die from their metastatic disease. It is therefore necessary to identify better site-specific biomarkers, or better yet, subtype-specific prognostic and predictive biomarkers to enhance individualization of therapy.

While several studies have shown that primary tumors and their metastases share similar copy number changes [15, 16] and gene expression profiles [17, 18], differences between these matched tumor pairs were not reported, most likely due to small sample sizes in these studies. Utilizing experimental mouse models and limited series of clinical metastatic biopsies, genes and signatures predicting the propensity of a primary breast tumor to relapse in bone [19], lung [20, 21], brain [22] and liver [23] have been published. However, because the experimental model systems incompletely capture the relevant genetic complexity of the tumors and the contribution of the host microenvironment, studies using clinical biopsies from metastases are required to validate and extend these findings. In addition, clinically relevant site-specific metastasis genes may be more readily identified by directly profiling metastatic lesions from breast cancer patients as this approach will take advantage of the natural selection of tumor cells exhibiting preferential tissue tropisms and the ability to grow as overt metastases at the specific distant site.

The aim of this study was to provide an independent description of the similarities and differences in clinico-pathological factors and transcriptional landscapes between breast cancer liver metastases compared with breast cancer metastases from other anatomical sites. In addition, we sought to identify breast cancer liver-selective genes differentially expressed in primary breast tumors with different metastatic site tropisms, to investigate their association with molecular and pathological characteristics of the primary tumors and test their potential to predict outcome after primary diagnosis.

Materials and Methods

Ethics statement

The regional ethics committees at all participating centers approved this study.

Patients and tumors

The study cohort consisted of 304 women diagnosed with locally advanced (in-operable) and metastatic breast cancer and enrolled in a randomized phase III trial (TEX) conducted between 2002 and 2007 across different treatment centers in Sweden. As first line treatment for metastatic disease, patients received a combination of an anthracycline (Epirubicin) and a taxane (Paclitaxel) with or without the addition of capecitabine (Xeloda). Conditions for trial exemption included brain metastases, indication for HER2-targeted therapy, or other malignancies diagnosed within five years of trial commencement. Clinical and pathological data were collected from the central clinical trial database. The initial metastatic sites (which could be single or multiple) were recorded and patients were classified into four main metastatic categories (loco-regional: locally advanced or regional metastases in the lymph nodes or skin; bone: skeletal metastases with or without loco-regional metastases; lung: plural metastases with or without skeletal and loco-regional metastases; liver: hepatic metastases with or without plural, skeletal or loco-regional metastases). The median follow-up for post-recurrence survival was 45 months (range 9 -135 months) for patients alive at last update. Detailed information regarding the design and outcome of the trial has been published [24]. Fine-needle aspirates (FNA) of at least one metastatic lesion were collected before commencement of treatment whenever possible. In addition, archival formalin-fixed paraffin-embedded primary tumor blocks were collected for tissue microarray (TMA) construction and central assessment of biomarkers by immunohistochemistry and *in situ* hybridization techniques where applicable. Using a combination of four biomarkers (ER, PR, HER2 and Ki67), surrogate molecular subtypes were assigned to primary tumors according to the recommendations of the 13th St Gallen International experts' consensus on primary therapy for early breast cancer [25]. Table 1 shows the distribution of clinico-pathological factors and adjuvant treatment in the cohort.

RNA extraction and hybridization

Tumor cellularity of FNAs was assessed by a pathologist on Giemsa stained, ethanol-fixed, cytospin preparations and total RNA was extracted from samples with high (>50%) tumor cell content using TRIzol (Invitrogen, Stockholm, SE)

following the manufacturer's recommendations. RNA quantity and integrity were analyzed using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) respectively, and biotin labelling of cDNA was performed using the NuGen 50ng amplification protocol (Covance Genomics Laboratory, Princeton, NJ). Labelled cDNA probes were hybridized onto custom-made whole genome Affymetrix HuRSTA-2a520709 gene chips following the GeneChip Hybridization, Wash, and Stain Kit according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Data pre-processing and normalization were performed using the robust multichip average (RMA) algorithm. After normalization, a presence filter was applied to select only probes present in $\geq 90\%$ of assays, while probes with intensities below the median intensity for Y chromosome gene probes were filtered out. The data were \log_2 transformed and transcripts showing high variance across assays were selected (variance filter $sd \geq 1$), leaving a final dataset with 8,339 features representing 5,232 unique gene variants for further analyses. All processes were performed using packages in R [26] and the TM4 microarray software suite [27]. The final dataset included 91 samples from 85 patients [liver (n=16), bone (n=5), lung (n=2), lymph node (n=39), local [breast (n=11) and skin (n=17), and ascite (n=1)]. The distribution of baseline clinico-pathological features in the original study cohort (n=304) and the subpopulation included in the transcriptional profiling experiment (n=85) is presented in Table 1. Raw and processed data are available in the Gene Expression Omnibus (GSE46141).

Multi-variable data analyses

Unsupervised analyses

Principal component analysis (PCA) was performed using SIMCA P version 13.0.2 software package (Umetrics AB, Umeå, Sweden). The dataset was mean-centered across rows (genes), unit variance scaled and model complexity was estimated by leave-one-out cross-validation. Unsupervised hierarchical clustering (HCL) was performed using the Pearson correlation distance metric and average linkage.

Supervised analyses

The intrinsic molecular subtypes of the metastases were determined by the PAM50 nearest centroid algorithm as previously described [28]. To identify genes differentially expressed between liver metastases and other sites, an unpaired two-class significance analysis of microarray (SAM; [29]) analysis was performed. The liver-selectivity of the differentially expressed genes was verified in an external breast cancer metastasis dataset (GSE14018, [30]). In addition, gene ontology enrichment analysis was performed in the DAVID [31, 32] database. Differences in the transcriptional biology of metastases associated to the site of relapse were further uncovered by comparing the activity of eight gene expression-based modules representing relevant breast cancer-specific biological processes (stroma, lipid, immune response, mitotic progression, mitotic checkpoint, basal, early response and steroid response; [33]). For each gene module, a score was computed for every sample by taking the average expression of all the genes in the module. Candidate genes among the genes differentially expressed in liver metastases which may serve as potential biomarkers associated with the liver metastatic potential of a primary tumor were validated in an external dataset of 192 primary breast tumors (GSE12276) [18]. Finally, associations between the candidate genes and outcome in early breast cancer were independently tested in the Gene expression-based Outcome for Breast cancer Online (GOBO; [34]), an online tool for validation of the prognostic value of single genes or sets of genes on breast cancer survival in a pooled dataset of primary breast cancers (n=1,881).

Survival analyses

Kaplan-Meier plots were generated and the Log-rank test was used to check for statistically significant differences between target groups of patients. Univariate and multivariate Cox-proportional hazards models were used to evaluate the independent prognostic significance of the site of recurrence, adjusting for conventional prognostic factors. Proportional hazards assumptions were checked by graphical methods. The statistical software package IBM SPSS Statistics 19 (IBM Corporation, NY) was used. *P*-values correspond to two-sided statistical tests and values <0.05 were considered significant.

Results

Liver relapse is associated with adverse clinico-pathological features and inferior outcome

We recently reported that the site of metastasis is prognostic for survival after recurrence in the present cohort [7], with liver metastases significantly associated with the poorest post-recurrence survival. However, there have been reports suggesting that patients with oligo-metastases in the liver experience longer survival compared to patients harboring metastases in other sites parallel to the liver [5, 8]. Our investigations corroborated these reports, as we found that patients harboring single metastatic deposits in the liver experienced survival rates comparable to patients without hepatic involvement, and that the inferior outcome was specific to patients with liver metastases occurring in parallel with metastases in other organs (Figure 1, Log-rank $P=0.01$, Multivariate Cox model $P<0.001$).

Next, we investigated associations between primary tumor clinico-pathological factors and the site/s of metastases (Table 1). ER positivity was found to be associated with bone and liver recurrences, while negative ER status correlated with loco-regional and lung relapses (Fisher's exact $P=0.002$). Loco-regional and bone metastases were often detected as oligo-metastases, while liver and lung metastases were often diagnosed in parallel with deposits in other sites ($P<0.001$).

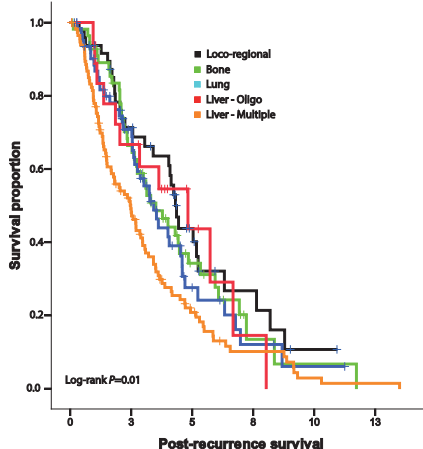


Figure 1. Post-recurrence survival according to metastatic category. Patients were categorized according to the most advanced metastatic site (loco-regional, locally advanced or regional metastases in the lymph nodes or skin; bone, skeletal metastases with or without loco-regional metastases; lung, plural

metastases with or without skeletal and loco-regional metastases; liver, hepatic metastases with or without plural, skeletal or loco-regional metastases). In addition, patients with liver recurrences were further stratified into two groups based on the number of sites involved (oligo, n=1 and multiple, n>1). A significantly inferior survival was observed for patients with liver metastases occurring parallel with metastatic deposits in other organs.

A similar distribution was observed when only the subset of ER positive tumors were considered ($P<0.001$). Furthermore, low histological grade (grades 1 and 2) was associated with bone and liver recurrences, while high grade (grade 3) correlated with loco-regional and lung relapses ($P=0.03$), but no significant association between tumor histological grade and metastatic site was observed when ER positive tumors were analyzed separately ($P=0.58$). When the primary tumor surrogate (IHC-based) molecular subtype was considered, bone and hepatic recurrences were associated with luminal (A and B) tumors, while relapses to the lung and loco-regional sites were associated with the triple-negative subtype (Fisher's exact test $P=0.01$). Consistently, sub-analyses within ER positive tumors revealed a borderline association of bone and liver metastases with the luminal A and luminal B subtypes, respectively ($P=0.05$). Overall, these results confirm that pathological biomarkers and the molecular subtypes provide important insights into a primary tumor's preferential site/s of relapse, and that liver metastases are commonly observed in pathological subgroups associated with poor prognosis. Nevertheless, there seems to be a remarkable prevalence of liver metastases even among subgroups associated with a favorable outcome.

Identification of shared and specific transcriptional portraits of liver metastases compared with metastases from other anatomical sites

The transcriptional landscape of breast cancer metastases has generally been inferred from primary tumors due to scarcity of clinical biopsies from metastases to perform independent studies. PCA analyses revealed that the first two principal components in the transcriptional fingerprints of breast cancer metastases were mainly associated with tumor biological factors, including ER expression in the primary tumor (Figure 2A) and the intrinsic molecular subtypes of the metastases

(Figure 2B). In addition, liver metastases were tightly clustered in the PCA score plot (Figure 2C), indicating unique transcriptional characteristics of liver metastases relative to other metastases. A similar clustering pattern was observed by unsupervised hierarchical clustering of the samples using the top 3,000 most variable features (Figure 2D). Of note, all biological replicates (independent metastatic biopsies from the same patient) clustered together pair-wise and adjacent to each other in the sample dendrogram, confirming that transcriptional profiles of intra-individual tumors are more similar than inter-individual profiles.

To provide more insight into the biology of site-specific metastases, we compared the activity of eight cancer modules representing key biological aspects specific to breast cancer [33]. We observed significant differences in the expression of four modules between the site-specific metastatic biopsies (Figure 3A-D). Relatively lower expression of the 'stroma' (Kruskal-Wallis $P=0.004$), 'basal' ($P=0.045$) and 'early response' ($P=0.008$) modules and high expression of the 'steroid response' ($P=0.006$) module was characteristic of liver metastases. Following the notion that the transcriptional profiles of multiple tumors from one individual are highly similar, the activity of the gene modules were compared between groups of patients stratified according to the most advanced metastatic site recorded, irrespective of the specific site from which the FNA biopsy profiled was obtained. Interestingly, similar distributions of the four modules were seen (Figure 3E-H). Low expression of the 'stroma' module was observed in tumors from patients with hepatic recurrences ($P=0.021$). In addition, the 'basal' module was high in the lung and local relapse categories ($P=0.024$), while 'steroid response' was higher in the liver and bone relapse categories ($P=0.003$). These results further confirm the association between the site of metastasis and intrinsic subtypes since the 'basal' and 'steroid response' modules were very strongly associated with the basal-like and luminal subtypes, respectively. In addition, a novel association between the expression of stromal and early response genes and breast cancer hepatic recurrences was uncovered.

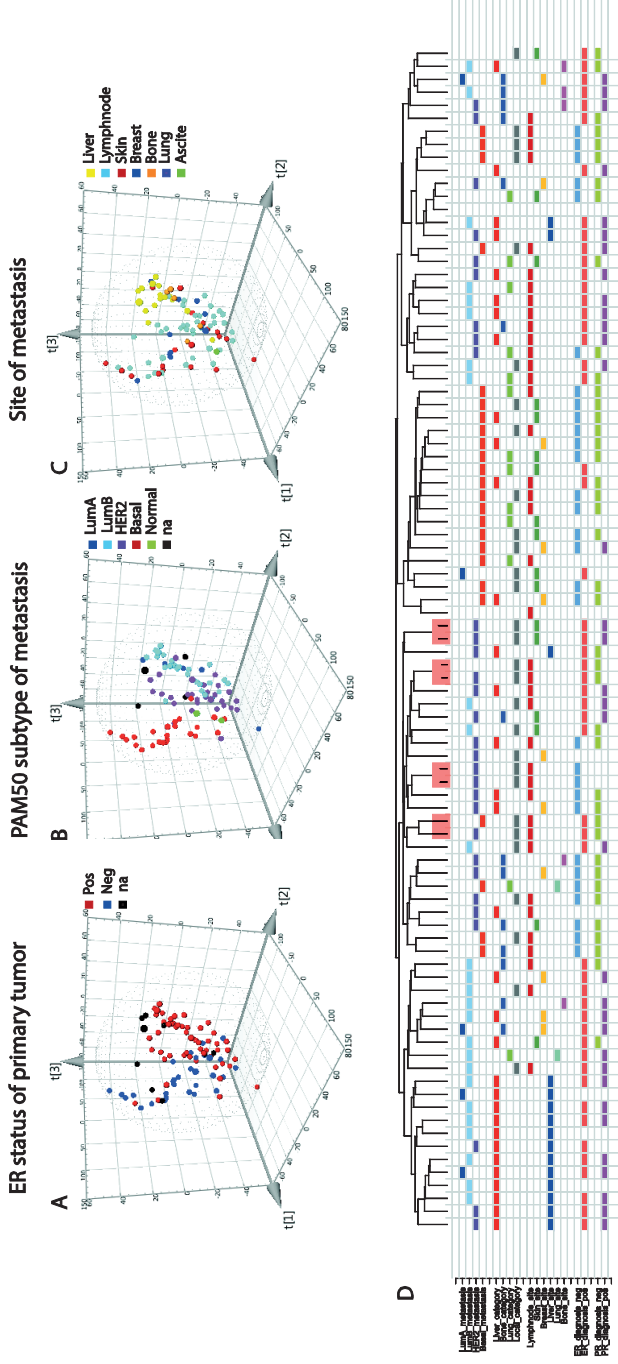


Figure 2. Unsupervised analyses of transcriptional similarities and differences between breast cancer site-specific metastases in the test cohort. PCA analyses of breast cancer metastases showing differences in global transcriptional profiles. Each circle represents the global gene expression profile of a sample and samples with similar profiles cluster close to each other in the three-dimensional space. Samples in the PCA score plots are colored according to **A**) ER status of the primary tumor, **B**) intrinsic subtype of the metastasis and **C**) specific site of the metastatic biopsy profiled. The contribution of the first three components in explaining the observed variation in the data is as follows: $PC1=(1)=15.1\%$, $PC2=(2)=8.52\%$, and $PC3=(3)=4.38\%$. (Overall Model coefficients: $R2X=(3)=0.512$ and $Q2=$ variation from cross-validation $=0.261$). **D**) Dendrogram showing HCL of samples using the top 3,000 most variable probes. Data were mean-centered across samples and clustering was performed using the Pearson correlation distance metric and average linkage. Highlighted samples in the tree represent biological replicates.

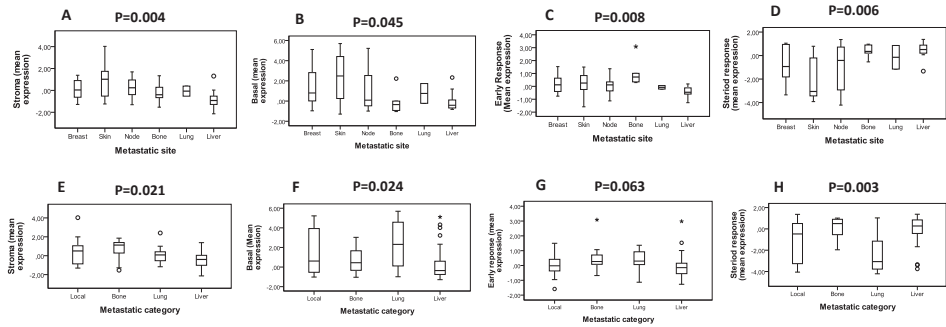


Figure 3. Associations between key breast cancer-specific biological gene modules and site of metastasis in the test cohort. A-D represents comparisons between specific metastatic sites and E-H represents comparisons between patient metastatic categories. *P*-values are from Kruskal-Wallis tests. The open circles and asterisks represent mild and extreme outliers respectively for each group in each comparison. All statistical tests are two-sided.

Table 1. Associations between the first site(s) of metastasis and clinico-pathological characteristics of primary tumors and metastases in the test cohort. Patients were categorized according to the most advanced metastatic site affected (loco-regional, locally advanced or regional metastases in the lymph nodes or skin; bone, skeletal metastases with or without loco-regional metastases; lung, plural metastases with or without skeletal and loco-regional metastases; liver, hepatic metastases with or without plural, skeletal or loco-regional metastases). *P* values are from Fisher's exact tests.

	All tumors						ER positive tumors					
	N	Metastatic category				<i>P</i>	N	Metastatic category				<i>P</i>
		Loco-regional	Bone	Lung	Liver			Loco-regional	Bone	Lung	Liver	
Primary tumor characteristics												
ER status												
Negative	68	20	7	19	22	0.002						
Positive	213	29	43	40	101							
PR status												
Negative	81	18	10	18	35	0.21	44	8	5	7	24	0.32
Positive	110	16	25	21	48		107	16	25	20	46	
HER2 status												
Negative	179	32	32	37	78	0.76	143	23	29	24	67	0.39
Positive	17	2	2	5	8		8	0	1	3	4	
Number of metastatic sites												
Oligo (n=1)	76	25	23	9	19	<0.001	57	17	19	5	16	<0.001
Multiple (n>1)	226	25	33	54	114		156	12	24	35	85	
Histological grade												
Grade 1/2	80	9	17	12	42	0.03	72	9	15	10	38	0.58
Grade 3	105	24	15	26	40		67	10	13	15	29	
Adjuvant endocrine therapy												
No	147	27	30	37	53	0.05	73	8	19	17	29	0.17
Yes	154	22	26	26	80		140	21	24	23	72	
Age at primary diagnosis												
<50 years	152	17	28	37	70	0.08	107	12	19	24	52	0.38

≥50 years	149	32	28	26	63		106	17	24	16	49
Metastasis-free interval											
≤24 months	80	15	15	16	34	0.91	55	7	11	11	26
>24 months	221	34	41	47	99		158	22	32	29	75
Nodal status											
N0	91	14	17	22	38	0.69	65	7	12	16	30
N+	202	35	37	37	93		145	22	30	23	70
Tumor size											
≤20 mm	119	14	26	21	58	0.21	82	9	20	12	41
>20 mm	178	33	30	40	75		128	18	23	27	60
Molecular subtype (St Gallen)											
Luminal A-like	65	9	19	11	26	0.01	65	9	19	11	26
Luminal B-like	81	13	9	16	43		81	13	9	16	43
HER2 positive	9	2	1	2	4						
Triple negative	24	8	2	9	5						

To identify other transcriptional differences specific to liver metastases, but outside the scope of the pre-defined gene modules, a two-class SAM analysis was performed using the site of the profiled metastasis (liver vs. others) as supervising variable. This analysis was restricted to metastases from patients with ER positive primary tumors, since the majority (12/16) of the profiled liver metastases were of this phenotype. We found 358 genes to be differentially expressed between liver compared to other metastases (309 up-regulated and 49 down-regulated, FDR=0.1; Supplementary Table 2); from here-on these genes are referred to as ‘breast cancer liver metastasis-selective genes’. An external dataset of breast cancer metastases [30] was used to analyze the expression pattern of these 358 genes. Through one-way HCL, we observed a very similar pattern of expression of these genes in the liver metastases (Supplementary Figure 1), thus confirming their liver selectivity.

Next, the ‘breast cancer liver metastasis-selective genes’ were subjected to functional/pathway enrichment analysis [31, 32]. Among the genes up-regulated in liver metastases, we observed biological processes and molecular functions associated to extracellular space, endopeptidase inhibitor activity, complement activation and blood coagulation, immune response, steroid metabolism as well as other molecular processes commonly occurring in the normal liver (Supplementary Table

3). Conversely, processes associated with extracellular matrix, adhesion, skeletal system development, and blood vessel development were enriched among the genes down-regulated in liver metastases (Supplementary Table 4). To ascertain that the enrichment of liver-related biological functions among the up-regulated genes was not associated with the potential of normal tissue contamination due to tumor cell content heterogeneity, we performed unsupervised HCL of samples in our dataset using genes previously reported to be relatively over-expressed in normal breast and normal liver tissue [35], as well as in breast cancer [36], respectively. Reassuringly, even though the liver metastases formed a distinct cluster in the sample dendrogram when clustered on the normal liver genes (Supplementary Figure 2A), no separation of the samples based on metastatic site was seen upon clustering with the normal breast (Supplementary Figure 2B) or breast cancer specific genes (Supplementary Figure 2C). Instead, clustering correlated with other biological characteristics, such as ER expression and molecular subtype. These results confirm that the liver metastases display an expression profile consistent with the site of origin of the tumor cells (breast), and in addition share other transcriptional features associated with the host microenvironment (liver).

Associations between breast cancer liver metastasis-selective genes and primary tumor patho-biological factors and clinical outcome

Robust tissue-specific biomarkers [gene(s)] of metastasis may be detectable in the primary tumors of patients who eventually develop metastases in the corresponding target organ, as revealed by studies reporting site-specific signatures of breast cancer metastasis for lung, bone and brain recurrences [19, 20, 22]. Using an external primary breast cancer dataset including only patients with metastatic disease and for which the annotation of the site(s) of metastasis could be retrieved [18, 22] we performed a two-class SAM analysis comparing tumors with a predilection to relapse in the liver with tumors with other metastatic site preferences. This analysis was restricted to the subset of ER positive tumors (n=119). 347/358 of the 'breast cancer liver metastasis-selective genes' identified in our test dataset could be mapped across platforms using official gene symbols as identifiers. We found

17/347 genes to be differentially over-expressed in tumors that relapsed in the liver. Figure 4 shows the expression of these 17 genes in our test (metastases) cohort. This list was enriched for genes involved in the cadherin and integrin signaling pathways, as well as in skeletal system development. Surprisingly, 14/17 liver metastasis-selective genes displayed an inverse expression pattern between primary tumors with liver metastatic potential and liver metastases, *i.e.* a relatively higher expression in primary tumors but low expression in liver metastases. However, 3/17 genes (*CDH2*, *CYP39A1*, *CAMK2N1*) were concordantly over-expressed in both the primary tumors metastasizing to the liver and the liver metastases. Of note, 6/17 (*CDH11*, *COL11A1*, *FBN1*, *MFAP5*, *SFRP4*, *SPON1*) genes overlapped with the previously described 'stroma' module, and a high Spearman correlation coefficient (median $r > 0.7$) was obtained when comparing the expression of genes from the two signatures.

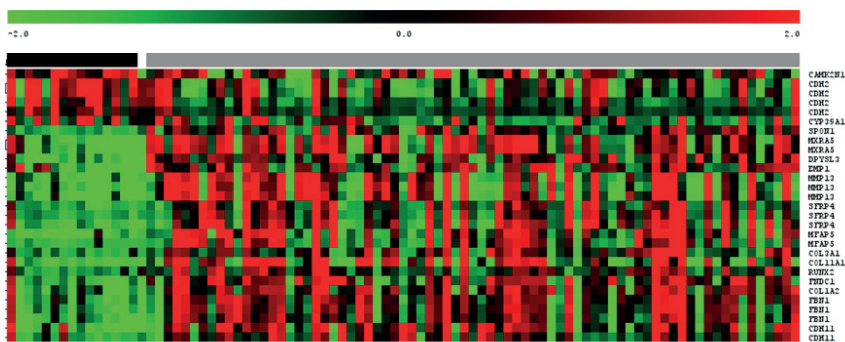


Figure 4. Expression of liver-selective genes in metastases in the test cohort. The heatmap shows the expression in the metastases of the 17 liver-selective genes found to be differentially expressed in primary tumors with a predilection to metastasize to the liver compared to other sites. Red corresponds to up-regulated genes and green corresponds to down-regulated genes. The color scale represents the mean centered log₂ expression. Black in the top bar represents liver metastases and gray represents metastases from other anatomical sites.

Finally, we sought to identify relevant associations between the collective expression of the 17-gene signature and other primary tumor pathological factors and prognosis in a large collection of primary breast cancers using the online tool GOBO. Associations between pathologically and molecularly defined patient groups were investigated by stratifying patients based on gene expression quantiles (using median expression). We

observed a lower expression of the 17-gene signature in the luminal B compared to the luminal A subtype (Figure 5A-B, Anova $P < 0.00001$) and a negative correlation with histological grade (Figure 5C-D, $P < 0.00001$). Remarkably, low expression of the 17-gene signature was significantly associated with shorter recurrence-free survival (RFS; Figure 6A, log-rank $P = 3 \times 10^{-5}$, Supplementary table 5; multivariate Cox model $P < 0.001$) and overall

survival (OS; Figure 6C, log-rank $P=0.00927$, Supplementary table 5; multivariate Cox model $P=0.026$) in patients with ER positive primary tumors. More importantly, the 17-gene signature remained significantly and independently prognostic for RFS when the subset of luminal A tumors were analyzed separately (Figure 6B, log-rank $P=0.00097$, Supplementary table 5; multivariate Cox model $P=0.004$). A trend toward poor OS for patients with luminal A tumors with low expression of the 17-gene signature was observed in univariate analysis (Figure 6D, log-rank $P=0.083$, Supplementary table 5; multivariate Cox model $P=0.29$). Exploratory analyses also confirmed the poor prognostic significance of the signature when all samples were included in the analyses, irrespective of ER status (Supplementary Figure 3).

Discussion

In this study we identified a 17-gene signature enriched for extracellular matrix/stroma genes, the majority of which are selectively down-regulated in breast cancer liver metastases and whose expression in primary tumors revealed clinically important associations with tumor biological factors and the ability of a primary breast tumor to spread. To discover this signature, we followed a pipeline of initially comparing global transcriptional profiles of metastatic biopsies to identify liver metastasis-selective deregulated transcripts, and subsequently by using an external primary tumor dataset, we performed further data mining to identify a subset of the liver metastasis-selective genes associated with liver metastatic potential in primary tumors. Our analysis was restricted to genes in common across the different microarray platforms used in the various studies, and is based on the assumption that differential expression of all site-specific metastasis genes is already overtly manifest in the bulk of the primary tumor. Importantly, this approach allowed us to exclude expression patterns

that may be associated with cross-platform or technical variability and improved our ability to identify robust liver metastasis-selective genes which may be useful to guide disease monitoring, prognosis and early identification of patients most likely to experience disease recurrence in the liver. Liver metastases are by far the most deleterious and account for early death from MBC in patients with ER positive tumors [2-7]. We observed significant associations between poor tumor biological characteristics, including the luminal B molecular subtype, high histological grade and high tumor burden (multiple metastatic sites) with the predilection to metastasize in the liver. Importantly, a considerable overlap of the prevalence of liver relapses was observed between all subgroups, indicating low specificity and sensitivity of these predictors. Since liver relapse is indicative of inferior post-recurrence survival, there is an urgent need for more specific and independent biomarkers to identify patients at risk. Recently, we demonstrated that CLDN2, which is significantly up-regulated in liver metastases, is an independent prognostic factor for early liver recurrence in breast cancer [7]. Our analyses of post-recurrence survival according to tumor burden showed that patients with solitary liver metastases experienced a better outcome compared to patients harboring liver metastases in addition to metastases in other organs. This finding is corroborated by other independent studies [5, 8]. The controversial clinical question of whether more radical treatment, such as surgical excision of the metastasis, may improve the survival of patients with solitary liver metastases has been raised. Our results, in combination with other reports [5, 8] support a proposal for more radical management of this sub-group of patients, which may improve the quality of life given the extended median survival observed with the current standard of care. In fact, a few small investigations have reassuringly indicated survival benefits after surgical removal of solitary liver metastases [37, 38].

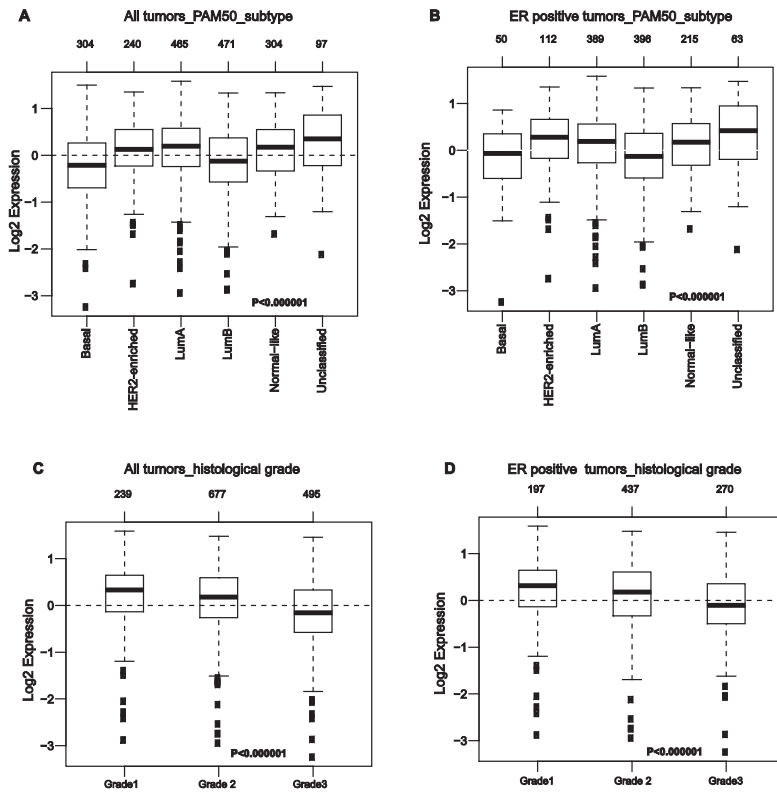


Figure 5. Expression of liver-selective genes in primary breast cancer in the validation cohort (GOBO). The boxplots illustrate the median expression of the 17 liver metastasis-selective genes in primary breast tumors. Tumors were stratified according to the PAM50 intrinsic subtypes: **A)** all tumors and **B)** ER positive tumors; and tumor histological grade: **C)** all tumors and **D)** ER positive tumors. *P*-values are from anova tests. The filled squares in the figures represent mild outliers for each group in each comparison. All statistical tests are two-sided.

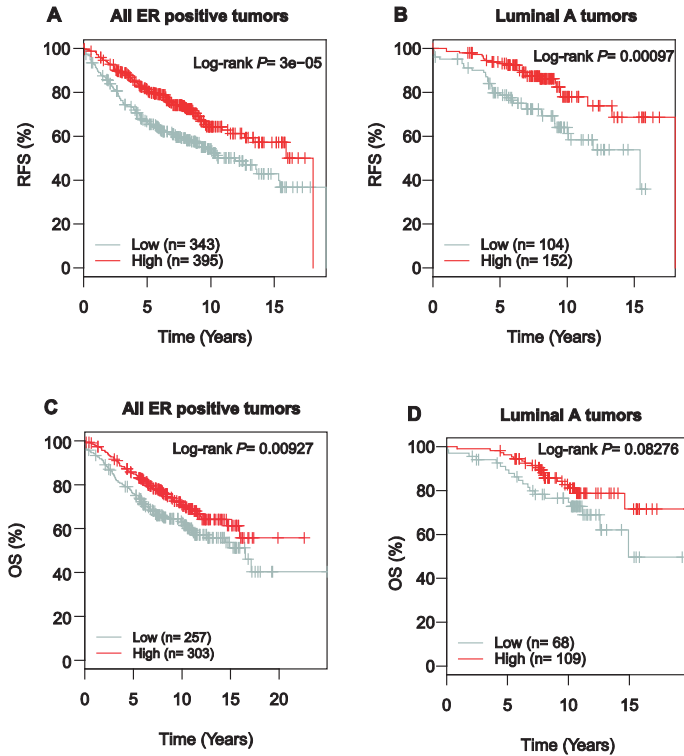


Figure 6. Liver-selective genes and survival in the validation cohort (GOBO). Relapse-free survival (RFS) and overall survival (OS) in patients with ER positive disease included in the tumor collection within the GOBO repository are shown. **A)** RFS for all ER positive tumors, **B)** RFS for luminal A (PAM50) tumors only, **C)** OS for all ER positive tumors, and **D)** OS for luminal A tumors only.

Transcriptional profiling has increased our understanding of the biology of organ-specific metastases and has led to the identification of site-specific metastasis genes and signatures [19, 20, 22, 23]. PCA and unsupervised HCL analyses of the metastases in our test dataset revealed that the major variation across samples was strongly associated with tumor pathological characteristics, an observation consistent with the conventional understanding of breast cancer biology. This similarity underscores the conservation of molecular fingerprints across tumor progression stages. Interestingly, we observed minor but significant site-specific differences at the transcriptional level, which may indicate further traits that cancer cells must acquire to thrive in the foreign milieu as they evolve into manifest metastases in secondary organs. Gene set enrichment analyses revealed a significant down-

regulation of extracellular matrix genes and genes involved in cell adhesion and the development of blood vessels and the skeletal system, which are all processes that have been linked with invasion and metastasis in breast cancer [39]. Of note, the top down-regulated gene was the epithelial mesenchymal transition inducer *PRRX1*, recently reported to play an important role in metastatic colonization through repression of its expression to favor reversion of the mesenchymal phenotype necessary for the outgrowth of metastases at distant sites [40]. On the other hand, an enrichment of genes and processes common in normal liver was observed among up-regulated genes. Importantly, most of these genes correspond to signaling peptides commonly found in the extracellular space, further highlighting the importance of the tumor microenvironment in metastatic colonization. The deregulation of genes which mimic target organ

functions is a theme commonly observed in other independent organ-specific metastasis research: differential expression of genes important for ossification in bone metastases [19, 41], brain metabolism in brain metastases [22], pulmonary function in lungs [20, 21] and liver function in liver metastases [23] have been reported. This phenomenon can be interpreted, within the confinements of the “seed and soil theory” of tumor invasion and metastatic colonization [42], as further adaptive measures undertaken by tumor cells to survive and grow in specific microenvironments. Of note, mimicry of target-organ properties was observed when pure tumor cell line populations displaying distinct site-specific preferences were used [19, 20, 22, 23], suggesting that part of this expression profile is indeed intrinsic to the tumor cells. Furthermore, our observation that samples did not cluster according to metastatic site when subjected to HCL on breast cancer-selective genes or normal breast selective genes also supports the notion that part of our observed expression pattern is indeed tumor cell intrinsic. The possibility of normal tissue contamination can however not be completely ruled out.

Currently, prediction of the prognosis of an ER positive breast cancer at the transcriptional level is limited to the expression of proliferation-related genes, but proliferation alone is not sufficient to account for all the recurrences observed among patients with ER positive breast cancer, especially those with luminal A tumors which generally are of a low proliferating phenotype. Our 17-gene signature was inversely associated with proliferation, since we observed lower expression of the signature in high histological grade and luminal B tumors compared to luminal A subtype. Remarkably, low expression was an independent predictor of shorter time to recurrence and shorter overall survival among ER positive tumors. Importantly, the 17-gene signature remained independently prognostic among patients with luminal A tumors in multivariable analyses adjusting for conventional prognostic factors. The fact that all 17 genes in our signature were found to be over-expressed in the group of primary tumors from patients who subsequently developed liver metastases compared to patients with other metastases is surprising since the majority of them were under-expressed in the liver metastases. However, low expression as observed in the liver metastases was prognostic of a poor outcome. The scarcity of datasets with annotations for the

metastatic site(s) hindered an evaluation of the ability of this signature to specifically predict breast cancer liver recurrence. Diagnosis of oligo-metastatic disease is uncommon. Hence, the classification of patients into different metastatic categories as presented in this study is confounded by intra-individual overlap of metastatic sites and may restrict our ability to identify an independent site-specific signature. The ideal way to confirm the ability of these 17 genes to predict the liver-specific metastatic potential would therefore require a well-annotated and sufficiently large independent cohort of patients with oligo-metastatic disease, which is difficult to achieve given the rarity of patients presenting with oligo-metastasis in the liver. Notwithstanding this limitation, our analysis pipeline beginning with analyzing clinical samples of metastases enabled us to identify an important gene set and independently validate its clinical relevance in a large cohort of primary breast cancer. Predicting the future metastatic site of a primary breast cancer is multifaceted and challenging. In their recent study aimed at unraveling how bone-specific metastatic traits arise in the primary tumor, Zhang and colleagues [41] showed that stromal signals resembling those of the distant target organ play very important roles at the primary tumor site to prime tumor cells for colonization of a specific metastatic niche. Also, three independent gene modules enriched for extracellular matrix (*i.e.* stroma) genes were among the 11 gene-modules recently identified to shape the transcriptional landscape of breast cancer [43]. More interestingly, in this study [43], only the expression of the ECM modules showed significant associations with site of metastasis, although liver metastases were not included among the sites considered. Our 17-gene signature was enriched for stroma-related genes and was significantly correlated to the stroma module [33] and consistent with our results, Fredlund *et al.* [33] found that low expression of the stroma module was associated with shorter distant metastasis free survival among patients with luminal A primary tumors. Furthermore, an independent study by Bergamaschi and colleagues [44] identified four extracellular matrix gene modules (ECM1 – ECM4) with prognostic significance in luminal breast cancer, but unfortunately their analyses did not address the luminal A subtype as a separate entity in survival analyses. Down-regulation of several genes in our signature was characteristic of the ECM1 module from [44], which was associated with the poorest

outcome. Taken together, these studies highlight the possibility of harnessing the heterogeneity in the expression of extracellular matrix/stroma genes to improve prognostication in hormone receptor positive disease.

We have identified a 17-gene signature enriched for genes selectively under-expressed in breast cancer liver metastases, with a remarkable ability to significantly and independently identify patients with luminal A primary breast cancers who may benefit from closer disease monitoring and additional therapeutic intervention. Further studies are warranted to validate our results in more recent patient series to adjust for modern advances in primary breast cancer management.

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Supplementary material

Supplementary Table 1. Baseline clinical and pathological characteristics in the test cohort. Data are shown for all patients with metastatic breast cancer included in the TEX clinical trial and the subset of patients included in whole genome transcriptional analyses.

Supplementary Table 2. Differentially expressed genes between liver metastases and metastases from other anatomical sites in the test cohort. Two-class SAM analyses were performed within the subset of ER positive tumors using the site (liver vs. other) of metastasis as supervising variable. Genes were considered significant at FDR=0.1.

Supplementary Table 3. GO analyses. Enrichment in biological processes (GO terms) among the up-regulated breast cancer liver metastasis-selective genes.

Supplementary Table 4. GO analyses. Enrichment in biological processes (GO terms) among the down-regulated breast cancer liver metastasis-selective genes.

Supplementary Table 5. Multivariable Cox proportional hazards analyses for relapse-free survival (RFS) and overall survival (OS) among patients with ER positive primary tumors in GOBO. Separate analyses are shown for all ER positive tumors and the subset of luminal A tumors. $P < 0.05$ was considered significant.

Supplementary figure S1. Unsupervised HCL of an external breast cancer metastasis dataset (GSE14018) using the 358 "breast cancer liver metastasis-selective genes" derived from SAM analyses of metastases in the test dataset.

Supplementary figure S2. Unsupervised HCL of the test cohort on normal tissue and breast cancer selective genes (gene lists from [35]): **A)** Normal Liver, **B)** Normal Breast; and **C)** breast cancer selective genes (gene list from [36]).

Supplementary figure S3: Associations between the 17 liver metastasis-selective genes and **A)** RFS and **B)** OS for all patients included in GOBO irrespective of ER status.

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Supplementary Table 1

Factor	All Patients	Transcriptional profiling
	(N=304)	(N=85)
	N (%)	N (%)
Age at diagnosis		
Median (Range)	50 (27-71)	51 (30-69)
< 50 years	154 (51%)	36 (43%)
≥ 50 years	149 (49%)	48 (57%)
Missing/unknown	1	1
Primary tumor ER status		
Positive	215 (76%)	50 (63%)
Negative	68 (24%)	29 (37%)
Missing/unknown	21	6
Primary tumor PR status		
Positive	151 (57%)	34 (47%)
Negative	114 (43%)	39 (53%)
Missing/unknown	39	12
Primary tumor histological grade		
Grade 1/2	121 (49%)	22 (41%)
Grade 3	125 (51%)	32 (59%)
Missing/unknown	58	31
Primary tumor size		
≤ 20 mm	119 (40%)	30 (37%)
> 20 mm	180 (60%)	51 (63%)
Missing/unknown	5	4
Primary tumor nodal status		
N0	92 (31%)	24 (30%)
N+	203 (69%)	57 (70%)
Missing/unknown	9	4
Adjuvant chemotherapy		
Yes	150 (49%)	38 (45%)
No	152 (50%)	46 (55%)
Missing/unknown	2	1
Adjuvant endocrine therapy		
Yes	156 (52%)	39 (46%)
No	147 (48%)	45 (54%)
Missing/unknown	1	1
Adjuvant radiotherapy		
Yes	214 (71%)	50 (60%)
No	87 (29%)	34 (40%)
Missing/unknown	3	1
Metastatic category		
Loco-regional	50 (17%)	24 (28%)
Bone	56 (18%)	14 (16%)

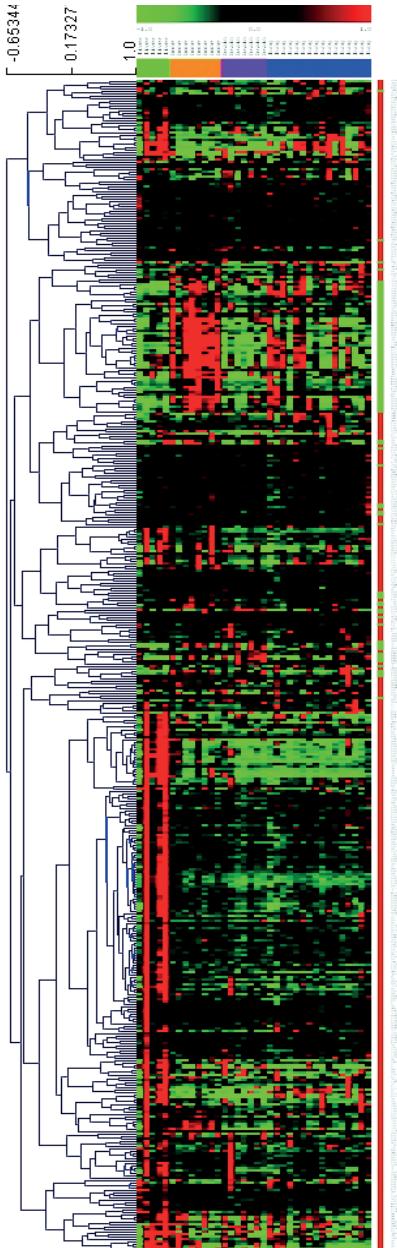
Lung	63 (21%)	14 (16%)
Liver	133 (44%)	33 (39%)
Missing/unknown	2	
No of metastatic sites		
Oligo metastasis (n=1)	76 (25%)	17 (20%)
Multiple metastases (n>1)	226 (75%)	68 (80%)
Missing/unknown	2	0
Metastasis-free interval		
≤ 24 months	80 (26%)	30 (36%)
> 24 months	223 (74%)	54 (64%)
Missing/unknown	1	1
Molecular subtype		
Luminal A	65 (37%) ^a	5 (6%) ^b
Luminal B	81 (45%) ^a	26 (31%) ^b
HER2-enriched	9 (5%) ^a	27 (32%)
Triple-negative ^a /Basal-like ^b	24 (14%) ^a	24 (29%) ^b
Normal-like ^b	n.a.	2 (2%) ^b
Unclassified/missing	125	1

a= St Gallen subtype, b=PAM50 intrinsic subtype

Supplementary Table 5

Factor	Relapse-free survival (RFS)						Overall survival (OS)					
	All ER Positive tumors			Luminal A tumors			All ER Positive tumors			Luminal A tumors		
	HR	CI	P	HR	CI	P	HR	CI	P	HR	CI	P
17-gene signature (low vs high)	1.5	1.2-2.0	0.001	2.2	1.3-3.9	0.004	1.4	1.0-1.9	0.0260	1.4	0.74-2.7	0.2937
Nodal status (Neg vs pos)	0.69	0.52-0.92	0.012	0.89	0.47-1.7	0.716	0.45	0.33-0.63	0.0000	0.5	0.25-1.0	0.0499
Histological grade (3 vs 1 and 2)	1.1	0.80-1.5	0.608	0.24	0.03-1.8	0.163	1.6	1.0-2.1	0.0323	na	na	na
Age at diagnosis (>50 vs ≤50)	0.78	0.59-1.0	0.079	0.98	0.52-1.8	0.943	1.7	1.2-2.3	0.0028	6.2	1.9-20.5	0.0027
Tumor size (>2cm vs ≤2cm)	2.1	1.6-2.7	0.000	2.4	1.4-4.3	0.002	1.8	1.3-2.5	0.0002	2.3	1.2-4.5	0.0122

na; not computed due to very few cases in one group



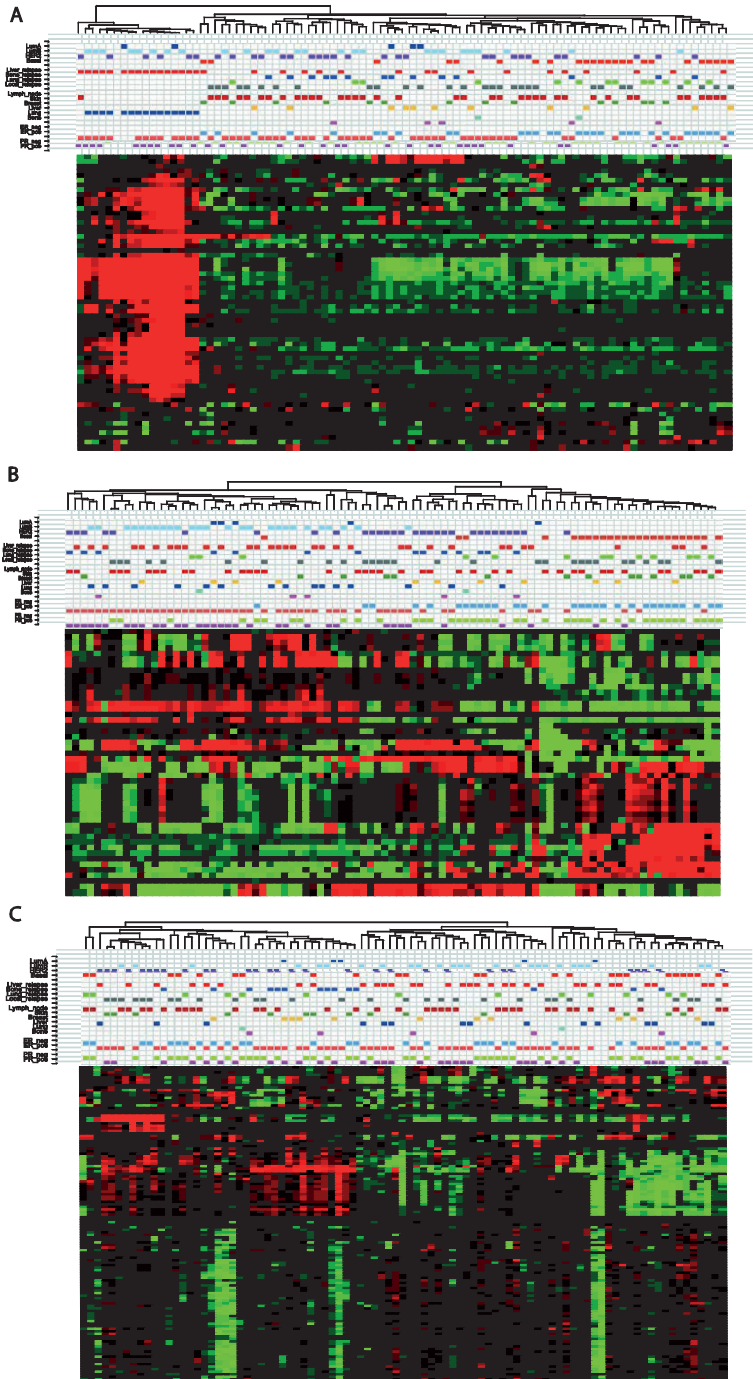
Samples

- Liver
- Bone
- Brain
- Lung

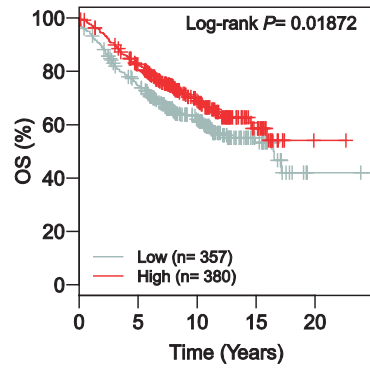
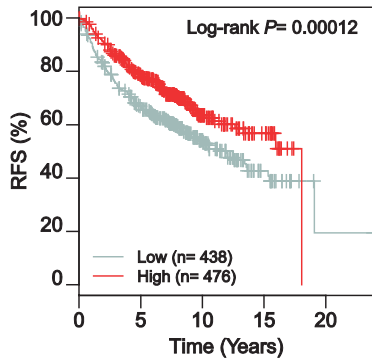
Genes

- Down-regulated in test cohort
- Up-regulated in test cohort

Supplementary figure S1



Supplementary figure S2



Supplementary figure 3

Paper III

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Claudin-2 is an independent negative prognostic factor in breast cancer and specifically predicts early liver recurrences



Siker Kimbung^{a,b}, Anikó Kovács^c, Pär-Ola Bendahl^a, Per Malmström^{a,d},
Mårten Fernö^a, Thomas Hatschek^e, Ingrid Hedenfalk^{a,b,*}

^aDivision of Oncology, Department of Clinical Sciences, Lund, Lund University, Sweden

^bCREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden

^cDepartment of Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden

^dSkåne Department of Oncology, Skåne University Hospital, Lund, Sweden

^eDepartment of Oncology and Pathology, Karolinska Institutet and Karolinska University Hospital, Sweden

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ABSTRACT

Background: Predicting any future metastatic site of early-stage breast cancer is important as it significantly influences the prognosis of advanced disease. This study aimed at investigating the potential of claudin-2, over-expressed in breast cancer liver metastases, as a biomarker for predicting liver metastatic propensity in primary breast cancer.

Methods: Claudin-2 expression was analyzed in two independent cohorts. Cohort 1 included 304 women with metastatic breast cancer diagnosed between 2002 and 2007, while cohort 2 included 237 premenopausal women with early-stage node-negative breast cancer diagnosed between 1991 and 1994. Global transcriptional profiling of fine-needle aspirates from metastases was performed, followed by immunohistochemical analyses in archival primary tumor tissue. Associations between claudin-2 expression and relapse site were assessed by univariable and multivariable Cox regression models including conventional prognostic factors. Two-sided statistical tests were used.

Results: CLDN2 was significantly up-regulated ($P < 0.001$) in liver metastases compared to other metastatic sites. Claudin-2 protein was more frequently expressed in primary tumors from patients who subsequently developed liver metastases ($P = 0.02$) and high expression was associated with a shorter metastasis-free interval (cohort 1, HR = 1.4, 95% CI = 1.0–1.9; cohort 2, HR = 2.2, 95% CI = 1.3–3.5). Specifically, a significantly shorter interval between primary tumor diagnosis and liver-specific recurrence was observed among patients with high levels of claudin-2 expression in the primary tumor (cohort 1, HR = 2.3, 95% CI = 1.3–3.9).

Conclusion: These results suggest a novel role for claudin-2 as a prognostic biomarker with the ability to predict not only the likelihood of a breast cancer recurrence, but more interestingly, the liver metastatic potential of the primary tumor.

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Abbreviations: BCSS, breast cancer specific survival; CI, confidence interval; CLDN2, claudin-2 mRNA; ER, estrogen receptor; HR, hazard ratio; IHC, immunohistochemistry; LiMFS, liver metastasis-free survival; LNM, lymph node metastasis; OR, odds ratio; PR, progesterone receptor; RFS, relapse-free survival; TMA, tissue microarray.

* Corresponding author. Division of Oncology, Department of Clinical Sciences, Lund University, Medicin Village, SE-22381 Lund, Sweden. Tel.: +46 46 2220652; fax: +46 46 147327.

E-mail address: Ingrid.Hedenfalk@med.lu.se (I. Hedenfalk).

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1. Introduction

Despite advances in management and the favorable prognosis of patients with early breast cancer, metastases are frequently diagnosed and the anatomical location of the metastases is correlated to the length of survival after recurrence (Imkampe et al., 2007; Largillier et al., 2008; Yardley, 2010). With the exception of the brain, recurrence in the liver is prognostic of the worst outcome relative to loco-regional, bone or lung relapses (Goldhirsch et al., 1988; Imkampe et al., 2007; Pentheroudakis et al., 2006; Yardley, 2010). Approximately 50% of all patients diagnosed with metastatic breast cancer develop hepatic metastases (Mano et al., 2005; Singletary et al., 2003; Solomayer et al., 2000) and there is evidence purporting an increasing trend in breast cancer liver metastases (Kennecke et al., 2010). However, the molecular determinants of site-specific metastatic preferences and factors accounting for heterogeneity in response to treatment and outcome are yet to be comprehensively established. A better understanding of these factors will likely influence decisions about surveillance and adjuvant therapy, as well as treatment of advanced disease.

Conventional clinico-pathological markers are used to assess the risk of recurrence. In addition, gene expression signatures stratifying patients according to recurrence risk (reviewed in Sotiriou and Pusztai, 2009) and more specifically, predicting the propensity of relapsing in bone (Kang et al., 2003), lung (Minn et al., 2005) and brain (Bos et al., 2009) have been published. However, because experimental models incompletely capture the relevant genetic complexity and the contribution of the host tumor microenvironment, studies using biopsies from metastases may be more suitable for identifying site-specific predictive biomarkers. Recently, we performed comparative genome-wide transcriptional profiling of a consecutive series of breast cancer metastases with one of the specific objectives being to identify potential liver metastasis genes (Kimbung et al., unpublished results). Remarkably, we observed that contrary to the down-regulation of many genes involved in cell adhesion and matrix re-modeling in liver metastases, *CLDN2*, a member of the same gene family, was significantly over-expressed. Over-expression of *CLDN2* was also recently observed in an experimental mouse model of breast cancer liver metastases (Tabaries et al., 2011), as well as in a limited series of clinical samples of breast cancer liver metastases (Tabaries et al., 2012), with accompanying data supporting the involvement of claudin 2 in the establishment and out-growth of breast cancer cells in the liver microenvironment. These data motivated the design of the present study, which was aimed at investigating if the high expression of *CLDN2* observed in liver metastases, is also a trait of primary breast cancers that recur in the liver. Furthermore, we sought to explore associations with conventional prognostic factors for breast cancer and patient outcome, with particular focus on the potential of claudin-2 as a biomarker for predicting liver metastatic propensity in primary breast cancer.

2. Materials and methods

2.1. Patients and tumors

This study was approved by the regional ethics committees at all participating sites.

2.1.1. Cohort 1

The test cohort consisted of 304 women with metastatic breast cancer who were enrolled in a randomized phase III trial conducted between 2002 and 2007 in Sweden, comparing two different first-line chemotherapy regimens (Hatschek et al., 2012). Patients with brain metastases, HER2 amplified tumors, or other malignancies diagnosed within five years of enrollment were excluded from the trial. Complete information on the study design, patient characteristics and trial outcome has been reported (Hatschek et al., 2012). The median follow-up for the endpoints relapse free survival (RFS) and breast cancer specific survival (BCSS) was 6.0 and 9.7 years respectively, for patients alive at last update.

2.1.2. Cohort 2

The prognostic value of claudin-2 was further evaluated in an independent cohort of 237 premenopausal women with early-stage lymph-node negative breast cancer included in a prospective study evaluating the prognostic value of the S-phase fraction (Malmstrom et al., 2001). Adjuvant treatment was administered to only 29 (12%) patients. Detailed information on treatment and evaluation of tumor pathological markers has been previously reported (Klinton et al., 2010; Malmstrom et al., 2001). Median follow-up was 10.6 and 18.3 years for RFS and BCSS, respectively.

2.2. Transcriptional analyses

Fine-needle aspirates from metastatic lesions from different anatomical sites were collected prior to treatment of metastatic disease whenever possible (cohort 1) and subjected to whole-genome transcriptional profiling. Tumor cellularity was assessed by a pathologist on Giemsa stained, ethanol-fixed, cytospin preparations and only samples with high (>50%) tumor cell content were included in the final analyses. Total RNA was extracted using the Qiagen RNA Mini kit (Qiagen, Valencia, CA), integrity analyzed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and hybridized onto custom made Affymetrix HuRSTA-2a520709 gene chips. Raw intensity gene expression levels were processed and normalized using the robust multichip average (RMA) algorithm. After normalization, a probe presence filter was applied to select only probes present in $\geq 90\%$ of assays. Probes expressed below the median intensity of Y-chromosome genes were filtered out of the dataset and gene-specific expression intensities were summarised by merging probes based on gene symbol. Finally, data were Log₂ transformed and mean-centered across the entire dataset. All data processing and normalization steps were performed in the R environment (www.r-project.org). Ninety-one out of 120 samples passed all quality assessments and were included in

subsequent analyses. Differentially expressed genes and biological processes between the liver metastases and other metastatic sites were identified using the Significance Analysis of Microarrays (SAM) and DAVID tools (Huang da et al., 2009a, 2009b), respectively. The gene expression data are available in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under the accession number GSE46141.

2.3. Tissue microarrays (TMAs) and immunohistochemistry (IHC)

Archival formalin-fixed paraffin-embedded (FFPE) primary tumor blocks were collected. Two representative 0.6 (cohort 1) or 1.0 (cohort 2) mm cores were extracted from the donor blocks and assembled in separate TMA blocks. Regional lymph node metastases (LNMs) from patients in cohort 1 were similarly assembled in a TMA. Whenever pathological markers were examined, both core biopsies were evaluated, and results from the core with the highest/strongest positivity were recorded. Investigators were always blinded to outcome.

2.4. Evaluation of standard pathological markers

Estrogen (ER) and progesterone (PR) receptor status were analyzed by IHC and cytosol based biochemical assays for cohort 1 and 2 respectively, as previously described (Chebil et al., 2003; Malmstrom et al., 2001). Antibodies were purchased from Ventana (ER, clone SP1; PR, clone 1E2) and staining was performed with the Ventana Benchmark ULTRA (Ventana Medical Systems, Tucson, AZ). Re-evaluation of histological grade was performed following the Elston and Ellis criteria as described (Malmstrom et al., 2001). Proliferation was assessed by the Ki67 index, using the MIB-1 antibody (K5001, Dako, Copenhagen, Denmark). A cut-off of $\geq 20\%$ was used to indicate high Ki67 (Klintman et al., 2010). All scorings were performed independently by board certified breast pathologists.

2.5. Claudin-2 immunohistochemistry

A mouse monoclonal antibody specific for claudin-2 (12H12, Invitrogen, Sweden) was used at a 1:400 dilution. This antibody has previously been used for the evaluation of claudin-2 expression by IHC in several studies (Dhawan et al., 2011; Kim et al., 2008; Soini, 2004, 2005; Szasz et al., 2010). Immunohistochemical reactions were performed following the manufacturer's protocol and the Envision horseradish peroxidase rabbit/mouse kit and the Dakocytomation Autostainer (DAKO) system was used. Staining was detected as a membranous and cytoplasmic granular reaction. Non-neoplastic human kidney tissue was included as positive control. Each sample was given a semi-quantitative score from 0 to 2 for the proportion of tumor cells staining positive [0 (<10%), 1 (11–50%), and 2 (>50%)] and 0–3 for the intensity of tumor cell staining [0 (absent), 1 (weak), 2 (moderate), and 3 (strong)]. The proportion and intensity scores were combined by addition to obtain a final score ranging from 0 to 5. No consensus for choice of cut-off for claudin-2 scoring was found in the literature. Therefore, in this study, a total score of ≥ 3 was

considered as high expression and scores <3 as low expression, representative of the majority of these studies (Dhawan et al., 2011; Soini, 2005; Szasz et al., 2010; Tabaries et al., 2011, 2012).

2.6. Statistical analyses

Patients and tumor characteristics were compared across the claudin-2 expression groups using the χ^2 and Mann–Whitney U or one-way analysis of variance tests for categorical and continuous variables, respectively. Odds ratios (OR) were computed by logistic regression modeling and the McNemar test was used to assess differences between paired primary tumors and regional LNMs. RFS, liver metastasis-free survival (LiMFS) and BCSS were the primary, secondary and tertiary end-points, respectively. RFS included recurrence to any site, LiMFS included only liver recurrences, and BCSS included breast cancer specific death as an event. The differences between the claudin-2 groups for each end-point were summarized using hazard ratios estimated in both univariable and multivariable Cox-proportional hazards models (see Appendix Methods A.1 for further details). Proportional hazards assumptions were checked by graphical methods. All P-values correspond to two-sided statistical tests and values <0.05 were considered significant. The statistical software package IBM SPSS Statistics 19 (IBM Corporation, NY) was used.

3. Results

3.1. Patient and tumor characteristics

Flow charts of the cohorts and a summary of primary tumor characteristics for patients in cohort 1 are presented in Appendix Figure A.1 and Appendix Table A.1. Figure 1A illustrates an inferior post-recurrence survival in patients with liver compared to non-liver recurrences in cohort 1 (Log-rank; $P = 0.006$). The poor outcome for patients with liver metastases remained significant (Figure 1B; Log-rank $P = 0.02$) after stratifying the patients with non-liver metastases into three groups based on the most advanced metastatic site recorded (loco-regional, bone and lung, respectively). Liver recurrences were rare (18 cases) in cohort 2, thus the distributions of patient and tumor characteristics by claudin-2 expression but not by site of relapse were explored in this cohort.

3.2. Claudin-2 expression and associations with clinicopathological characteristics

A total of 91 breast cancer metastases from 6 specific anatomical sites [liver ($n = 16$), bone ($n = 5$), lung ($n = 2$), lymph node ($n = 39$), local [breast ($n = 11$) and skin ($n = 17$)], and ascite ($n = 1$)] were included in the search for differentially expressed genes associated with hepatic recurrence. SAM analyses revealed 733 (423 up-regulated and 307 down-regulated) significantly differentially expressed genes between liver metastases and other sites. There was an enrichment of genes associated with cell adhesion and matrix re-modeling among the significantly down-regulated

genes in the liver metastases (Figure 2). In contrast, *CLDN2* expression was found to be significantly up-regulated in liver metastases compared to other sites (Figure 3A; Mann–Whitney; $P < 0.001$, and Figure 3B; Kruskal–Wallis; $P = 0.007$). Following the notion that transcriptional profiles of primary tumors and metastases from a patient are very similar (Harrell et al., 2012; Weigelt et al., 2003), we investigated if *CLDN2* was up-regulated in metastases derived from patients diagnosed with liver metastases compared to non-liver involvement irrespective of the anatomical location of the metastatic lesion that was profiled. *CLDN2* was thus found to be significantly over-expressed in metastases from patients with liver involvement compared to those without (Figure 3C; Mann–Whitney $P = 0.001$, and Figure 3D; Kruskal–Wallis $P = 0.06$).

Next, we investigated (in cohort 1) if the high *CLDN2* expression observed in the hepatic metastases could be a trait acquired from the primary tumors, potentially priming them for selective colonization of the liver. Of the 191 evaluable cases, 134 (70%) were classified as high claudin-2 expressing (Table 1 and Figure 4). Notably, a significant association between high claudin-2 expression in the primary tumor and liver relapse was found (OR = 2.1, 95% CI = 1.1–4.0).

Other associations between claudin-2 and conventional breast cancer prognostic factors were then explored. High expression of claudin-2 was found to be significantly associated with positive nodal status (OR = 2.1, 95% CI = 1.1–3.9) in cohort 1, while significant positive associations between claudin-2 expression and high histological grade (grade 3; OR = 3.0, 95% CI = 1.6–5.7), high proliferation (high Ki67; OR = 4.4, 95% CI = 2.3–9.0), and younger age (<50 years; OR = 2.0, 95% CI = 1.1–3.7) were observed in cohort 2 (Table 1).

3.3. Claudin-2 expression and tumor progression: correlation between primary and lymph node metastasis

Paired data from primary tumors and LNs were available from 107 cases in cohort 1. Discordant claudin-2 expression was observed in 32 pairs [30% (McNemar; $P = 0.02$)], the majority of which changed from low expression in the primary tumor to high expression in the LNM [23/32 (72%)]. Subgroup analyses revealed that significant discordant expression was only demonstrated among ductal carcinomas ($n = 83$, McNemar; $P = 0.02$). In contrast, no difference in the expression pattern was observed in lobular carcinomas (McNemar; $P = 0.5$), as 15/17 evaluable cases displayed concordant high expression.

3.4. Claudin-2 expression in relation to recurrence and breast cancer death

Uni- and multivariable Cox proportional hazards ratio estimates of the difference between the claudin-2 groups for RFS, LiMFS and BCSS, respectively are shown in Tables 2–4. Twenty-year survival estimates are reported.

The median RFS was significantly shorter (3.6 years vs 5.7 years) for the high claudin-2 group in both univariable (HR = 1.4, 95% CI = 1.0–1.9) and multivariable analyses (Tables 2 and 3) in cohort 1. Histological grade, ER status, tumor size, axillary lymph node status and age at primary diagnosis were other independent factors significantly correlated with a shorter RFS in multivariable models. In cohort 2, high claudin-2 expression was prognostic for shorter RFS (HR = 2.2, 95% CI = 1.3–3.5) in univariable analyses. Age, HER2 status and histological grade were also significant in univariable analyses, with age and HER2 status remaining significant independent factors in multivariable models (Table 4).

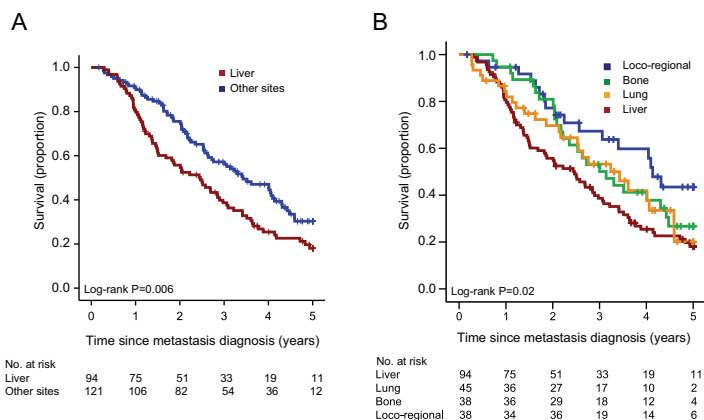


Figure 1 – Kaplan–Meier representation of post-recurrence survival according to site of relapse in cohort 1. A) Patients were stratified by presence (liver) or absence (other) of liver metastases. B) Patients with non-liver metastases (breast, lymph-node, skin, bone, lung and ascite) were further stratified into three groups according to the most distant metastatic site. P values are from two-sided Log-rank tests.

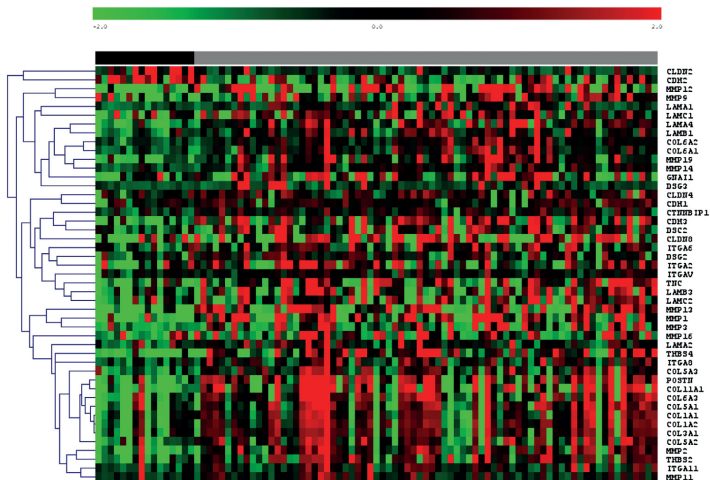


Figure 2 – Supervised analysis comparing transcriptional profiles of liver metastases to non-liver metastases (breast, lymph-node, skin, bone, lung and ascite). A summary of significantly differentially altered cell adhesion and matrix-remodeling genes is presented. Red corresponds to up-regulated genes and green corresponds to down-regulated genes within the heatmap. The color scale represents the mean centered Log₂ expression of the genes. Black in the top bar represents liver metastases and gray represents other metastases.

Next, we investigated if claudin-2 expression in the primary tumor was prognostic for the diagnosis of liver metastases in cohort 1. Univariable analyses revealed a substantial decrease in the median time to liver metastasis diagnoses from 12.1 years in the low expressing group to 5.9 years in high expressing groups (Tables 2 and 3, HR = 2.3, 95% CI = 1.3–3.9). Claudin-2 remained the strongest independent liver metastasis risk factor in multivariable analyses (HR = 2.0, 95% CI = 1.1–3.8).

In addition, there was a trend toward higher risk of death from breast cancer among patients with high claudin-2 expression in univariable analyses (cohort 1: Appendix Table A.2; HR = 1.4, 95% CI = 0.98–2.1 and cohort 2: Table 4; HR = 1.3, 95% CI = 0.76–2.3).

4. Discussion

Our study reveals that CLDN2 is frequently over-expressed in breast cancer liver metastases, and in addition conclusively demonstrates that primary tumors from patients who are diagnosed with hepatic recurrences also frequently express high levels of claudin-2 protein. Most importantly, for the first time, we provide evidence that claudin-2 is a potential prognostic factor for predicting the likelihood of a breast tumor to relapse specifically in the liver, and is furthermore a general predictor of early breast cancer recurrences.

While it is known that cancer cells preferentially metastasize to specific organs, the molecular mechanisms driving this organ-specific tropism are not well understood. Gene expression signatures that predict bone (Kang et al., 2003), lung (Minn et al., 2005) and brain (Bos et al., 2009) metastases

from breast cancer have been published, but no signature for liver metastasis is currently available despite the adverse clinical outcome of patients with hepatic metastases as demonstrated by us herein, and others (Imkampe et al., 2007; Largillier et al., 2008; Yardley, 2010). Although these gene signatures have contributed greatly to the understanding of metastasis organotropism, there is a need to identify the most informative and robust candidate genes among these signatures, which may be used as surrogate biomarkers in more convenient assays such as IHC. In concordance with previous experimental mouse model studies of breast cancer (Erin et al., 2009; Tabaries et al., 2011) we observed that decreased expression of cell adhesion and tight junction genes (including DSG2, CLDN4, CLDN8, POSTN, THBS2) may be a trait of breast cancer liver metastases. Interestingly however, like Tabaries et al., we show that claudin-2 is over-expressed in breast cancer liver metastases, highlighting a potentially important role of claudin-2 in the development of liver metastases in these patients. Importantly, our study further demonstrates that this is an attribute of primary tumors, as a significantly higher proportion of patients with liver metastases also displayed high claudin-2 levels in their primary tumors. Additionally, Tabaries et al. (2011, 2012) provided the functional evidence characterizing CLDN2 as a breast cancer liver metastasis virulence gene that endows circulating breast cancer cells with enhanced capacity to adhere, survive, and proliferate in the hepatic microenvironment. Taken together, these studies compel us to propose that claudin-2 is a novel and functionally relevant biomarker for predicting liver metastases.

In order for circulating tumor cells to seed metastases, interactions between tumor cells and the microenvironment are

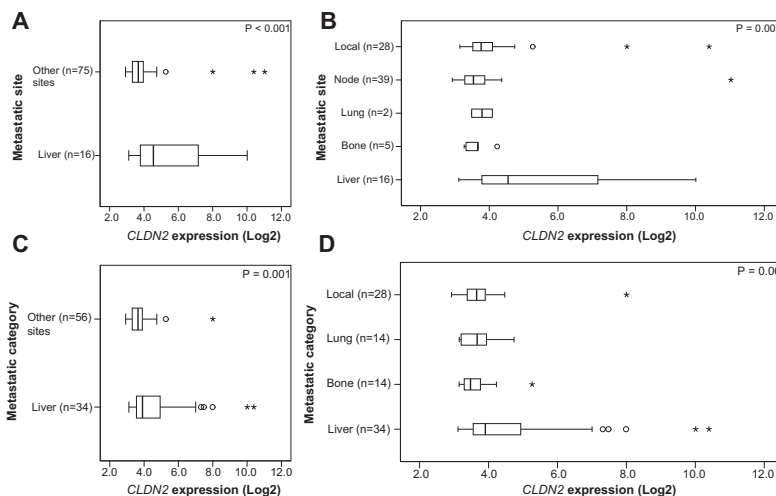


Figure 3 – Claudin-2 mRNA expression. A–B) Box plots comparing *CLDN2* expression between liver and non-liver (breast, lymph-node, skin, bone, lung and ascite) metastatic lesions in cohort 1. The specific anatomical location of the profiled metastases was taken into consideration. C–D) Box plots comparing *CLDN2* expression between patients presenting with liver metastases vs non-liver metastases. Patients were categorized into four groups associated with prognosis and this stratification considered only the most advanced metastatic site recorded and not the specific anatomical location of the metastatic lesion profiled [local; locally advanced or regional metastases in the lymphnodes or skin, bone; skeletal metastases with or without loco-regional metastases, lung; plural metastases with or without skeletal and loco-regional metastases, liver; hepatic metastases with or without plural, skeletal or loco-regional metastases]. The open circles and asterisks in the figures represent mild and extreme outliers respectively for each group in each comparison. All statistical tests are two-sided.

critical. Claudin-2 is a unique member of the claudin family of transmembrane cell adhesion proteins and is selectively expressed in leaky epithelia (Escaffit et al., 2005; Reyes et al., 2002). Available data indicate that it is highly expressed and play a role in the onset and progression of colorectal cancer (Dhawan et al., 2011), lung cancer (Peter et al., 2009), and inflammatory bowel disease (Ridyard et al., 2007; Weber et al., 2008). There are limited but controversial data on the expression of claudin-2 in breast cancer, and its role in disease progression and prognosis has not been extensively studied. While it is reported to be expressed in about 50% of primary breast carcinomas (Soini, 2004, 2005; Thakur et al., 2007), one study reported down-regulation of claudin-2 in up to 93% of primary breast cancers compared to adjacent normal breast tissue (Kim et al., 2008). The recently described poor prognosis claudin-low subtype of breast cancer is characterized by down-regulation of claudins 3, 4 and 7, and is enriched with triple-negative tumors (Prat et al., 2010). We found claudin-2 to be expressed in 70% of tumors in cohort 1 and 51% of tumors in cohort 2. The distribution of claudin-2 in cohort 2 in our study is in line with previous studies (Soini, 2004, 2005; Thakur et al., 2007) and in addition, we found a significant positive association between high claudin-2 expression and poor prognostic factors including high histological grade, younger age and high proliferation, confirming the negative prognostic effect of its expression in breast cancer. The higher proportion of claudin-2 positive tumors seen in cohort 1 reflects the conservative selection bias of the clinical trial, resulting in an

enrichment of patients with an inferior prognosis within this cohort. On the one hand, this provided sufficient statistical power to study the liver metastatic potential of the biomarker, while on the other hand, because the exclusion criteria of the trial are linked to prognosis, this may have confounded the statistical estimates toward the null hypothesis, partly explaining the absence of a significant statistical association between claudin-2 expression and other poor prognostic factors in cohort 1.

Claudin-2 expression in matched primary tumors and lymph node metastases in relation to clinico-pathological features and outcome has been previously studied (Szasz et al., 2010), showing loss of expression in the LNMs among lobular cancers only. Similarly and consistent with another previous study (Soini, 2004), we did not observe any significant differences in expression in ductal vs lobular, amongst primary tumors. In contrast to the previous study however, increased expression of claudin-2 in LNMs compared to primary tumors was observed among ductal tumors. This could suggest that claudin-2 may facilitate ductal breast cancer dissemination, a hypothesis supported by results from studies in colorectal (Dhawan et al., 2011) and lung cancer (Peter et al., 2009). Claudin-2 facilitates the conversion of tight junctions from a compact to a leaky strand phenotype (Furuse et al., 2001; Singh et al., 2007), suggesting that over-expression may increase the permeability of epithelial structures, thereby enabling access to factors in the microenvironment necessary for tumor growth, invasion and metastasis. It remains to be investigated if claudin-2 can be targeted

Table 1 – Associations between claudin-2 protein expression and other breast cancer prognostic factors in cohorts 1 and 2.

Prognostic factor	Cohort 1% in high CLDN2 (N high/N total)		Cohort 2% in high CLDN2 (N high/N total)	
	CLDN2+70% (134/191)	P	CLDN2+51% (107/208)	P
Age				
<50 years	73% (62/85)	0.45	56% (86/154)	0.03
≥50 years	68% (72/106)		39% (21/54)	
ER				
Positive	71% (107/150)	0.93	49% (67/136)	0.39
Negative	71% (24/34)		56% (40/72)	
PR				
Positive	64% (67/104)	0.06	51% (74/145)	0.86
Negative	78% (59/76)		52% (33/63)	
Tumor size				
≤2.0 cm	68% (54/79)	0.61	49% (77/156)	0.30
>2.0 cm	72% (79/110)		58% (30/52)	
Nodal status				
N0	60% (37/62)	0.03	51% (107/208)	–
N+	75% (94/125)		0	
Histological grade				
1/2	78% (56/72)	0.16	43% (61/143)	<0.001
3	68% (66/97)		69% (43/62)	
Ki67				
High	65% (41/63)	0.29	77% (44/57)	<0.001
Low	73% (85/117)		43% (56/129)	
Site of relapse				
Liver	79% (66/84)	0.02	50% (9/18)	0.90
Non-liver	64% (68/107)		52% (98/190)	

Abbreviations: CLDN2, claudin-2; ER, estrogen receptor; PR, progesterone receptor. P = P-value from χ^2 test for association in 2 × 2 tables. Cases with missing data were not included in the analyses. Numbers in bold represent statistically significant differences.

therapeutically to prevent dissemination and outgrowth of liver metastases. Of interest, preclinical studies have shown that claudin-2 expression can be down-regulated by inhibition of EGFR and PI3K using specific antibodies and inhibitors (Bos et al., 1997; Dhawan et al., 2011), providing additional support for the use of these compounds, many of which are currently being evaluated in clinical trials. However, because of the limited number of cases with matched primary tumor and LNM data in our study (n = 107) and that of Szasz et al. (n = 97), larger studies are required to better understand the significance of these findings.

Notably, we observed a positive association between high claudin-2 expression in the primary tumor and a significantly shorter relapse-free interval, and a trend toward higher risk of death was noted. Importantly, claudin-2 remained a significant independent prognostic factor for RFS in multivariable analyses. The prognostic value of claudin-2 expression in primary breast tumors has been previously studied (Szasz et al., 2010), but no significant association with survival was observed. Cohort 1 in the present study included only patients with advanced disease, biasing the effect estimates toward the null hypothesis. Notwithstanding, the negative prognostic power of claudin-2 was confirmed in the independent cohort of premenopausal women with early-stage node-negative disease.

Most importantly, for the first time, we present data showing that high expression of claudin-2 in primary tumors predicts shorter time to develop liver metastases. Associations between site of relapse and molecular subtype have been reported (Kennecke et al., 2010; Smid et al., 2008), but the significant overlap between relapse sites across subtypes compromises their predictive power and warrants the identification of supplementary site-specific biomarkers. In multivariable analyses (cohort 1) including ER status, histological grade, nodal status, age at primary diagnosis and tumor size, only claudin-2 and tumor size remained independently significant for liver metastases. While we observed a marginal increase in the liver metastatic risk among patients with larger tumors, Kennecke et al. (2010) reported a significant association between large tumor size and lower risk of liver and brain

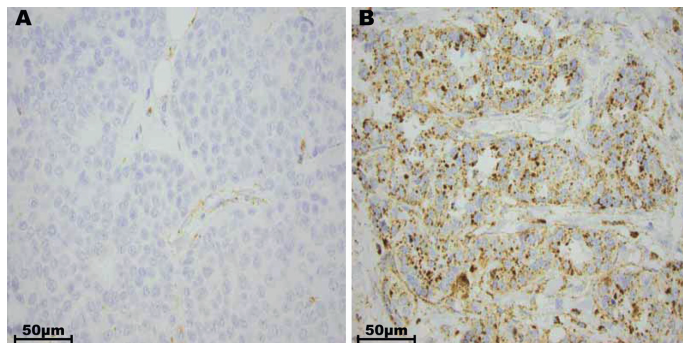


Figure 4 – Claudin-2 protein expression. Representative images of immunohistochemical staining of primary breast cancers showing A) deficient (<10% positive tumor cells) and B) high (>50% positive tumor cells) claudin-2 expression, respectively.

Table 2 – Median survival in relation to the expression of claudin-2 in cohort 1.

	n	Events	Median (yrs)	95% CI	P
RFS					0.03
Low CLDN2	55	55	5.7	4.5–6.9	
High CLDN2	126	126	3.6	2.9–4.2	
LiMFS					0.002
Low CLDN2	55	11	12.1	8.3–15.8	
High CLDN2	126	63	5.9	3.8–7.9	
BCSS					0.06
Low CLDN2	57	41	10.6	7.6–13.5	
High CLDN2	134	97	6.6	5.4–7.8	

Abbreviations: CLDN2, claudin-2; RFS, relapse-free survival; LiMFS, liver metastasis-free survival; BCSS, breast cancer specific survival; CI, confidence interval; yrs, years.
Numbers in bold represent statistically significant differences.

seeding. Although our findings are consistent with the metastatic model purporting that an aggressive potential can be reflected by a large volume (Norton and Massague, 2006), it does not explain the propensity for liver-specific colonization. Importantly, claudin-2 was the strongest predictor for time to liver recurrence. It remains to be verified if it is also

functionally important in mediating the early stages of tumor invasion or whether it only serves as a passenger biomarker for the liver metastatic potential of a tumor at the primary site. We found claudin-2 expression to have limited value in predicting liver metastatic potential in colorectal cancer, most likely due to high overall levels of expression in colorectal carcinomas (data not shown).

Despite improvements in breast cancer survival, distant recurrences are not uncommon and remain incurable. Our data provide evidence projecting claudin-2 as a novel breast cancer prognostic biomarker with application for predicting not only the likelihood of a tumor to recur, but more interestingly its liver metastatic potential. We have uncovered novel correlations, corroborated previous data and observed important discrepancies. The inconsistencies between our results and some of the previous studies may be partly attributed to differences in the patient cohorts with respect to clinicopathological characteristics and follow up time, sample size, as well as the choice of analytical and statistical methods. Nevertheless, the analogous negative prognostic effect of claudin-2 observed in the two cohorts despite their clinical differences, and the significance of our results for improving personalized management of breast cancer warrants further investigation in larger population based cohorts which better capture the heterogeneity in biology and outcome of breast cancer.

Table 3 – Relapse-free survival (RFS) and liver metastasis free survival (LiMFS) in cohort 1.

	RFS						LiMFS					
	Univariable			Multivariable			Univariable			Multivariable		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
CLDN2 (high vs low)	1.4	1.0–2.0	0.03	1.5	1.0–2.2	0.03	2.3	1.3–3.9	0.003	2.0	1.1–3.8	0.03
Age (>50 yrs vs ≤50 yrs)	2.3	1.7–3.2	<0.001	2.4	1.7–3.5	<0.001	1.6	1.0–2.5	0.04	1.4	0.81–2.3	0.23
ER (Neg vs Pos)	2.0	1.4–3.0	<0.001	2.0	1.3–3.3	0.004	1.2	0.58–2.4	0.68	1.3	0.58–3.1	0.49
Histological grade (3 vs 1/2)	1.6	1.2–2.2	0.002	1.6	1.1–2.3	0.01	1.1	0.70–1.7	0.66	1.3	0.79–2.2	0.29
Nodal status (N+ vs N0)	1.7	1.2–2.2	0.001	1.4	1.0–2.1	0.05	1.5	0.94–2.3	0.09	1.2	0.69–2.0	0.54
Tumor size (>2.0 cm vs ≤2.0 cm)	1.6	1.2–2.2	0.001	1.4	1.0–2.0	0.04	1.4	0.92–2.2	0.12	1.7	1.0–2.9	0.04

Abbreviations: HR, hazards ratio; CI, confidence interval; CLDN2, claudin-2; ER, estrogen receptor.
Numbers in bold represent statistically significant differences.

Table 4 – Relapse-free survival (RFS) and breast cancer specific survival (BCSS) in cohort 2.

	RFS						BCSS					
	Univariable			Multivariable			Univariable			Multivariable		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
CLDN2 (high vs low)	2.2	1.3–3.5	0.002	1.4	0.83–2.4	0.20	1.3	0.76–2.3	0.32	0.78	0.42–1.4	0.42
Age (years)	0.92	0.88–0.96	<0.001	0.93	0.89–0.98	0.004	0.9	0.87–0.95	<0.001	0.92	0.87–0.97	0.002
ER (Neg vs Pos)	1.5	0.98–2.4	0.06	1.4	0.86–2.4	0.17	1.3	0.78–2.3	0.3	1.35	0.73–2.5	0.34
Histological grade (3 vs 1/2)	1.9	1.2–3.0	0.004	1.3	0.77–2.2	0.32	2.3	1.4–3.9	0.002	1.9	0.98–3.5	0.06
HER2 (Pos vs Neg)	2.8	1.6–5.1	0.001	2.1	1.1–4.0	0.02	3.9	2.0–7.5	<0.001	2.9	1.5–5.8	0.003
Tumor size (>2.0 cm vs ≤2.0 cm)	1.2	0.7–1.9	0.56	1	0.56–1.8	0.99	1.3	0.75–2.4	0.33	1.2	0.6–2.4	0.59

Abbreviations: HR, hazards ratio; CI, confidence interval; CLDN2, claudin-2; ER, estrogen receptor.
Numbers in bold represent statistically significant differences.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molonc.2013.10.002>.

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Paper IV



Co-targeting of the PI3K pathway improves the response of *BRCA1* deficient breast cancer cells to PARP1 inhibition

Siker Kimbung^{a,b}, Ewa Biskup^{a,b,c}, Ida Johansson^{a,b}, Kristina Aaltonen^{a,b}, Astrid Ottosson-Wadlund^d, Sofia Gruvberger-Saal^{a,b}, Heather Cunliffe^e, Bengt Fadeel^d, Niklas Loman^{a,b}, Pontus Berglund^{a,b}, Ingrid Hedenfalk^{a,b,*}

^a Department of Oncology, Clinical Sciences, Lund University, Lund, Sweden

^b CREATE Health Strategic Center for Translational Cancer Research, Lund University, Lund, Sweden

^c Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

^d Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

^e Translational Genomics Research Institute, Phoenix, AZ, United States

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ABSTRACT

Although pre-clinical and clinical studies on PARP1 inhibitors, alone and in combination with DNA-damaging agents, show promising results, further ways to improve and broaden the scope of application of this therapeutic approach are warranted. To this end, we have investigated the possibility of improving the response of *BRCA1* mutant breast cancer cells to PARP1 inhibition by co-targeting the PI3K pathway. Human breast cancer cell lines with or without the expression of *BRCA1* and/or *PTEN* were treated with PARP1 and PI3K inhibitors as single agents and in combination. PARP1 inhibition induced DNA damage conferring a G2/M arrest and decrease in viability, paralleled by the induction of apoptosis. PI3K inhibition alone caused a G1 arrest and decreased cell growth. Most importantly, sequential combination of PARP and PI3K inhibitors interacted synergistically to significantly decrease growth compared to PARP inhibition alone. Global transcriptional profiling revealed that this decrease in growth was associated with down-regulation of macromolecule biosynthesis and the induction of apoptosis. Taken together, these results suggest an improved treatment strategy for *BRCA1*-mutant and possibly also triple-negative breast cancers with similar molecular defects.

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1. Introduction

Major improvements in breast cancer therapy have been achieved with the development of treatments targeting estrogen receptor signaling (anti-estrogens and aromatase inhibitors) and epidermal growth factor receptors (e.g. HER2 signaling using monoclonal antibodies and tyrosine kinase inhibitors), respectively. However, "triple-negative" (i.e. ER, PR and HER2 negative) tumors remain a treatment challenge and the survival of these patients is poor compared to patients with other subtypes of breast cancer. Systemic treatment for patients with triple-negative disease is currently limited to chemotherapy. Gene expression profiling and immunohistochemical studies have repeatedly classified most *BRCA1*-associated tumors together with the triple-negative and basal-like tumors [1–3]. This suggests a common pathogenesis for *BRCA1*-associated cancers and a subset of triple negative

cancers, considering the marked heterogeneity within the triple negative class. Furthermore, this indicates the possibility of a common therapeutic approach for these patients. The inherently aggressive behavior, poor prognosis and limited therapeutic options urgently warrant new, targeted therapies for this group of patients.

Poly(ADP-ribose) polymerase 1 (PARP1), is the most abundant and well-studied member of the PARP family of proteins. It plays a crucial role in the repair of both single- and double-stranded DNA breaks (SSB and DSB) by binding to the DNA lesions and activating downstream repair proteins [4]. PARP inhibitors gained ground as potential anti-cancer agents due to their ability to sensitize tumor cells to DNA damaging radio- and chemotherapy [5]. More interestingly, the demonstration that PARP inhibitors could selectively kill cells with defective DSB repair (such as *BRCA1/2* mutated cells) [6,7] when used as a single agent inspired their rapid development and prompt entry into a series of clinical trials [8–10]. The proposed rationale for the selective sensitivity of *BRCA1/2* deficient cells to PARP1 inhibition is their increased dependency on PARP1 for efficient repair of spontaneous SSB to maintain genomic integrity and stability. In the past 5 years several

* Corresponding author at: Department of Oncology, Clinical Sciences, Lund University, BMC C13, SE-22184 Lund, Sweden. Tel.: +46 46 2220652; fax: +46 46 147327.

E-mail address: Ingrid.Hedenfalk@med.lu.se (I. Hedenfalk).

studies have been published reporting promising effects of combining PARP inhibitors with radiotherapy and conventional DNA damaging chemotherapy (reviewed in [11]) as well as when used as single agents for targeting *BRCA*-associated tumors [9]. However, as for most single agent cancer therapies, the obvious risk that some patients may acquire resistance cannot be neglected and needs to be addressed. Also, because the consequence of long-term treatment and the overall effect of PARP inhibitors on other cellular pathways are widely unknown it is important to investigate options for limiting toxicity and establishing a more rational design of the treatment course.

BRCA1-dependent breast tumors, like all triple-negative breast tumors, harbor a variety of de-regulated pathways and it has been suggested that management of these tumors with multiple targeted therapies may be a superior therapeutic approach [12]. Specifically, informed combination treatments targeting different key pathways have the potential of both increasing the efficacy and reducing the risk of resistance. Aberrant signaling of the phospho-inositol-3 kinase (PI3K) pathway is frequently observed in many cancer types. Over-activation of this pathway in breast cancer may result from genetic abnormalities including gain of function of oncogenes (e.g. *PIK3CA*) or the loss of function of tumor suppressor genes (e.g. *PTEN*). Tumorigenic advantages driven by inappropriate activation of the PI3K/AKT pathway include cell transformation, proliferation, increased migration, angiogenesis, evasion of apoptosis and genome instability [13]. It has been suggested that among other mechanisms, increased signaling through the PI3K/AKT pathway may constitute a mechanism of resistance to cancer therapeutic agents (reviewed in [13]).

PTEN is a tumor suppressor gene and its function is crucial for regulating and maintaining accurate PI3K/AKT signaling. More recently, a nuclear role of *PTEN* has been elucidated, in which dysfunctional *PTEN*, e.g. by mutations, contributes to defective homologous recombination [14] resulting in increased sensitivity to PARP inhibition [15]. Intriguingly, gross *PTEN* mutations have been shown to be a specific and recurrent oncogenic consequence of a malfunctioning DNA repair pathway and are strongly associated with *BRCA1* mutations [16]. It has further been shown that *BRCA1* is able to bind to phosphorylated AKT, thereby functioning as a negative regulator of AKT activity [17]. In the same study, loss of *BRCA1* expression was found to increase AKT activation. These separate links between *BRCA1* and the PI3K pathway would then provide a mechanistic explanation to the negative correlation between *BRCA1* expression and AKT phosphorylation found in human breast cancers [18]. Taken together, this suggests an addiction of *BRCA1* deficient tumors to aberrant PI3K/AKT signaling. This implies that a combination of PI3K inhibitors with PARP inhibitors for targeting *BRCA1* mutant cells should be more effective than targeting a single molecular abnormality. It is also possible that this combination may circumvent the development of resistance without compromising the specificity of the treatment. Furthermore, rational design of the combination regimen may also avoid the exacerbation of toxicity.

We hypothesized that the effect of PARP inhibition on *BRCA1* mutant cells would be potentiated by co-targeting PI3K signaling. As an experimental model, we have used the human breast cancer cell lines MDA-MB-436, SUM149, HCC1937 and L56Br-C1, all of which harbor *BRCA1* mutations [19,20] and, with the exception of L56Br-C1, gross *PTEN* mutations [16]. MCF7 cells, with wild type *BRCA1*, served as control. The drugs tested in combination were the PARP inhibitors AG14361 or AG014699 and the PI3K inhibitor LY294002. Our *in vitro* results suggest that the cytotoxic effect of combining a PARP inhibitor and a PI3K inhibitor in a sequential regimen is superior over PARP inhibition alone and may represent an improved selective targeted treatment strategy for breast cancers with concomitant DNA damage repair defects and

de-regulated PI3K signaling, and potentially also for sporadic tumors with a "BRCAness" [21] phenotype.

2. Materials and methods

2.1. Drugs

AG14361 was synthesized by Istvan Jablonksi, Institute of Biomolecular Chemistry, Hungarian Academy of Science and a 78 mM stock solution was prepared. AG014699 and AZD2881 (olaparib) were purchased from Selleck Chemicals (Houston, TX) and 20 mM stocks were prepared. LY294002 was purchased from Invitrogen (Carlsbad, CA) and diluted to a 10 mM stock solution. All stock solutions were prepared using dimethyl sulfoxide (DMSO) as solvent and stored at -20°C .

2.2. Cell lines

The human breast cancer cell lines MDA-MB-436, HCC1937 and MCF7 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). SUM149 was purchased from Asterand (Detroit, MI) and L56Br-C1 was established at the Department of Oncology, Lund University [20]. Cells were expanded and frozen down and a new vial of cells was taken up after 40 passages. MDA-MB-436 cells were maintained in McCoy's 5A medium (Invitrogen) supplemented with 10% v/v fetal bovine serum (FBS) (HyClone, Logan, UT) and 500 U/ml penicillin/streptomycin (Invitrogen). SUM149 cells were cultured in HAM'S F12 medium (Fisher Scientific, Gothenburg, Sweden) supplemented with 5 $\mu\text{g}/\text{ml}$ insulin (Invitrogen), 1 $\mu\text{g}/\text{ml}$ hydrocortisone (Sigma-Aldrich, Stockholm, Sweden), 10 mM HEPES (Fisher Scientific) and 5% FBS and 50 U/ml penicillin/streptomycin. MCF7 and L56Br-C1 were grown in RPMI 1640 supplemented with 10 $\mu\text{g}/\text{ml}$ insulin (Invitrogen), 5% FBS and 50 U/ml penicillin/streptomycin. MDA-MB-436, HCC1937 and SUM149 cells harbor the 5396 + 1G > A (spliced donor site of exon 20), 5382insC and 2288delT (exon 11) *BRCA1* mutations respectively [19] and both have gross mutations in *PTEN* [16]. L56Br-C1 harbors a germ-line *BRCA1* nonsense mutation (1806C > T; Q563X) [20], while MCF7 cells on the other hand express wildtype *BRCA1* but harbor activating mutations in the *PIK3CA* gene [22]. All cells were cultured at 37°C in a humidified environment in the presence of 5% CO_2 .

2.3. Cytotoxicity assay

The growth inhibitory effects of the PARP inhibitors (AG014699, AG14361 and AZD2881), and the PI3K inhibitor (LY294002) alone or in combination were studied. Exponentially proliferating cells were seeded into 12-well culture plates and incubated overnight to allow cells to adhere. Continuous treatment with different concentrations of the PARP inhibitors for 7 days or the PI3K inhibitor for 3 days as single agents was administered to determine the concentration of the respective drugs required to achieve 50% growth inhibition (GI_{50}). For combination treatments, a continuous regimen (combination of both PARP and PI3K inhibitors for the entire treatment period) and a sequential regimen (treatment with PARP inhibitor alone for 72 h followed by a combination of PARP and PI3K inhibitors for a further 96 h) were tested. Combination treatments were performed for either one (7 days) or two (14 days) cycles. Cytotoxicity was measured using the sulphorodamine-B (SRB, Sigma-Aldrich) assay as previously described [23]. Subsequently, by using the dose-response curves and GI_{50} calculated, the combination index (CI) values were determined by implementing the Chou-Talalay method [24]. CI values = 1, >1, and <1 indicate additive, antagonistic, and synergistic interactions, respectively.

2.4. DNA double-strand breaks

The phosphorylated histone H2AX (γH2AX) focus formation is a marker for double-strand breaks (DSB) in DNA and was used to evaluate the effect of PARP inhibition on DNA. The presence of DNA DSBs can be visualized using antibodies against the amino acid Ser-139 in the carboxy terminal of H2AX, which is phosphorylated to generate γH2AX in the event of a DSB [25]. SUM149 cells seeded onto cover slips were exposed to AG14361 for 48 h after which they were fixed in ice-cold methanol for 30 min. As a positive control for DNA damage, cells were exposed to 100 μM etoposide (Sigma-Aldrich) for 2 h before fixation. Following fixation, cells were permeabilized with 2% NP40 for 20 min, blocked in 5% goat serum for 1 h and primary monoclonal anti- γH2AX (1:200 dilution; rabbit anti-human γH2AX Cell Signalling, *in vitro* AB, Stockholm, Sweden) was applied for 2 h. This was followed by a 1 h incubation with Alexa Fluor-488 labeled secondary antibody (1:500 dilution; anti-rabbit IgG, Invitrogen). The cover slips were mounted onto slides using 4',6'-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Burlingame, CA). The γH2AX associated fluorescence was visualized on an AXIOPLAN 2 IMAGING inverted fluorescence microscope (Zeiss, Hamburg, Germany) and images were analyzed using the Metasystem software (Zeiss).

2.5. Cell cycle analysis

Flow cytometric analysis was performed to evaluate the effect of the different treatment conditions on cell cycle phase distribution. Following each specific treatment, cells were harvested by trypsinization and fixed with ice-cold methanol for at least 1 h at -20°C . After fixation, cells were rinsed once with phosphate buffered saline (PBS) and DNA was stained with propidium iodide (PI) nuclear isolation medium (PBS containing 100 $\mu\text{g}/\text{ml}$ PI, 0.6% Nonidet P-40, and 100 $\mu\text{g}/\text{ml}$ RNase A) [26]. Flow cytometric analysis was carried out with the FACScalibur using the Cellquest software (BD Biosciences Immunocytometry Systems, San Jose, CA). The proportions of cells in the different cell cycle phases were determined manually using Winlist software (version 5.0; Verity Software House, Topsham, ME).

2.6. Western blot analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, 1% NP-40, 1% deoxycholate, 0.1% SDS, supplemented with protease inhibitors (Roche GmbH, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma-Aldrich) and sonicated. Equal amounts of total protein lysates were resolved by SDS-PAGE, transferred onto PVDF membranes and probed with the following primary antibodies: anti-PARP1 (1:2000), anti-p-AKT Ser 473 (1:5000), anti-AKT (1:5000), anti-GAPDH (1:10,000) and anti-ACTB (1:1000). All antibodies were obtained from Cell Signaling, except the anti-ACTB antibody, which was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.7. RNA interference

MDA-MB-436 cells were transiently transfected with 50 nM of PARP1 siRNA (Thermo Scientific, cat no J-006656-06-0020) or non-targeting control (Thermo Scientific, cat no D-001810-10-20) using DharmaFECT siRNA transfection reagent (Thermo Scientific) in accordance to the manufacturer's instructions. Following an incubation period of 96 h, fresh growth medium containing 5 μM LY294002 was added and the SRB assay was performed after an additional 72 h incubation. For Western blot analysis, cells were harvested 48 h post-transfection.

2.8. Apoptosis assays

Following one cycle of combination treatment, cells were harvested and apoptosis induction was determined. Caspase-3-like activity was assessed using the method described previously [27]. In brief, cell lysates of treated or control cells were combined with DEVD-AMC, a fluorogenic substrate, in 1 \times HEPES buffer (20 mM HEPES, pH 7.5, 10% glycerol, and 2 mM dithiothreitol) and real-time measurements of the release of AMC catalyzed by caspase-3-like enzymes was measured using the Tecan Infinite F200 automated plate reader (Mannedorf, Switzerland). Fluorescence values for each sample were converted to picomoles of AMC release using a standard curve generated with free AMC and the rate of AMC release was calculated. The fraction of apoptotic cells was also estimated after treatment by staining cells with the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis kit with Alexa Fluor 488 annexin V and PI for flow cytometry (Invitrogen) following the manufacturer's instructions. Apoptosis was also assessed by immunoblotting for the cleaved fragment of PARP1 and in addition, the trypan blue exclusion assay was used to determine the total fraction of dead cells following treatment.

2.9. Gene expression profiling

Total RNA was extracted using the Qiagen RNA Mini kit (Qiagen, Valencia, CA), integrity analyzed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and then hybridized onto the Illumina HumanHT-12 v4.0 microarray platform (Illumina Inc, San Diego, CA).

Data normalization and management were performed using BioArray Software Environment (BASE) [28] and R [29]. The data were qQuantile normalized (which is inspired by the "Cubic Spline" normalization in Illumina Beadstudio and the work by Workman et al. [30]). Probes with a p -value greater than 0.01 were filtered out in BASE and thereafter the data were \log_2 transformed and mean centered across experiments in R. Differentially expressed genes and biological processes between the different treatments were identified using the Significance Analysis of Microarrays (SAM) and DAVID [31,32] tools, respectively. Gene expression data is available in the National Center for Biotechnology Information Gene Expression Omnibus (GEO); GSE34817.

2.10. Statistical analyses

Statistical comparisons of mean values were calculated using the two-sided student's t -test with the assumption of unequal variances. $p < 0.05$ was considered significant. All data are presented as mean \pm s.d. ($n = 3$) unless otherwise stated.

3. Results

3.1. Sensitivity to PARP inhibition

Initially, we were interested to validate the observation that *BRCA1* mutated cells are sensitive to PARP inhibition. Following continuous treatment with the PARP inhibitor AG014699 alone for seven days, three out of four *BRCA1* deficient breast cancer cell lines (MDA-MB-436, L56Br-C1 and SUM149) were more sensitive to the treatment compared to the *BRCA1* expressing MCF7 cell line (Fig. 1A). Growth inhibition of approximately 50% was reached at 0.07 μM , 2 μM and 2.8 μM for MDA-MB-436, L56Br-C1 and SUM149 cells, respectively. Similar results were obtained after treating MDA-MB-436 cells with three different PARP inhibitors: AG14361, AG014699 and AZD2881 (Supplementary Fig. 1). In contrast, HCC1937 and MCF7 cells reached 50% growth inhibition at concentrations $>10 \mu\text{M}$ (Fig. 1A). To further ascertain that the growth inhibitory effects due to PARP inhibition were associated with increase in DNA DSBs, we assayed γH2AX foci formation after 48 h treatment with AG14361 in SUM149 cells (Fig. 1B). A dose-dependent increase in the number of γH2AX foci formed in the AG14361 treated cells compared to DMSO controls was observed. Treatment with 100 μM of etoposide for 2 h resulted in the induction of massive DNA damage (positive control). These results clearly indicated that AG14361 inhibits cell growth through mechanisms involving the induction of DNA DSBs.

3.2. Sensitivity to PI3K inhibition

Following treatment of the different cell lines with increasing concentrations of LY294002 for three days, no considerable differential sensitivity was observed between MDA-MB-436, SUM149 and MCF7 cells (Fig. 1C). At concentrations between 10 and 20 μM , LY294002 inhibited the growth of MDA-MB-436, SUM149 and MCF7 cell lines by approximately 50%. L56Br-C1 and HCC1937 cells were less sensitive to LY294002, with 50% growth inhibition attained at concentrations $>20 \mu\text{M}$. To confirm that the effects of LY294002 treatment were associated with inhibition of the PI3K pathway, Western blot analysis to determine the levels of AKT phosphorylation at Ser-473 was performed following 24 h of treatment. The levels of Ser-473 phosphorylation significantly decreased upon treatment with LY294002 in a dose dependent manner (Fig. 1D), confirming that the observed growth inhibition was associated with inhibition of the targeted PI3K pathway.

3.3. Co-targeting PI3K and PARP

We aimed at investigating if the inhibition of the PI3K pathway could potentiate the growth inhibitory effects of PARP inhibition. To this end, we first studied the effects of the single drugs on the cell cycle phase distribution and found that the inhibition of PARP and PI3K affected the cell cycle differently. DNA histogram analysis revealed that PI3K inhibition resulted in an accumulation of cells in G1 concomitant with a decreased S-phase fraction. Inhibition of PARP1 on the other hand, induced a G2/M accumulation (Fig. 2). For PARP inhibition to be effective, cells must pass through S-phase and replicate their DNA. We therefore reasoned that sequential addition of the PARP inhibitor followed by the PI3K inhibitor would most likely be an optimal combination strategy. This sequential treatment resulted in an intermediate cell cycle phase distribution compared with the single drug treatments using the corresponding concentrations (Fig. 2).

Next, we investigated if significant growth inhibition could be observed upon combination of sub-optimal doses (doses of $\leq\text{GI}_{50}$) of the different drugs. When used as a single agent over 72 h, 5 μM

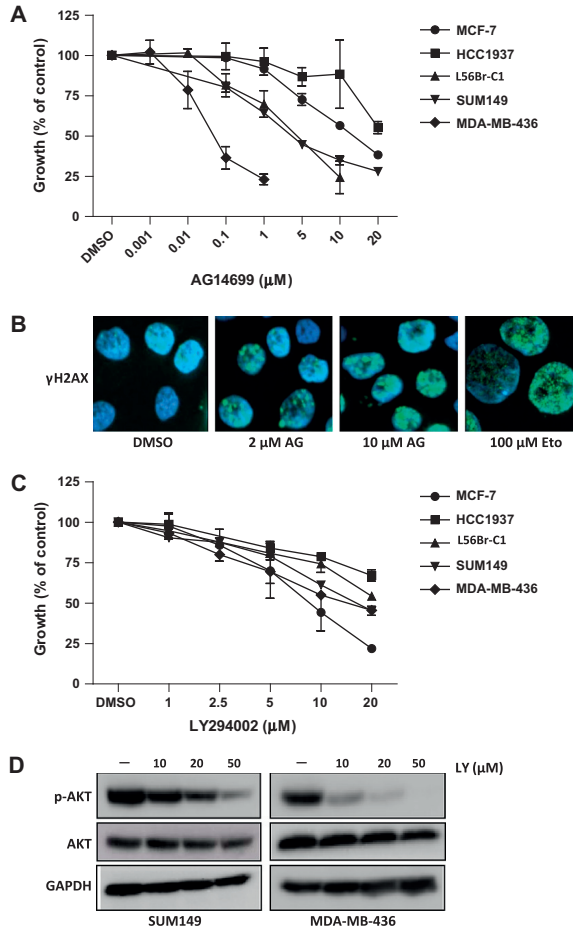


Fig. 1. Cytotoxic and growth inhibitory effects of single agent PARP1 and PI3K inhibitors. (A) Cells were treated with increasing concentrations of the PARP1 inhibitor AG014699 for 7 days and the surviving fraction was assessed using the SRB assay. MDA-MB-436, SUM149, L56Br-C1 and HCC1937 cell lines all harbor *BRCA1* mutations while MCF7 has wild type *BRCA1*. Data plotted are the mean \pm s.d. of three independent experiments. (B) DNA DSB accumulation (γ H2AX phosphorylation) after 48 h treatment of SUM149 cells with different concentrations of the PARP1 inhibitor AG14361. Etoposide (Eto) was used as positive control. Cell nuclei are stained in blue while γ H2AX foci are stained in green. (C) Cells were treated with increasing concentrations of the PI3K inhibitor LY294002 for 3 days and the surviving fraction was assessed using the SRB assay. Data plotted are the mean \pm s.d. of three independent experiments. (D) Western blot showing down-regulation of PI3K signaling via reduction in p-AKT levels after 24 h of treatment with LY294002. GAPDH was used as loading control.

of LY294002 reduced the growth of all five cell lines by approximately 20–30% (Fig. 1C); hence, this dose was selected as the fixed single concentration for use in combination treatments. Similarly, sub-optimal doses for the PARP inhibitor AG014699 for the respective cell lines were selected for combination treatments. After one cycle of sequential combination of AG014699 and LY294002, significant chemo-potential was observed for both the MDA-MB-436 ($p < 0.01$; Fig. 3A) and the SUM149 ($p = 0.01$; Fig. 3B) cell lines. LY294002 improved the efficacy of AG014699 by further increasing the growth inhibition by approximately 20% compared

to the effect of AG014699 alone. A similar trend in growth inhibition was observed after transient knock-down of PARP1 with siRNA in MDA-MB-436 cells, confirming the specificity of the PARP inhibitors. PARP1 silencing and sequential treatment with 5 μ M LY294002 resulted in a significant reduction of cell growth ($p < 0.01$) compared to PARP1 knock-down alone (Supplementary Fig. 2). In addition, calculation of the CI using the median-effect method revealed a clear synergistic interaction between AG014699 and LY294002 in combination ($CI < 1$) in MDA-MB-436 and SUM149 cells at all doses tested (Table 1). L56Br-C1 cells were also

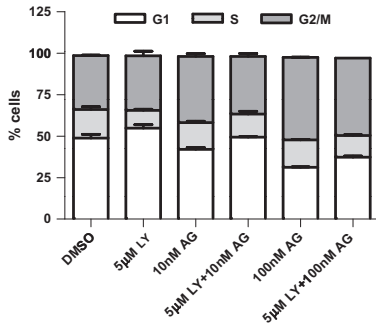


Fig. 2. Cell cycle analysis after one cycle of sequential combination of PARP1 and PI3K inhibitors. The proportions of cells in the different phases of the cell cycle were estimated after one cycle (7 days) sequential combination of the PARP inhibitor AG14361 and the PI3K inhibitor LY294002. Data shown is from one representative experiment on MDA-MB-436 cells. Error bars are s.d. from triplicate determinations.

more sensitive to the combination of LY294002 and AG14699 (Fig. 3C) but the increase in sensitivity did not reach statistical significance when compared to AG14699 alone. However, based on calculations of the combination index, the two drugs interacted in a synergistic manner ($CI < 1$) in this cell line as well (Table 1). The combination of AG14699 and LY294002 in MCF7 cells also yielded a significant decrease in viability (Fig. 3E). While 1 μ M of AG14699 by itself did not affect the viability of MCF7 cells, in combination with LY294002 a significant increase ($p < 0.01$) in growth inhibition compared to AG14699 was observed. Importantly however, drug interaction calculations revealed an antagonistic effect of the combination ($CI > 1$). No significant differences were observed

Table 1

Calculation of combination indices for AG14699 with LY294002 using the Chou–Talalay method. Different concentrations of the PARP inhibitor AG14699 were combined sequentially with a fixed dose of the PI3K inhibitor LY294002. The combination index (CI) values for two concentrations of the PARP inhibitor for MDA-MB-436, SUM149, L56Br-C1 and a single concentration for MCF7 are reported. FA represents the fraction affected by the combination expressed as a percentage. $CI < 1$, $CI = 1$ and $CI > 1$ indicate synergism, additive effect and antagonism, respectively.

	Cell line	FA	CI
1 cycle	MDA-MB-436	50	0.45
		81	0.18
	SUM149	32	0.85
		52	0.55
	L56BR-C1	25	0.68
		29	0.75
2 cycles	MCF7	30	1.12
	MDA-MB-436	60	0.92
		80	0.70
	SUM149	58	0.69
		77	0.83
	L56BR-C1	28	0.86
		67	1.81

for any comparisons in the HCC1937 cell line (Fig. 3D), precluding the calculation of a CI.

We also compared the effects of a sequential versus continuous combination treatment strategy in the MDA-MB-436, SUM149 and MCF7 cell lines. A continuous combination regimen was more effective in inhibiting growth in MDA-MB-436 and MCF7 cell lines, but not in SUM149 cells (Supplementary Fig. 3).

Most intriguing was the pronounced potentiation in growth inhibition observed after two cycles (14 days) of sequential combination treatment. In MDA-MB-436 cells, growth inhibition reached 80% ($p < 0.05$) for the combination of 10 nM AG14699 with 5 μ M LY294002, corresponding to about twice the effect observed after one cycle of treatment. Furthermore, doses as low as 5 nM AG14699, which did not show any growth inhibitory effects of

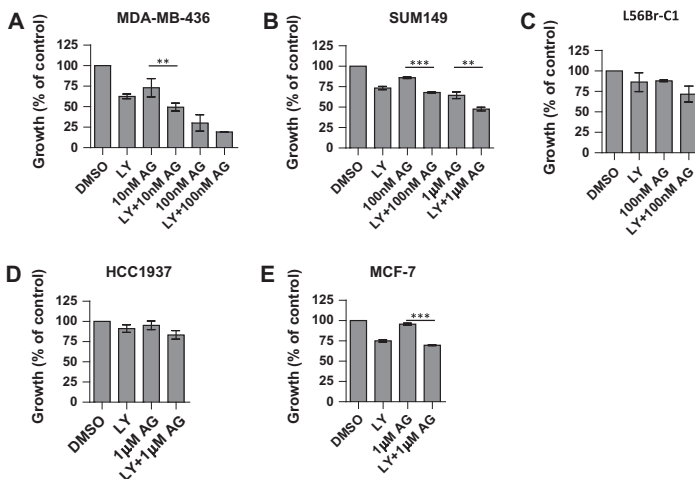


Fig. 3. Cytotoxic effects after one cycle of sequential combination of PARP1 and PI3K inhibitors. Cells were seeded into 12-well plates and treated with the PARP inhibitor AG14699 (AG) alone for 72 h followed by a combination of AG with the PI3K inhibitor LY294002 (LY) for a further 96 h. (A) MDA-MB-436, (B) SUM149, (C) L56Br-C1, (D) HCC1937 and (E) MCF7 cells. Data are the mean \pm s.d. of three independent experiments. Statistically significant differences as determined by the student's *t*-test are represented as; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

their own after one cycle of treatment, resulted in an inhibition of approximately 60% ($p < 0.01$; Fig. 4A) following two cycles of sequential combination with 5 μM LY294002. In addition, in MDA-MB-436, two cycles of treatment with 10 nM AG014699 alone increased growth inhibition by approximately 45%, while

5 μM LY294002 alone resulted in only a 7% increase in growth inhibition after two cycles. Similar effects were observed after two cycles of combination treatment with sub-optimal doses of AG014699 and LY294002 in the SUM149 cells, with up to 80% growth inhibition attained by treating with a combination of

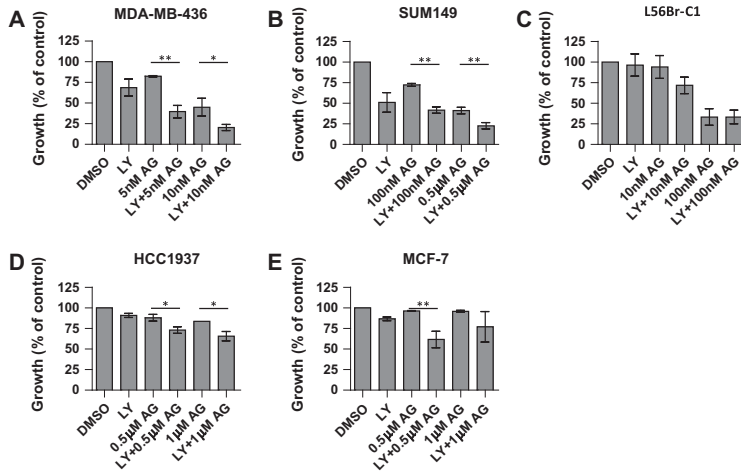


Fig. 4. Cytotoxic effects after two cycle sequential combination of PARP1 and PI3K inhibitors. Cells were seeded into 12-well plates and treated with the PARP inhibitor AG014699 (AG) alone for 72 h followed by a combination of AG with the PI3K inhibitor LY294002 (LY) for a further 96 h. This was repeated for another cycle. (A) MDA-MB-436, (B) SUM149, (C) L56Br-C1, (D) HCC1937 and (E) MCF7 cells. Data are the mean \pm s.d. of three independent experiments. Statistically significant differences as determined by the student's *t*-test are represented as: * $p < 0.05$ and ** $p < 0.01$.

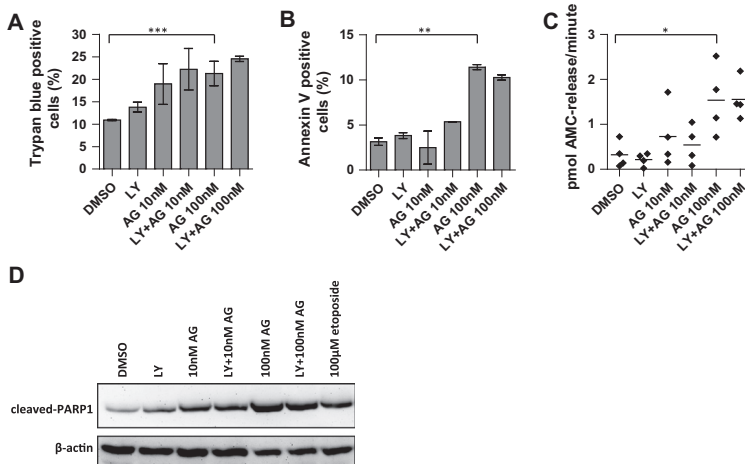


Fig. 5. Effects of treatment on apoptosis markers. Induction of apoptosis in MDA-MB-436 cells following one cycle of combination treatment with the PARP inhibitor AG014699 (AG) and the PI3K inhibitor LY294002 (LY): (A) trypan blue exclusion assay, (B) Annexin V staining, (C) Caspase-3-like activity measured by DEVD-AMC cleavage. Data plotted are the means \pm s.d. from at least two independent experiments. Statistically significant differences as determined by the student's *t*-test are represented as: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (D) Western blot analysis for PARP1 cleavage after one cycle of combination treatment. 50 μg of total protein lysates was electrophoresed. Etoposide treated (100 μM for 24 h) cell lysate was used as a positive control and β -actin served as loading control.

0.5 μM AG014699 and 5 μM LY294002 ($p < 0.01$; Fig. 4B). The significant enhancement of growth inhibition by the combination in these cell lines was associated with combination indices < 1 , confirming that the drugs interact in a synergistic manner (Table 1). After two cycles of treating MCF7 cells, no significant increases in growth inhibition were observed for AG014699, LY294002 or the combination compared to the effects after one treatment cycle (Fig. 4E). L56Br-C1 was extremely sensitive to single agent AG014699 after two treatment cycles, with up to 67% growth inhibition reached after treating with 0.1 μM AG014699 (Fig. 4C). A synergistic interaction ($CI > 1$) was observed at 10 nM AG014699, but not at 0.1 μM (Table 1). HCC1937 cells, which did not show any considerable changes after one cycle of treatment, were significantly more sensitive to the combination compared to each single agent (Fig. 4D).

3.4. Assessment of apoptosis

While it is well established that treatment of BRCA1 deficient cells with PARP inhibitors results in the increase of DNA DSBs and decrease in survival, data confirming that cell death induction is also involved in reducing viability are limited. We therefore attempted to delineate if apoptosis induction was involved in decreasing viability in the MDA-MB-436 cell line following PARP and PI3K inhibition by interrogating established cell death markers such as trypan blue exclusion, annexin V staining, caspase-3 activity and PARP1 cleavage. Firstly, trypan blue exclusion assays revealed a significant increase in the proportion of dead cells after

PARP inhibition relative to the control (from approximately 11% in the DMSO control to 25% in the 100 nM AG014699 combination, $p < 0.001$; Fig. 5A). Next, the percentage of cells positive for annexin V was greater upon AG014699 treatment compared to the DMSO controls. The fraction of annexin V positive cells increased from approximately 3% in the DMSO control to 11% in the 100 nM AG treated samples ($p < 0.01$; Fig. 5B). Similarly, caspase-3 activity increased from 0.32 pmol AMC release/min in the DMSO control to > 1.5 pmol AMC release/min after 100 nM AG014699 treatment ($p < 0.05$; Fig. 5C). Finally, Western blot analysis revealed a dose-dependent increase in PARP1 cleavage upon AG014699 treatment (Fig. 5D). In general, the effects of the combination of AG014699 with LY294002 on all the apoptosis markers were similar to those observed with the corresponding doses of AG014699 alone. However, LY294002 alone did not result in any major increase in apoptosis compared to the DMSO controls.

3.5. Transcriptional effects of PARP and PI3K inhibition

To identify genes and pathways differentially altered after treatment of MDA-MB-436 with the PARP inhibitor AG014699 as a single agent or in combination with LY294002, SAM followed by gene ontology analyses were performed. Several genes and biological processes were found to be differentially altered between PARP inhibitor treated cells and untreated controls (Fig. 6A). Amongst these was the up-regulation of apoptosis (Fig. 6B) and inflammation (immune response) genes. In addition, there was an up-regulation of genes involved in the negative regulation of

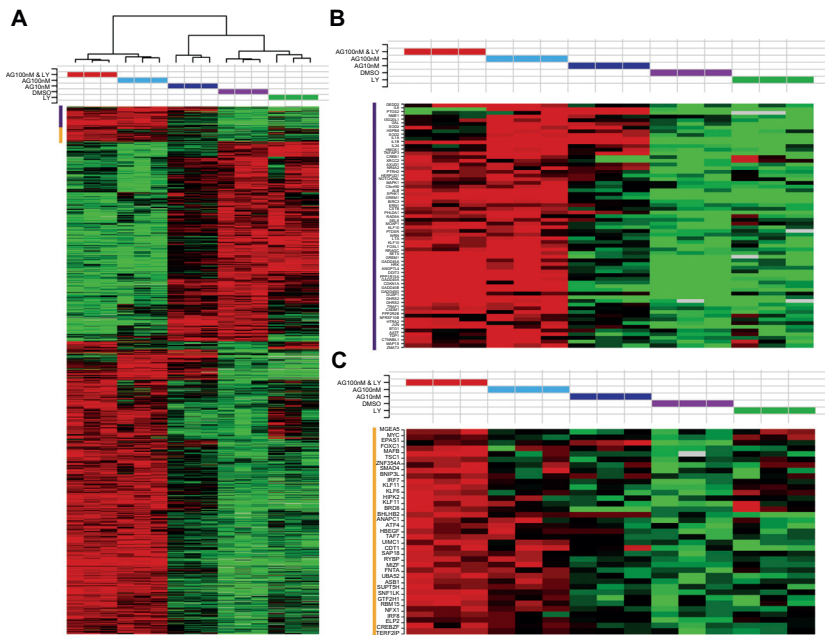


Fig. 6. Transcriptional effects of PARP and PI3K inhibition. (A) Hierarchical clustering of the 1555 most significantly differentially altered genes between PARP inhibitor treated cells and untreated controls using Pearson correlation distance and complete linkage. (B) A zoom-in of the heat map in (A) of genes involved in apoptosis, which are significantly up-regulated in the PARP inhibitor treated cells. (C) A zoom-in of the heat map in (A) of genes involved in negative regulation of macromolecule biosynthetic processes, which are more significantly up-regulated in the combination treatment compared to PARP inhibitor alone.

macromolecule biosynthetic processes in the combination compared to AGO14699 alone (Fig. 6C).

4. Discussion

PARP inhibitors are showing great promise as a selective targeted therapy for breast and ovarian cancers with mutations in the *BRCA* genes [8–10]. At the same time, these clinical trials clearly show that there is significant variation in the response to these agents even in selected (*i.e.* *BRCA1/2* mutation positive) patient cohorts. It is possible that prior exposure to chemotherapy may affect the biological behavior and responsiveness to PARP inhibition. In fact, patients with previous platinum-based chemotherapy were less sensitive to the PARP inhibitor olaparib (AZD2881), but the response was not limited by the extent of previous non-platinum chemotherapy [10]. These discrepancies warrant a refinement of this treatment strategy so as to increase the number of responders as well as to improve the durability, and potentially later even to aid in selecting an optimal regimen for studies of adjuvant (curative) treatment in *BRCA*-associated cancer. Herein, we have investigated the possibility of potentiating the response of *BRCA1* mutated cells to PARP1 inhibition by co-targeting PI3K signaling. Because the response to most targeted therapies when used as single agents frequently is not durable, current efforts towards treatment enhancement focus on identifying optimal ways of targeting combinations of specific molecular defects present in each cancer, *i.e.* individualized treatment. To our knowledge, the present *in vitro* study is the first to show beneficial effects of co-targeting the PI3K signaling pathway and PARP1. Our results clearly indicate that combination of the PARP inhibitor AGO14699 with the PI3K/AKT inhibitor LY294002 interacts synergistically to increase growth inhibition specifically in the *BRCA1* mutated cell lines compared to wildtype cells after extended exposure, albeit to a varying extent. This specific molecular targeting differs from the more traditional approach of combining targeted agents like PARP inhibitors with radiotherapy or conventional chemotherapy. Genomic alterations in *PTEN* are frequently associated with *BRCA1* deficiency [16,33] and predict sensitivity to both PI3K [34] and PARP [15] inhibition. Furthermore, there is evidence suggesting that inhibition of PARP1 results in activation of the PI3K/AKT pathway [35], thereby promoting resistance to chemotherapy. Taken together, these observations support a therapeutic strategy based on co-targeting of PARP1 and the PI3K pathway, an approach which is strengthened by the data presented herein.

Of importance, we report the effects of very low doses of PARP inhibitors in combination with an equivalent low dose of a PI3K inhibitor. The extensive therapeutic responses achieved using sub-micromolar concentrations of PARP inhibitors in sequential combination with a low dose of LY294002 provides a foundation for further exploration of this therapeutic strategy in *BRCA1* deficient tumors. This study was designed as a proof-of-concept study to test the hypothesis that co-targeting PI3K will enhance the effect of PARP inhibition in *BRCA1* deficient cells. Even though the pharmacological specificity of the LY294002 compound for PI3K may be of concern, this drug has no doubt played a key role in unraveling of the function of PI3K signaling. Further studies using new generation PI3K inhibitors that are more specific and well tolerated clinically are warranted to validate and extend these results.

An important challenge of combinatorial treatment design is to establish optimal doses and treatment schedules to optimize efficacy while minimizing toxicity and avoiding over-treatment. Consistent with previous data [36], cell cycle analysis revealed that treatment of MDA-MB-436 cells with 5 μ M LY294002 resulted in accumulation of cells in G1, supporting a sequential combination regimen for PI3K and PARP inhibitors. Even though a stronger

growth inhibitory effect was observed in some cell lines after a continuous combination treatment strategy, prolonged continuous obstruction of the PI3K pathway is likely more toxic and compromises the specificity of the PARP inhibition treatment more than the sequential regimen, thereby hypothetically out-weighting the growth inhibitory advantage. This sequential treatment regimen nonetheless achieved potent effects *in vitro* (up to 80% growth inhibition after two treatment cycles) and is likely less toxic than a continuous regimen since the cumulative exposure to the PI3K inhibitor is reduced. Hence, the proposed combination treatment strategy described herein may minimize any potential unspecific and adverse effects of inhibiting the PI3K pathway.

In concordance with the results of Drew et al. [37] and Mendes-Pereira et al. [15] we also show that *BRCA1* and *PTEN* deficient breast cancer cell lines are selectively sensitive to PARP inhibition. However, based on our selected cell lines and the data we present, it is not clear if this selectivity is driven by only one or a combined effect of both mutations. Also in line with earlier pre-clinical as well as clinical studies, we observed significant heterogeneity in the sensitivity of the *BRCA1* deficient cell lines to PARP inhibition. The MDA-MB-436 cells were more sensitive to PARP inhibition compared to the SUM149 and L56Br-C1 cells, even though neither cell line expresses *BRCA1*. These three cell lines most certainly harbor unique properties with evident consequential influence on phenotype and sensitivity to treatment, similar to what has been observed in the clinical setting [8–10]. Despite the difference in sensitivity, these cell lines are very sensitive to PARP inhibition when compared to MCF7 cells with wildtype *BRCA1*. While several of the previous studies have only used non-human *BRCA*-null cell lines [6,7] the only studies that have made use of human *BRCA* mutated breast cancer cells [37,38] to study their response to PARP inhibition have also demonstrated similar variation in the level of sensitivity. Interestingly, the often-used *BRCA1* mutant cell line HCC1937 was the least sensitive after one cycle of treatment; however, similar to the other *BRCA1* mutant cell lines, there was an enhancement in sensitivity upon prolonged combination treatment. In support of this observation, Lehmann et al. [38] recently showed that HCC1937 cells were resistant to the PARP inhibitor olaparib as well as the PI3K inhibitor NVP-BE235 after short-term treatment, suggesting that these cells may require longer exposure for the synthetic lethal effects of PARP inhibition to be effective, or, alternatively, may require other contributions from the tumor microenvironment to elicit a response. Notwithstanding, LY294002 potentiated the effects of the PARP inhibitors in all *BRCA1* mutant cell lines tested. The current biomarkers used for selection of patients for PARP inhibition therapy include genetic mutations in the *BRCA* genes, a triple negative phenotype and more generally defects in the homologous recombination biological process [39]. There is a need for better characterization of the properties that predict sensitivity to PARP inhibitors and for the identification of new biomarkers that can be further used for optimal patient and dose stratification.

Even though many studies have consistently shown very encouraging responses to PARP inhibition in targeted cell populations, information on the molecular mechanisms driving sensitivity to treatment is limited. To our knowledge, there are no published reports that have shown that PARP inhibition leads to cell death through apoptosis in *BRCA* mutated breast cancer cells. Most studies have limited their focus to the characterization of the DNA damage response defects and measurements of PARP activity [6,15,37]. To gain more insight into the molecular mechanisms involved in decreasing viability following treatment, we investigated several markers of apoptosis. To this end, PARP inhibition was consistently found to increase apoptosis in MDA-MB-436 cells as revealed by annexin V staining, caspase-3 activity and caspase-mediated PARP1 cleavage. Transcriptional profiling

corroborated this by revealing a significant up-regulation of apoptosis associated genes following PARP inhibition. The differences in apoptosis induction were modest but the cumulative effects observed may nevertheless contribute to the pronounced net effect of treatment (80% reduction in viability). Similar data showing a modest increase in caspase-3 cleavage upon PARP inhibition *in vivo* have been reported [40]. Importantly, while most regimens depend on exogenously induced DNA damage (chemotherapy or radiotherapy), which can be controlled by dosing, the current approach only takes advantage of endogenously generated DNA damage and the use of low doses of the PARP inhibitor. As unsynchronized cells progress through the cell cycle and acquire spontaneous DNA damage, PARP inhibition will prevent their repair and only target the fraction of cells with sufficient levels of damage to undergo apoptosis. The immunofluorescence, cell cycle, apoptosis assays and transcriptional profiling taken together, suggest that the cytotoxic effects of PARP inhibition are partially achieved through the accumulation of DNA DSBs, thereby blocking cells in G2/M, and leading to apoptosis. On the other hand, LY294002 at the 5 μ M dose used here did not result in any significant change in the levels of these cell death markers, implying that low concentrations of LY294002 only prevents cells from progressing through G1. Interestingly, there was a down-regulation of macromolecule biosynthetic processes in the combination compared to PARP inhibition alone. The fact that the combination of PARP and PI3K inhibitors did not result in any further significant increase in apoptosis compared to PARP inhibition alone as measured in the four assays should be considered in the context of significantly increased growth inhibition as a result of decreased macromolecule biosynthesis, *i.e.* fewer cells remain that may undergo apoptosis. Furthermore, only anti-proliferative effects were observed upon LY294002 treatment, similar to previous reports indicating that PI3K inhibition alone does not induce apoptosis, but rather induces growth arrest [36]. Taken together these results suggest that the molecular mechanisms driving the decrease in viability can in part be explained by decrease in macromolecular biosynthesis followed by apoptosis induction.

In conclusion, we have shown that a sequential combination of PARP and PI3K inhibitors is superior over PARP inhibition alone for targeting *BRCA1* deficient breast cancer cells. As it is unlikely that tumor cells are dependent on a single pathway for survival, a strategy targeting the crucial molecular defects in a tumor, in the present case *BRCA1* mutation and PTEN loss, is more likely to be effective, and warrants further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2012.01.015.

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