General introduction and scope of the thesis
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Breast cancer epidemiology
Breast cancer is the leading cancer diagnosis in women worldwide. Approximately 1.7 million global cases were diagnosed in 2012, over 464,000 cases in Europe in 2012 and over 14,500 cases in the Netherlands in 2015. This translates into a cumulative risk of breast cancer to age 90 years of about 12–13%. In other words, roughly one out of eight women will be affected. The incidence of breast cancer has increased for the 1980–2002 interval, most likely due to changes in reproductive factors (e.g. menarcheal age, interval from menarche to first pregnancy, parity, number of children, oral contraceptives, etc), the prescription of menopausal hormone replacement therapy (HRT) and the implementation of preventive breast cancer screening. Following a decline in 2002–2003 because of the reduction in the use of HRT, breast cancer incidence has remained relatively stable afterwards in western countries. In contrast, the global cases of breast cancer diagnosed in women will continue to rise to 2.4 million per year in 2030, an increase of 41% from the present levels, and this increase is believed to be attributed to aging population and a growing number of breast cancer cases in women from developing countries. Breast cancer accounts for more than 500,000 cancer-related deaths annually worldwide being the most common cause of cancer death in women. The breast cancer death, primarily in western countries, has been decreasing since 1990 despite the increasing global incidence of breast cancer. This decrease in mortality may be attributed to the early detection of breast cancer at potentially curable stage and an improvement in treatment modalities.

Treatment for advanced breast cancer
Current management of early-stage breast cancer encompasses surgery, radiotherapy and a variety of (neo)adjuvant systemic treatment options, such as endocrine therapy, cytotoxic agents and molecular agents targeting aberrant pathways. Patients usually receive various treatment modalities depending on stage of disease and breast cancer subtype. The clinical outcome of patients with breast cancer after curative therapy has improved dramatically over the last decades. However, about 20% of patients with early-stage breast cancer will develop distant relapse, although the recurrent rates differ among subtypes of breast cancer. Another 5–10% present with stage IV breast cancer at the time of diagnosis. The choice of first-line treatment for patients with locally recurrent or metastatic breast cancer is dependent on patient’s general health, tumor characteristics, the site of recurrence, prior received therapy and patient’s preferences. Treatment generally consists of systemic therapy, including endocrine therapy, cytotoxic agents and targeted therapy, while salvage surgery and/or palliative radiotherapy may be given in case of local recurrence. Decisions on the initiation and selection of palliative systemic therapy are currently based on testing of estrogen receptor (ER), progesterone receptor (PgR) and HER2 status, further supported by extent of disease, comorbidities and choice of the patient. Most
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Patients cannot be cured, because of which symptom palliation and prolongation of life with maintenance of its quality are major aims. A flowchart for treatment of patients with metastatic breast cancer is shown in Figure 1.

**Figure 1. A flowchart for treatment of patients with metastatic breast cancer (adapted from Boven 2014 39).**

Patients with hormone receptor-positive metastatic breast cancer with slow progression or low tumor burden are eligible for endocrine therapy: a non-steroidal aromatase inhibitor *i.e.* anastrozol and letrozol, and or a steroidal aromatase inhibitor *i.e.* exemestane for postmenopausal women and for premenopausal women tamoxifen plus ovarian ablation or suppression. Following progression on first-line endocrine therapy on anastrozol or letrozol, a switch to tamoxifen/fulvestrant or to exemestane with or without the addition of an mTOR inhibitor may be considered. According to the current Dutch guideline 8, first-line palliative chemotherapy is recommended in case of 1) the presence of visceral crisis (e.g. lymphangitic carcinomatosis, bone marrow involvement, carcinomatous meningitis or significant liver metastases), 2) hormone receptor-negative metastatic breast cancer, or 3) hormone receptor-positive metastatic breast cancer progressing on palliative endocrine therapy. An anthracycline-based or taxane-based regime can be chosen as first-line palliative chemotherapy. In case of taxanes, paclitaxel administered weekly is more effective than a three-weekly schedule 14,15, whereas three-weekly compared with weekly docetaxel is equally effective but less toxic 15,16. Regarding the choice for combination chemotherapy...
(A + B) versus sequential therapy (A → B), recent meta-analysis has shown that combination chemotherapy resulted in a significantly better tumor response and progression-free survival, and a moderate survival benefit, but at the expense of significant more toxicity. Therefore, combination therapy is usually reserved for patients with rapid disease progression and those, who require rapid control of symptoms and/or disease. The sequence of cytotoxic agents following progression on first-line therapy is still under debate, because of which recommended second-line treatment may differ according to national guidelines. Several cytotoxic agents have been registered for use in metastatic breast cancer, such as capecitabine, vinorelbine and eribulin mesylate.

For HER2-positive, hormone receptor-negative metastatic breast cancer, recommended first-line treatment consists of trastuzumab in combination with cytotoxic agents i.e. a taxane such as paclitaxel or docetaxel considering a better tumor response, progression-free survival and overall survival compared with the cytotoxic agent alone. Another combination regime of trastuzumab, pertuzumab and docetaxel has been registered as first-line palliative therapy. Patients may also benefit from an anthracycline + cyclophosphamide. For HER2-positive, hormone receptor-positive metastatic breast cancer, anti-hormonal therapy can be combined with trastuzumab or lapatinib. Trastuzumab emtansine (T-DM1) is available following progression on trastuzumab-containing therapy.

Lastly, patients with triple-negative metastatic breast cancer are candidates for chemotherapy, because anti-hormonal and anti-HER2 agents are not considered effective. Currently, no specific regime is preferred as standard first-line. Therefore, treatment is selected from a number of agents approved for general use in breast cancer. Platinum-based therapy may be beneficial in triple-negative breast cancer occurring in BRCA1 mutation carriers. Moreover, a meta-analysis of three phase III trials indicated that a significant improvement in progression-free survival was observed for bevacizumab-containing therapy versus chemotherapy alone in patients with triple-negative breast cancer.

**Molecular targeted agents**

In the past years, molecular techniques have demonstrated the presence of aberrant pathways sustaining tumor growth in various types of breast cancer. As a consequence, a number of agents has been developed or is under development that may be of benefit in patients with tumors containing such aberrant pathways.

For hormone receptor-positive metastatic breast cancer, drug development is focused on tumors with relative endocrine resistance. Activation of the PI3K-Akt-mTOR route is occurring rather frequently in this subtype. Everolimus, an inhibitor of mTORC1, is the first drug registered and is given in combination with exemestane to postmenopausal patients that previously received a non-steroidal aromatase inhibitor. Many other drugs directed against the PI3K-Akt-mTOR route are in clinical trial. Alterations in several cell cycle regulatory proteins have been described in hormone receptor-positive breast cancer, be-
cause of which inhibitors targeting the cyclin-dependent kinases (CDK4/6 inhibitors) are under development\textsuperscript{26,27}. First results on palbociclib, either combined with letrozol\textsuperscript{28} or with fulvestrant\textsuperscript{29} are very promising.

Since many years, patients with HER2-positive metastatic breast cancer have gained benefit from the introduction of inhibitors of HER2. Trastuzumab, a humanized monoclonal antibody against HER2 and to be combined with chemotherapy, has consistently shown improvement in overall survival\textsuperscript{30,31}. Trastuzumab can also be combined with endocrine therapy in patients that have HER2-positive, hormone receptor-positive disease. Lapatinib, an oral HER2 inhibitor, can be combined with endocrine therapy or with capecitabine. Pertuzumab has been registered in combination with trastuzumab and docetaxel in first-line metastatic disease. T-DM1 can be given to patients that progressed on previous HER2-inhibiting agents\textsuperscript{30,32}.

Bevacizumab, a humanized monoclonal antibody capturing circulating vascular endothelial growth factor A, represents an agent targeting tumor angiogenesis. Bevacizumab added to first-line paclitaxel has demonstrated encouraging improvement in clinical outcome for treatment of metastatic breast cancer in the pivotal E2100 phase III trial\textsuperscript{33,34}. The initial optimism has been tempered, because the magnitude of clinical improvement for the addition of bevacizumab was less than anticipated in follow-up phase III trials (AVADO\textsuperscript{35}, RIBBON-1\textsuperscript{36}). Nonetheless, paclitaxel and bevacizumab may still be considered in selected patients\textsuperscript{8}.

Triple-negative breast cancer is most aggressive and results in a worse prognosis when compared to other breast cancer subtypes. In recent years it has been shown that patients with tumors deficient in\textit{BRCA1/2} or other homologous recombination DNA repair proteins may benefit from treatment with an inhibitor of poly(adenosine diphosphate-ribose) polymerase (PARP), an enzyme involved in the recognition and repair of DNA breaks\textsuperscript{37}. A recent finding is the possible usefulness of immune checkpoint inhibitors in metastatic triple-negative breast cancer, such as demonstrated for pembrolizumab, a humanized monoclonal antibody targeting PD-1\textsuperscript{38}.

**Biomarkers for systemic therapy in advanced breast cancer**

According to the definition of National Institutes of Health, a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention”\textsuperscript{40}. Biomarkers could serve for different purposes based on clinical needs, such as diagnosis, prognosis estimation and prediction of treatment response. In breast cancer, diagnostic biomarkers are utilized to detect or exclude its presence and to improve current diagnostic workup. These biomarkers may also be implemented in a screening setting for early breast cancer detection. Prognostic biomarkers reflect the likely course of disease irrespective of treatment allowing better risk stratification\textsuperscript{41}. This is helpful in particular in patients with
early-stage disease, because adjuvant treatment may be given for those with features of poor prognosis. Predictive biomarkers can be used to predict treatment response, may point towards drug resistance and have the potential for guidance of treatment with the goal of survival improvement. Moreover, biomarkers may indicate an increased risk of developing toxicities from treatment and their use may improve patients’ quality of life.

In breast cancer, tumor markers including ER, PgR and HER2 are the only established biomarkers for prognosis and response to therapy. ER and PgR status have prognostic significance in which hormone receptor-positive breast tumors have better prognosis in comparison with receptor-negative breast tumors. Future research has demonstrated that hormone receptor status can guide endocrine therapies including tamoxifen and aromatase inhibitors being predictive markers. Similarly, HER2 status is a prognostic as well as predictive marker, in which overexpression is indicative of an aggressive breast cancer phenotype with poor prognosis and it also represents a target for anti-HER2 therapy.

It has increasingly been recognized that heterogeneity exists in treatment effects among breast cancer patients with apparently similar tumor characteristics. For example, overall response rates of first-line taxane-based chemotherapy in HER2-negative metastatic breast cancer are reported to be 30–70% (Chapter 2 appendix 7). In view of this example, it is reasonable to assume that prognosis differs among traditional clinical subtypes caused by factors yet to be elucidated and that current selection of eligible patients may need further refinement. Moreover, the treatment landscape of advanced breast cancer has changed considerably with increasing availability of newer anti-cancer agents entering the clinical arena. Therefore, there is a posing need for biomarkers, that can be used in conjunction with ER, PgR and HER2 status, for guidance of palliative systemic therapy and to improve clinical outcome, quality of life or cost effectiveness. No biomarkers have been validated for these purposes.

**Aims and thesis outline**

In this thesis, we aimed to investigate potential biomarkers for optimizing current therapy in the metastatic setting of HER2-negative breast cancer requiring first-line chemotherapy. Furthermore, the potential use of proteomics technology for the discovery of clinically useful biomarkers was studied.

In part I of this thesis, the potential role of biomarkers with respect to efficacy or toxicity from treatment was addressed. Currently, the identification of a patient subgroup with optimal treatment benefit is challenging. General aspects taken into account are not only response rate, progression-free survival or overall survival, but also the occurrence of treatment-related adverse events. In Chapter 2 a multicentre, open-label, randomized controlled phase 2 trial has been described in which the efficacy and toxicity of the addition of capecitabine to first-line paclitaxel and bevacizumab was evaluated in HER2-negative
advanced breast cancer patients (ATX trial). A prognostic risk score for predicting clinical outcome in the context of bevacizumab-containing therapy was validated. Since blood samples were collected at baseline and after one cycle of treatment, this cohort provided the opportunity to assess potential biomarkers. In Chapter 3 we measured angiogenesis- and hypoxia-related proteins at baseline and their changes after short-term treatment for their association with clinical outcome. Measurement of proteins at baseline may provide additional prognostic information, in conjunction with patient and tumor characteristics. Dynamic changes of these biomarkers during short-term bevacizumab-containing therapy may provide a means of treatment response monitoring.

Genetic polymorphisms as hereditary factors could potentially explain individual treatment response or susceptibility to toxicity. In Chapters 4 and 5, we reviewed the current evidence on the use of genetic variants as biomarker for outcome or toxicity in patients receiving taxane-based or capecitabine-based therapy. On this basis, genetic variants were selected for further analysis in the ATX trial cohort. Chapter 6 illustrates the evaluation of selected genetic variants for the prediction of paclitaxel-induced peripheral neuropathy. In Chapter 7, we analyzed the possible presence of an association between genetic variants and capecitabine efficacy and toxicity.

Part II of the thesis has its focus on the use of proteomics technology as a novel strategy to identify putative biomarkers of breast cancer. Recent advancements in this technique have yielded protein markers useful for further refinement of breast cancer classification and findings are reviewed in Chapter 8. In Chapter 9, the potential role of cancer-released proteins constituting the secretome as noninvasive biomarkers for cancer management is reviewed. In Chapter 10, we analyzed the cancer secretome harvested from breast tumors of genetically engineered mouse tumor models by mass spectrometry to identify released proteins related to BRCA1 deficiency. In order to explore clinical relevance, two proteins namely TOP1 and CDH3 were validated in a large panel of human breast carcinomas. The results of this thesis and future perspectives are discussed in Chapter 11.
CHAPTER 1

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