Receptor tyrosine kinases and circulating tumor cells as new prognostic biomarkers in breast cancer with special focus on TNBC

Jansson, Sara

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with special focus on TNBC

Sara Jansson

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Receptor tyrosine kinases and circulating tumor cells as new prognostic biomarkers in breast cancer, with special focus on TNBC

Abstract

About 8000 Swedish women are diagnosed with breast cancer each year, and around 1500 die from the disease. Triple-negative breast cancer (TNBC) constitutes 7-14% of all breast cancer and is characterized by an aggressive phenotype with poor prognosis and no targeted therapy available. The aim of this thesis was to evaluate new potential prognostic biomarkers in breast cancer, and with special focus on TNBC.

In paper I and II the importance of the four receptors cKIT, VEGFR2, PDGFRα and PDGFRβ, and of ligand PDGF-CC was explored in a prospectively gathered cohort of primary breast cancer patients. In both papers, tissue micro arrays and immunohistochemistry was used to evaluate protein expression, and in paper I, we also investigated gene copy number using fluorescence *in situ* hybridization. In paper I, we found that high tumor cell protein expression, but not elevated gene copy number, of cKIT, VEGFR2 and PDGFRα was associated to TNBC. 74% of TNBC tumors displayed high expression of at least one of these three receptors compared to 30 % of non-TNBC. In paper II, we showed that high expression of the PDGF receptors α and β, and ligand PDGF-CC correlated to several prognostic patient and tumor characteristics related to tumor inherent biological aggressiveness (e.g. hormone receptor negativity and higher tumor grade). Neither of the receptors investigated in paper I or II were associated to survival in TNBC but interestingly, in the whole cohort we found that patients with high expression of ligand PDGF-CC in the primary tumor had increased risk of 5-year distant-recurrence.

In papers III and IV, we investigated circulating tumor cell (CTC) count and morphologic CTC characteristics as prognostic markers in patients with newly diagnosed metastatic breast cancer (MBC) scheduled for 1st line systemic therapy. The CellSearch system was used for CTC isolation and characterization. In paper III, only patients with baseline (BL) CTC count ≥5 before initiation of therapy were included (N=52). We found that presence of apoptotic CTCs and CTC-clusters during treatment (but not at BL) was associated with a significantly worse prognosis. We also found that at BL, TNBC and HER2+ patients had CTC-clusters present more frequently than hormone receptor positive patients. In paper IV, 156 patients were included (irrespective of BL CTC count), and we showed that CTC count ≥5, and presence of CTC-clusters were prognostic for PFS and OS at BL and during the first 6 months of systemic therapy following diagnosis of MBC. Also, changes in CTC count during therapy significantly correlated to response evaluation and survival. Finally, both factors independently added value at all time points to a prognostic model based on clinicopathological variables.

In conclusion, paper I and II present support for the involvement of cKIT, VEGFR2, PDGFRα and PDGF-CC in TNBC. These receptors are not prognostic markers in TNBC, but they are upregulated and further studies are encouraged to elucidate their values as predictive markers and possible drug targets in TNBC. Paper III and IV show the clinical value of CTC count and CTC-cluster detection before and during 1st line systemic therapy for prognosis and treatment monitoring in patients with newly diagnosed MBC. Our results highlight the importance of serial monitoring of these variables as the prognostic value of both CTC count and CTC-cluster detection increased over time.

Key words: breast cancer, triple-negative breast cancer, receptor tyrosine kinases, circulating tumor cells

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Receptor tyrosine kinases and circulating tumor cells as new prognostic biomarkers in breast cancer

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Sara Jansson

LUND
UNIVERSITY

Author Sara Jansson
e-mail: sara.jansson@med.lu.se

Principal supervisor: Professor Lisa Rydén (MD)
Department of Clinical Sciences Lund,
Division of Surgery, Lund University, Sweden

Co-supervisors: Associate professor Kristina Aaltonen
Department of Clinical Sciences Lund,
Division of Oncology and Pathology,
Lund University, Sweden

Professor Kristian Pietras
Department of Laboratory Medicine,
Division of Translational Cancer Research,
Lund University, Sweden

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Faculty of Medicine
Department of Clinical Sciences Lund, Division of Oncology and Pathology

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To my beloved family

Non scholae, sed vitae discimus – Vi lär inte för skolan utan för livet

Content

List of included papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. The three receptor tyrosine kinases c-KIT, VEGFR2 and PDGFRα, closely spaced at 4q12, show increased protein expression in triple-negative breast cancer

Sara Jansson, Pär-Ola Bendahl, Dorthe Aamand Grabau, Anna-Karin Falck, Mårten Fernö, Kristina Aaltonen, Lisa Rydén. PLoS One, 2014. Vol 9 (7):e102176

II. The PDGF pathway in breast cancer is linked to tumour aggressiveness, triple-negative subtype and early recurrence

Sara Jansson, Kristina Aaltonen, Pär-Ola Bendahl, Anna-Karin Falck, Maria Karlsson, Kristian Pietras, Lisa Rydén. Manuscript submitted

III. Prognostic impact of circulating tumor cell apoptosis and clusters in serial blood samples from patients with metastatic breast cancer in a prospective observational cohort

Sara Jansson, Pär-Ola Bendahl, Anna-Maria Larsson, Kristina Aaltonen, Lisa Rydén. BMC cancer, 2016. Vol 16:433

IV. Longitudinal CTC and CTC-cluster evaluation improves prognostication and monitoring in metastatic breast cancer patients starting 1st line systemic treatment

Anna-Maria Larsson*, **Sara Jansson***, Pär-Ola Bendahl, Charlotte Levin Tykjær Jørgensen, Niklas Loman, Cecilia Graffman, Charlotte Lundgren, Kristina Aaltonen, Lisa Rydén. Manuscript

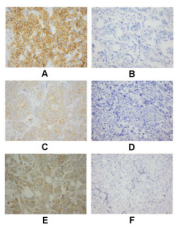
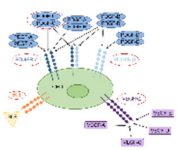
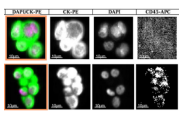
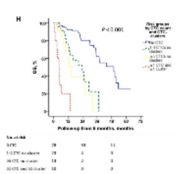
*these authors contributed equally to this work

List of non-included papers

I. Systemic Monocytic-MDSCs Are Generated from Monocytes and Correlate with Disease Progression in Breast Cancer Patients

Bergenfelz, C., Larsson, A. M., von Stedingk, K., Gruvberger-Saal, S., Aaltonen, K., **Jansson, S.**, Jernstrom, H., Janols, H., Wullt, M., Bredberg, A., Ryden, L., Leandersson, K.. PLoS One, 2015. Vol 10 (5):e127028

Dissertation at a glance

Study	Aim	Methods	Results and conclusion
Paper I 	To elucidate if there is a correlation between the protein expression of the three receptor tyrosine kinases cKIT, VEGFR2 and PDGFRα, their gene copy number, and prognosis in triple-negative breast cancer (TNBC) compared to non-TNBC.	Protein expression was investigated by immunohistochemistry (IHC) and gene copy number by fluorescence *in situ* hybridization (FISH). All staining was done on tissue micro arrays (TMAs).	High tumor cell expression, but not elevated gene copy number, of cKIT, VEGFR2 and PDGFRα was associated to TNBC. No association was found to survival in TNBC. 74% of TNBC had high expression of ≥1 receptor compared to 30% of non-TNBC. cKIT, VEGFR2 and PDGFRα are potential drug targets in TNBC.
Paper II 	To evaluate the protein expression of PDGFRα, PDGFRβ and ligand PDGF-CC in breast cancer in relation to molecular breast cancer subtypes and prognosis.	Protein expression was investigated in primary tumors, synchronous lymph node metastasis and asynchronous recurrences by IHC on TMAs.	High expression of PDGFRα, PDGFRβ and PDGF-CC was associated to patient and tumor characteristics that indicate tumor inherent biological aggressiveness. High tumor cell PDGF-CC was associated to TNBC, and increased risk of 5-year distant recurrence. Our findings support an active role of the PDGF signaling in tumor progression and suggest that strategies to target this pathway could be beneficial in breast cancer.
Paper III 	To explore whether apoptotic circulating tumor cells (CTCs), CTC-clusters and WBC-CTCs are associated with breast cancer subtype and prognosis at baseline (BL) and during first six months of follow-up in metastatic breast cancer (MBC) patients	CTCs were isolated by the CellSearch system. Morphologic evaluation of CTCs was performed on CTC-galleries exported from the CellTracks Analyzer. No further staining was added.	Patients with apoptotic CTCs and CTC-clusters present during treatment have worse prognosis. TNBC and HER2+ patients have CTC-clusters present more often in their blood than patients with hormone receptor positive breast cancer. Morphologic characterization of CTCs and CTC-clusters in the blood during treatment may be an important prognostic marker.
Paper IV 	To evaluate if longitudinal enumeration of CTCs and CTC-clusters could improve prognostication and monitoring of patients with MBC starting 1st line systemic therapy. A prospective observational trial.	CTCs were isolated by the CellSearch system. Blood samples were collected at BL, 1, 3 and 6 months. Primary end-point was progression-free survival (PFS) and secondary end-point overall survival (OS).	CTC count ≥5, and presence of CTC-clusters were prognostic for PFS and OS at BL and during the first 6 months of systemic therapy. Both variables independently improved a clinicopathological prognostication model. Changes in CTC count during therapy correlated to response evaluation and survival. The prognostic value of CTC count and CTC-cluster evaluation increased over time, suggesting that dynamic changes of CTCs and CTC-clusters are more clinically relevant than BL evaluation only.

Abbreviations

CI	confidence interval
CK	cytokeratin
CNS	central nervous system
CTC	circulating tumor cell
DRFi	distant recurrence-free interval
DTC	disseminated tumor cell
EMT	epithelial-to-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ER	estrogen receptor
FISH	fluorescence *in situ* hybridization
HER2	human epidermal growth factor receptor 2
HR	hazard ratio
IHC	immunohistochemistry
MBC	metastatic breast cancer
NHG	Nottingham histological grade
OR	odds ratio
OS	overall survival
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PFS	progression-free survival
PR	progesterone receptor
RTK	receptor tyrosine kinase
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
TNBC	triple-negative breast cancer
VEGFR2	vascular endothelial growth factor receptor 2
WBC	white blood cell

Introduction

Breast cancer is the most common female cancer and approximately 1.67 million women were diagnosed worldwide in 2012.[1] In Sweden, breast cancer represents nearly 30% of all female cancer and in 2015, a total of 7929 women received a breast cancer diagnosis.[2] The 5-year survival rate after primary breast cancer is almost 90% today, and it has increased over the last decades as a result of improvements in diagnostics and treatments.[3,4] Most breast cancer patients are cured by primary surgery; and additional radiotherapy and adjuvant systemic treatment(s) decrease the risk of recurrence. Nevertheless, approximately one in three breast cancer patients will recur with metastatic disease during their lifetime. For the metastatic breast cancer (MBC) patients, the 5-year survival has also increased over the last decades, and it is now around 27%.[5]

Of note is that the incidence of breast cancer in Sweden has increased by 1.7% annually over the last 20 years.[2] Possible explanations for this are increasing exposure to female hormones, e.g. use of hormone replacement therapies, lower age at menarche and higher age at first pregnancy, and life-style changes such as increased prevalence of obesity.

Biomarkers

Biomarkers are important tools in oncology to help evaluate tumor characteristics and to guide choice of treatment. The term "biomarker" has been defined by amongst others the Biomarkers Definitions Working Group in 2001 as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[6] In other words, it is a measurable sign (e.g. a protein) that can be accurately and reproducibly determined within a person and that gives an indication of the medical condition of that person. This thesis focuses on receptor tyrosine kinases (RTKs) (proteins) and circulating tumor cells (CTCs) (cancer cells in the blood stream) as potential new prognostic biomarkers in breast cancer.

Breast cancer development, risk factors and diagnosis

The normal breast is composed of fatty tissue, connective tissue and glandular tissue; and the proportion between these constituents vary amongst individuals. The glandular tissue is organized into a tree-like structure starting inside the breast with small lobules containing milk-producing apical luminal epithelial cells and surrounded by basal myoepithelial cells. The lobules unite into lobes and later ducts leading out to the nipple. A continuous basement membrane surrounds the breast epithelium.[7]

It is not known why a normal breast cell transforms into a malignant cell but there are background concepts explaining how a cancer in general is formed, "the Hallmarks of Cancer".[8,9] There are also some known risk factors for developing breast cancer.

Hallmarks of Cancer

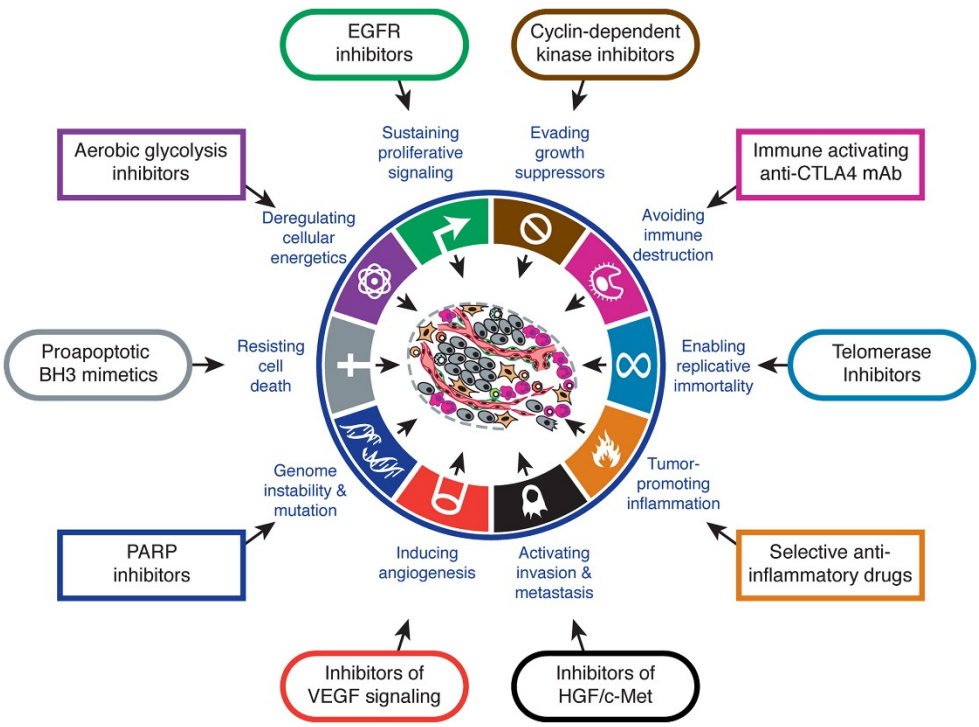


Figure 1. The Hallmarks of Cancer and possible targets for therapy
Reprinted from The Cell, volume 144, issue 5, Hanahan D, Weinberg RA, Hallmarks of Cancer: The Next Generation, 646-674. Copyright (2011), with permission from Elsevier (Hanahan & Weinberg, 2011).

The concept of "Hallmarks of Cancer" was initially described in a review article by Hanahan and Weinberg in 2000,[8] and later refined in a second review by the same authors in 2011.[9] The hallmarks of cancer are a collection of important biological attributes that a normal cell must acquire in order to become a malignant cell. The six core traits that were proposed in 2000 included self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, replicative immortality, sustained angiogenesis, and tissue invasion and metastasis.[8] In 2011, four new traits were added; genome instability and mutation, avoidance of immune destruction, tumor promoting inflammation and deregulation of cellular energetics.[9] The hallmarks of cancer were assembled to describe the process of tumor development, but they also provide an overview of distinct tumor promoting mechanisms that can be targeted by anti-cancer therapies, see Figure 1.

Risk factors for breast cancer

The risk of developing breast cancer increases with age and reaches a peak in women 65-79 years.[2] There is an association between exposure to female sex hormones and increased risk of breast cancer. Important risk factors are early menarche, late menopause, low parity and high age at first full-term pregnancy.[10] Hormone replacement therapy[11] and to some degree oral contraceptives[12] has also been shown to increase the risk of breast cancer. Other known risk factors are previous benign breast disease[13], high breast density[14], postmenopausal obesity[15], high alcohol intake[16] and exposure to radiation at young age.[17]

In addition to the risk factors described above, a family history of breast cancer and genetic inheritance are also known factors associated with increased risk.[18] Two high risk genes for breast cancer were discovered in the 1990s; *BRCA1* and *BRCA2*.[19] Persons with BRCA-mutations can have a life-time risk of breast cancer development of up to 80%, and the disease usually occur at younger age.[20]

Diagnosis

Diagnosis of breast cancer is performed by triple diagnostics as a golden standard. Triple diagnostics is a combination of 1) clinical examination of the breast and loco-regional lymph nodes, 2) core needle biopsy for pathological evaluation and if needed also fine-needle aspirate for cytological evaluation, and 3) imaging diagnostics (usually mammography and ultrasound, and for selected cases magnetic resonance imaging (MRI)).[21]

Since 1997, all regions of Sweden have a mammography screening program and about half of all breast cancers are detected by screening mammography.[22] The screening program includes women age 40-74 years and within this age group, 64% of all breast cancers are detected by screening. It has been estimated that screening mammography reduces the relative risk of breast cancer mortality by 16-25%.[23]

Metastasis

Tumor metastasis is the leading cause of death amongst cancer patients.[24] Metastasis is a multistep process where tumor cells leave the primary tumor, travel through the lymphatic and/or hematogenous systems to distant locations in the body where they exit and start to proliferate to eventually create metastatic lesions. Different tumors have different sites of preference, breast tumors principally spread to lymph nodes, bone, lungs, liver and brain.[25] Metastases located in visceral organs (e.g. lung, liver, pleura) are often referred to as visceral metastases whereas metastases in the bones, lymph nodes, skin etc. are termed non-visceral. If metastasis is only present in the bone, it is usually termed bone-only and these patients have a better prognosis than patients with metastasis in other locations.[26]

Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is thought to be an important step in cancer progression and metastasis. Most cancers originate in epithelial cells and these cells are characterized by inherent polarity, tight cell-cell junctions and non-motile behavior.[27] EMT is a process in which an epithelial tumor cell is suggested to lose its epithelial characteristics and conversely gain invasive mesenchymal and stem cell-like features. The cell thereby remarkably changes its protein expression leading to changes in cell architecture (e.g. shape and cytoskeletal organization) and behavior (e.g. gain ability to migrate and invade the surroundings) .[28] Examples of epithelial proteins that are downregulated during EMT are epithelial cell adhesion molecule (EpCAM) and cytokeratins (CKs), and of mesenchymal protein that are upregulated are vimentin and twist. EpCAM is a transmembrane protein in epithelial tissues that mediates cell-cell adhesion and CKs are important for organization of the cytoskeleton. Vimentin is expressed in mesenchymal cells, and expression of this protein in cancer cells increase their growth and invasiveness. Twist is active during cell differentiation and expression of twist in breast cancer cells results in resistance to paclitaxel.[29]

Tumor cell migration and invasion is facilitated by EMT in three major ways; weakened cell-cell cohesion, boosted ability to degrade the surrounding matrix and modified cytoskeleton. Examples of EMT related transcription factors are Snail, Twist1, and FOXC2. Important signaling pathways for EMT include TGF-β, Notch and Wnt.[30]

Diagnosis of metastatic breast cancer

When a patient presents with symptoms that raise the suspicion of metastatic breast cancer, the patient undergoes examination with imaging diagnostics (normally a CT scan of the thorax and abdomen, and a bone scintigraphy), blood tests and a tissue biopsy of the suspected metastasis. The biopsy is important to verify the presence of a malignant lesion and its origin.[21] In breast cancer, it is also important to re-evaluate

biomarker status on the biopsy to help guide choice of treatment since studies have shown it is not uncommon with a shift in breast cancer subtype between the primary tumor and later metastatic relapse.[31,32] This is thought to be part of the tumor progression process.

Prognostic and predictive factors in breast cancer

Prognostic and predictive factors are key elements in the clinical treatment of breast cancer.[33] These factors comprise patient characteristics as well as tumor biology features. A prognostic factor predicts the natural course of the disease within the untreated patient whereas a predictive factor predicts how a patient will respond to a given therapy. Some factors can be both prognostic and predictive (e.g. the estrogen receptor (ER)).[34] Below is a description of the most commonly used prognostic and predictive factors in clinical practice today.

Patient characteristics

Age

The median age at breast cancer diagnosis in Sweden is approximately 65 years[22] and about 4% of patients are younger than 40 years.[35] Younger age at diagnosis is a prognostic factor for unfavorable outcome[35-37] and younger patients more often have aggressive tumors with high grade, high proliferation index and no expression of ER.[38] The age limit to define younger age for when this prognostic effect is seen has varied amongst studies, and it has been proposed at <35 years[37] or <40 years[35].

Menopausal status

The menopausal status is a predictive factor important for the choice of endocrine treatment in women with hormone receptor positive tumors. Premenopausal women have a considerable estrogen production in their ovaries and because of that, aromatase inhibitors are not effective in these women.[39]

Tumor characteristics

TNM classification

The TNM classification system describes the clinical stage of the breast cancer disease and it is based on tumor size and invasiveness (T), extent of lymph node involvement (N) and the presence of distant metastasis (M).[40] Tumor size, and presence and number of axillary lymph node metastasis are both independent prognostic factors for breast cancer recurrence and survival. An increase in tumor size increases the likelihood of metastasis formation, and number of axillary lymph node metastasis is in direct relation to risk of metastasis.[41,42]

The TNM classification is well established world-wide and it provides prognostic information to help guide decisions regarding clinical treatment.

Histological classification

Breast tumors are assessed morphologically and divided into different histological subtypes according to a classification system defined by the WHO.[43] Around 70% of invasive breast tumors are classified as "no special type" (NST), a subgroup previously known as ductal carcinomas. The remaining 30% are classified as carcinomas of special subtype and these are grouped into amongst others lobular carcinoma, tubular carcinoma, carcinoma with medullary features and metaplastic carcinoma. Invasive lobular carcinoma is the largest group within the special subtypes representing 5-15% of invasive breast tumors.[43] Mucinous, medullary and tubular carcinomas have a better prognosis compared to NST, but it is important to note that most histological subtypes are very rare occurring at only 1-2%, and in general the prognostic impact of histological subtype is limited.[44]

Nottingham Histological Grade (NHG)

NHG is based on microscopic evaluation of tumor tissue. It was constructed to summarize the aggressiveness of a tumor and it is strongly correlates to prognosis.[45] The system was initially proposed by Bloom and Richardson[46] in 1957, and it was later revised by Elston and Ellis[47] in 1991. Briefly, NHG is composed of assessment of three components; tubule formation, nuclear atypia and mitotic count in a defined field area. Each component is given a score from 1-3 and all three components are then summarized to a total score between 3-9. Tumors are graded based on their total score as grade I (score 3-5), grade II (score 6-7) or grade III (score 8-9). NHG measures the degree of differentiation, i.e. how much the tumor cells resemble normal breast epithelial cells. Grade I has a close resemblance (high differentiation) and grade III has poor resemblance (low differentiation).

Ki67

Ki67 is often used in oncology as a proliferation marker since it is universally expressed by proliferating cells but is absent in quiescent (G0 phase) cells. It is a nuclear protein whose exact function is unknown but it is elevated in all stages of the cell cycle except G0 and it reaches a peak at mitosis.[48] Further, blocking of Ki67 prevents proliferation.[49] The clinical use of Ki67 is debated because there is so far no international consensus on how to stain and assess it. It is included in the St Gallen guidelines for subtype discrimination[50] but it is not yet included in the ASCO guidelines[51] due to the difficulties in reproducibility.

High expression of Ki67 is related to poor prognosis[52] and its independent prognostic utility has been shown in the group of ER-positive and in grade II tumors.[53]

Molecular and intrinsic subtypes

To further characterize breast tumors and understand the heterogeneity of this disease, different subclassification systems have been proposed. Molecular subtyping is based on evaluation of protein expression and information on molecular subtype is important for both prognostication and choice of treatment in the clinical setting. This subclassification system is further described in the next section, "Breast cancer subtypes".

Studies using gene expression profiling has revealed that breast tumors can be categorized into distinguished intrinsic subtypes with different gene expression profiles, clinical features and prognosis. Several multigene prognostic tests that use the information from gene expression to guide breast cancer treatment have been developed, but most of them are limited to certain subgroups of patients e.g. ER-positive breast cancers.[54] In Sweden, thus far no prognostic multigene tests are approved for clinical use.[21] In the latest ASCO-guidelines, the 21 gene recurrence score, the 12-gene risk score, the PAM50 ROR*, and the Breast Cancer Index* were accepted for dividing the ER/PR-positive, HER2-negative breast cancers into different risk groups.[51] The 70 gene signature was accepted for use in ER/PR-positive, HER2-negative breast cancer patients with high clinical risk and up to three positive lymph nodes.[55] In the latest St Gallen guidelines, the 21 gene recurrence score, the 70 gene signature, the PAM50 ROR score*, the EpClin score*, and the Breast Cancer Index* were all endorsed for the use of guiding adjuvant chemotherapy in ER-positive, node-negative tumors. However further data, and/or other assays were warranted before the use in ER-positive, node-positive patients.[50]

Hormone receptor status

The connection between breast cancer and hormones has been known since 1896,[56] and the ER was identified in 1958.[57] There are two known types of ER, ERα and ERβ[58], where ERα is the most studied and the one used in routine clinical assessment. The ERs are activated by estrogens and function as DNA binding intracellular transcription factors mainly located in the nucleus where they induce transcriptional signaling involved in cell growth and survival.[59] ER expression varies with ethnicity, in a large statistical report on female breast cancer in the United States, white women had the highest rates of ER+ breast tumors whereas African American women had the lowest.[60] In Sweden, it is estimated that about 85%, i.e. the majority, of breast cancers are positive for ER.[22] ER expression has been associated to better prognosis, especially in the first five years after diagnosis[61], but it is also a predictor of late relapse.[33,62]

More than 50% of tumors expressing ER also express the progesterone receptor (PR)[63] and PR expression has a prognostic value resembling that of ER.[64] Tumors expressing only PR and not ER are rare and they have been suggested to mirror errors in hormone receptor assessment.[65]

Both ER and PR are prognostic factors in breast cancer, but ER is also a predictive factor for endocrine treatment.[61]

HER2 status

HER2 is a transmembrane receptor tyrosine kinase located on the cell surface. It is encoded by the proto-oncogene *ERBB2* and belongs to the HER-family of epidermal growth factor receptors which also includes HER1 (EGFR), HER3 and HER4. These receptors are involved in cell adhesion, proliferation, differentiation and survival.[66] *ERBB2* was first reported to be amplified in breast cancer in the late 1980s[67] and amplification leads to overexpression of the HER2 receptor. In Sweden, approximately 13-14% of breast cancers overexpress HER2 and are termed HER2-positive.[68,69] HER2-positive breast cancer is associated to shorter relapse-free interval and poorer survival. However, the treatment of HER2-positive breast cancer has been revolutionized by the development of monoclonal antibodies that target the HER2 receptor (e.g. trastuzumab). Thus the HER2 receptor is both a prognostic and a treatment predictive factor.[69]

Breast cancer subtypes

Breast cancer is a complex heterogeneous disease which can be subdivided into distinct intrinsic subtypes based on gene expression profiles.[70-72] This classification gives important clinical information about prognosis and prediction of therapy response.[71,73] The main gene expression profiling based breast cancer subtypes recognized today by the WHO are luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-like, basal-like breast cancer (BLBC), normal epithelial-like and claudin-low.[74] Amongst the intrinsic breast cancer subtypes, luminal A breast cancers have the best overall prognosis and basal-like the worst.[71] In clinical practice, immunohistochemistry (IHC) staining for ER, PR and Ki67 in addition to evaluation of HER2 overexpression and/or *ERBB2* amplification is used as a surrogate translation of gene expression profiles to allocate breast tumors into different so called molecular subtypes.[75,76] Classification based on IHC does not completely correspond to that of gene expression profiling but it is more available in clinical practice.[50] The exact definition of the IHC derived breast cancer subtypes according to the international St Gallen consensus guidelines has varied over the years[50,77-79] but according to the latest guidelines from 2017 there are in broad clinical terms four recognized breast cancer subtypes that demand different treatment approaches. These are triple-negative tumors, HER2-positive tumors (regardless of ER status) and two types of ER-positive tumors.[50]

In the papers included in this thesis, the subtype guidelines form 2013 have been applied. According to the 2013 classification the following five subtypes are formed: luminal A-like (ER+, PR >20%, Ki67 low and HER2-), luminal B-like HER2- (ER+, PR ≤20% and/or Ki67 high and HER2-), luminal B-like HER2+ (ER+ and/or PR+, any Ki67 and HER2+), HER2+ (non-luminal) and triple-negative breast cancer (TNBC) (ER-, PR-, HER2- and any Ki67).[78] These five subtypes are used in clinical practice in Sweden today. Figure 2 shows the approximate distribution of the different St Gallen 2013 subtypes within cohorts of European women diagnosed with primary breast cancer.[31,80-82]

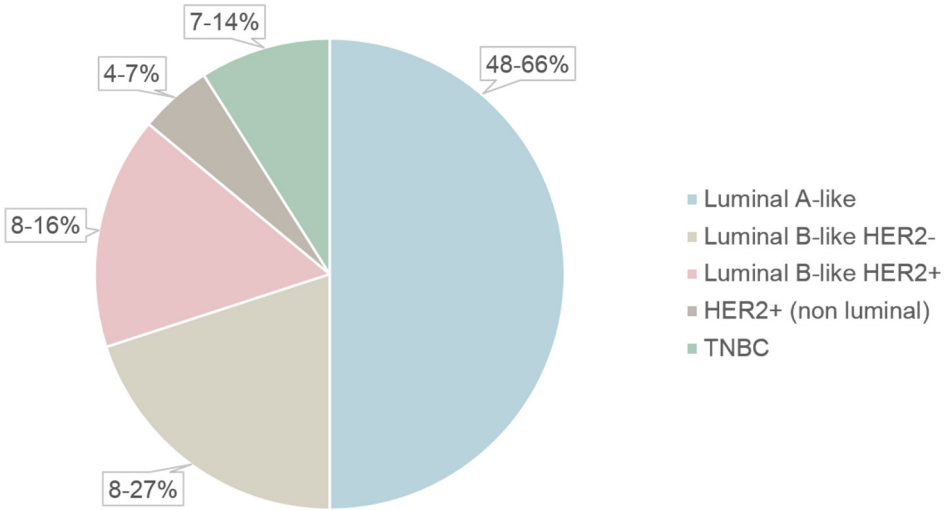


Figure 2.
Approximate distribution of 2013 St Gallen breast cancer subtypes in european women with primary breast cancer.

Triple-negative breast cancer (TNBC)

A subtype of breast cancer that is based on results of IHC is TNBC, which is defined as being negative for ER, PR, and HER2.[83] The incidence of TNBC varies somewhat between different places in the world but it is estimated to represent 7-14% of female breast cancers. Patients diagnosed with TNBC are often younger, have tumors with a higher histologic grade and they tend to present with larger tumors at diagnosis. TNBC patients are also more frequently *BRCA1* mutation carriers than those diagnosed with other breast cancer subtypes.[84,85] TNBC tumors often respond well initially to chemotherapy but the overall prognosis is poor, both in the primary and in the metastatic breast cancer setting. Primary TNBC has a higher risk of both local and distant recurrence, and an early peak in distant recurrence is seen three years after diagnosis. Furthermore, in the metastatic setting, TNBC patients more often present with metastasis to visceral organs and/or brain.[86] There is currently no targeted therapy available for this subgroup of patients but potential new drugs are under development.[86]

In 2011, a study was published by Lehmann *et al.* showing that TNBC can be further subdivided into six different subtypes using gene expression profiling. A dataset including 587 TNBC was used and the following subtypes were identified: basal-like (BL) 1, BL2, immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR).[87] This study highlighted the

heterogeneity of tumors within the TNBC subgroup and suggested this subtype information should be taken into consideration when searching for new treatment targets in TNBC.[88]

TNBC is closely related to BLBC. TNBC is defined by IHC (protein expression) whereas BLBC is defined by gene expression profiling. These two breast cancer groups largely overlaps, about 77 % of BLBC are also TNBC, and about 71 % of TNBC are also BLBC.[84] BLBC is characterized by the expression of genes related to basal epithelial cells such as keratin 5, keratin 17, integrin-β4 and laminin.[70]

In this thesis, there is a sub-focus on the difficult-to-treat TNBC breast cancer subtype.

Prognosis in primary and metastatic breast cancer

Papers I-II in this thesis are about primary breast cancer, and papers III-IV about metastatic breast cancer.

Primary breast cancer

Primary breast cancer, sometimes referred to as early breast cancer or non-metastatic breast cancer, is a term used to describe an invasive breast tissue derived tumor within the breast, with or without axillary lymph node involvement but without distant metastasis. A patient with primary breast cancer has an estimated 5-year survival of approximately 90%. However, breast cancer is a disease known to have a substantial risk of relapse, sometimes occurring decades after the primary diagnosis, and the overall risk for a breast cancer patient to eventually develop distant metastasis is around 30%.[5]

Metastatic breast cancer

Metastatic breast cancer (MBC), occasionally also called advanced or secondary breast cancer, is a term used to describe breast cancer that has spread to distant locations in the body. MBC is incurable and the treatment is palliative.[21] The development of metastasis is still an unsolved challenge in cancer care, and metastasis is the main cause of death in cancer patients.[89] Only 6% of all breast cancer patients present with metastatic disease at diagnosis.[90] Still, in Sweden about 1500 women develop MBC every year[21] and for these women, the 5-year survival is between 15-27%.[5,91]

In this thesis, two cohorts of breast cancer patients were included, one with primary breast cancer and one with MBC. Figure 3 presents survival curves for the different cohorts, stratified by breast cancer subtype, to illustrate the differences in survival.

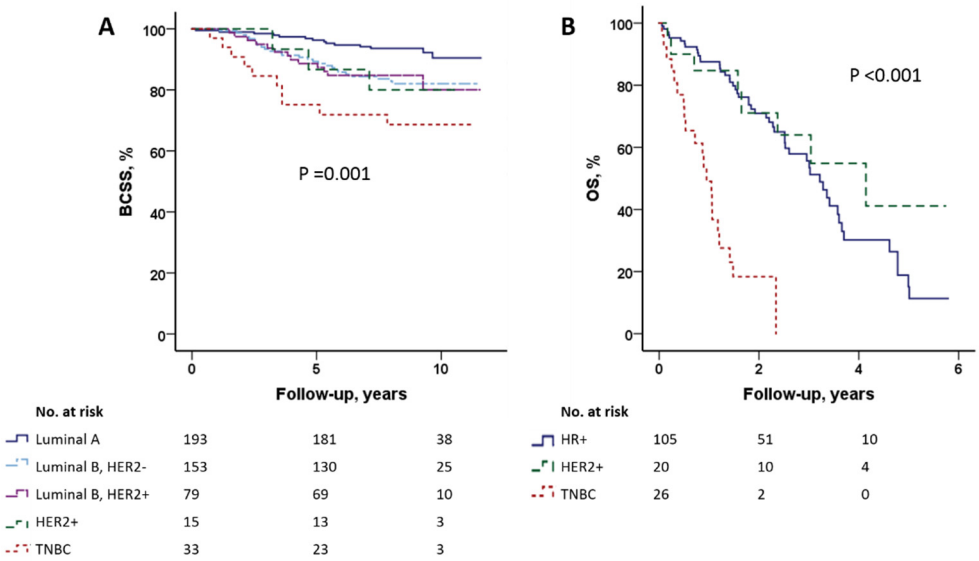


Figure 3. Outcome by breast cancer subtype in primary breast cancer (A) and simplified breast cancer subtypes in MBC (B). Results from the two cohorts included in this thesis
Note the scale on the X-axis.
Abbreviations: BCSS, breast cancer specific survival; OS, overall survival; HR, hormone receptor; TNBC, triple-negative breast cancer.

Breast cancer treatment

Primary breast cancer

Modern adjuvant therapy in the primary breast cancer setting aims to eradicate micro-metastases and thereby preventing cancer recurrences and disseminated disease. In Sweden, the choice of treatment is based on national guidelines and it depends on tumor characteristics and extent of the disease.[21] There is a growing arsenal of cancer treatment options, from different types of chemotherapy to new targeting drugs used for subpopulations of cancer patients.[9] To choose the right treatment for the right patient, all breast cancer patients in Sweden are discussed on multidisciplinary conferences with presence of oncologists, pathologists, surgeons, radiologists and contact nurses.[92]

Surgery

Successful surgical techniques to remove breast tumors were developed during the second half of the 19th century, after aseptic techniques and inhalation anesthesia became available. Initially, breast cancer surgery was performed as radical mastectomy, a large operation where the breast, pectoral muscles and axillary lymph nodes were removed.[93] In the middle of the 20th century this technique was replaced by modified radical mastectomy sparing the pectoral muscles[94] and in the 1980s, breast-conserving surgery followed by radiation therapy became the treatment of choice if possible based on patient and tumor characteristics (in particular tumor size), and patients choice.[95]

In the 1990s, the sentinel node (SN) technique was developed which decreased the axillary lymph node clearance. Breast cancer often spread to the lymph nodes as a first step in the metastatic process. The SN is the first lymph node (or first few nodes) to receive the lymphatic drainage from the primary breast tumor site.[96] The SN is identified by injecting a radioactive isotope and a blue dye close to the tumor location. The isotope and the dye is then transported by the lymphatic system to the SN which can be identified and removed during surgery of the primary tumor. The SN was previously sent immediately to a pathologist for a quick preliminary evaluation of signs of metastasis on frozen sections and an answer was given before the end of the

operation. If metastasis was found in the SN, axillary lymph node clearance was performed within the same operation.[21] Today, quick diagnostics on frozen section of SN during primary surgery is on its way out from the clinical practice as lymph node clearance has been shown beneficial only for patients with macro metastasis (size >2 mm) in the axillary nodes.

Radiotherapy

Radiotherapy is delivered postoperatively to eradicate any remaining tumor cells and thereby reduce the risk of loco-regional recurrences and improve survival. Several studies including three large meta-analyses by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) in 2005, 2011 and 2014 have showed that postoperative radiotherapy after breast conserving surgery and mastectomy decreases the relative risk of loco-regional recurrences by up to 50% and increases the breast cancer specific survival.[97-99] According to international guidelines by EUSOMA and ASTRO, postoperative radiotherapy should be recommended to patients who have an estimated 10-year risk of local recurrence >20%.

The national clinical guidelines in Sweden recommend that tangential radiotherapy to the breast is given to patients operated with breast-conserving surgery, radiotherapy to the thoracic wall is given to patients operated with mastectomy and with a tumor lager than 50 mm, and locoregional radiotherapy is delivered to regional lymph nodes is given to patients with axillary lymph node metastases.[21]

Systemic treatment

Systemic treatments affect the entire patient and include chemotherapy, endocrine treatment, anti-HER2 drugs and other targeted therapies. "Targeted therapy" is a term used to describe treatments that are designed to disrupt specific disease driving molecules or pathways. The number of drug targets and also of targeting drugs have increase rapidly over the last decade and targeted therapy is now an important cornerstone in modern individualized cancer treatment for many cancer types. In breast cancer, anti-HER2 drugs are the most known targeting drugs.[100] The text below will focus on conventional chemotherapy, endocrine treatment and anti-HER2 drugs used in the clinic in Sweden today. A section on receptor tyrosine kinase inhibitors (RTKi) can be found in the next section on receptor tyrosine kinases, under the headline "Targeting RTKs", these are new potential targeting drugs for breast cancer and are part of the focus in two of the studies included in this thesis.

Chemotherapy

Chemotherapy primarily targets dividing cells and has been shown to eradicate micro metastasis and improve outcome for patients with breast cancer.[101,102] Poly-chemotherapy (i.e. the combination of two or more cytotoxic agents) is more effective than single agent therapy and adjuvant poly-chemotherapy has been shown to reduce breast cancer mortality by approximately one third.[33,102] It is though that by combining different cytotoxics, they provide a synergistic effect and moreover, if they have different toxicity profiles, more intensive treatment can be delivered.

Within the group of ER+, node negative patients without risk factors such as young age, there is currently a debate concerning which patients should receive chemotherapy (and which should not) as part of their breast cancer treatment. In some low risk breast cancers there is no or very little benefit from chemotherapy and it is important to avoid overtreatment in this group.[103,104] At the same time, it is vital to not withhold chemotherapy from the patients who can derive an increase in survival by this treatment.[103] Many patients have clinical risk profiles that place them in the border zone between chemotherapy versus (vs) no chemotherapy and further studies are ongoing in search of tools to separate these patients.

In Sweden, chemotherapy is recommended for patients with high risk of breast cancer recurrence. These are in general patients with one or more of the following risk factors: young age, lymph node metastasis, tumors with high proliferation rate, high histological grade, ER-negativity and HER2-positivity.[21] The chemotherapy recommended usually consists of an anthracycline-based combination (e.g. (F)EC; F = 5-fluorouracil, E = epirubicin and C = cyclophosphamide or TAC; T = taxotere, A = doxorubicin (adriamycin), C = cyclophosphamide) followed by a taxane (e.g. docetaxel or paclitaxel).[21]

Endocrine treatment

The majority (80-85%) of invasive breast cancers express the ER and can be treated with endocrine treatment[22]. ER expression is assessed by IHC staining of tumor tissue and the limit for positivity has varied over the years.[60,105] Current Swedish guidelines recommend a tumor is assigned as ER positive if the expression of ER is >10%. However, international guidelines by St Gallen[50] and ASCO[51] currently recommend a lower limit at >1% ER-positive cells for a tumor to be assigned as ER-positive. The ER signaling pathway is a main driver of tumor development in patients carrying ER-positive tumors and endocrine treatment aims to block this pathway. Endocrine treatment significantly reduces breast cancer recurrence rates and improves survival in ER-positive patients.[33] In contrast, patients with ER-negative tumors have no benefit from endocrine treatment.[33]

There are three principal ways to target the ER pathway, 1) by blocking the ER (tamoxifen), 2) by degrading the ER (fulvestrant) or 3) by preventing ER ligand (i.e.

estrogen) production by degrading the aromatase enzyme which converts testosterones to estrogens (aromatase inhibitors (AIs))[39]. In addition, estrogen production can also be blocked irreversibly by oophorectomy or bilateral ovarian irradiation, or reversibly by gonadotropin-releasing hormone (GnRH).[106] Tamoxifen is effective in all women irrespective of menopausal status whereas aromatase inhibitors only have effect in postmenopausal women as it fails to block the ovarian estrogen production which is present in premenopausal women.[39]

According to current Swedish guidelines, all patients with hormone receptor positive breast cancer should be offered endocrine treatment. An exception can be made for patients with small (<10 mm) node negative luminal A-like tumors. Postmenopausal women should receive five years of AI treatment. Pre- and perimenopausal women should receive tamoxifen for five years. Amongst these patients, all who had lymph node positive disease at diagnosis should be offered an additional five years of treatment thereafter with either tamoxifen (if still pre- or perimenopausal) or AI if they converted to postmenopausal.[21]

Anti-HER2 treatment

Trastuzumab was amongst the first anti-HER2 drugs to be developed and it is a monoclonal antibody that targets the extracellular component of the HER2 receptor and thereby blocks receptor signaling. Trastuzumab greatly improves both disease-free and overall survival in patients with HER2 positive breast cancers.[107,108] HER2 status is determined by IHC with complementary *in situ* hybridization (ISH) in borderline cases. In Sweden, all patients with HER2-positive tumors (13-14%) are recommended one year of adjuvant trastuzumab unless they have a tumor with very good prognostic factors (e.g. size ≤5 mm, ER-positive, low grade).[21]

There are more HER2-targeting drugs under evaluation, and two of them have received approval for clinical use in Sweden, namely pertuzumab and lapatinib. These drugs are however only approved for treatment in the locally advanced or metastatic setting.[21]

Metastatic breast cancer

For women with MBC, the aim of treatment is improving quality of life, symptom prevention, palliation and survival prolongation. Treatments available are to a large extent the same as in primary breast cancer but with focus on systemic therapies, i.e. chemotherapy, endocrine therapy and anti-HER2 therapy. Choice of treatment in MBC is complex and depends on patients' health status, risk factors and tumor characteristics.[109] For patients with recurrent breast cancer, time to distant recurrence is an important factor. As written in the introduction above, metastases are biopsied if possible and patient treatment is guided by biomarker expression on the metastases.[21]

In general, first line treatment for patients with hormone receptor positive metastatic breast cancer is endocrine therapy. Choice of endocrine therapy depends on amongst others if the patient has received any previous adjuvant endocrine therapy, when, what drug and how the patient responded to that drug. Postmenopausal women usually receive an AI and premenopausal women receive tamoxifen in combination with an LHRH-analogue. Patients with a biologically aggressive and/or triple negative tumor are offered chemotherapy as first line treatment. In MBC, sequential monotherapy is recommended, compared to combination therapy in the primary breast cancer setting. Combination therapy is only used in selected MBC cases where an urgent response is vital. Patients with HER2-positive disease should be given an addition of a HER2-targeting drug in combination with chemotherapy, or sometimes endocrine therapy.[21]

Receptor tyrosine kinases

Papers I and II in this thesis has focused on exploring the importance of the four receptor tyrosine kinases (RTKs) c-KIT, VEGFR2, PDGFRα and PDGFRβ, and the PDGF receptor ligand PDGF-CC as potential new biomarkers in primary breast cancer. The genes encoding cKIT, VEGFR2 and PDGFRα are all closely located on the 4q12 chromosomal segment.[110]

General background

There are 89 known tyrosine kinases and they can be divided into RTKs and non-RTKs, Figure 4. Tyrosine kinases are proteins important for control of development and growth of multicellular organisms. They regulate processes such as cellular proliferation, differentiation, survival, metabolism, migration and control of the cell-cycle.[111] Dysregulation of these proteins is common in cancer; about 25% of the tyrosine kinases were actually discovered as oncogenes and currently, over 50% of RTKs are documented as oncogenes.[112]

A RTK contain three parts; an extracellular region including a ligand binding domain, a transmembrane helix, and a cytoplasmic region that includes the tyrosine kinase domain. Most RTKs are activated by growth factor binding, which induces receptor dimerization.[113] Receptor dimerization leads to tyrosine kinase activation and autophosphorylation of the receptor. The exact mechanisms for how this is accomplished varies between the different receptors and it is not yet fully elucidated for all RTKs. After the autophosphorylation, several downstream signaling molecules are recruited and activated by the RTK. The complexity of the signaling networks controlled by RTKs remains partially unclear. It has been proposed that the network resembles a "bow tie" or "hourglass" network where multiple RTKs transmit input to a limited number of "core processes", such as phosphoinositide 3-kinase (PI-3K) signaling, MAPK signaling, and Ca^{2+} signaling, which then translate into changes in e.g. cellular growth and behavior.[111]

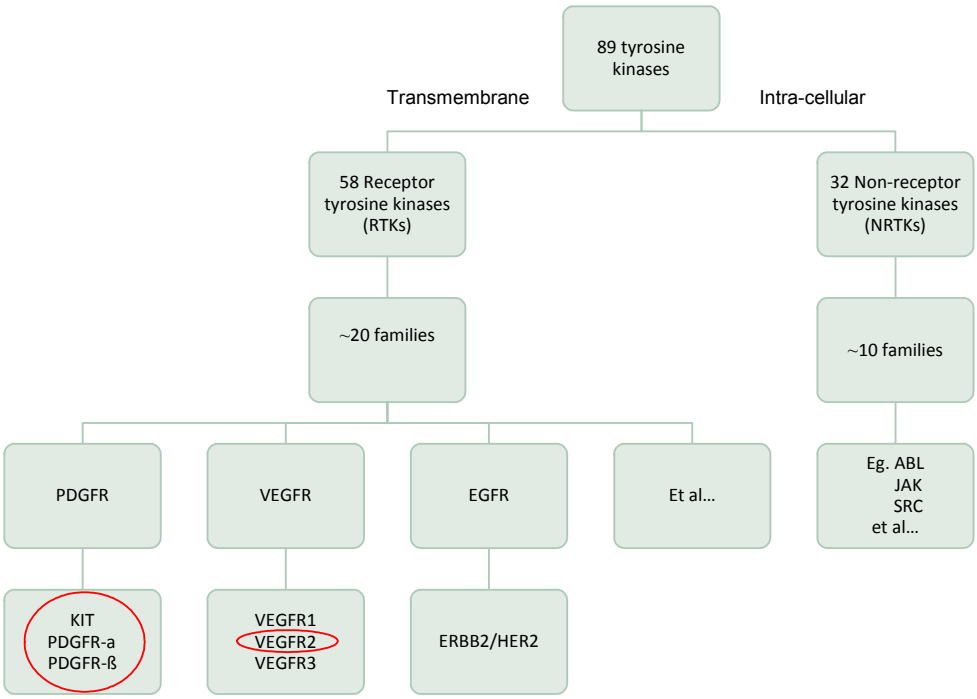


Figure 4.
Overview of the tyrosine kinases including the RTKs studied in this thesis as well as some of the other most well known members.

The tumor microenvironment and angiogenesis

The importance of the tissue microenvironment (TME) in tumorigenesis has gained increasing attention over the last decades. Today, a tumor is viewed as a complex tissue that in addition to neoplastic cells also consists of carcinoma associated fibroblasts (CAFs), endothelial cells, pericytes, various immune cells and extracellular matrix (ECM).[114,115] The TME surrounding tumor cells supports tumor growth, invasion and metastasis.[116] One important result of the interaction between tumor cells and their TME is the establishment of a vascular network, a process called angiogenesis. A vascular network is essential for a tumor to exceed a certain size. Angiogenesis is regulated by several different signal transduction pathways. In breast cancer, the VEGF, the FGF and the PDGF families with relevant receptors have been identified as the most common promotors or inhibitors of angiogenesis.[117]

Figure 5 shows an overview of the receptors that are being investigated in papers I and II in this thesis, and their respective ligands.

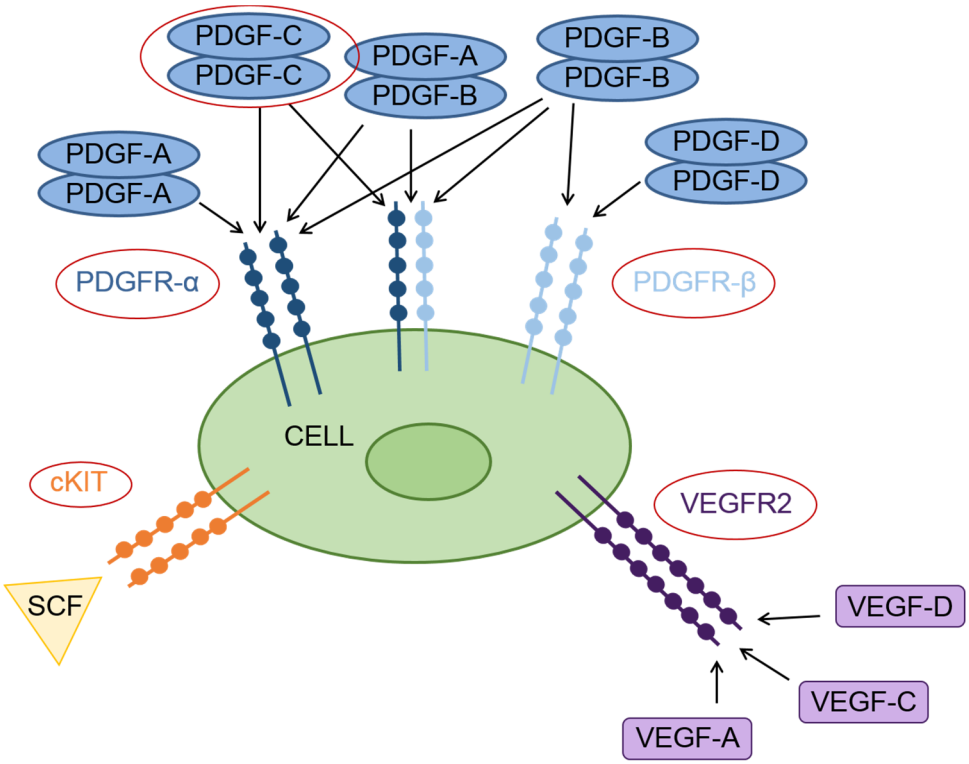


Figure 5. Overview of the RTKs included in this thesis and their respective ligands
All investigated biomarkers are outlined with a red ellipse. Abbreviations: PDGF(R), platelet-derived growth factor (receptor); VEGF(R), vascular endothelial growth factor (receptor); SCF, stem cell factor.

cKIT

cKIT, also known as CD117, is a RTK expressed by several cell types (e.g. mast cells, some hematopoietic stem cells, germ cells, melanocytes, some cerebellar neurons, Cajal cells of the gastrointestinal tract, and epithelial cells in the breast), and normal signaling through this receptor regulates processes such as cell adhesion and differentiation, apoptosis, and proliferation. Only one ligand is known for the cKIT receptor, the stem cell factor (SCF).[118]

cKIT was originally discovered as a proto-oncogene and dysregulation of cKIT signaling is involved in several cancers. Gain-of-function mutations in the *KIT* gene leads to pathologic receptor activation and neoplasia in cKIT-dependent and cKIT-positive cell types. Examples of tumors driven by this mechanism are gastro-intestinal stromal tumors (GISTs), acute myeloid leukemia and seminoma.[118]

cKIT and breast cancer

High expression of cKIT has previously been linked to TNBC.[119-122] There are conflicting results whether cKIT is related to prognosis in breast cancer. In one study, high cKIT protein expression was reported to be associated to poor cancer specific survival and disease-free survival.[120] In another study however, high cKIT protein expression showed no association to survival but increased gene copy number of *KIT* and/or *VEGFR2* was associated to worse breast cancer specific survival.[121]

The VEGF-family

The VEGF-family consists of five ligands that acts through three receptors; VEGFR1, VEGFR2 and VEGFR3. The VEGFs are important regulators of vascular development, and also of blood and lymphatic vessel function under normal and pathological conditions. In addition, different members of the VEGF-family have been shown to play an important role in tumorigenesis. VEGFs can be secreted by tumor associated stromal cells (e.g. macrophages, fibroblasts and endothelial cells) but also by the tumor cells themselves. VEGFs secreted by tumor cells can function in an autocrine manner and promote tumor cell EMT which leads to invasion and survival.[123]

In this thesis, VEGFR2 (sometimes also called KDR or flk-1) is investigated. Under normal conditions, VEGFR2 is mainly expressed on vascular endothelial cells[124] and experiments on mice have shown that lack of VEGFR2 leads to early embryonic death due to impaired hematopoietic and endothelial cell development.[125] VEGFR2 is however also expressed by many types of tumor cells, including amongst others breast[126], colon[127], lung[128], ovarian[129] and prostate.[130]

VEGFR2 and breast cancer

High expression of VEGFR2 has been found to be significantly associated to poor survival in univariable but not in multivariable analysis in a large cohort of 642 patients with primary breast carcinomas. It has also been found to be significantly correlated TNBC, and to decreased breast cancer specific survival in TNBC.[131]

In addition, increased gene copy number of KIT and/or VEGFR2 has been associated to an aggressive phenotype and impaired prognosis in primary breast cancer.[121]

The PDGF-family

There are two known PDGF receptors, PDGFRα and PDGFRβ. They both belong to the type III tyrosine kinase receptor family, which also include the c-KIT receptor amongst others. Five different ligands bind to and activate the PDGF receptors (see

Figure 5). These ligands are secreted by several different cell types such as activated platelets (where the first ligand of the family was initially discovered), endothelial, epithelial, glial and inflammatory cells. PDGFs mostly act on neighboring cells in a paracrine manner. The PDGF receptors are normally located on cells with mesenchymal origin, e.g. fibroblasts and vascular smooth muscle cells.[132] In embryogenic development, PDGF signaling is involved in many processes such as creation of a normal craniofacial anatomy, formation of lung alveoli, proper investment of mural cells (e.g. pericytes) in blood vessels and normal kidney development.[132]

The PDGF signaling pathway is also important in cancer and autocrine or ligand independent stimulation of PDGF receptors has frequently been observed in various neoplasms such as gliomas,[133] GISTs[134], dermatofibrosarcoma protuberans (DFSP)[135] and chronic myelomonocytic leukemia.[136] In addition, dysregulation of paracrine PDGFR signaling can cause extracellular matrix remodeling in a tumor-promoting way to facilitate migration, invasion, angiogenesis and possibly also lymph angiogenesis.[137,138]

This thesis focus on the two PDGF receptors and one of the ligands, PDGF-CC. PDGF-CC was discovered towards the end of the 1990s and it has been shown to be involved in tumor growth by paracrine signaling through PDGFRα in malignant melanoma[139] and cervical carcinoma.[140]

PDGFRα, PDGFRβ and PDGF-CC in breast cancer

Expression of PDGFRα has been found both in stroma and in tumor cells. High tumor cell PDGFRα expression has been associated to lymph node metastasis, HER2-positivity,[141] high histologic grade, ER-and PR-negativity, and TNBC whereas high stromal expression of PDGFRα has been linked to HER2-positivity and high Ki67.[142] Expression of PDGFRβ in breast cancer has only been reported in stroma and high expression has been associated to HER2-positivity, high Ki67,[142] high histologic grade, ER-negativity, and shorter survival.[143]

The role of PDGF-CC in breast cancer is largely unknown. We have recently shown that tumor cell derived PDGF-CC acts on neighboring tumor stromal cells in mouse models, and proposed that the PDGF signaling pathway is a regulator of breast tumor subtype where high expression of PDGF-CC drives breast tumors towards a more basal-like phenotype (Roswall *et al.*, manuscript submitted).

Targeting RTKs

RTKs are emerging targets in anti-cancer therapy and many tyrosine kinase inhibitors (TKIs) are currently being developed and tested. The best example of a successful TKI is imatinib (Glivec®) that inhibits both c-KIT and PDGFRα, and it is currently used for treating amongst others GISTs and chronic myeloid leukemia (CML).[144] Two other examples are Sunitinib (Sutent®) and Sorafenib (Nexavar®), which are multi-TKIs that target for example c-KIT, VEGFR2 and PDGFRβ.[100]

Sunitinib

The effect of sunitinib on metastatic breast cancer has been studied in several clinical trials and the results have been partially conflicting. In two pilot studies, metastatic TNBC showed promising response rates to sunitinib.[145,146] However, several subsequent studies on MBC evaluating both single agent sunitinib and combinations with cytotoxic agents, have shown no survival benefit.[147-150] One trial evaluating sunitinib as a single treatment in MBC was even aborted ahead of schedule since preliminary data indicated a lower PFS in the patients receiving sunitinib.[147] In addition, in some studies reported higher frequency of adverse events in patients receiving sunitinib.[148,150]

Sorafenib

In 2016, a review was published evaluating the role of sorafenib in breast cancer. The authors found 21 published trials on sorafenib, of which 16 were performed in breast cancer stage IV, 2 in stage III-IV and 3 in early breast cancer. The effect or sorafenib has been investigated both as single agent and as combination therapy together with cytotoxics, endocrine therapy and radiation therapy. The authors conclude that sorafenib was in general well tolerated amongst the patients. Given together with gemcitabine, capecitabine or tamoxifen, the addition of sorafenib showed somewhat promising results but additional clinical trials were encouraged to further clarify the role of sorafenib in breast cancer before any recommendation could be made on clinical use.[151]

Circulating tumor cells

Papers III and IV in this thesis has focused on exploring the importance of circulating tumor cells (CTCs) as biomarkers in patients with newly diagnosed metastatic breast cancer (MBC).

General background

A key step in metastasis is intravasation, i.e. the entrance of tumor cells into the hematologic or lymphatic system, and subsequent hematogenous and/or lymphatic spread. Carcinoma-derived tumor cells circulating in the bloodstream are termed CTCs and their presence in a patient with metastatic cancer was observed for the first time in 1869.[152] Development of techniques to isolate and detect CTCs increased rapidly in the beginning of the 21st century but hitherto, only one system has been approved for this purpose by the American Food and Drug Administration (FDA), namely the CellSearch system.[153] This system allows for isolation and enumeration of CTCs in a blood sample of 7.5 ml of whole blood from patients with various carcinomas (see methods for further technical information on how the system works).[154] Enumeration of CTCs by the CellSearch system has been shown to carry additional prognostic information to standard clinical tumor and patient characteristics in patients with metastatic breast[155], colon[156] and prostate[157] cancer.

The liquid biopsy

A blood sample collected from a patient for the purpose of detecting CTCs or other tumor derived biologic material e.g. pieces of circulating tumor DNA (ctDNA) is often referred to as a "liquid biopsy". It is non-invasive and easily accessible with limited risk of complications (discomfort from the blood draw, small risk of bleeding and of infection) in comparison to taking a standard tissue biopsy. Furthermore, an ordinary tumor tissue biopsy reflects a momentary state of the tumor at one location in the body. This biopsy may not properly reflect the entire disease and furthermore, tumors change with time due to selection pressure from therapy. In addition, tumor tissue biopsies can be difficult to obtain due to the location of the tumor. In contrast, liquid biopsies are non-invasive and can be repeated regularly, which allow serial monitoring of real-time

tumor evolution in a way that would be difficult to achieve by repeated needle biopsies since these are invasive procedures. Also, CTCs and other tumor derived biologic material detected in the blood are considered to originate both from the primary tumor and from metastatic locations when present. They are therefore thought to offer a better proxy for the whole cancer disease than a single tumor tissue biopsy from a selected tumor location, as is the standard approach in the clinic today. Liquid biopsies thus holds promise for improved cancer diagnostics, prognostics, and treatment monitoring and prediction.[158,159]

Some limitations to keep in mind with the liquid biopsy is that the half-life of a CTC is very short, probably measured in hours.[160] Also, not all CTCs are capable of initiating a metastasis. It is estimated that only 0.2% of disseminated tumor cells are able to successfully seed metastasis.[161] Furthermore, all current CTC isolation and detection methods have their limitations and might miss detection of certain subpopulations of CTCs.[153] Figure 6 presents an illustration of hematogenous spread of tumor material including CTCs.

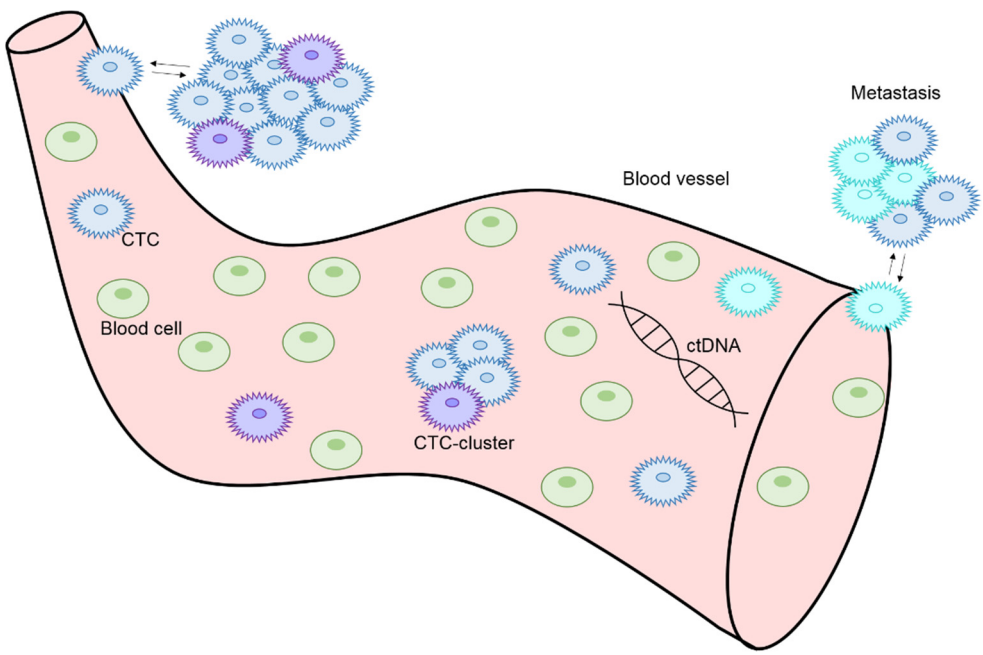


Figure 6. The metastatic process
The figure above shows how CTCs, CTC-clusters and ctDNA leaves the primary tumor site and travel through the hematologic system. CTCs and CTC-clusters may exit at distant sites in the body to form metastatic leasons, and they may also recirculate from these metastatic sites.[162] CTCs are displayed in several different nuances of blue-purple to illustrate tumor heterogeneity, while blood cells are depicted in green.

CTCs and disseminated tumor cells

When CTCs exit the bloodstream at different locations in the body they are called disseminated tumor cells (DTCs).[163] The presence of CTCs and DTCs is considered a sign of minimal residual disease (MRD). MRD is the existence of undetectable (by conventional imaging and laboratory testing), potentially metastasis-initiating, malignant cells residing in distant organs after curative surgery of the primary tumor.[164] Approximately 30% of patients with primary breast cancer have micrometastasis (DTCs) in the bone marrow at diagnosis and it has been shown to be an independent factor of poor prognosis.[165]

CTCs in breast cancer, presence and prognostic implication

Primary breast cancer

The cut-off threshold for CTC-positivity in primary breast cancer has been proposed at 0 vs ≥1 CTC[166,167] in comparison to <5 vs ≥5 CTCs in MBC using the CellSearch system.[155,168] At this limit, approximately 20% of primary breast cancer patients are positive for CTCs.[167]

Presence of CTCs in primary breast cancer detected by RT-PCR, the CellSearch system and other ICC-based methods have been shown to be associated with worse prognosis according to a meta-analysis from 2012.[169] The independent prognostic value of CTCs in average-to-high risk primary breast cancer patients has also been shown in a study from 2014 by Rack *et al.* using the CellSearch system. This study included 2026 non-metastatic breast cancer patients and showed that presence of CTCs both before and after adjuvant chemotherapy was associated with worse prognosis.[166] Last year (2016), another large meta-analysis including 3173 women with stage I-III (non-metastatic) breast cancer evaluated by the CellSearch system concluded that presence of ≥1 CTC at primary diagnosis was an independent factor of poor outcome.[167]

Metastatic breast cancer

The prognostic value of CTC enumeration by the CellSearch system in patients with metastatic breast cancer was first shown in 2004.[168] The authors of this pioneering study showed that presence of five or more CTCs in the blood of patients with MBC before start of a new line of treatment was associated with worse PFS and OS. Several studies have been published since then in support of these results[170-182], and in 2014, a large meta-analysis confirmed the prognostic value of CTC count in MBC and deemed it to have reached level one evidence of clinical validity in MBC.[155] Furthermore, CTC count during treatment has also been shown to be associated with worse prognosis.[155,181,183]

To note however is that some studies have questioned the cut off at five CTCs. Bidard *et al.* 2010 found that a cut off of three or more CTCs but not at five or more was prognostic in MBC patients starting 1st line chemotherapy in combination with bevacizumab.[184] Also, two studies have proposed that CTCs should be divided into additional risk groups at several cut off values, or that CTCs should be analyzed as a continuous variable.[172,185]

Table 1 presents an overview of the studies evaluating the clinical validity of CTC count in MBC by the CellSearch system.

Table 1.Clinical relevance of CTC count in MBC, assessed by the CellSearch system

Author, year	No. MBC	Sampling time	Incl. criteria	Study design	BL CTC cutoff	Outcome	Results
Cristofanilli, 2004[168]	177	BL + 1st FU	New line	Pros	≥5 CTC	PFS + OS	CTC count ≥5 CTC before a new line of therapy associated to worse survival
Cristofanilli, 2005[186]	83	BL + 1/m for 6m	1st line	Pros	≥5 CTC	PFS + OS	CTC count ≥5 CTC before 1st line therapy associated to worse survival
Hayes, 2006[183]	177	BL + 1, 2, 3, 4 m	New line	Pros	≥5 CTC	PFS + OS	CTC count ≥5 CTC at any time point during therapy associated to worse survival
Budd, 2006[187]	138	1m	New line	Pros	≥5 CTC	OS + PD	CTC count is an earlier and more reproducible marker of disease status than imaging
Cristofanilli, 2007[170]	151	BL	New line	Retro	≥5 CTC	OS	CTCs have superior and independent prognostic value of tumor burden and disease phenotype
Nolé, 2008[172]	80	BL + 1, 2, 3m, then every 2m	New line	Pros	≥5 CTC	PFS	BL CTCs are prognostic and changes in CTC count during therapy may indicate clinical response
Yagata, 2008[188]	38	BL	New line	Pros	≥5 CTC	PFS + OS	BL CTC count before start of a new line of treatment is prognostic
Dawood, 2008[171]	185	BL	1st line	Retro	≥5 CTC	OS	BL CTC count is a strong independent predictor of survival in newly diagnosed MBC
Liu, 2009[189]	74	BL + 1/m	New line	Pros	≥5 CTC	PFS + PD	Strong correlation between CTC count and radiographic disease progression
Botteri, 2009[185]	80	BL	New line	Pros	Continu-ous	PFS + OS	Worse survival with increasing CTCs as a continuous variable. Rate of increase in risk was reduced after 5 CTCs
Bidard, 2010[184]	67	BL + after 2 cycles of treat	1st line chemo +bevacizumab	Pros	≥3 CTC and ≥5 CTC	TtP	BL CTC ≥5 CTC was not a prognostic marker for TtP (but ≥3 CTC was). Changes in CTC count during treatment were not a surrogate for TtP. Bevacizumab + 1st line chemo may modify the predictive value of CTC during treatment
Nakamura, 2010[173]	119	BL + 1, 3m	New line	Pros	Multiple cutoffs tested	OS	Change in CTC number highly correlated with results of imaging before and after therapy. CTCs proposed as earlier predictors or treatment effect than imaging
Hartkopf, 2011[174]	58	BL + after 3 cycles	New line	Pros	≥5 CTC	OS + treat response	Changes in CTC levels during chemo is useful to monitor therapy efficacy, and correlates to OS

Giuliano, 2011[175]	235	BL	1st line	Retro	≥5 CTC	PFS + OS	CTC count may be useful in patient stratifications and therapy selection, especially amongst pats with ≥5 CTC
Giordano, 2012[190]	517	BL	New line	Retro	≥5 CTC	PFS + OS	BL CTC count was strongly predictive of survival in all MBC subtypes except HER2+ who had received targeted therapy
Pierga, 2012[176]	267	BL + before cycle 2+3	1st line chemo	Pros	≥5 CTC	PFS + OS	CTC count has an independent prognostic value compared to serum markers (CA15-3, CEA and LD). High CTC count before cycle 2 is an early predictive marker for poor PFS and OS
Müller, 2012[191]	254	BL	New line	Pros	≥5 CTC	PFS + OS	CTC count was prognostic for OS but not PFS at BL
Martín, 2013[177]	117	BL + before cycle 2	1st line chemo	Pros	≥5 CTC	PFS + OS	CTC count at BL and day 21 was prognostic for OS and PFS. CTC count before cycle 2 seem to be an early and strong predictor of treatment outcome
Smerage, 2014[179]	595	BL + at day 21	1st line chemo	Pros	≥5 CTC	PFS + OS	Confirms the prognostic significance of CTCs in patients receiving chemo
Giuliano, 2014[192]	492	BL	New line	Retro	≥5 CTC	Time to NMS/NML/ PD	BL CTC count can be used as an early predictor of metastatic potential in MBC patients with limited metastatic disease
Wallweiner, 2014[178]	393	BL + after 1 cycle	New line	Pros	≥5 CTC	PFS + OS	CTC count at BL and 1m, and change in CTC count, were all predicate of outcome in MBC
Bidard, 2014[155]	1944	BL + 1, 2m	New line	Pooled analysis	≥5 CTC	PFS + OS	Confirm the independent prognostic effect of CTC count on PFS and OS at BL and FU. Addition of CTC count to a clinicopathological predictive model improves prognostication
Peeters, 2014[193]	154	BL	1st line	Retro	≥1 CTC and ≥5 CTC	PFS + OS	No significant difference in detection of CTCs, or in CTC positivity rate (≥1 and ≥5 CTCs) between five St Gallen IHC subtypes. CTC count was prognostic in all subtypes except HER2+
Mu, 2015[180]	115	BL	1st line, stage III+IV	Pros	≥5 CTC	PFS	BL CTC count ≥5 CTC was associated to worse PFS
Wang, 2017[181]	128	BL + 1st FU + every 2 m	New line	Pros	≥5 CTC	PFS + OS	Elevated CTC count at BL and first FU was significantly associated with worse PFS and OS

Abbreviations; FU, follow-up; pros, prospective; retro, retrospective; BL, baseline; treat, treatment; 1/m, Once per month; m, month; PFS, progression-free survival; OS, overall survival; PD, progressive disease; TtP, time to progression, pats; patients; NMS, new metastatic site; NML, new metastatic lesion; IHC, immunohistochemistry

CTCs and apoptosis

Apoptosis means programmed cell death, and the role of apoptotic CTCs in cancer patients remains elusive. Presence of apoptotic CTCs has been shown to be associated to worse PFS and OS in small-cell lung cancer,[194] and in MBC.[195] However, opposing results have also been reported. Paoletti *et al.* investigated 52 metastatic TNBC and found that high frequency of apoptotic CTCs did not predict PFS, neither at BL, nor at day 15 or 29 of systemic treatment.[182]

In patients with primary breast cancer, presence of apoptotic DTCs in the bone marrow at the time of primary surgery has been shown to correlate to a significantly shorter overall survival.[196]

CTCs, DTCs and the immune system

The presence of DTCs in the bone marrow of patients with primary breast cancer is predictive of later metastatic relapse, but still, many patients with DTCs present will never have a cancer recurrence.[165] It is hypothesized that DTCs could recirculate from the bone marrow and return to the site of the original tumor, or to other sites in the body, and start to expand to form new tumors. The immune system is thought to be involved in processes that can both suppress and promote CTCs and DTCs in the blood and bone marrow.[197] To date, little is known about the interaction between CTCs and immune cells in the blood stream.

CTCs and targeted therapy

Enumeration of CTCs, and also characterization of phenotypical, physical and biological aspects is anticipated to improve prediction and monitoring of anti-cancer therapy, and provide a tool to further personalize therapy.[153,198,199] Several studies are currently ongoing to evaluate the clinical application of CTCs in breast cancer (Table 2).[200]

The first large randomized controlled trial (RCT) using CTCs to guide choice of therapy, the SWOG 0500, has been completed and the results were published in 2014. This study was a RCT including MBC patients scheduled for 1st line chemotherapy. Patients with persistent CTC count ≥5 after one cycle of therapy were randomized to either continue the initial therapy, or to change to an alternative chemotherapy of the clinicians' choice. No difference was seen in OS between the two randomized arms and it was concluded that early switch to an alternate cytotoxic 21 days after initiation of 1st line chemotherapy was not effective to improve survival.[179] Various explanations

have been proposed for the negative results of this study. For example, patients who did not experience a decrease in CTCs by chemotherapy could represent cancers with a general chemoresistance and thus just changing to another chemotherapy will most likely not be effective for these patients.[201]

Table 2.
Overview of selected, currently ongoing or recently completed, interventional trials on CTCs in breast cancer[200]

Study	Patients	Design	Endpoint and results
Metastatic breast cancer			
SWOG 0500[179]	Included 595 MBC patients scheduled for 1st line chemotherapy	RCT, patients with persistent CTC count ≥5 after one cycle of chemo were randomized to remain on initial therapy or switch to another cytotoxic of the clinicians choice	Completed. No difference in OS was seen between patients with elevated CTC count who remained on initial therapy compared to those who had an early switch to another cytotoxic[179]
CirCe01	To include 304 MBC patients with high CTC count (≥5) before start of 3rd line systemic therapy	RCT, patients will be randomized to control arm or CTC arm upon inclusion. In the CTC arm, control of CTC count after first therapy cycle of all subsequent therapies (3rd, 4th, 5th, line and so forth). If no decrease to <5 CTCs, therapy will be switched, otherwise it will be continued until signs of progression	Primary endpoint: OS Estimated completion: beginning 2018
STIC CTC META-BREAST	To include approx. 1,000 HR+ MBC	RCT, 1st line systemic chemotherapy or endocrine therapy based on clinicians choice or CTC-driven (<5 CTC, endocrine, ≥5 CTC, chemo)	Primary endpoint: PFS Estimated completion: end 2018
DETECT III	To include 228 MBC patients with HER2- primary tumor and HER2- metastasis, and with ≥1 HER2+ CTC	RCT, patients will be randomized to planned treatment, or to planned treatment + lapatinib	Primary endpoint: PFS Estimated completion: beginning 2020
Primary breast cancer			
Treat CTC	To include 174 patients with HER2-non-amplified primary breast cancer and ≥1 CTC after completion of adjuvant therapy and surgery	RCT, eligible patients will be randomized to 6 injections with trastuzumab or observation. New CTC count week 18 for all patients	Primary endpoint: CTC detection rate at 18 weeks, secondary endpoint: RFS Recently terminated ahead of time due to slow inclusion rate and negative results; equal number of CTCs at 18 weeks in the observation and the treatment group

Circulating tumor cell clusters

General background

CTC-cluster definition and biological background

There is hitherto no existing standard definition of a CTC-cluster (sometimes also referred to as circulating tumor microemboli, circulating micrometastasis or circulating tumor aggregates), but most studies have described them as groups of ≥2 or ≥3 CTCs clustered together with intact cytoplasm and with non-overlapping nuclei. Arguments for using a 3-cell definition has been to avoid erroneously appointing a dividing CTC as a cluster.[194] However, studies using the 2-cell definition have shown that 2-cell CTC-clusters have different features and prognostic implication compared single CTCs and that they should be included in the cluster definition.[202] Most CTC enrichment and detection methods were designed to capture single CTCs. It is not known to what extent they also capture and present intact CTC-clusters.[203]

CTC-clusters have been shown to originate from oligoclonal multicellular groupings of tumor cells, held together by plakoglobin-dependent intercellular adhesions, that break loose together from a malignant tumor and collectively enter the vascular system.[202] It has also been demonstrated that CTC-clusters are not formed by intravascular aggregation events.[202] Circulating CTC-clusters have for a long time been assumed to rapidly get trapped in capillaries because of their size.[204] However, a recent paper showed that a striking majority of clusters with a size of up to 20 cells could successfully traverse small blood vessels of 5-10μm in diameters. It was demonstrated that the clusters reorganize into single-file chain like formations to pass these narrow passages.[205] These intriguing data suggest that CTC-clusters are likely to have an important role in the hematogenous spread of cancer cells in the metastatic process.

Involvement in disease progression

CTC-clusters are rare; they are estimated to constitute approximately 2-5% of all CTC events detectable in the circulation. However, they have been shown to possess an up to 50-fold metastatic potential compared to single CTCs[202] and a few clinical studies (including us) have shown that CTC-cluster presence in patients with metastatic cancer is associated with worse survival.[181,194,206]

EMT is considered an important process in cancer cell development to acquire a more invasive and migratory phenotype, as described in the introduction of this thesis. CTC-clusters have been shown to contain CTCs with a more mesenchymal phenotype.[29,207] Additionally, no or very few apoptotic CTCs are found within clusters suggesting these cells have a survival advantage.[194] It has been hypothesized that clustered CTCs avoid anoikis by being in direct contact with each other and thereby generate survival signals that are missing in single CTCs.[194] Furthermore, one study on MBC patients using the Cluster-Chip for CTC isolation found that about half of the CTCs within CTC-clusters were proliferating as determined by Ki67 protein expression.[208] However, a study on small-cell lung cancer found no proliferation in clustered CTCs and proposed that absence of proliferation might protect these cells from cytotoxic agents targeting dividing cells.[194]

CTC-clusters in breast cancer, presence and prognostic implication

The presence and significance of CTC-clusters in breast cancer remains largely unknown. Presence of CTC-clusters (≥2 CTCs) identified by the CellSearch system has been related to poor outcome in stage III-IV breast cancer.[180] In a study on metastatic TNBC, detection of CTC-clusters (≥3 CTCs) added prognostic information in follow-up samples during treatment. Recently, a study was published reporting on serial sampling of CTCs and CTC-clusters before start of a new line of therapy in 128 patients with MBC and concluded that both CTC count and presence of CTC-clusters were significantly associated to PFS and OS at baseline and during follow-up. In this study, it was also suggested that the size of the clusters matters; 3-cell clusters had higher HR for OS compared to 2-cell clusters.[181]

Aim of the studies

Overall aim

There are currently insufficient biomarkers and possible drug targets available for the TNBC subgroup of breast cancer patients and this subgroup lack the benefit of today's available targeted cancer therapies.[209] The overall aim of this thesis was to evaluate new potential prognostic biomarkers in breast cancer, and with special focus on TNBC.

Paper I

Study I aimed to elucidate if there is a correlation between the protein expression of three RTKs c-KIT, VEGFR2 and PDGFRα, their gene copy number, and prognosis in TNBC compared to non-TNBC.

Paper II

Study II aimed to explore the expression of PDGFRα, PDGFRβ and ligand PDGF-CC in breast cancer to elucidate if these proteins are associated with molecular surrogate subtypes, type of metastatic location and prognosis in breast cancer. A secondary aim was to explore the relation to tumor progression by investigating changes in protein expression between primary tumor, synchronous lymph node metastases and asynchronous recurrences.

Paper III

Study III aimed to explore whether apoptotic CTCs, CTC-clusters and leukocytes attached to CTCs are associated with breast cancer subtype and prognosis at baseline and during first six months of follow-up in MBC patients scheduled for 1st line systemic therapy.

Paper IV

Study IV aimed to evaluate if longitudinal enumeration of CTCs and CTC-clusters could improve prognostication and monitoring of patients with MBC starting 1st line systemic therapy.

Patients

Paper I-II

The cohort for study I-II included women (N = 569) diagnosed with unifocal, primary invasive breast cancer in the South Swedish Health Care Region (Lund, Landskrona and Helsingborg) between June 1999 and May 2003. The cohort was originally assembled for a prospective observational study with the aim of evaluating the presence and prognostic value of DTCs in the bone marrow.[163] All included patients gave a written informed consent and the study was approved by the Lund University ethics committee (LU699-09, LU75-02). Further information has been published elsewhere.[31,163,210]

Detailed information on routine prognostic factors and clinical data was assembled as described in Falck *et al.* 2013 and 2016.[31,210] The patients were treated surgically with mastectomy or breast-conserving operation based on pre-operative disease stage and characteristics. Axillary lymph node dissection was performed on patients with lymph node metastasis detected before surgery or by sentinel-node biopsy. Less than 1% of the patients received neo-adjuvant treatment. Adjuvant systemic treatment and radiation therapy was given to patients according to Regional Guidelines. Data on breast cancer related death was retrieved from the Swedish Register of Causes of Death (Central Statistics Office) and the latest review of patient charts to evaluate recurrence status was performed in 2015 (all events until November 2015 were documented).

In both paper I and II, a subset of patients was included from the original cohort. Paper I included all patients (N = 464) with known breast cancer subtype according to the St Gallen classification from 2013[78] and tissue remaining from the primary tumor. Paper II included all patients (N = 550) who met the original inclusion criteria and who had not been excluded at later follow-up due to discovery of no invasive cancer, previous breast cancer diagnosis, bilateral cancer, treatment diverging from Regional Guidelines or missing data. Figure 7 presents a flow-chart of the cohort and the biomarkers evaluated.

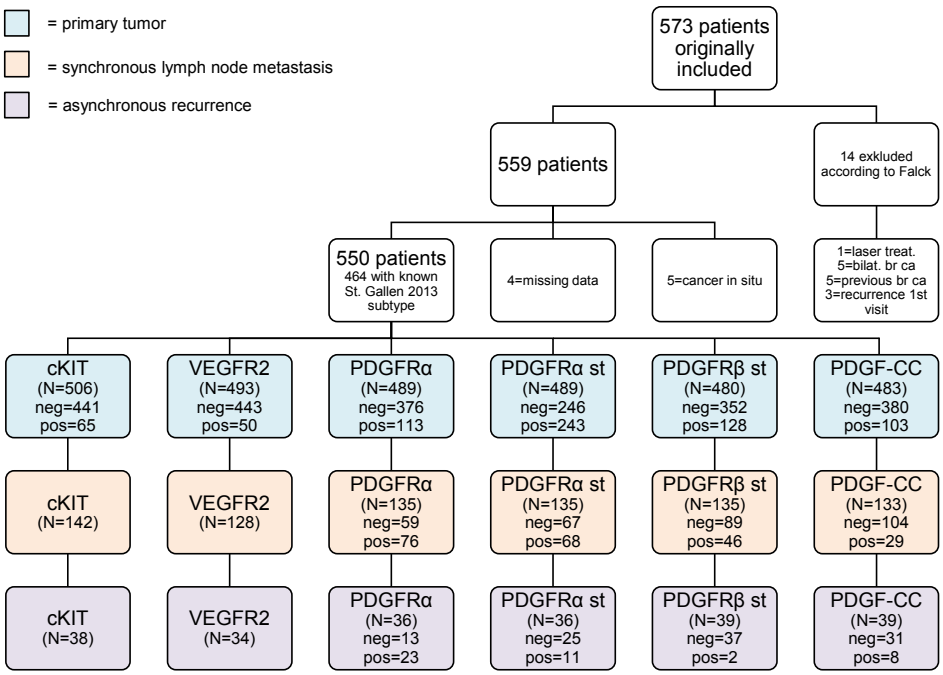


Figure 7Flowchart over the patient cohort and RTK staining for study I-II
Abbreviations; br ca, breast cancer; bilat, bilateral; neg, negative; pos, positive; st, stroma.

Paper III-IV

Patients (N=156) diagnosed with a first MBC event between April 2011 and June 2016, and scheduled for 1st line systemic treatment in Lund, Malmö and Halmstad were enrolled onto a prospective monitoring trial (Clinical Trials NCT01322893) conducted at the Department of Oncology and Pathology of Lund University, Lund, Sweden. The study was approved by the Lund University Ethics committee (LU 2010/135) and all included patients provided a written informed consent. Patients included were age ≥18 years, had an Eastern Cooperative Oncology Group (ECOG) performance status of 0-2 and a predicted life expectancy of >2 months. Exclusion criteria were prior systemic therapy for metastatic disease, other malignant disease in the last 5 years and inability to understand the study information. The aim of the study was to enumerate and characterize CTCs from serial blood samples before and during treatment.

Patient and tumor characteristics were retrieved from the patients' medical charts and data on routine prognostic biomarker assessments were collected from clinical

pathology reports. Each participating study site had a research nurse responsible for blood sample collection, regular monitoring and reporting of patient physical status, treatment received and results of clinical evaluations. After inclusion, patients started 1st line systemic therapy for MBC according to national guidelines and the study results were blinded to the treating physicians. Samples of whole blood, plasma and serum were collected from each patient at baseline (BL) and after 1, 3, 4 and 6 months depending on the treatment regimen, or until disease progression. Patients who experienced treatment failure and changed therapy from 1st to 2nd line within 6 months of inclusion were offered to enter a 2nd line blood sample series with a new BL (before start of 2nd line therapy), and new 1, 3, 4 and 6 months' sample during 2nd line therapy. We followed 23 patients for 2nd line. The blood samples were continuously examined for circulating tumor cells (CTCs) using the CellSearch system. Structured clinical and radiological evaluation was performed every 3rd month or at the discretion of the treating physician. Figure 8 presents a flowchart over the cohort and blood samples collected during the study.

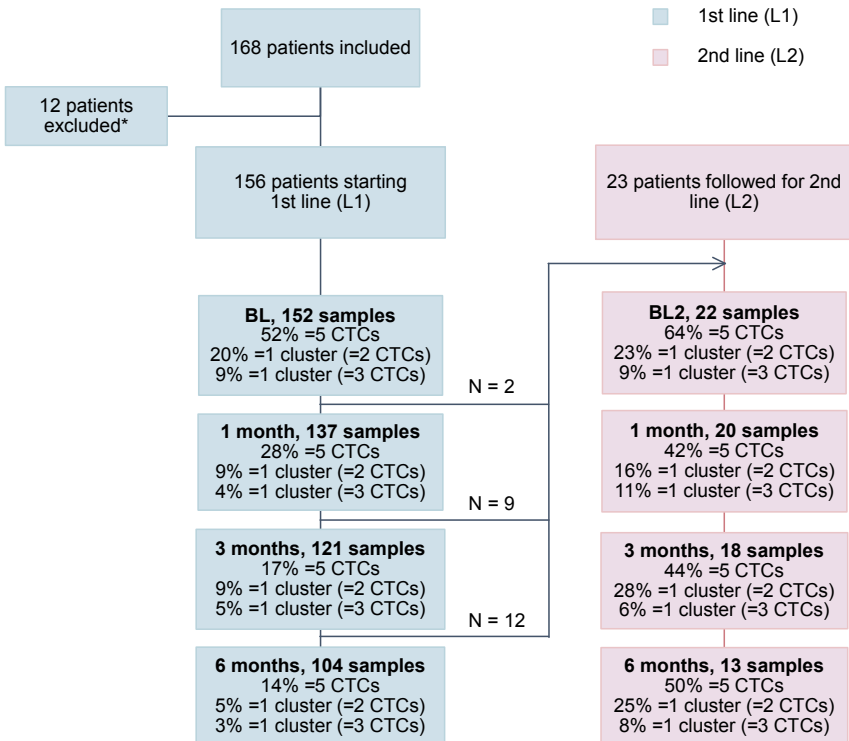


Figure 8. Flowchart over patients and samples at different time points during 1st and 2nd line treatment.

Methods

Tissue microarray

Tissue microarrays (TMAs) were introduced in the end of the 1990s and enabled simultaneous staining and rapid evaluation of large numbers of tumor samples.[211] A TMA is a paraffin block into which multiple cores of paraffin embedded tissue are assembled in a structured fashion (Figure 9). The diameter of the cores varies but is usually between 0.6-2 mm. Smaller biopsies save tissue but increase the risk of missing important features of the complete tissue sample of interest due to heterogeneity. To decrease the risk of such non-representative results, two core biopsies from different locations are usually taken from each tissue sample. Several studies have been performed to investigate the risk of missing important information by the use of TMA and they have found a strong correlation between assessment of whole tissue specimens and TMAs.[212-215]

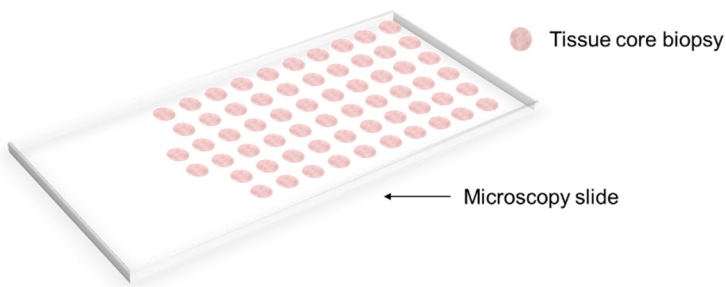


Figure 9. Schematic picture of a TMAslice mounted on a microscopy slide
Tissue core biopsies are punched out from paraffin embedded tissue biopsies and assembled into a recipient paraffin block. Thin slices of TMAs are then transferred to glass slides for staining and microscopy evaluation.

In this thesis, a TMA was constructed and used for biomarker assessment in the cohort of patients included in paper I-II. Briefly, tissue core biopsies of 1.0 mm in diameter were punched out from representative areas of invasive cancer using a tissue array machine (TMArrayer Pathology Devices, INC.). Two cores were taken from each patient tumor sample. The cores were then mounted into a recipient block and stored dark at room temperature until glass slide transfer and staining.

Immunohistochemistry and fluorescence *in situ* hybridization

Immunohistochemistry

Immunohistochemistry (IHC) is a tissue staining method used for the detection of antigens, usually proteins. It is performed using antibodies (immunoglobulins) that bind to specific antigens. The antibodies can be directly linked to a reporter molecule (e.g. a fluorochrome, enzyme, colloidal gold) in the case of direct IHC, or more commonly be indirectly detected by secondary antibodies directed against the primary antibodies bound to the antigen, called indirect IHC. Indirect IHC has a higher sensitivity because the number of secondary lables per primary antibody is higher, increasing the intensity of the staining.[216] Staining intensity and frequency can then be evaluated by microscopy, and reveals the presence and distribution of the antigen of interest.

There are several factors that can influence the IHC results. Some examples are pre-analytical handling of the tissue samples (time to and method of fixation, storage before staining etc.), antigen retrieval, selection and preparation of the antibody used, and last but not least, the staining procedure.[217] Regarding the antibodies used for antigen detection, they can be monoclonal or polyclonal. Monoclonal antibodies are homogeneous antibodies derived from a single B-cell clone and directed against a specific antigen epitope. They have a high specificity and reduced background reactivity. A drawback is that they are technically challenging and expensive to produce. Polyclonal antibodies are generated by many different B-cell clones and contains a heterogeneous mix of antibodies against different epitopes of the same antigen. They are inexpensive and easy to produce but can have a variable specificity and risk producing a false IHC result due to cross reactions with similar epitopes.[216] Below is a table over the antibodies used in paper I-II.

Table 3.
Overview of antibodies used for IHC in papers I-II

Target	Antibody	Type of antibody	Manufacturer	Dulution
c-KIT	#A4502	pAb rabbit	DAKO	1:400
VEGFR2	#2479	mAb rabbit	CellSignaling	1:100
PDGFRα	#3164	pAb rabit	CellSignaling	1:100
PDGFRβ	#3169	mAb rabbit	CellSignaling	1:100
PDGF-CC	-	mAb	Karolinska Institute	1:2000

Abbreviations; pAb, polyclonal antibody; mAb, monoclonal antibody

IHC staining for all biomarkers evaluated in papers I-II was performed as follows. TMA sections 3-4 µm thick were transferred to glass slides (Menzel Super frost plus, Thermo

Scientific, Germany), dried at room temperature and then baked in a heat chamber at 60°C for 2 hours. After deparaffinisation and antigen retrieval, IHC staining was performed with an Autostainer *Plus* (Dako Denmark A/S, Glostrup, Denmark). A Rabbit Link K8009 (Dako Denmark A/S, Glostrup, Denmark) was used to amplify the signal of the primary PDGFRα antibody and a visualization kit K801021-2 (Dako Denmark A/S, Glostrup, Denmark) was used for all stainings. Finally, all slides were counterstained with Mayer's Haematoxylin applied for two minutes.

IHC assessment

IHC staining was assessed in invasive tumor cells for c-KIT, VEGFR2, PDGFRα and PDGF-CC, and in tumor associated stroma for PDGFRα and PDGFRβ. Only TMA core biopsies with >100 invasive tumor cells were included. Two TMA cores were assessed for each tumor and the highest value was used for statistical analysis.

c-KIT, VEGFR2 and tumor cell PDGFRα was assessed by two independent investigators. Stainings were evaluated for intensity 0-3 (0=negative, 1=weak, 2=intermediate and 3 =strong) and fraction stained tumor cells (0-100%). According to common practice, a tumor was considered positive for c-KIT whenever ≥1% of the tumor cells were stained.[218] For VEGFR2 and tumor cell PDGFRα, no consensus exists on how to assign tumors as positive or negative. We searched the literature for assessment protocols and found two protocols based on histoscores. For VEGFR2, the percentage of stained cancer cells were grouped in 4 groups (<5%=0, 5-33%=1, 34-66%=2, 67-100%=3). A score was calculated by multiplying the fraction (0-3) with the intensity (0-3) resulting in a product between 0-9. All tumors with a final score >6 were considered positive.[219] For PDGFRα, the percentage of stained cancer cells were grouped in 5 groups (0%=0, 1-9%=1, 10-50%=2, 51-80%=3, 81-100%=4). A score was calculated by multiplying the fraction (0-4) with the intensity (0-3) resulting in a product between 0-12. All tumors with a final score ≥5 were considered positive.[220]

Stromal PDGFRα, PDGFRβ and tumor cell PDGF-CC were assessed by a clinical pathologist and scored for staining intensity 0-3 (0=negative, 1=weak, 2=intermediate and 3=strong). All tumors with a high intensity (score=3) were considered positive and tumors with negative to intermediate intensity (score 0-2) were considered negative.[143] Figure 10 shows representative photos of the different IHC stainings.

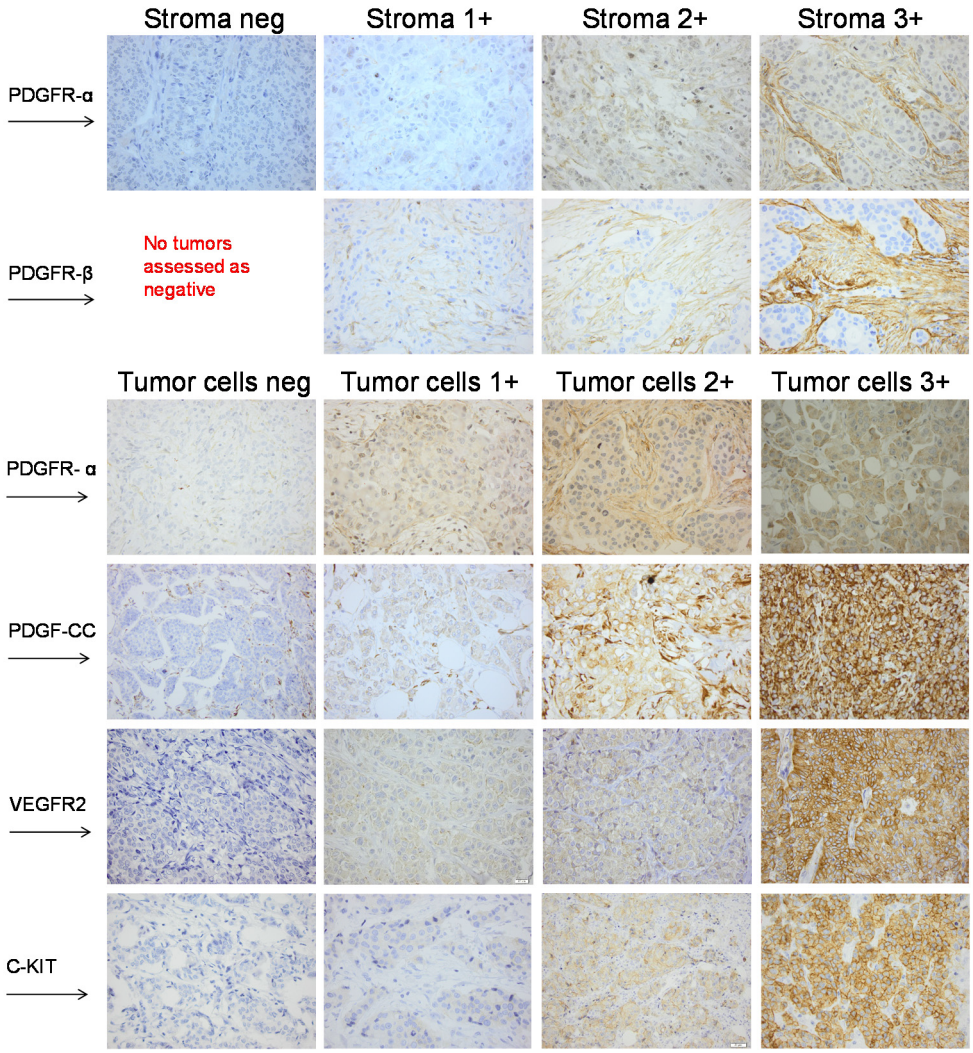


Figure 10. Examples of IHC staining on primary tumor tissue.
Columns represents intensity from 0 (negative) to 3+ (strong). Original magnification x40.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) is a method that can be used to detect gene amplification and deletion, chromosome number and translocations. In this method, a fluorescently labeled probe complementary to a specific DNA or RNA sequence is added to a tissue sample. The probe binds to its complementary sequence and can then be evaluated by fluorescent microscopy.

In paper I, FISH was used to evaluate gene amplification and/or gain of c-KIT, VEGFR2 and PDGFRα. The protocol for the FISH staining is described in detail in the publication.[221] FISH staining using a quadruple probe was performed on TMAs of paraffin embedded tissue.

FISH assessment

Only one of the two TMA cores were assessed for each patient and a total of 30 invasive cancer cells were evaluated per core. The number of gene copies and of chromosome 4 control regions were counted in each cell. If ≥4 gene copies of the same gene were detected, the cell was considered to have a gain of that gene. Any cell with a ratio between gene copies and chromosome 4 control region >2 was considered to have an amplification. If a TMA core had ≥5 cells with gains and/or amplifications, it was considered FISH positive.[222]

CTC enrichment and detection technologies

CTCs generally occur at very low concentrations ranging from 1-10 cells per 10 ml of peripheral blood in most cancers.[223] It is estimated that for each CTC, there are approximately a billion normal blood cells (consisting of leukocytes, erythrocytes, platelets and other hematopoietic cells)[202] making is difficult to detect the rare CTCs. Thus, the majority of CTC detection methods starts with an enrichment step to increase the concentration of CTCs. In general, this is done by exploiting different biological or physical properties of the CTCs (Figure 11).[223-225] Below is a description of the major enrichment and detection methods.

CTC enrichment

Biological enrichment approaches rely on cell-specific markers expressed by CTCs or by the surrounding blood cells that can be detected by antibodies and used for positive or negative selection of cells. A commonly used marker for positive selection is the EpCAM, which is a cell-surface protein expressed by epithelial cells (carcinoma cells) and which is absent in blood cells. It is also possible to use negative selection and deplete the blood cells to detect remaining CTCs. Negative selection is often performed using antibodies that target CD45, a leukocyte antigen that is not expressed by carcinoma cells.[223,225]

Physical approaches make use of differences in the inherent physical properties between CTCs and blood cells (e.g. size, density). Examples of isolation methods using physical properties are cell filtration and centrifugation.[223,225]

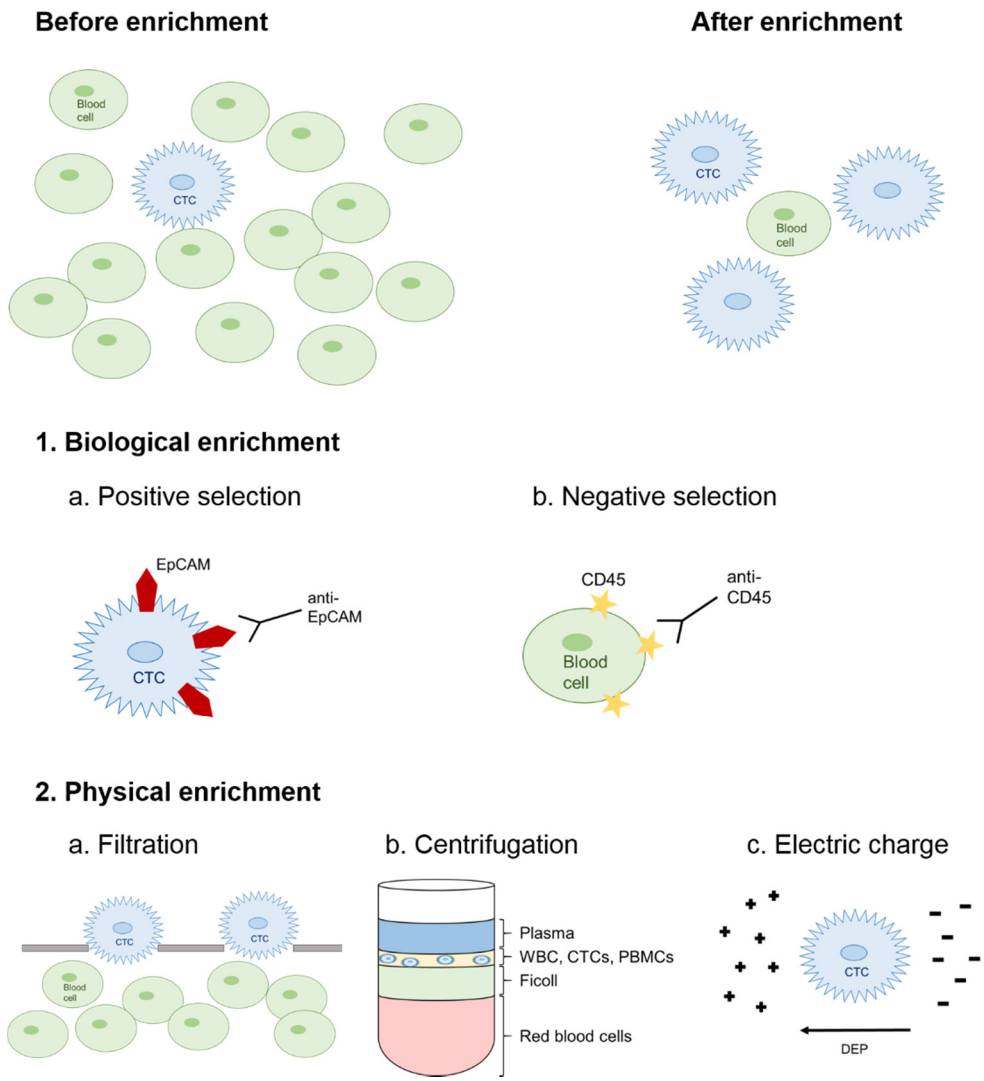


Figure 11.
Examples of CTC enrichment by biological (1) or physical (2) properties.

CTC detection

After the enrichment step, all collected cells need to be identified to distinguish CTCs from possible contaminating blood cells. Briefly, CTCs can be detected by immunological or molecular techniques. Immunological techniques include flow cytometry and immunocytochemistry as e.g. by the CellSearch system using fluorescent antibodies against CKs and CD45, as well as 4',6-diamidino-2-phenylidole (DAPI)

staining of the nucleus. Molecular techniques are RNA-based and an example is reverse transcription-quantitative polymerase chain reaction (RT-qPCR).[223]

The CellSearch system

The CellSearch system (Menarini Silicon Biosystems, Bologna, Italy) is a semi-automated system for isolation and detection of CTCs. It contains two parts; the CellTracks AutoPrep System for isolation and staining of CTCs, and the CellTracks Analyzer II for evaluation and enumeration of CTCs. Here is a brief summary of the technique.

A blood sample of 7.5-10 ml of blood is drawn into a CellSave tube (Menarini Silicon Biosystems). Samples can be stored at room temperature after collection for a maximum of 96 hours before analysis. For analysis, 7.5 ml of blood is mixed with a dilution buffer, centrifuged and then placed in the CellTracks AutoPrep System. The system removes the plasma and dilution buffer, and adds anti-EpCAM antibodies coated with ferrofluids, which labels all EpCAM positive cells (i.e. epithelial tumor cells). Following immunomagnetic labeling, the sample is incubated and labeled cells are separated with magnetic forces. Unbound cells and remaining plasma is removed, the remaining cells are then re-suspended in buffer and stained with fluorescent nuclear dye (DAPI) and fluorescent antibodies against CK 8, 18 and 19; and against CD45 (an antigen expressed by blood cells and absent in epithelial cells) to enable later microscopic evaluation. Thereafter, the sample is once more incubated and subjected to magnetic separation. Unbound staining reagents are removed and a cell fixative is added. The sample is subsequently automatically transferred to a cartridge which is placed in a magnetic holder (MagNest). All immunomagnetically labeled cells are then oriented by magnetic forces to enable subsequent analysis by the CellTracks Analyzer II.[226]

The CellTracks Analyzer II is a semi-automated fluorescence microscope that scans the MagNests containing the cells enriched by the CellTracks AutoPrep System. Each cartridge is scanned 4 times with automatic change of fluorescent filter between the scans. All scanned objects fulfilling certain predefined criteria are assembled in a gallery for manual selection of which cells are defined as CTCs and which cells are not. CTCs are CK+/DAPI+/CD45- cells at least 4 μm in diameter and with morphologic characteristics of a cell (e.g. a visible nucleus within the cell).[226]

The accuracy, precision and linearity of the CellSearch system was tested in 2004 by Allard *et al.* Using blood samples spiked with known numbers of tumor cells derived from cell lines, they could demonstrate that this system has a high accuracy and reproducibility. CTC recovery rate for spiked samples was >85% and it was linear over the range in CTC counts usually detected in metastatic carcinoma patients. Furthermore, they also tested healthy volunteers and women with non-malignant

diseases, and only one of 344 samples investigated contained >1 CTC.[154] Hitherto, the CellSearch system is the only CTC isolation and detection system to be approved by the FDA.[159]

CTC isolation, detection and evaluation by the CellSearch system in the patient cohort included in this thesis

In the patient cohort for paper III-IV, blood samples were collected at baseline before start of 1st line systemic therapy, and after 1, 3, 4 and 6 months of therapy. The 4 months' sample was not included in any analyses in the papers included in this thesis.

Blood samples were drawn into 10 ml CellSave Preservation tubes, stored at room temperature and processed within 96 hours using the CellSearch system. Two investigators trained and certified in the CellSearch technology independently assessed all gallery events and selected CTCs. Any event where the assessment differed between the investigators was re-evaluated and a consensus decision was reached.

Using the built in export function in the CellTracks Analyzer II system, all selected CTCs were grouped in a pdf gallery and exported. No additional staining was added after the CellSearch analysis. CTCs in the galleries were assessed for apoptosis, CTC-clusters and WBC-CTCs. Apoptotic CTCs were identified as CTCs with characteristic fragmented and condensed nuclear morphology as defined by a clinical pathologist and according to previous publications.[227] CTC-clusters were evaluated both as two cell clusters (paper IV) and as three cell clusters (paper III). Two cell clusters were defined as ≥2 CTCs clustered together with non-overlapping nuclei. Three cell clusters were defined as ≥3 CTCs clustered together with non-overlapping nuclei. WBC-CTCs were defined as ≥1 CTC clustered with ≥1 leukocyte. Figure 12 shows examples of apoptosis, CTC-clusters and WBC-CTCs as detected in the CellSearch galleries.

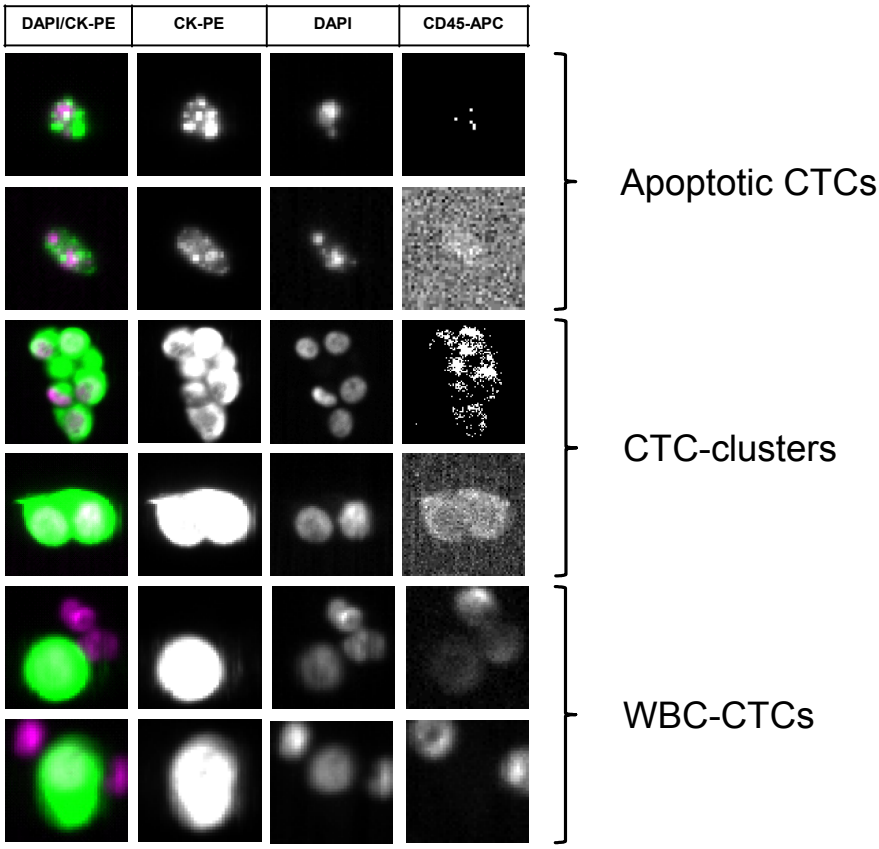


Figure 12. Morphologic characteristics of CTCs.

Statistics

All statistical calculations were performed using SPSS versions 21.0-24.0 (SPSS, Chicago, IL and IBM, Armonk, NY, USA) and Stata versions 12.1-15.0 (StataCorp LP, College Station, TX, USA).

Categorical or categorized patient and tumor characteristics were compared using Pearson's chi-squared test or, if expected counts in one or more cells was <5, Fishers exact test. Ordinal variables were compared using Pearson's chi-squared test for trend and variables measured on a continuous scale by the Mann-Whitney U test or Kruskal-Wallis test if a variable contained more than two categories.

Binary logistic regression analysis, with and without adjustment for different tumor characteristics, was performed in paper I to quantify the effect of each RTK and in paper IV to assess the association between CTC count and outcome at first evaluation.

Change in biomarker expression between primary tumors, synchronous recurrences and asynchronous relapses in paper II was assessed by the McNemar test. Ordered differences between receptor and ligand status was evaluated by the Jonckheere Terpstra test.

In paper IV, likelihood ratio (LR) statistics in Cox regression models was used to evaluate the added value of CTC count and CTC-clusters to a prognostic clinicopathological model developed in this paper, using the model by Bidard *et al.* as a reference.[155]

Survival analysis

Kaplan-Meier estimated and the log-rank test

Kaplan-Meier survival plots and log-rank tests were used to evaluate survival. The Kaplan-Meier method is a non-parametric method for estimation of survival probabilities. These probabilities are often plotted versus time in a so-called survival plot.[228] The log-rank test can be used to test differences in survival between two or more groups within the Kaplan-Meier plot.[229] There are many ways to define the time period and the endpoints. Start of time can be diagnosis of a disease, date of surgery, inclusion in a study, collection of first study sample etc. In paper I-II, time zero was date of primary surgery. In paper III-IV, it was date of blood sample collection. End of the time period is when the endpoint is reached, or at censoring. The endpoint can be e.g. recurrence of a disease (local and/or distant), disease specific death, death from any cause etc.[230] In this thesis, the endpoints chosen differed between the different papers. For all time variables included in this thesis, censoring of time periods for patients without event was done at last medical follow-up visit.

Endpoints

Paper I and II use the same patient cohort, but with a slightly different selection of patients. There are also different endpoints used for these two papers. In paper I, breast cancer mortality (BCM, i.e. death from breast cancer) was chosen as endpoint. This is a good choice of endpoint for studies with old breast cancer patients and/or long follow-up since the effect of the factors studied will be diluted by deaths unrelated to breast cancer if overall survival (OS) was chosen as endpoint. The total mortality can be divided into mortality due to breast cancer (BCM) and the competing event "mortality due to other causes". As cohort 1 consisted of breast cancer patients with good prognosis and had long follow-up, it was estimated that death from other causes than breast cancer probably contributed to a non-negligible part of the overall mortality, i.e. there was a competing risk situation between breast cancer related death and death from other causes. However, in paper II we wanted to increase power and also capture early breast cancer events in the endpoint. We investigated primary tumors, synchronous lymph node metastasis and asynchronous recurrences, and wanted the recurrences to be part of the endpoint. It was thus decided to use distant recurrence-free interval (DRFi) as endpoint. DRFi is defined as the time from a specific starting point (in this paper, date of primary surgery) until distant recurrence or breast cancer related death.[230]

In papers III and IV, progression-free survival (PFS) and OS were used as endpoints. These are common endpoints in metastatic cancer where the competing mortality is low. Time to progression (PFS) or death (OS) was calculated from date of baseline blood draw until date of progression or death from any cause.

Cox regression

Cox regression is a method to quantify relative effects, hazard ratios (HRs) of prognostic factors. In Cox regression, proportional hazards are assumed, which means the ratio of the hazards comparing different groups are maintained constant over time.[231] In the papers in this thesis, uni- and multivariable HRs for selected potential predictors of survival outcome were determined by Cox proportional hazards regression.

Landmark analysis and extended Cox model

In papers III and IV, survival analysis was used to evaluate not only the effect of variables measured at baseline (time zero) but also the effect of the same variables measured at 1, 3 and 6 months of follow-up. Introduction of time-dependent so-called internal variables complicates the analysis somewhat. Two approaches were used to handle this extra complexity. First, by subtracting the time from baseline blood draw to the 1-month follow-up blood draw from all the survival times we redefined time zero to be the date of the 1-month blood draw. For analysis of survival, conditional on survival up to the 1-month blood draw, standard methods of survival analysis, like the Kaplan-Meier method, the log-rank test and Cox regression, can be used. This approach, which was used also for variables measured at the 3- and 6-month blood

draw, is known as landmark analysis or the clock reset method. Second, an extension to the Cox model which can handle time varying variables was used. The HR for a binary variable X in a model of this kind measures the relative effect of the variable on outcome for episodes with X=1 compared to that of episodes with X=0. This means that a patient can contribute person-years to both the numerator and the denominator.

Strengths, limitations and potential bias

A summary of the strengths, limitations and potential sources of bias for the papers included in this thesis is presented in Table 4.

Both patient cohorts which the papers in this thesis are built upon are prospective observational cohorts, and both cohorts have relatively long follow-up in relation to the patient subpopulation included and their respective prognosis. The term prospective observational study means that the study objects (here breast cancer patients) are included at baseline (here at breast cancer diagnosis) when the factor of interest for the study (here biomarkers) can be evaluated and the patients are then observed over time to investigate how these factors relates to a certain outcome of interest (here tumor and patient characteristics, and survival). In prospective observational studies, no interventions are made to the study population. This study design is ideal for the aims of the papers in this thesis.

Patient cohort 1 included only patients with operable primary breast cancer who did not receive neoadjuvant therapy and these patients had a good prognosis in line with contemporary data. Hence the results are not generalizable to all breast cancer patients but rather to this selected sub-population of somewhat less aggressive tumors with a low fraction of the typically aggressive TNBC.

The pace of inclusion was slower than expected for cohort 2 (MBC). Inclusion was estimated to take 2 years but took 5 years. Possible explanations for this are strict inclusion criteria and insufficient screening routines at the clinic. However, the long inclusion is a possible source of bias if e.g. only patients with certain characteristics were asked to join.

In general, this thesis has sub focus on TNBC. TNBC is a relatively rare breast cancer subtype occurring at only about 7-14% of primary breast cancers. It is thus difficult to gather a sufficiently large breast cancer patient cohort to enable enough statistical power for subgroup analysis on TNBC. Cohort 1 with primary breast cancer had only 34 (7.3%) TNBC, and cohort 2 with MBC had 26 (17%) TNBC. In both cohorts, additional analyses were made to assure that the TNBC patients had tumor and patient characteristics representative of a typical TNBC (e.g. poor prognosis, younger age and more aggressive tumors) and so they did.

Table 4.
Strengths, limitations and potential bias for the papers included in this thesis.

	Strengths	Limitations and potential bias
Paper I	Large prospective observational cohort with long follow-up time Detailed patient and tumor data available. All tumor markers evaluated by two independent reviewers enabling e.g. St Gallen classification	Difficulties with the FISH staining, <50% of samples assessable No standardized assessment of VEGFR2 and PDGFRα available – difficult to compare studies Only patients with operable primary breast cancer and good prognosis included, results not generalizable to unselected breast cancer Few TNBC (7.3%)
Paper II	Large prospective observational cohort with long follow-up time Detailed patient and tumor data available. All tumor markers evaluated by two independent reviewers enabling e.g. St Gallen classification Assessments of PDGF-CC and stromal PDGFRs performed by clinical pathologist Tumor tissue available from PT, N and R	Limited tissue remaining from N and R R included both locoregional and distant recurrence, no data available on the origin of R-tissue Only patients with operable primary breast cancer and good prognosis included, results not generalizable to unselected breast cancer No standardized assessment of PDGFRs and PDGF-CC available – difficult to compare studies Few TNBC (7.3%)
Paper III	Prospective observational cohort, study results blinded to treating physicians Detailed patient and tumor data available Newly diagnosed MBC scheduled for 1st line systemic therapy, regardless of therapy planned Longitudinal blood sampling and long follow-up, few patients left the study prematurely CellSearch system used for CTC enumeration, this is the most used system in other similar trials and thus enable good comparison	Small cohort, limited statistical power, especially for subgroup analyses Only patients with ≥5 CTCs at BL included Long inclusion period Strict inclusion criteria, this can however also be a strength
Paper IV	Prospective observational cohort, study results blinded to treating physicians Power calculation performed before study to assure sufficient number of patients to be included Detailed patient and tumor data available Newly diagnosed MBC scheduled for 1st line systemic therapy, regardless of therapy planned Longitudinal blood sampling and long follow-up, few patients left the study prematurely Thorough study monitoring and data collection during study period CellSearch system used for CTC enumeration, this is the most used system in other similar trials and thus enable good comparison	Limited statistical power for subgroup analyses due to few HER2+ and TNBC patients Long inclusion period Strict inclusion criteria, this can however also be a strength

Abbreviations: PT, primary tumor; N, lymph node metastasis; R, recurrence; CTC, circulating tumor cell; BL, baseline

Results

Paper I

A subset of 464 patients with known breast cancer subtype according to St Gallen 2011 were included.[77] Thirty-four (7.3%) had TNBC, and these patients had typical characteristics of TNBC (e.g. younger age, higher grade and Ki67, larger tumors and poor prognosis).

Tumor cell expression of c-KIT, VEGFR2 and PDGFRα in TNBC vs non-TNBC

c-KIT and VEGFR2 showed a significantly higher expression in TNBC compared to non-TNBC (P<0.001), and the same tendency was seen for PDGFRα (P=0.07). The unadjusted odds ratios (ORs) of positive expression of these biomarkers in TNBC vs non-TNBC were 8.9, 5.8 and 2.0 for c-KIT, VEGFR2 and PDGFRα, respectively. The ORs adjusted for histopathological type, grade, tumor size >20 mm, and lymph node engagement were 6.8, 3.6 and 1.3, respectively.

High expression of ≥1 of the 3 receptors was seen in 25 (73.5%) of the TNBC tumors compared to 129 (30.0%) of the non-TNBC (P<0.001) and high expression of ≥2 of the 3 receptors were seen in 12 (35.3%) of the TNBC compared to 25 (5.8%) of the non-TNBC (P<0.001). Three tumors in total were positive for all three markers, one of them was a TNBC.

Gene copy number in TNBC vs non-TNBC, and comparison of high receptor expression and increased gene copy number

Only 193 (42%) of tumors had sufficient quality of FISH staining for assessment. Approximately 12% of the patients in both the TNBC group and the non-TNBC group had increased gene copy number of the investigated genes. No correlation was found between increased gene copy number and high protein expression for either c-KIT, VEGFR2 nor PDGFRα. Table 5 summarizes the results of IHC and FISH evaluations in TNBC vs non-TNBC.

Table 5.
Overview of tumor cell expression and gene copy numbers of c-KIT, VEGFR2 and PDGFRα in TNBC vs non-TNBC.

	non-TNBC (N=430, 93%)	TNBC (N=34, 7%)	*P*-value
Protein expression (IHC)	**Pos marker, N (%)**	**Pos marker, N (%)**	
c-KIT	41 (10)	16 (49)	<0.001
VEGFR2	32 (6)	11 (32)	<0.001
PDGFRα	83 (19)	11 (32)	0.07
Gene copy number (FISH)			
c-KIT	19 (11)	2 (11)	1.0
VEGFR2	20 (11)	2 (11)	1.0
PDGFRα	22 (12)	2 (11)	1.0

Abbreviations: Pos, positive; IHC, immunohistochemistry; FISH, fluoresence *in situ* hybridization.

Prognosis in relation to tumor cell expression and gene copy number of c-KIT, VEGFR2 and PDGFRα in TNBC vs non-TNBC

Non-TNBC patients with tumors positive for VEGFR2 had a lower BCM compared to patients with negative tumors (log rank *P*=0.03). The corresponding HR indicated a notably lowed BCM, HR=0.04 (95% CI 0.001-3.3), but it was not significant (*P*=0.16). Patients with tumors positive vs negative for c-KIT and PDGFRα had no differences in survival or HR for mortality, neither in the TNBC nor the non-TNBC group. In addition, no difference in BCM was seen between patients with increased gene copy numbers compared to those with normal, neither in TNBC nor in non-TNBC.

Paper II

This paper included 550 patients, median age at primary breast cancer diagnosis was 57.8 years and median follow-up time was 13.7 years. In total, 473 patients had a known breast cancer subtype according to St Gallen 2013.[78]

Patient and tumor characteristics in relation to primary tumor expression of PDGFRα, PDGFRβ and PDGF-CC

High expression of tumor cell PDGFRα was significantly associated to increasing NHG, high Ki67, TNBC and expression of CK5/6+. High expression of tumor cell PDGF-CC was significantly associated to young age (< 50 years), large tumor size, increasing NHG, high Ki67, TNBC, ER-, PR-, CK5/6+ and EGFR+. High expression of stromal cell PDGFRα was significantly associated to young age, increasing NHG, high Ki67, HER2+, ER- and EGFR+. High expression of stromal cell PDGFRβ was significantly associated to young age, increasing NHG and high Ki67. In summary,

high expression of the investigated members of the PDGF-family correlated to several prognostic patient and tumor characteristics that indicate tumor inherent biological aggressiveness.

Seventy-seven patients developed distant recurrence during follow-up (bone-only=28, visceral=43 and CNS=6). Recurrence within CNS was more common in patients with high expression of tumor cell PDGFRα in the primary tumor; 4/15 (27%) vs 2/58 (3%) (*P*=0.01).

Biomarker expression and tumor progression

There was a shift in biomarker expression from primary tumor to lymph node and later recurrence for a substantial number of tumors. In particular, PDGFRα expression in tumor cells was significantly up-regulated in lymph node metastases and recurrences; and stromal PDGFRβ expression was significantly down-regulated in recurrences.

Concomitant expression of ligand PDGF-CC and the PDGF-receptors.

A total of 80 primary tumors (18%) had concomitant high expression of ligand PDGF-CC in tumor cells and at least one of the PDGF-receptors in tumor and/or stromal cells. Nearly all tumors with high stromal cell PDGFRβ and high tumor cell PDGF-CC also had high PDGFRα, either in stromal or tumor cells. Contrarily, >50% of the tumors with high PDGF-CC and high PDGFRα expression in tumor or stromal cells, displayed low PDGFRβ. Concomitant PDGFRα and PDGF-CC expression varied markedly between the molecular subtypes. TNBC displayed co-expression in 59% of the primary tumors whereas the Luminal subtypes only displayed co-expression in 5% (Luminal A) to 19% (Luminal B HER2+).

Primary tumor biomarker expression and prognosis

Survival analysis showed no significant difference in outcome for patients positive vs negative for PDGFRα, PDGFRβ or PDGF-CC. However, the survival curves for PDGF-CC indicated a prognostic effect over the first years after primary breast cancer diagnosis. DRFi was thus divided into three time intervals, 0-5 years, >5-10 years and >10 years. This revealed a significant increased risk of early breast cancer event (recurrence or breast cancer related death within 5 years of primary diagnosis) in the group of patients with tumors positive for PDGF-CC (HR=1.77; 95% CI: 1.03 – 3.04, Figure 13). This increase did however not remain significant in multivariable analysis adjusted for age, tumor size, node status, NHG and St Gallen molecular subtype (HR=1.14; 95% CI: 0.59-2.19). For late events (occurring >10 years after primary breast cancer diagnosis), there was an increased risk amongst patients with tumors negative for PDGF-CC.

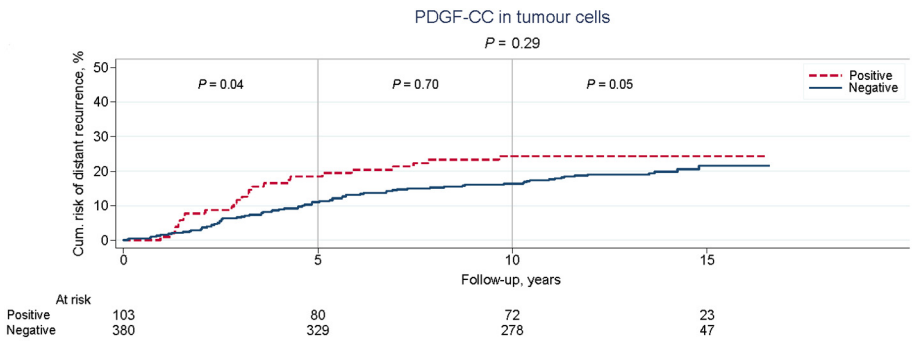


Figure 13.
Kaplan-Meier survival curve showing distant recurrence-free interval (DRFi) (years) in relation to expression of PDGF-CC in tumour cells, dichotomized into positive vs negative.

Paper III

This study included a subset of 52 MBC patients with ≥5 CTCs present at baseline before start of 1st line systemic therapy. Median age at diagnosis with MBC was 60 years, and 39 patients had HR+, 7 had HER2+ and 4 had TNBC.

Apoptotic CTCs

No significant difference in proportion of patients with apoptotic CTCs present was seen between HR+, HER2+ and TNBC patients. Presence of apoptotic CTCs was not associated to survival at BL, but worse outcome was seen in patients with apoptotic CTCs in follow-up samples at 1-3 and 6 months. After adjustment for CTC count, breast cancer subgroup (HR+, HER2+ or TNBC), age at diagnosis, time to recurrence, type and number of metastasis; the presence of apoptotic CTCs was significantly related to increased HR_{PFS} at 1-3 months, and to HR_{OS} at 1-3 and 6 months.

CTC-clusters

In this paper, CTC clusters were defined as ≥3 CTCs clustered together with non-overlapping nuclei. HER2+ and TNBC patients had CTC-clusters present more frequently than HR+ patients at BL (P=0.01). At 1-3 months, CTC-clusters remained more frequent in TNBC but at 6 months, no significant difference was observed. Similar to the results for apoptotic CTC, no difference in survival was seen at BL for patients with CTC-clusters present. However, at 1-3 and 6 months, CTC-cluster presence was significantly associated to worse PFS and OS. HR for OS at 6 months was not even definable as all patients in the CTC-cluster group died before any patient

in the non-cluster group, i.e. perfect prediction. However, when HRs for PFS and OS at 1-3 and 6 months comparing CTC-cluster presence vs no presence were adjusted for CTC count and other prognostic factors, there was a tendency to increased HRs but no significant remaining effects were observed on outcome.

WBC-CTC

Presence of WBC-CTC did not differ between the breast cancer subtypes at any time point and no significant difference was observed in survival at BL or 1-3 months. At 6 months, presence of WBC-CTC was associated with inferior OS in uni- and multivariable Cox regression analysis.

Paper IV

In total, 156 women with newly diagnosed MBC scheduled for 1st line systemic therapy were included in this study. Median follow-up time from BL for patients alive at last medical visit was 25 (7-69) months. Breast cancer subtype was defined by analysis of the metastasis primarily, and primary tumors secondly; 105 (70%) patients had HR+, 20 (13%) had HER2+ and 26 (17%) had TNBC.

Prediction of Outcome in Relation to CTCs and CTC-clusters

At BL, 79 (52%) patients had ≥5 CTCs and 30 (20%) patients had ≥1 CTC-cluster, and both factors were significantly associated with poor survival. During treatment, a time-dependent increase in HR_{PFS} and HR_{OS} was observed by landmark analysis, predicting worse survival for CTC count ≥5 and presence of CTC-clusters (Table 6). Stratification of patients based on CTC count and CTC-clusters revealed four risk groups at all time points (0 CTC, 1-4 CTCs, ≥5 CTCs, ≥1 CTC + CTC-clusters), where patients with CTC-clusters had significantly worse survival from all measured time points compared to patients with no clusters. Changes in CTCs during treatment were significantly correlated to response evaluation and survival.

Cox regression analysis of OS with time-varying covariates showed that mortality was increased for episodes with CTCs ≥5 and CTC-cluster presence (5.74 and 5.14 respectively. Including both factors in the same model, the mortality was 7.8 times higher for episodes with both CTC-count ≥5 and CTC-cluster presence.

Prognostication by a Clinicopathological Model Including CTC Count and CTC-clusters

CTC count and CTC-clusters were incorporated into a clinicopathologic prognostication model including breast cancer subtype, histologic grade, performance status (ECOG), age, metastasis-free interval, visceral metastases and number of metastatic locations. It was observed that CTC count and CTC-cluster presence

independently improved the survival prognostication of the model at all time points for both PFS and OS. Notably, at 3 and 6 months, CTC count and CTC-cluster presence enhanced the models' C-index to >0.70 for PFS and >0.80 for OS.

Table 6.
Univariable and multivariable Cox regression HRs for CTC count ≥5 vs <5, and for presence vs absence of CTC-clusters (≥1 cluster of ≥2 CTCs) at BL and during 1st line systemic therapy.

	PFS		OS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
BL				
Unadjusted				
CTC ≥5	1.75 (1.19-2.57)	0.004	2.55 (1.54-4.22)	<0.001
Clusters present	1.71 (1.08-2.71)	0.02	2.33 (1.36-3.97)	0.002
Adjusteda				
CTC ≥5	2.30 (1.43-3.71)	0.001	3.92 (2.09-7.36)	<0.001
Clusters present	2.64 (1.46-4.78)	0.001	4.07 (1.99-8.31)	<0.001
1 month				
Unadjusted				
CTC ≥5	2.11 (1.38-3.24)	0.001	4.24 (2.49-7.20)	<0.001
Clusters present	3.31 (1.70-6.44)	<0.001	4.17 (2.02-8.62)	<0.001
Adjustedb				
CTC ≥5	2.30 (1.23-4.32)	0.009	4.39 (2.04-9.43)	<0.001
Clusters present	3.37 (1.51-7.55)	0.003	5.67 (2.30-13.95)	<0.001
3 months				
Unadjusted				
CTC ≥5	2.08 (1.11-3.93)	0.02	3.10 (1.61-6.00)	0.001
Clusters present	4.00 (1.96-8.13)	<0.001	4.82 (2.27-10.22)	<0.001
Adjustedb				
CTC ≥5	2.95 (1.44-6.06)	0.003	5.93 (1.54-7.51)	<0.001
Clusters present	3.04 (1.35-6.84)	0.007	3.55 (1.44-8.77)	0.006
6 months				
Unadjusted				
CTC ≥5	4.07 (1.94-8.51)	<0.001	8.58 (3.70-19.9)	<0.001
Clusters present	6.26 (2.12-18.50)	0.001	10.64 (3.27-34.62)	<0.001
Adjustedb				
CTC ≥5	6.43 (2.30-17.94)	<0.001	15.72 (3.79-65.17)	<0.001
Clusters present	7.17 (2.03-25.36)	0.002	21.65 (5.06-92.63)	<0.001

Abbreviations: PFS, progression-free survival; OS, overall survival; HR, hazard ratio; BL, baseline; CTC, circulating tumor cell
[a]Adjusted for the variables included in the clinicopathological model
[b]Adjusted for the variables included in the clinicopathological model and for BL CTC count (<5 vs ≥5)

Discussion

The survival of patients with primary as well as metastatic breast cancer has increased over the last decades, but further treatment improvements are warranted since 1500 women still die from this disease every year in Sweden.[232] TNBC represents a small subgroup of breast cancer but it attracts attention as it more frequently affects younger patients, it is characterized by an aggressive tumor phenotype and behavior, and it has a poor prognosis both the primary and the metastatic setting.[84-86] Further, no targeted therapy is hitherto available for TNBC.[233]

The aim of this thesis was to explore new potential prognostic biomarkers in breast cancer, with a special focus on TNBC. Two patient cohorts were investigated, one with primary breast cancer and one with MBC. Importantly, the treatment goals differ between these two stages of breast cancer. In primary breast cancer, the goal is to eradicate minimal residual disease and thus prevent disease recurrence and metastasis. In MBC, the goal is palliation, symptom control and survival prolongation. Biomarkers may have different roles in these different settings, and there may also be a need for different biomarkers. In the primary setting, biomarkers are needed that can e.g. predict which patients are at high risk of recurrence and death from the disease, to enable more aggressive treatments in these patients. In the metastatic setting, biomarkers are needed to e.g. monitor response to treatment to minimize exposure to useless and potentially harming treatments.

Primary breast cancer, paper I and II

In the first two papers, the importance of the four tyrosine kinase receptors cKIT, VEGFR2, PDGFRα and PDGFRβ, and of ligand PDGF-CC was explored. The receptors have recently been implicated in the pathogenesis and evolvement of breast cancer, and emerged as possible drug targets.[149] In paper I, we found that cKIT and VEGFR2 showed higher expression in TNBC, and a tendency towards higher expression of PDGFRα was also observed. Moreover, it was found that more than two thirds of TNBC tumors displayed high expression of at least one of these three receptors. Our results confirm previous studies showing upregulation of cKIT and VEGFR2 in TNBC.[119-121,131] In paper II, we wanted to further explore the expression of selected members of the PDGF-family. Here, we showed that high expression of the PDGF receptors α and β, and ligand PDGF-CC correlates to several prognostic patient and tumor characteristics related to tumor inherent biological aggressiveness, such as

negative ER and PR, and increasing NHG. This has previously been shown for the PDGF receptors[141-143] but not for ligand PDGF-CC. Also, concomitant expression of PDGFRα and PDGF-CC was highest in TNBC (59%) and lowest in the luminal subtypes (5-19%). Together, the results of study I and II supports an involvement of cKIT, VEGFR2, PDGFRα and ligand PDGF-CC in TNBC. Neither of these receptors were associated to survival in TNBC but interestingly, in the whole cohort we found that patients with high expression of ligand PDGF-CC had increased risk of 5-year distant-recurrence. The results of paper II also proposes that PDGFRα is upregulated during tumor progression. Previous studies have shown up-regulation of the members of the PDGF-pathway during EMT[234], and experiments in mouse models have proposed that an autocrine PDGF/PDGFR loop contribute to tumor progression and metastasis in vivo.[235] These studies conclude that the PDGF-pathway is involved in cancer progression, as is also supported by our results.

Based on these findings in paper I and II, it would be appealing to try to target these RTK signaling pathways in TNBC. Indeed, some treatment attempts have been made with known multi-TKIs e.g. sunitinib but the results have in general been disappointing showing more adverse events and no survival benefit.[147-150] Sorafenib is another multi-TKI that has showed slightly better results in combination therapy. However, more studies are needed to clarify the potential of sorafenib as a treatment in breast cancer.[151] Notably, the larger part of the studies on both sunitinib and sorafenib have been performed in MBC. Little is known on their potential as adjuvant treatments in the primary breast cancer setting.

It has been recognized in many articles on TNBC that finding both biomarkers and actionable drug targets is difficult in this breast cancer subtype due to the large heterogeneity that exists between different TNBC tumors.[86,88] So far, no single oncogenic driver has been found in TNBC, which is part of the problem to design a successful targeted therapy to treat these patients.[236] As written in the background of this thesis, TNBC can be further subdivided into several subtypes using e.g. gene expression profiling as described by Lehmann *et al*. In the same article it was shown that this genomic classification could identify putative therapeutic targets based on the genetic abnormalities within the different TNBC subtypes identified.[87] A retrospective analysis on 146 TNBC treated with chemotherapy showed that classifying TNBC into subtypes based on gene expression predicted rate of pathological complete response (pCR). The BL1 subtype had the highest pCR (52%), and BL2 and LAR the lowest (0% and 10% respectively). This study confirmed the clinical relevance of further subtyping TNBC tumors to improve treatment outcome for these patients.[237]

Additional systems have been proposed for the subclassification of TNBC, and a review was published in 2015 which tried to identify major groupings within TNBC that can be useful for clinical trial development. In this review, the major biological pathways behind each subgroup were presented, as well as potential ways to target these.[238]

However, a problem with subdividing an already small subgroup as TNBC into even smaller subgroups is associated with practical issues of study design. As demonstrated in paper I and II in this thesis, the number of TNBC is limited (here 34 patients, 7.3%) and very large cohorts would be needed to attain enough statistical power. The cohort used in paper I and II was not designed to evaluate TNBC and consequently, the power was limited. Moreover, in our papers we used a prospectively gathered cohort but the design of our study was retrospective. We thus also had the problem of limited tissue material remaining, especially for the synchronous lymph node metastasis and asynchronous recurrences evaluated in paper II. In summary, to study TNBC and particularly subgroups on TNBC very large cohorts are needed, or cohorts with high number of TNBC.

To conclude, TNBC comprises a small but very heterogeneous subgroup of tumors, and there is currently intense research focused of identifying possible drug targets in this subtype.[238] Many clinical trials are ongoing at present, however few of them are currently investigating pathways involving growth factor overexpression. This could partially be explained by discouraging results from previous exploratory trials, and also that some of these pathways are overexpressed only in a very limited set of tumors.[86] The most promising targeting drugs in TNBC at the moment seems to be PARP-inhibitors, antiandrogen therapy and different immunotherapy based approaches.[86]

Metastatic breast cancer, paper III and IV

In papers III and IV, the focus was on CTCs as prognostic markers in MBC. CTCs are not (yet) used in clinical treatment of MBC, but the numbers of studies supporting the prognostic value of CTC count by the CellSearch system in MBC are accumulating. In 2014, a large pooled-analysis confirmed that a CTC count ≥5 is an independent prognostic factor for worse PFS and OS, and deemed it to have reached level one evidence of clinical validity.[155] However, hitherto most studies have included MBC patients regardless of prior line(s) of systemic therapy and/or have investigated only baseline blood samples drawn before start of therapy. Thus, the dynamic and value of CTCs before and during 1st line systemic therapy in newly diagnosed MBC patients remains largely unknown. In addition, a growing number of studies have been performed evaluating molecular characterization of CTCs but so far, morphologic characterization of these cells remains relatively unexplored.

In paper III we evaluated the prognostic value of apoptotic CTCs, CTC-clusters and WBC-CTCs in patients with newly diagnosed MBC and CTC count ≥5 before start of therapy. We found that presence of apoptotic CTCs and CTC-clusters during treatment (but not at baseline before treatment initiation) was associated with a significantly worse prognosis. We also found that at baseline, TNBC and HER2+ patients had CTC-clusters present more frequently than hormone receptor positive patients.

In paper IV we investigated if longitudinal enumeration of CTCs and CTC-clusters could improve prognostication and monitoring of patients with MBC starting 1st line systemic therapy. We showed that CTC count ≥5, and presence of CTC-clusters were prognostic for PFS and OS at BL and during the first 6 months of systemic therapy following diagnosis of MBC. Also, changes in CTC count during therapy significantly correlated to response evaluation and survival. Finally, both factors independently added value at all time points to a prognostic model based on clinicopathological variables.

The results of paper IV support the clinical validity of serial CTC and CTC-cluster detection for monitoring treatment and predicting prognosis in patients with MBC starting 1st line systemic therapy. This is in line with previous findings[155], but our study also is unique as it to our knowledge is the first study to describe the longitudinal dynamics and independent prognostic value of CTC count and CTC-clusters within a prospective cohort of newly diagnosed MBC patients starting 1st line systemic therapy. Previous studies on newly diagnosed MBC patients are few, and their main focus has been on evaluating the prognostic value of CTC count at baseline[180] or first follow-up.[177] Also, some studies have been retrospective,[171,175] or have included only patients with a certain subtype[182] or scheduled for a specific therapy.[184]

CTCs are part of the "liquid biopsy" that has gained much attention over the past few years.[239] The liquid biopsy is non-invasive and holds promise for improved cancer diagnostics, prognostics, treatment monitoring and therapy guidance.[159] However, despite highest level evidence for clinical validity of CTC count in MBC, no study has thus far provided evidence for its clinical utility which has hindered the clinical use.[201] SWOG 0500, the first clinical trial evaluating the clinical utility of CTC count in MBC was published in 2014, and showed negative results.[179] In this trial, patients with persistent high CTC count after one cycle of chemotherapy were switched from one cytotoxic to another at the discretion of the treating physician. There has been critique against this study design since persistent CTCs during chemotherapy could be a sign of chemoresistance, and thus simply switching to another chemotherapeutic agent would not affect outcome in that case.[201] However, several other studies with different designs are still ongoing (Table 2).

Some findings differ between study III and IV although they are based on the same patient cohort, but not the same selection of patients. In paper III, no prognostic effect was seen for CTC-clusters at baseline. This was not true for paper IV, where CTC-clusters were associated to both worse PFS and OS at baseline. Furthermore, in paper III, TNBC and HER2+ patients were found to have CTC-clusters present in their blood significantly more frequently than hormone receptor positive patients, which was not seen in paper IV. These diverging findings could be explained by differences in study design. Paper III was an exploratory pilot study comprising only a subset of the patients included in final cohort, and it was conducted before the finalization of the

main study, described in paper IV. Only patients with ≥5 CTCs at baseline were included in paper III which gave a different population of MBC within the reference group for the CTC-cluster negative patients. Moreover, we changed the definition of a CTC-cluster from containing ≥3 CTCs in paper III to ≥2 CTCs in paper IV. Reasons for changing our definition was compiling evidence that two-cell clusters are indeed clusters and not single CTCs dividing in the circulation, and that 2-cell CTC-clusters are important for prognosis in MBC.[180,181,202] We have however also collected data on 3-cell clusters within the entire cohort, and we have performed preliminary exploratory analyses using this definition but the results are not presented in this thesis.

Previous reports on the prognostic value of apoptotic CTCs and CTC-clusters in MBC has been conflicting. This could probably in part be explained by the same factors that affected the results of our two studies, i.e. which patients were included, how the reference groups were appointed, power of the study, and which definitions were used for apoptotic CTCs and CTC-clusters. Paoletti *et al.* showed that presence of CTC-clusters (≥3 CTCs) in patients with TNBC at day 15 and 29 during systemic treatment were associated to worse PFS, while no difference in survival was seen at baseline. In the same study, no association was seen between apoptotic CTCs and survival at any time point.[182] In contrast, Mu *et al.* showed that CTC-cluster (≥2 CTCs) presence in stage III and IV breast cancer patients indicated worse PFS before start of 1st line treatment.[180] These results were also supported by a recent study by Wang *et al.* who showed that presence of CTC-clusters (≥2 CTCs) added prognostic value to CTC enumeration alone in MBC patients before start of a new line on treatment, and at first follow-up.[181]

Two reviews have recently been published on the importance of CTC-clusters in cancer progression and metastasis. Both reviews acknowledge the significance of CTC-clusters in metastasis development and their potential in prognostication and disease monitoring but warrant further studies to elucidate the clinical potential of CTC-cluster detection. Important issues remaining are e.g. to agree on a standard definition of a CTC-cluster and also to validate the techniques for capturing clusters, as most techniques used today were developed for isolation of single CTCs.[203,240]

We used the CellSearch system for enrichment and morphologic characterization of CTCs and CTC-clusters. This system is limited by the use of EpCAM for extraction of CTCs. EpCAM is a surface protein on epithelial cells (such as carcinoma cells) known to be downregulated during EMT and concerns have been raised regarding the sensitivity of the CellSearch system, and the risk of missing a potentially highly malignant subpopulation of more mesenchymal like CTCs lacking EpCAM-expression.[241,242] Also, the sensitivity of cluster detection by the CellSearch system (and any other technique using a label based enrichment system) has been questioned. CTC-clusters have a small surface area to volume ratio which is believed to decrease the efficacy of antibody capture methods.[203] However, so far, CellSearch is the most

validated system for CTC enumeration and the prognostic value of CTC detection by this system has been proven in many epithelial cancers.[201] We performed the morphologic evaluation of CTCs on galleries exported from the CellTracks Analyzer II, without further staining of the CTCs. This can be considered both a strength and a limitation. A previous study in patients with small-cell lung cancer has supported the feasibility of morphologic characterization of CTCs following isolation and detection using the CellSearch system. In this study, the authors performed blood-spiking experiments using a cluster prone cell line to prove that CTC-clusters found in the CellTracks Analyzer galleries are not artifacts. Furthermore, visual morphologic characteristics of apoptosis in CTCs in CellTracks Analyzer galleries was verified using an antibody to caspase-cleaved cytokeratin to prove the feasibility of visual evaluation of apoptosis.[194]

The overall aim of this thesis was to evaluate new potential prognostic biomarkers in breast cancer, and with special focus on TNBC.

Paper I and II have presented support for the involvement of cKIT, VEGFR2, PDGFRα and PDGF-CC in TNBC. In conclusion, these receptors are not prognostic markers in TNBC, but they are upregulated in this breast cancer subtype and further studies are encouraged to elucidate their values as predictive markers and possible drug targets in TNBC. Ligand PDGF-CC was also highly expressed in TNBC. In addition, it was a prognostic marker for early breast cancer relapse in the whole cohort of patients, but little is still known about its role in breast cancer.

Paper III and IV showed the clinical value of CTC count and CTC-cluster detection before and during 1st line systemic therapy in newly diagnosed MBC patients. Our results highlight the importance of serial monitoring of these variables as the prognostic value of both CTC count and CTC-cluster detection increased over time.

Conclusions

TNBC patients had the worst prognosis of all breast cancer subtypes in the two modern cohorts investigated in this thesis, reflecting the urgent need for better treatment options for these patients. Papers I and II propose new potential drug targets and biomarkers in TNBC. Papers III and IV show the prognostic value of CTC enumeration and morphologic characterization of CTCs before and during treatment of MBC, not specified by breast cancer subtype.

Paper I

High tumor cell protein expression, but not elevated gene copy number, of cKIT, VEGFR2 and PDGFRα is associated to TNBC

Expression of cKIT, VEGFR2 or PDGFRα does not correlate to survival in TNBC, and these are thus not prognostic biomarkers in this subgroup of patients

A remarkable high expression of at least one, and at least two, of the three investigated RTKs was seen in TNBC compared to non-TNBC

In summary our results support the involvement of these receptors in TNBC and suggest that they are possible candidate biomarkers for targeted therapy

Paper II

High expression of PDGFRα, PDGFRβ and ligand PDGF-CC is significantly associated to several prognostic patient and tumor characteristics that indicate tumor inherent biological aggressiveness

High tumor cell expression of PDGF-CC is associated to TNBC, and it also increase the risk of 5-year distant recurrence in breast cancer

PDGFRα is commonly up-regulated in lymph node metastases and asynchronous recurrences

In summary, our findings support an active role of the PDGF signaling pathway in tumor progression and suggest that strategies to target this pathway could be beneficial since evidence is compiling for its involvement in breast cancer progression

Paper III

Before start of treatment, TNBC and HER2+ patients have CTC-clusters present in the blood more frequently than HR+ patients

MBC patients with apoptotic CTCs and CTC-clusters present during treatment have a significantly worse prognosis

The impact of WBC-CTC clusters on survival is unclear. Our results indicate a possible association to inferior survival at 6 months

In summary, our results support that morphologic characterization of CTCs and CTC-clusters present in the blood during treatment may be an important prognostic marker, in addition to CTC count

Paper IV

CTC count ≥5, and presence of CTC-clusters are prognostic for PFS and OS at BL and during the first 6 months of systemic therapy following diagnosis of MBC

Changes in CTC count during therapy significantly correlates to response evaluation and survival

CTC count and CTC-cluster presence independently improve the survival prognostication (PFS and OS) of a clinicopathological model at all time points

In summary, our data support the clinical value of longitudinal CTC and CTC-cluster evaluation for prognostication and treatment monitoring in patients with newly diagnosed MBC starting 1st line systemic therapy. Moreover, the prognostic value of CTC count and CTC-cluster evaluation increases over time, and thus suggests that dynamic changes of CTCs and CTC-clusters are more clinically relevant than baseline evaluation only

Future perspectives

Many advances have been made in the treatment of breast cancer over the last decades and the survival for patients with both primary and metastatic breast cancer has increased considerably. However, TNBC remains a challenge to treat and despite strong efforts, no targeted therapy has been discovered for this patient subgroup. Chemotherapy is currently the cornerstone in the treatment of TNBC.

Targeting TNBC

In paper I and II we found that cKIT, VEGFR2, PDGFRα and PDGF-CC showed higher expression in TNBC compared to non-TNBC, and proposed them as potential drug targets in TNBC. However, previous attempts to target these pathways have not been clinically successful. Possible explanations for this is the selection of patients and the drugs used in these studies. It would be interesting to e.g. retrospectively stain tumors from patients treated with sunitinib or sorafenib for the markers above and to correlate protein expression to patient response to treatment. Also, in any future trial with TKIs targeting cKIT/VEGFRs/PDGFRs, it would be interesting to design the study to stratify patients to different treatment groups based on staining results of these proteins. Few studies have so far been performed to evaluate the use of these targeted therapies in patients with primary breast cancer, and the value of the treatment in this setting might be different from the metastatic setting. Today, TNBC patients only receive chemotherapy as adjuvant therapy. An interesting study design would be to investigate the added value of a combination treatment including chemotherapy plus a TKI in the adjuvant setting. Ideally, the selected patients would be TNBC positive for at least one of the three RTKs evaluated in paper I. This would translate into approximately 75% of TNBC, and thus about 5% of all patients with primary breast cancer. Power would be a challenge but not impossible. As a final comment, the drugs currently existing to target these tyrosine kinase signaling pathways might be inefficient in breast cancer and/or these pathways may not be as important for tumor progression in breast tumors. There is also crosstalk recognized between different tyrosine kinase signaling pathways and perhaps wider targeting is needed. This would however increase the risk of toxicities.

In summary, identification of new biomarkers for prognosis and treatment prediction in TNBC is challenging due to the large intertumor heterogeneity within this group. Furthermore, many new promising drug targets occur at a very low frequency. Since TNBC already is a small subgroup of breast cancer, dividing it into even smaller subgroups would render it difficult to power clinical trials. Still, the TNBC is in urgent

need for targeted treatment that could improve the prognosis for patients with this aggressive type of breast cancer. The upregulation of RTKs as discussed in this thesis suggests an activation of these pathways primarily within TNBC tumors. Better therapeutics in combination with marker directed selection of patients might provide a future opportunity for targeted therapy also in TNBC.

Liquid biopsy and CTCs in MBC

Tumors display both spatial and temporal heterogeneity. A liquid biopsy is a promising tool to capture and follow the evolution of this heterogeneity. It contains the potential to help monitor treatment response and to guide clinicians to the right choice of therapy. In this thesis, we showed the prognostic value of longitudinal CTC and CTC-cluster evaluation in newly diagnosed MBC. Despite clinical validity and strong evidence for the potential of CTCs to monitor disease progression in breast cancer, it has not been incorporated into clinical practice. This is largely due to the lack of clinical utility, i.e. the ability to improve patient outcome with this test. A challenge in proving clinical utility is to find an effective treatment for the patients with persistent high CTC count. I expect that CTCs and the liquid biopsy will eventually enter the clinic, but first we might need additional treatment options, and/or better tests to distinguish which patients will respond to a given therapy. It will probably be important to further characterize the CTCs, e.g. with DNA, RNA and/or protein analysis, and to incorporate this information in addition to CTC count to design a successful CTC trial. In the future, CTCs might also be used *in vitro* in cell cultures, or in xenograft mouse models, to test drug sensitivity. It is known that protein expression of e.g. hormone receptors and HER2 can differ between the primary tumor, CTCs and metastases. The DETECT III trial is a study currently ongoing that investigates the effectiveness of HER2 targeted therapy in patients with HER2 positive CTCs but HER2 negative primary tumor and metastasis. The results of this study will be highly interesting. Ongoing research is also trying to identify additional drug targets on CTCs, which could be e.g. proteins or DNA mutations. I think this research will lead to many future exciting trials and hopefully a major improvement in patient survival based on information from liquid biopsies.

Finally, in paper IV we showed the importance of longitudinal CTC evaluation during treatment and future CTC studies should take this information into account and plan for repeated CTC enumeration. The optimal frequency of blood samples for CTC evaluation, and also the duration of monitoring patients for CTCs is currently unknown and should be evaluated in future clinical trials. An extension of study IV is indeed ongoing, where selected MBC patients are followed after the initial study period of 6 months with additional CTC evaluation approximately every 6 months lifelong. Lastly, exploratory results from study IV question the optimal prognostic cut off for CTCs in newly diagnosed MBC and future studies should be conducted to clarify this question.

Populärvetenskaplig sammanfattning

Bröstcancer drabbar ca 8000 personer i Sverige varje år och det är den vanligaste cancersjukdomen hos kvinnor i världen. 5-årsöverlevnaden har stigit de senaste decennierna tack vare bättre diagnostik och behandling, och ligger idag på ca 90 % hos kvinnor med primär bröstcancer. Det är dock inte alla som kan botas utan en allt större andel lever med metastatisk, även kallad spridd eller kronisk, bröstcancer som hålls under kontroll med mediciner. För kvinnor med metastatisk bröstcancer är 5-årsöverlevnaden endast ca 27%.

Bröstcancer är en heterogen sjukdom. Det betyder att olika brösttumörer kan vara väldigt olika varandra i sin biologi och prognos. Detta är viktigt att känna till när man skall behandla patienter med bröstcancer i kliniken då vissa typer av tumörer svarar bra på en sorts av behandling, men kanske inte alls på en annan. Man har därför tagit fram olika system för att dela in bröstcancer i subgrupper som skall vara mer lika varandra. Det vanligaste indelningssystemet kallas för St Gallen, och kortfattat går det ut på att man färgar primärtumören för hormonreceptorer (östrogen och progesteron) samt för HER2-receptorn och för Ki67, ett protein som indikerar celltillväxt. Men denna information kan man sedan dela in alla brösttumörer i fem olika subtyper. Antalet subtyper och deras exakta definitioner har varierat något genom åren. En subtyp som fått extra uppmärksamhet är trippelnegativ bröstcancer (TNBC). TNBC kännetecknas av att den inte uttrycker några hormonreceptorer eller HER2. Den utgör ca 7-14% av all bröstcancer och drabbar oftare yngre personer samt har en dålig prognos. För de övriga fyra bröstcancersubtyperna finns idag målriktad läkemedelsbehandling som ofta är mycket framgångsrik, men man har tyvärr ännu inte hittat någon sådan behandling för TNBC.

Biomarkörer är faktorer som går att mäta på ett säkert och upprepningsbart sätt i en person, och som säger något om den personens medicinska tillstånd. Inom onkologi kan det vara exempelvis proteiner i en tumör som är kopplade till prognos och förväntat svar på behandling (prediktion). Ett exempel i bröstcancer är östrogenreceptorn. Högt uttryck av östrogenreceptorer är kopplad till bra prognos och indikerar att tumören är känslig för antihormonell behandling.

Syftet med min avhandling är att undersöka nya möjliga biomarkörer i bröstcancer, framför allt TNBC subgruppen, som skulle kunna kopplas till prognos.

Arbete I och II

Arbete I och II handlar om tyrosinkinasreceptorer (RTKs). Detta är proteinreceptorer som sitter på cellytan på exempelvis cancerceller och som är viktiga för cellernas överlevnad, tillväxt och delning. Vi har valt att titta på RTKs bland annat då tidigare studier visat att de är inblandade i cancer, och för att det finns tillgängliga mediciner som är riktade mot dessa.

I arbete I undersökte vi tre olika RTKs; cKIT, VEGFR2 och PDGFRα. Vi fann ett högt uttryck av cKIT och VEGFR2 i TNBC. Uttrycket av PDGFRα var på gränsen till förhöjt. Totalt hade 74% av TNBC ett högt uttryck av minst en av de tre RTKs jämfört med 30% av icke-TNBC. Vi tittade även på antalet genkopior av de gener som kodar för de tre RTKs vi undersökte. Vi hittade ett förhöjt antal genkopior av respektive RTK i ca 12% av alla brösttumörer, oavsett subtyp. Slutligen undersökte vi om det fanns någon koppling till överlevnad men varken proteinuttryck eller avvikande antal genkopior av RTKs var kopplade till prognos i TNBC.

I arbete II tittade vi vidare på PDGFRα, och lade till två andra markörer som tillhör samma familj; PDGFRβ (också en receptor) och PDGF-CC (en ligand, d.v.s. ett protein som aktiverar en receptor). Vi fann att ett högt uttryck av alla dessa tre proteiner var kopplat till flertalet patient- och tumörkaraktäristika som indikerar tumöraggressivitet. Vidare såg vi att simultant högt uttryck av både PDGFRα och dess ligand PDGF-CC var vanligare i TNBC jämfört med hormonreceptorpositiv bröstcancer (59% vs 5-19%).

Sammanfattningsvis så ger resultaten från arbete I och II stöd för att cKIT, VEGFR2, PDGFRα och PDGF-CC är inblandade i TNBC och att de skulle kunna fungera som mål för målriktad läkemedelsbehandling. Det har gjorts en del studier i bröstcancer de senaste åren där man provat behandla med målriktade mediciner mot dessa RTKs. Tyvärr har resultaten varit mestadels negativa och inte visat någon bättre överlevnad, men däremot en ökad mängd biverkningar.

Arbete III och IV

Arbete III och IV handlar om cirkulerande tumörceller (CTCs) i metastatisk bröstcancer.

Sedan ca 15 år kan man på ett mer standardiserat sätt utvinna tumörceller, så kallade CTCs, från blodet hos bröstcancerpatienter. Den vanligaste metoden för att isolera och detektera CTCs i ett blodprov kallas för CellSearch. Den går ut på att fästa magnetiska antikroppar på tumörcellernas yta och sedan dra ut dem från blodprovet med en magnet. Genom att räkna antalet CTC har man kunnat konstatera att 5 CTCs eller fler innebär dålig prognos i metastatisk bröstcancer. Med mikroskop kan man dock titta närmare på cellernas utseende, vilket vår forskargrupp har gjort, för att se om där finns värdefull information för prognos och behandlingsval. Vi valde att undersöka

betydelsen av bl.a. tumörcellskluster (definierat som antingen minst 2 eller minst 3 CTCs som sitter ihop) och apoptos (celldöd). Detta har tidigare gjorts för lung- och prostatacancer men är relativt outforskat inom bröstcancer.

I arbete III undersökte vi blodprover från 52 olika kvinnor med spridd bröstcancer som hade minst 5 CTCs vid provtagning före behandling. Upprepade prover togs för flertalet av patienterna efter ca 3 månaders samt efter ca 6 månaders behandling. Alla patienter fick standardbehandling för metastatisk bröstcancer under studietiden. Vi fann att de patienter som hade tumörcellskluster eller apoptotiska CTCs i blodet i uppföljningsprover under behandling hade en mycket dålig prognos. Dessa resultat stämmer väl överens med resultat från studier kring lung- och prostatacancer. Man har kunnat visa i studier på djurmodeller att tumörcellskluster är små tumörgrupper som har betydligt högre sannolikhet att ge upphov till metastaser än ensamma celler. När det gäller apoptos visar de flesta studier att en hög andel apoptotiska CTCs indikerar en dålig prognos. Detta är tvärtemot vad man kan tro, då de flesta tänker att celldöd borde vara ett gott tecken. Det finns dock teorier om att en hög andel celler med apoptos kan bero på aggressivare tumörceller med snabb tillväxthastighet. Då vi tittade på prognosen för våra patienter såg vi att de vars behandling lyckats få bort alla tumörcellskluster hade en bättre prognos än de med kvarvarande kluster vid flera mätpunkter.

I arbete IV undersökte vi den prognostiska betydelsen av antal CTC och närvaro av tumörcellskluster före behandlingsstart, samt vid upprepade tillfällen under behandling i kvinnor som nyligen diagnosticerats med metastatisk bröstcancer. I detta arbete fann vi att antal CTC och även tumörcellskluster kunde förutse prognosen för patienterna både före behandlingsstart samt under behandling. Vi skapade även en klinisk prognosmodell som tog hänsyn till flera av de vanligaste kända prognosfaktorerna i metastatisk bröstcancer och kunde visa att CTC antal och tumörcellskluster kunde förbättra denna modell ytterligare. Slutligen visade vi även att ändringar i CTC antal under behandling var kopplat till prognos, och det gick bättre för de patienter som snabbt blev av med sina CTCs efter start av behandling. CTC antal kunde även förutspå resultatet av röntgenutvärdering vid läkaråterbesök under behandling.

Sammanfattningsvis så hoppas vi att man i framtiden ska kunna förbättra överlevnaden hos bröstcancerpatienter genom att mäta och karaktärisera CTCs, både före behandlingsstart och sedan regelbundet under behandling. Flera kliniska studier pågår just nu runt om i världen där patienter med spridd bröstcancer får lämna blodprov regelbundet under sin behandling för mätning av CTCs. Deras behandling ändras sedan beroende på vad proverna visar. Kanske är detta ett steg i riktning mot en mer skräddarsydd cancerbehandling där man snabbt och enkelt med ett vanligt blodprov kan utvärdera behandlingseffekten och byta terapi i tid.

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