General discussion and future perspectives
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In this thesis, biomarkers are presented, that have the potential to improve treatment selection for patients with HER2-negative advanced breast cancer, as described in part one of this thesis. In part two of this thesis, a discovery strategy encompassing genetically engineered mouse tumor models and mass spectrometry is employed to identify putative protein biomarkers, which may have clinical utility in early detection of breast cancer.

Part I – Main findings and general discussion

First-line treatment for advanced breast cancer

In Chapter 2, we described the multicentre, open-label, randomized phase II ATX trial evaluating paclitaxel and bevacizumab (AT) or paclitaxel, bevacizumab and capecitabine (ATX) as first-line therapy in women with HER2-negative locally recurrent or metastatic breast cancer. Safety analysis showed an increase rate of grade 3–4 adverse events, e.g. hand-foot syndrome and neutropenia, as anticipated from the addition of capecitabine, but these generally did not affect the continuation of treatment. No unexpected adverse events were observed with the addition of capecitabine to AT. A significant improvement in progression-free survival, overall response rate and response duration was demonstrated for the treatment with ATX compared with AT. This trial showed that the addition of capecitabine to paclitaxel and bevacizumab is active and tolerable as a first-line regimen for HER2-negative metastatic breast cancer. This combination may be given to patients, who require rapid disease reduction. Despite improved progression-free survival and response rate, overall survival was similar in both treatment arms. The similarity in overall survival may be explained by the small sample size, the possibility of crossover to capecitabine therapy after progression on first-line paclitaxel and bevacizumab and the uncertain relationship between progression-free survival and overall survival. To address the potential impact of treatment crossover, we evaluated the outcome of AT-treated patients that received capecitabine as a later line. We demonstrated that capecitabine given as second or later line following disease progression on first-line paclitaxel therapy improved overall survival and post-progression survival. Previous retrospective analyses of phase III trial data have shown in patients with anthracycline-pretreated metastatic breast cancer that capecitabine versus other cytotoxic agents sequentially given following progression on docetaxel improved overall survival. In extension of previous observations, our findings suggest that capecitabine given at any time point in the course of disease has the potential to improve survival prospects of patients with HER2-negative metastatic breast cancer, although a prospective clinical trial for confirmation is required.

A better patient selection may improve current systemic therapy of metastatic breast cancer by maximizing efficacy. In Chapter 2 on the basis of a preliminary report, we validated a prognostic score constituting four clinical risk factors for poor overall survival (disease-
free interval following primary diagnosis of ≤24 months, the presence of liver metastases or three or more metastatic sites, triple-negative breast cancer, prior (neo) adjuvant therapy with a taxane and/or an anthracycline), which can be utilized to classify patients into three prognosis groups. We confirmed that an increasing number of poor risk factors was associated with impaired overall survival. This prognostic score including an additional clinical variable (ECOG performance status 2 or prior analgesic/corticosteroid treatment) has been fully reported in a similar patient cohort in which patients with three or more risk factors were at high risk for poor survival 6. One may speculate that this prognostic score could identify high risk patients that benefit from more intensive systemic therapy, i.e. the addition of bevacizumab. However, this could not be addressed in the ATX trial lacking a control arm without bevacizumab. A prospective clinical trial with stratification by prognostic risk groups before randomization to bevacizumab and chemotherapy or chemotherapy alone is warranted.

**Circulating factors**

The addition of bevacizumab to standard chemotherapy has demonstrated promising improvement in efficacy in patients with metastatic breast cancer as reported in the E2100 trial 7,8. However, accumulating phase III trial data (AVADO 9, RIBBON-1 10) have indicated a modest progression-free survival prolongation and no improvement in overall survival. Nowadays, it is evident that a beneficial effect from bevacizumab added to chemotherapy should be reserved for selected patients, presumably those with advanced triple-negative breast cancer 11. We made an attempt to identify potential biomarkers that may indicate candidates for bevacizumab-containing therapy. In Chapter 3, we investigated whether circulating proteins related to angiogenesis (VEGF-A, soluble VEGFR2 [sVEGFR2], angiopoietin 2 [ANG2], soluble TIE2 [sTIE2], IL6, IL8) and hypoxia (carbonic anhydrase 9 [CA9]) as well as tumor marker CA15-3 and their changes after short-term systemic therapy were associated with clinical outcome. Baseline levels and their changes after one cycle of therapy for all proteins were determined in 181 patients enrolled in the ATX trial being representative for the total trial cohort. Prior to the start of treatment, patients with high baseline levels of ANG2, IL6, IL8, CA9, and CA15-3 had a higher risk of progression with a hazard ratio (HR) ranging from 1.44 to 2.54, while patients with high baseline levels of ANG2, IL6, IL8, CA9, CA15-3 and sTIE2 had a higher risk of death (HR of 1.33–4.23). The associations were adjusted for clinical prognostic factors, which means that measurement of these proteins at baseline may add independent prognostic information beyond those reflected by traditional patient and tumor characteristics. Moreover, the concurrent presence of high baseline levels of multiple proangiogenic and hypoxic factors may indicate a particularly aggressive subset of breast carcinomas. After one cycle of treatment we found significant changes in protein levels suggesting a modulating effect of bevacizumab-containing therapy. Changes in circulating factors were measured after administration of VEGF inhibitors by other investigators and decrease or increase tended to be drug-specific 12-14. Based on these findings, it may be hypothesized that treatment-induced changes in circulating factors may represent surrogate markers of pharmacodynamics or even indicate efficacy. This hypothesis is
strengthened by our observation showing that the magnitude of protein changes indicated a reduction in tumor size, in which a relative increase in sVEGFR2 and a relative decrease in IL8 levels were associated with treatment response. Moreover, patients with a high CA9 rise of >2.9% had a significantly improved progression-free survival (HR = 0.45) and overall survival (HR = 0.54) than individuals with low or no rise.

Taken together based on our findings, measurement of circulating proteins prior to the start of treatment may provide additional information on patient’s prognosis. Highly expressed proteins, *i.e.* ANG2, IL6, IL8, and CA9 are even under investigation as potential drug targets. Moreover, assessment of treatment-induced changes in protein levels after one cycle, in particular sVEGFR2 and CA9, may provide an early method to predict efficacy from bevacizumab-containing therapy in metastatic HER2-negative breast cancer patients. In this thesis we measured protein changes after one cycle of therapy in order to determine potential biomarkers useful for early prediction of efficacy from bevacizumab-containing therapy. Repeated measurement of protein levels during the disease course and at the time of disease progression may improve our understanding of molecular changes promoting failure to anti-VEGF agents.

**Genetic polymorphisms**

Treatment with cytotoxic agents, including paclitaxel and capecitabine, is associated with a wide range of side-effects frequently requiring dose reduction, delay or even early discontinuation of treatment before disease progression (*Chapter 2*). Early toxicity leading to unnecessary discontinuation should be prevented. Although patients are monitored periodically after initiating therapy, identification of patients at risk to develop serious toxicities would enable the possibility to timely reduce drug doses without compromising efficacy. Genetic polymorphisms, so called single-nucleotide polymorphisms (SNPs), may provide a better understanding of the individual response to systemic therapy and susceptibility of toxicities and may, therefore, serve as biomarkers. *Chapters 4* and *5* were dedicated to the current pharmacogenetic research studying SNPs for the prediction of toxicities related to taxanes *i.e.* paclitaxel and docetaxel (*Chapter 4*) and for the efficacy and toxicities from capecitabine (*Chapter 5*). Genetic variants analyzed in relation with taxane-induced adverse events *i.e.* peripheral neuropathy are mostly located in genes potentially influencing taxane pharmacokinetics, such as drug transporters (*ABCB1*) and metabolizing enzymes (*CYP3A4*, *CYP3A5*, for paclitaxel *CYP2C8*). More recent research focus has shifted to putative genetic variants involved in microtubule function (*TUBB2A*) or neuronal regeneration (*EPHA5*, *EPHA6*). Several SNPs in these genes have been identified displaying novel associations with taxane-induced toxicities. In pharmacogenetic studies on capecitabine-treated patients, associations were found between the occurrence of capecitabine-related adverse events and several genetic variants involved in the 5-fluorouracil (5-FU) metabolic pathway, such as thymidylate synthetase (*TYMS*), methylenetetrahydrofolate reductase (*MTHFR*) and dihydropyrimidine dehydrogenase (*DPD*). Recent pharmacogenetic research has increasingly focused on genes encoding enzymes involved in capecitabine activation.
to 5-FU i.e. carboxylesterase (CES), cytidine deaminase (CDA) and thymidine phosphorylase (TYMP), of which some were demonstrated to be associated with the occurrence of adverse events. Apart from the well-known DPYD*2A variant, additional risk alleles of DPYD, i.e. *13, -2846A>T and -1236G>A/HapB3 have now been recognized to contribute to individual susceptibility to adverse events from fluoropyrimidines. A corresponding conclusion in Chapters 4 and 5 is that the reported associations varied across pharmacogenetic studies, because of differences in patient characteristics, sample size, study endpoints and treatment duration, but also because of the combination with other cytotoxic agents. Consequently, their clinical utility needs further testing in independent populations and in prospective studies.

Genetic polymorphisms, which have previously been described to play a potential role in the development of paclitaxel-related toxicities were selected for further investigation in a cohort of 188 patients from the ATX trial. The results are presented in Chapter 6. Genotyping of SNPs of genes encoding paclitaxel-metabolizing enzymes (CYP2C8*3 [-416G>A], CYP3A4*22 [-522-191C>T]) and novel targets (TUBB2A -101T>C, FGD4 -2044-236G>A and EPHA5 -2895G>A) was performed in serum obtained from these patients. Genetic variations were examined for their association with cumulative paclitaxel dose until grade ≥1 peripheral neuropathy or until first dose reduction from relevant toxicities. When adjusted for age, body surface area and total cumulative paclitaxel dose, CYP2C8*3 was associated with cumulative dose-dependent peripheral neuropathy showing an increased risk for *3 carriers versus non-carriers (HR = 1.59). Analysis of paclitaxel dose reduction revealed, that FGD4 -2044-236 A-allele carriers received a significantly lower cumulative dose of paclitaxel until first dose reduction with an estimated HR per A-allele of 1.38. This association was adjusted for the total cumulative paclitaxel dose. In conclusion, variants in genes involved in paclitaxel metabolism or cellular targets may point towards individual susceptibility to paclitaxel-related adverse events. These findings extend the current knowledge of genetic variations as potential risk factors for paclitaxel toxicity. Our study also underscores the need for independent validation as previously suggested associations for other SNPs could not be confirmed in the present study.

Chapter 7 concerns genetic polymorphisms, which may have a role in the interindividual variation in efficacy as well as toxicity from capecitabine. One major limitation of previous pharmacogenetic studies is the lack of predictive evaluation of genetic variants. The ATX trial design, in which patients were randomly assigned to a capecitabine-containing arm and a control arm, allowed identification of predictive biomarkers for capecitabine efficacy. Genetic variants of capecitabine-activating enzymes, including CES2 -823C>G, CDA -943del/insC and CDA -451C>T, as well as CDA enzymatic activity in serum were evaluated in a cohort of 188 patients. CDA enzymatic activity can affect cytotoxicity of capecitabine and high levels may induce life-threatening toxicity.16-19 In the total cohort and adjusted for hormone-receptor status, CDA -943del/insC showed a prognostic association with better overall survival for carriers of an insC-allele (HR = 0.66). A significant improvement in pro-
gression-free survival for treatment with ATX compared with AT was measured in patients with a CDA-943insC/del or del/del genotype (HR = 0.52), whereas this effect was not seen in patients with an insC/insC genotype. Similarly, a beneficial effect on progression-free survival from ATX relative to AT was found in patients with a CT or a CC genotype of CDA-451C>T (HR = 0.52) and a CES2 CC genotype (HR = 0.53) as compared to their counterparts. Patients with a high CDA level (defined as ≥median) experienced a significantly longer progression-free survival when treated with ATX compared with AT (HR = 0.43), which was not observed in patients with a low CDA level. The interaction tests were, however, not significant, probably due to the relatively small sample size. Using the subpopulation treatment effect pattern plot to explore a possible interaction between treatment and continuous CDA levels, increasing CDA levels seemed to be associated with increasing overall survival benefit from ATX relative to AT ($p_{interaction} = 0.013$). Finally, a significant interaction between treatment and CES2-823C>G was noted ($p_{interaction} = 0.004$), in which patients with a CG genotype seemed to derive more survival benefit from ATX (HR = 0.54) than CC wild-type carriers (HR = 1.09). None of the factors were predictive for capecitabine-related adverse events. Further studies are warranted to confirm the use of genetic variants in CES2 and CDA or CDA enzymatic activity for selecting patients with metastatic breast cancer, who are optimal candidates for capecitabine-based therapy.

**Part I – Future directions**

Part I of this thesis is dedicated to a well-characterized cohort of patients with advanced HER2-negative breast cancer that were candidates for first-line chemotherapy. Blood samples were taken to identify biomarkers that may be relevant for prognosis or may be predictive for efficacy or toxicity from treatment. Current research is focused on the clinical usefulness of biological markers, that are easily available and can be taken repeatedly.

To date, the optimal systemic therapy for individual women with an advanced stage of disease remains elusive, partly because of the absence of screening factors for patient selection. The need for clinically useful markers is emphasized because of the growing availability of effective anticancer agents as well as the accumulating knowledge of breast cancer subtypes making the clinical management of individual patients increasingly complex and complicated. Traditional tumor markers, including hormone receptors and HER2, have helped oncologists to determine patient’s prognosis and appropriate treatment. However, these three markers do not fully capture the heterogeneous clinical course of progressive disease, which is characterized by molecular changes in tumor cells due to unstable DNA and are aggravated by previous treatments.

There are several ways to refine the current assessment of patient’s prognosis, which might also be helpful in directing optimal treatment. As exemplified in this thesis in patients with advanced HER2-negative breast cancer that received bevacizumab-based the-
rapy, a prognostic score model derived from easily accessible clinical features may provide clinicians an objective, standardized classification system. The major advantage of a clinical prognostic score is an easy implementation in the current patient’s care. For patients with primary breast cancer, several prognostic tools are already available to decide on adjuvant systemic therapy for improvement of survival. For patients with advanced breast cancer, the question remains how to improve outcome once patients with poor prognosis have been identified.

Besides clinical characteristics, tumor tissue will undeniably provide the most in-depth information about its molecular characteristics, which will also be an important determinant for the selection of therapy. Biopsy of a first metastatic lesion is currently advocated for histological confirmation and tumor marker expression, because changes have been reported in comparison with the primary breast tumor. Serial biopsies of tumor tissue during treatment have demonstrated to improve our understanding of tumor heterogeneity and evolution during treatment and indicate molecular mechanisms underlying treatment failure. However, serial biopsies from breast cancer lesions are often not feasible (small size and deep-seated location) and there may be a risk for sampling errors. Moreover, patients have to be exposed to repeated invasive tests.

In addition to tumor tissue, circulating factors may provide similar information with respect to the nature of cancer, course of disease, and prognosis and might be of help in the selection of treatment. The use of circulating factors may be preferred given the ease of repeated sampling and the possibility of non-invasive assessment. A panel of multiple markers is generally considered to increase the accuracy of prognosis estimation compared to a single marker. Future studies should determine which circulating factors or combinations are clinically relevant for HER2-negative advanced breast cancer. Circulating factors may also be useful for treatment guidance of anti-VEGF agents as suggested by our findings and other retrospective biomarker analyses. Several randomized trials, i.e. the MERIDIAN trial (NCT01663727) in HER2-negative advanced breast cancer and the Triple-B trial (NCT01898117) in triple-negative advanced breast cancer are in progress to address whether circulating angiogenic factors, such as VEGF-A and sVEGFR2, could predict benefit from bevacizumab added to first-line chemotherapy. Results from these trials will hopefully provide a definitive answer to the role of bevacizumab in advanced breast cancer and the feasibility of using markers to enrich patients deriving the best clinical benefit from bevacizumab-containing therapy. Changes in circulating factors monitored early during treatment may also be a tool for noninvasive prediction of treatment response.

Pharmacogenetic analyses have identified predictive genetic markers of toxicities as well as treatment efficacy. Future studies are needed to confirm these association and to address their accuracy. Prospective genotype-directed trials are emerging to examine the use of genetic markers for treatment selection or initial drug dosing. With the advances in sequencing technologies, major progress has been made in reducing costs and analysis
time. Therefore, genetic testing is expected to be readily available for clinical use in the near future. The increasing availability of this technology will unravel more putative genetic variants displaying an association with toxicity or modulating drug pharmacokinetics. Reliable tests for functional assessment of novel variants are emerging. Alternative to genetic analysis, phenotypic classification of enzyme activity levels pivotal for drug metabolism or detoxification processes represents a promising surrogate marker for toxicity.

In the ATX population, we assessed circulating proteins related to angiogenesis and hypoxia as well as single nucleotide polymorphisms related to treatment with paclitaxel and capecitabine. Many other factors, such as miRNA, circulating tumoral DNA or circulating tumor cells can be used for the purpose of prognostic stratification or treatment selection in breast cancer populations. For example, measurement of circulating tumoral DNA may reveal tumor-specific characteristics, such as somatic mutations in PIK3CA, ESR1 and gene amplification in HER2 relevant for the selection of (targeted) therapy as well as therapy resistance. An increase in circulating tumoral DNA levels during treatment has been reported to reflect tumor progression. Similarly, quantitative testing of circulating tumor cells is making its way into breast cancer care and several clinical applications have been evaluated. For example, the presence of circulating tumor cells may predict metastatic spread in patients with early stage breast cancer. Moreover, ER-negative circulating tumor cells may indicate resistance to endocrine therapy in a previously ER-positive breast tumor.

For noninvasive assessment of tumor characteristics and changes during the course of treatment positron emission tomography (PET) imaging using novel tracers is also a promising approach. PET provides a technology for serial images to monitor in vivo protein expression in all tumor lesions. Several novel PET tracers displaying biological processes in breast cancer, such as ER or HER2 expression, tumor proliferation, DNA repair and angiogenesis are currently under investigation. For each tracer, important issues i.e. tumor uptake, biodistribution, sensitivity and specificity need to be addressed before entering clinical practice. As a disadvantage, serial PET imaging needs experienced personnel and comes at high costs. PET imaging with novel tracers is not expected to become readily available in the near future.

Part II – Main findings and general discussion

Proteomics for discovery of breast cancer biomarker

Breast cancer is traditionally considered a heterogeneous disease characterized by a considerable variation in disease course and treatment response. Over the last decade through gene expression studies, several breast cancer subgroups have been identified, each with distinct clinical course, histopathology, adjuvant systemic treatment response,
risk of disease relapse, metastatic pattern and molecular aberrant pathways (Chapter 8). These so-called intrinsic subtypes may aid in the optimal treatment selection. The original molecular classification of breast cancer is based on gene expression, while it has been shown that immunochemical testing of markers including estrogen receptor (ER), progesterone receptor (PgR) and HER2 can roughly approximate the intrinsic subtypes.30,31 Proteomics is a promising research field that has the aim of qualitative and quantitative profiling of proteins present in a biological system. With the use of mass spectrometry, protein expression profiles can accurately be determined providing novel insights into functional biological processes. Disease-specific biomarkers can also be identified. Protein markers are especially interesting because these may be readily implemented in routine clinical workflow and represent novel candidates for targeted therapy. In Chapter 8, an overview of current knowledge of proteomic research covering different clinical aspects of breast cancer subtypes is provided. It was discussed how proteomics technology can be employed for the discovery of subtype-specific proteins. Global phosphoproteomic analysis may provide new information on molecular processes contributing to drug resistance. Novel drug targets can also be identified. The quality of generated data is influenced by the use of sensitive, reproducible methods for protein quantification. This is in particular important for comprehensive profiling of protein expression in complex biological material, such as tumor tissue. The inclusion of a sufficient sample size and validation of preliminary preclinical findings in relevant patient cohorts are important factors to consider in view of their clinical relevance. The recent reports of comprehensive proteomic portraits of human breast cancer subtypes provide further insight into functional differences delineating specific breast cancer subtypes.32-34 In conclusion, proteomic technology is a promising research tool for the identification of putative protein markers, that have the potential to improve breast cancer care through a better diagnosis, prognosis or prediction of treatment response and/or resistance.

Secretome as an extracellular compartment containing secretory proteins from cells has been introduced as a source for discovery of disease-specific, noninvasive biomarkers. The main advantages of secretome is the relative enrichment of secreted proteins and the ease to harvest large sample amounts from cancer cell lines, the possibility to analyze the effect of different conditions, such as gene knockdown or drug response. An overview of recent proteomic studies aimed at the identification of secreted proteins useful as putative noninvasive biomarkers of breast cancer is provided in Chapter 9. Proteomic research on breast cancer has mostly been focused on identification of protein markers potentially useful in directing diagnosis and monitoring treatment response. Furthermore, secretome analysis may point towards molecular processes altered by gene mutations or the influence of the tumor microenvironment to improve our understanding of tumor progression. In Chapter 10, we illustrate the use of proteomics combined with breast cancer secretome harvested from genetically engineered mouse models (GEMMs) as a valid strategy for clinical biomarker discovery. These GEMMs developed breast tumors resembling human hereditary BRCA1-deficient breast carcinoma and BRCA1-proficient sporadic breast carcinoma. Cell line
secretome was harvested and analyzed by mass-spectrometry. A total of 2,107 proteins were identified. Distinct protein profiles were found between BRCA1-deficient and BRCA1-proficient groups suggesting that the proteomic portrait of secretome may be influenced by the BRCA1 gene knockdown status. A total of 215 proteins were highly released by the BRCA1-deficient cell line and about 75% of these proteins were detectable in human plasma. These 215 BRCA1-related proteins were subsequently mapped to a previously published mRNA dataset containing human breast carcinomas. This analysis showed that BRCA1-related proteins could cluster human BRCA1- and BRCA2-related breast carcinomas from sporadic breast carcinomas. Another interesting finding was the presence of multiple proteins of intracellular origin in secretome, that are not believed to be released from cells, suggesting that another nonconventional secretory pathway may be responsible for their release. Recent publications reported extracellular vesicles, most notably those of endosomal origin, the exosomes, as carriers of a variety of molecular factors reflecting the cells of origin. Consequently, an exploratory analysis of BRCA1-deficient and -proficient microvesicles was performed for the protein contents by mass spectrometry. We showed a considerable overlap of protein contents between secretome and extracellular vesicles/exosomes and that proteins upregulated in BRCA1-deficient microvesicles recapitulate the biology of BRCA1 deficiency, such as DNA replication, RNA degradation and RNA splicing. Lastly, given the quantitative data and literature reporting a relationship with BRCA1, two candidate-proteins, i.e. TOP1 and CDH3, were validated in a large panel of breast carcinomas from female carriers of a BRCA1- or BRCA2-mutation or from women without hereditary predisposition. These analyses confirmed the clinical relevance of our findings and underscore the secretome proteomics as a valid strategy to identify putative biomarkers related to breast cancer. These biomarkers might be useful for non-invasive breast cancer detection. A potential drawback of our study is the lack of validation in human biological fluids due to the limited availability of samples from relevant patient cohorts and good quality of antibodies for detection of novel candidate proteins. These proteins, such as TOP1 and CDH3, should be evaluated for their presence in biofluids from mutation carriers in future studies in order to confirm their potential as non-invasive biomarkers associated with BRCA1-deficiency.

Part II – Future directions

For breast cancer, proteomics offers several opportunities for further insight into breast cancer biology and novel candidate biomarkers as extensively reviewed in Chapters 8 and 9. Proteomic technology, in general, has generated a tremendous wealth of data for discovery of protein biomarkers that have the potential to alter clinical management. Careful selection of biomarkers for subsequent validation is mandatory as the first step to enrich promising candidates. In view of secreted proteins, potential candidate biomarkers are preferably be validated in biological fluids, i.e. plasma or serum obtained from representa-
tive patient cohorts with detailed clinical information. Currently, validation relies on sensitive and reliable antibody-based assays, thereby limiting the selection of putative protein markers for the validation phase. In the future, targeted-mass spectrometry may hold the promise as a novel technology with high degree of flexibility in assay design and ability to acquire quantitative protein information about complex biological samples with high sensitivity and throughput capacity.
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