LEENA TIAINEN

Metastatic Breast Cancer

*Efficacy of Bevacizumab-based
Chemotherapy and Prognostic Factors*

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ACADEMIC DISSERTATION
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ABSTRACT

Breast cancer is the most common malignancy in women worldwide. Five-year survival rates of breast cancer are high (91 %), but due to its high incidence, it is still the leading cause of cancer death in females. In 2017, breast cancer was diagnosed in 4974 patients, and 928 patients died of breast cancer, according to Finnish Cancer registry data.[1]

The prognosis of metastatic breast cancer has only modestly improved during the last few decades. This improved survival is mostly due to the implementation of effective anti-HER2 therapy in standard clinical care of metastatic HER2-positive breast cancer. During this century, the advances in the treatment of the other metastatic breast cancer subtypes, hormone receptor positive and triple-negative, have been minimal in terms of improving patient survival. The most aggressive subtype with the greatest treatment challenges is the triple-negative breast cancer, in which all three clinically significant breast cancer receptors, i.e., estrogen, progesterone and HER2, are not expressed. The oncological treatment options for metastatic breast cancer depend on the receptor status, tumor burden, prior adjuvant therapies, patient performance status, other comorbidities and patient preferences.

For hormone receptor-positive disease, endocrine therapy is recommended as a first-line treatment option. Chemotherapy should be considered as the first-line treatment only in cases of visceral crisis. In addition, all patients with advanced breast cancer can be treated with chemotherapy after disease progression on endocrine therapy. For triple-negative patients, endocrine therapy is not effective, so chemotherapy is the only valid option for these patients. The taxanes docetaxel and paclitaxel are the most common choices for the first-line chemotherapy treatment of metastatic breast cancer.

Bevacizumab is a monoclonal antibody targeting vascular endothelial growth factor A. Angiogenesis is one of the hallmark processes of malignant tissue, needed for its proliferation, and bevacizumab aims to inhibit tumor neovascularization. Several phase III trials have evaluated bevacizumab as a treatment for metastatic breast cancer in combination with several chemotherapy agents. These studies with bevacizumab have resulted in a few months' benefit in progression-free survival and

higher frequency of response rates. However, an overall survival benefit was not established in any of the studies.

This study was designed to evaluate the feasibility of bevacizumab in combination with taxane chemotherapy as first-line chemotherapy treatment of metastatic HER2-negative breast cancer, to evaluate biomarkers for their prognostic value in advanced breast cancer and to improve the sensitivity and specificity of the CA15-3 tumor marker in disease monitoring. The median progression-free survival, which was the primary endpoint in our trial, was 11.3 months. This is similar to other results from first-line bevacizumab combinations. The median overall survival of our patients reached almost three years, which can be considered a good outcome. The toxicity related to bevacizumab treatment was mostly manageable, although one patient died of treatment-related side effects. In the biomarker study, low plasma interleukin-8 level was associated with excellent long-term survival. In addition, high plasma Tie1 was found to be a novel factor for poor prognosis in metastatic breast cancer. In this study, patients with high levels of the extracellular fragment of the Tie1 receptor and angiopoietin-2 had the poorest survival. In the substudy aiming to improve CA15-3 as a breast cancer tumor marker, the new nanoparticle-lectin immunoassay CA15-3^WGA was significantly more sensitive than the conventional CA15-3 assay.

TIIVISTELMÄ

Rintasyöpä on naisten yleisin pahanlaatuinen sairaus. Rintasyövän viiden vuoden elossaoloennusteet ovat hyviä (91 %), mutta johtuen rintasyövän yleisyydestä se on silti naisten suurin syöpäkuolemien aiheuttaja. Suomen syöpärekisterin tilastojen mukaan vuonna 2017 rintasyöpä todettiin 4974 potilaalla ja 928 potilasta kuoli rintasyöpään.[1]

Tällä vuosisadalla levinneen rintasyövän ennuste on parantunut vain vaatimattomasti. Tehokkaat HER2-vasta-ainehoidot ovat nykyään standardikäytössä levinneen HER2-positiivisen rintasyövän hoitona, ja levinneen rintasyövän ennusteen paraneminen johtuu suurimmalta osin ainoastaan HER2-positiivisesta alatyypistä. Edistysaskeleet hormonireseptoripositiivisen ja kolmoisnegatiivisen rintasyövän hoidossa ovat olleet vähäisiä potilaiden elinajan pidentymisen suhteen. Kolmoisnegatiivisessa rintasyövässä kaikki kolme rintasyöpäreseptoria ovat negatiivisia: estrogeeni, progesteroni ja HER2. Kolmoisnegatiivinen tauti on kaikkein aggressiivisin ja sen hoidossa on eniten haasteita. Levinneen rintasyövän hoitovaihtoehtoihin vaikuttavat syövän reseptoristatus, kasvainkuorma, mahdolliset aiemmat liitännäishoidot, potilaan toimintakyky, muut sairaudet ja potilaan oma mielipide annettavista hoidoista.

Hormonireseptoripositiivisessa levinneessä taudissa endokriinista hoitoa suositellaan ensilinjan hoidoksi. Kemoterapiaa suositellaan ensilinjan hoidoksi vain tilanteissa, joissa potilaalla on uhkaavia sisäelinmetastaaseja. Muille potilaille kemoterapiaa harkitaan siinä vaiheessa, kun rintasyöpä on edennyt yhden tai useamman endokriinisen hoidon aikana. Kolmoisnegatiivisessa rintasyövässä hormonaaliset hoidot eivät ole tehokkaita ja siksi kemoterapia on ainoa hoitovaihtoehto tätä aggressiivista tautimuotoa sairastavilla potilailla. Taksaani, dosetakseli ja paklitakseli, on tavanomaisin valinta levinneen rintasyövän ensilinjan kemoterapiahoidoksi.

Bevasitsumabi on verisuonen endoteelin kasvutekijä-A:ta kohtaan vaikuttava monoklonaalinen vasta-aine. Verisuonten muodostuminen on yksi pahanlaatuiden kudoksen tunnusomaisista piirteistä, jotta tuumorikudos pystyy lisääntymään. Bevasitsumabi pyrkii estämään kasvaimen uudisverisuonimuodostusta. Useat vaiheen III tutkimukset ovat arvioineet bevasitsumabin tehoa kemoterapiaan

yhdistettynä levinneen rintasyövän hoitona. Bevasitsumabitutkimuksissa taudin etenemisvapaassa ajassa ollaan saavutettu muutaman kuukauden hyöty ja hoitovasteet ovat olleet yleisempiä. Kuitenkaan hyötyä kokonaiselossa-ajassa ei ole pystytty osoittamaan missään näistä tutkimuksista.

Tämän tutkimuksen tarkoituksena oli arvioida bevasitsumabihoidon soveltuvuutta levinneen HER2-negatiivisen rintasyövän ensilinjan kemoterapiahoidoksi yhdistettynä taksaanihoitoon, plasman biomerkkiaineita levinneen rintasyövän ennustetekijöinä sekä parantaa CA15-3 kasvainmerkkiainemenetelmän herkkyyttä ja tarkkuuta rintasyövän hoidon seurannassa. Tutkimuksemme ensisijainen päätetapahtuma, mediaani taudin etenemisvapaa-aika, oli 11,3 kuukautta, mikä on samaa luokkaa kuin muissa ensilinjan bevasitsumabihoitotutkimuksissa. Tutkimuspotilaidemme mediaani kokonaiselossaolo-aika saavutti kuitenkin lähes kolmen vuoden rajapyykin, mitä voidaan pitää hyvänä tuloksena. Bevasitsumabihoitoon liittyvät haittavaikutukset olivat enimmäkseen hallittavissa huolimatta siitä, että yksi potilas menehtyi bevasitsumabihoidon haittavaikutuksiin. Ennustetekijätutkimuksessa plasman matala interleukiini-8-pitoisuus oli yhteydessä erinomaiseen pitkäaikaisselviytymiseen. Lisäksi korkea plasman Tie1-pitoisuus osoittautui levinneen rintasyövän uudeksi huonon ennusteen merkiksi. Tässä tutkimuksessa huonoin ennuste oli niillä potilailla, joilla todettiin sekä korkea Tie1-reseptorin solunulkoisen osan pitoisuus että plasman korkea angiopoietiini-2-pitoisuus. CA15-3 määritysmenetelmän kehitystä selvittäneessä osatyössä uusi lektiinipohjainen CA15-3WGA menetelmä oli perinteistä CA15-3 määritysmenetelmää tilastollisesti merkitsevästi herkempi.

CONTENTS

ABBREVIATIONS

AI	Aromatase inhibitor
Ang	Angiopoietin
APBI	Accelerated partial breast irradiation
ALP	Alkaline phosphatase
AUC	Area under the curve
BRCA	Breast cancer susceptibility gene
CDK4/6	Cyclin-dependent kinases 4 and 6
CEA	Carcinoembryonic antigen
CEC	Circulating endothelial cell
cfDNA	Cell-free DNA
CI	Confidence interval
CNS	Central nervous system
CT	Computed tomography
CTC	Circulating tumor cells
ctDNA	Circulating tumor DNA
DCIS	Ductal carcinoma in situ
DFS	Disease-free survival
DIBH	Deep inspiration breath hold
Dll-4	Delta-like 4
ECOG	Eastern Cooperative Oncology Group
ECG	Electrocardiogram
e.g.	Exempli gratia, for example
ELISA	Enzyme-linked immunosorbent assay
FDA	United States Food and Drug Administration
FGF	Fibroblast growth factor
Gy	Gray
HER1-4	Human epidermal growth factor receptor 1-4
HIF-1	Hypoxia-inducible factor-1
HMGB1	High-mobility group box 1
HR	Hazard ratio

HRE	Hypoxia-response element
IDFS	Invasive disease-free survival
IHC	Immunohistochemistry
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-18	Interleukin-18
i.v.	intravenously
LHRH	Luteinizing hormone-releasing hormone
m	Median
mBC	Metastatic breast cancer
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MRI	Magnetic resonance imaging
MUC-1	Mucin-1
NCCN	National Comprehensive Cancer Network
NGS	Next generation sequencing
OFS	Ovarian function suppression
ORR	Overall response rate
OS	Overall survival
PARP	Poly ADP ribose polymerase
pCR	Pathological complete response
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
PDGF	Platelet derived growth factor
PI3K	phosphatidylinositol 3-kinase
PFS	Progression-free survival
PlGF	Placental growth factor
RECIST	Response Evaluation Criteria in Solid Tumors
ROC	Receiver operating characteristic
RR	Risk ratio
RS	Recurrence score
SIB	Simultaneously integrated boost
SNP	Single nucleotide polymorphism
TAM	Tumor associated macrophage
TIL	Tumor-infiltrating lymphocyte

TKI	Tyrosine kinase inhibitor
TNBC	Triple-negative breast cancer
TNM	Tumor-nodes-metastases
TPA	Tissue polypeptide antigen
TPS	Tissue polypeptide-specific antigen
TTF	Time to treatment failure
TTP	Time to progression
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vs.	Versus
WBI	Whole breast irradiation

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1   INTRODUCTION

Breast cancer is the most common cancer in females worldwide, with 2.09 million breast cancers diagnosed in 2018, making up 24.2% of all new cancer cases in women[2]. However, the prognosis of primary breast cancer is mainly good: in Finland, the five-year net survival rate is one of the highest in the world, and 88.5% [95% confidence interval (CI) 87.7-89.3] of the patients are alive five years after the breast cancer diagnosis[3]. However, due to the high incidence of breast cancer, it is the leading cause of cancer death in females worldwide and in Finland[1,4].

Over the last few decades, the prognosis of breast cancer has improved mainly due to improved detection and earlier diagnosis[4]. Additionally, due to adjuvant and neoadjuvant therapies with long-term follow-up data, patient survival has improved[5,6]. The population-based mammography screening program was initiated in Finland in 1987, and since 1992, the program covered the entire country. This organized mammography screening program has decreased breast cancer mortality. However, the studies have yielded varying estimates of the survival benefit (0-43 %). Criticism of national screening programs has been raised since results with no survival benefit have also been published.[7–10] Approximately 200 women need to be screened to avoid one breast cancer death, and all these screened women are predisposed to the fear of breast cancer and the possibility of overdiagnosis[11].

Although in general the prognosis of breast cancer is good, and most breast cancers will not recur, numerous women still face the diagnosis of incurable metastatic disease. In 2018, 626 679 women died of metastatic breast cancer (mBC)[2]. The prognosis of mBC has improved, but this is mainly limited to human epidermal growth factor receptor 2 (HER2)-positive breast cancer[12–14]. No significant improvement has been observed in the prognosis of other breast cancer subtypes, including hormone receptor-positive HER2-negative mBC and biologically aggressive triple-negative breast cancer (TNBC)[12–14].

Globally, remarkable regional variation exists in the prognosis of breast cancer. In low- and middle-income countries, breast cancer is more commonly diagnosed as metastatic stage IV disease, and these countries also have the highest mortality rates for breast cancer[15]. Up to 36.1% of newly diagnosed breast cancer cases were metastatic in black South African people compared to only 3.0% in Sweden[15]. There

is also considerable variation in the access of new treatment modalities and drugs. For example, trastuzumab, a monoclonal antibody targeting HER2, has been the standard treatment for HER2-positive breast cancer for almost two decades and has proven to improve patient survival[13,16]. However, in a web-based survey mapping the global use of HER2 testing and antiHER2 therapy, 20% of the Asian responders did not have HER2 testing routinely available, and 80-100% of Latin American, Asian and African responders had encountered a situation that due to treatment costs, patient did not receive recommended HER2-targeted adjuvant therapy[17].

Angiogenesis, the formation and maintenance of vascular structures, is essential for all human cells[18]. Angiogenesis is an essential process for tumor proliferation, progression and metastatic spread. Tumor vasculature is different from normal vasculature. Tumor blood vessels are disorganized and irregular[19]. In addition, the blood flow is mostly sporadic, resulting in a damaged capillary network[20]. Adipose tissue surrounding the malignant cells and the stromal cells are responsible for producing angiogenic growth factors, e.g., vascular endothelial growth factors A, B and C (VEGF-A, -B and -C), fibroblast growth factor, matrix metalloproteinases (MMPs) and interleukin-8 (IL-8)[21,22]. Vascular endothelial growth factors recruit vascular endothelial cells to propagate and form tube-like structures[23]. The precursor endothelial cells originate from bone marrow. They migrate through the circulation to a vascular niche and start to form new blood vessels in the presence of vascular growth factors: VEGFs, fibroblast growth factor and platelet-derived growth factor[20,24]. VEGF-A is the most highly expressed member of the VEGF family in many pathological processes[25,26]. The effect of VEGF-A on the target cells is mediated mainly by its membrane receptor, vascular endothelial growth factor receptor 2 (VEGFR-2), although it binds with higher affinity to vascular endothelial growth factor receptor-1 (VEGFR-1)[25–28]. VEGF-A binding to VEGFR-2 activates multiple intracellular signaling pathways that result in survival, proliferation, migration and remodeling of endothelial cells[28]. Both VEGF-A and VEFGR-2 are expressed in breast cancer tissue[29].

Bevacizumab is a monoclonal antibody to VEGF-A. Bevacizumab inhibits vascular endothelial cell proliferation and therefore tumor angiogenesis[30]. Bevacizumab is used in combination with chemotherapy regimens in various indications for malignant diseases, including metastatic colon cancer, ovarian cancer, non-small cell lung cancer and kidney cancer[31]. In the treatment of breast cancer, bevacizumab is indicated for the first-line treatment of metastatic disease in combination with either paclitaxel or capecitabine[31]. The combination with capecitabine can only be considered if the patient is not suitable for other

chemotherapy options, including taxanes or anthracyclines[31]. However, the Finnish national breast cancer guidelines do not recommend the use of bevacizumab as a treatment for mBC[32].

2 REVIEW OF THE LITERATURE

2.1 Treatment of early breast cancer

2.1.1 Diagnostics of primary breast cancer

Primary assessment of a breast tumor includes a trimodal approach: physical examination, imaging and a core-needle biopsy of the suspected lesion[33]. Imaging modalities include bilateral mammography and ultrasound of the breast and regional lymph nodes followed by magnetic resonance imaging (MRI) of the breast only in selected cases[34]. MRI can be considered in cases of a high risk of breast cancer, for example, breast cancer susceptibility gene (BRCA) gene mutation carriers, a lobular tumor histology or other reasons to suspect tumor multifocality, a discrepancy between mammography and ultrasound findings that might alter operative treatment decisions or a discrepancy between clinical and imaging findings[35]. Pathological assessment is based on a core-needle biopsy of the primary breast tumor to determine the histological type, grade and receptor status of the tumor. In addition, a fine-needle aspiration or a core biopsy must be performed on the suspected axillary lymph nodes[33].

2.1.2 Surgery

A multidisciplinary team with a breast cancer-specialized medical and radiation oncologist, at least one surgeon, a pathologist and a radiologist should be consulted pre- and postoperatively for each patient before coming to a treatment decision[36,37]. Most patients with operable breast cancer are referred to surgery. In the case of a locally advanced setting, a large primary tumor or inflammatory breast cancer, neoadjuvant therapies are considered, especially in more aggressive tumor types: triple-negative or HER2-positive breast cancer[38]. Surgery options for the breast include breast-conserving surgery or mastectomy, depending on patient choice, comorbidities, tumor size, location, multicentricity, prior chest wall radiotherapy and

contraindications to radiotherapy[33,38]. The surgery is considered sufficient if the microscopic margins are free of the invasive cancer and at least 2 mm from ductal carcinoma in situ (DCIS)[39,40]. Obtaining wider negative margins than required by no ink on the tumor is not indicated in routine practice[38,39,41]. An axillary lymph node dissection is performed for patients with clinically detected lymph node metastases; otherwise, sentinel node biopsy is a sufficient procedure [33,38]. Previously, the patients with positive sentinel lymph node metastases underwent an axillary lymph node dissection[42]. However, according to the results of the ACOSOG Z0011 trial, patients with 1-2 sentinel lymph node metastases did not benefit from axillary lymph node evacuation if they were treated with adjuvant radiotherapy and systemic therapy[43]. In 2019, new results were published from the AMAROS trial. According to the results, the patients with a tumor size smaller than 5 cm, no clinical signs of axillary lymph node metastases and a positive sentinel node diagnosed during breast cancer surgery did not benefit in terms of survival from a complete axillary lymph node dissection[44]. In this study, both the complete lymph node dissection group patients and the comparison group patients underwent radiotherapy according to standard clinical practice. The extensive axillary surgery group patients suffered more often from chronic lymph edema of the limb, as expected.[44]

2.1.3   Pathology

An accurate evaluation of the tumor by an experienced pathologist and a full pathology report are essential for further oncology treatment decisions on early breast cancer[45]. The pathological assessment should be made according to the tumor-node-metastasis (TNM) staging system (Supplementary Table 1) and to the World Health Organization (WHO) classification[46,47]. In addition to the TNM assessment, the report should include the histological type, grade(s) of the tumor(s), evaluation of the resection margins, vascular invasion, immunohistochemical (IHC) evaluation of estrogen and progesterone receptors and HER2 status[48,49]. Hormone receptor positivity is defined as estrogen and/or progesterone positivity ≥ 1%. HER2 may be determined from all invasive tumors by *in situ* hybridization or only for the tumors with an ambiguous IHC score of 2+[49]. The Finnish breast cancer group recommends verifying HER2 positivity with *in situ* hybridization for all tumors with IHC scores of 2+ or 3+[32]. Ki-67, a proliferation marker assessed by IHC, adds useful information about the aggressiveness of the tumor[50,51]. Additionally, for the treatment decision and for evaluation of the patient's prognosis, tumors should be

grouped into intrinsic subtypes based on the histology and the receptor status data (Table 1)[45]. Furthermore, high-risk patients are screened by computed tomography (CT) and a bone scan for distant metastases. The high-risk features include clinically positive axillary lymph nodes, large tumors (≥ 5 cm), aggressive biology and clinical signs of metastases[33].

Table 1.   Intrinsic subtypes of breast cancer based on the St Gallen consensus 2015[45]

	Receptor status			
	ER	PR	HER2	Ki-67*
Luminal A	+	≥ 20%	negative	low
Luminal B	+	+ / -	+ / -	high
HER2 overexpression	-	-	+	any
Basal-like	-	-	-	any

* Ki-67 should be interpreted based upon local laboratory values; the cut-off value for low vs. high Ki-67 is approximately 20%

Abbreviations: ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor

2.1.4   Gene expression profiling for early breast cancer

Several gene expression profiles were evaluated for their extra prognostic and predictive information and for the selection of patients who would more likely benefit from adjuvant chemotherapy. Most of the studies were done retrospectively, and only a few large prospective phase III trials were published: TAILORx, PlanB and MINDACT.

Oncotype DX is a 21-gene recurrence score (RS) assay that has been validated most extensively in prospective studies[41,52]. It includes the following genes: *Ki-67, STK15, Survivin, CCNB1, MYBL2, HER2, GRB7, MMP11, CTSL2, GSTM1, CD68, BAG1, ER, PGR, BCL2, SCUBE2, ACTB, GAPDH, RPLPO, GUS* and *TFRC*. These genes are associated with tumor proliferation, invasion, HER2 expression and the estrogen pathway. The expression of these genes is normalized to the reference genes, which are the last five genes in the list above.[53] In the TAILORx study evaluating the clinical utility of Oncotype DX, the aims of the study were to confirm that a low RS of 0 to 10 was associated with a low rate of distant recurrence even if patients were treated with endocrine therapy alone and whether patients with mid-range RS of 11 to 25 would benefit from chemotherapy[54]. The study enrolled

patients with axillary node-negative, hormone receptor-positive, HER2-negative breast cancer. On the basis of Oncotype DX RS, the patients were assigned to four treatment groups. The women with low RS (≤ 10) received only endocrine therapy, and those with high RS (≥ 26) were treated with chemotherapy and endocrine therapy. The patients with mid-range RS (11-25) were randomized to either endocrine therapy alone or both chemo- and endocrine therapy[54,55]. The patients with RS ≤ 10 had a very good prognosis: 5-year disease-free survival (DFS) was 94%[55]. After a 5-year follow-up, distant metastases were observed in 1% of the patients, and according to recent updated results after a 9-year follow-up, only 3% of the patients had a distant recurrence[54]. Only 8% of low-RS patients had tumors with size ≥ 3 cm, and only 7% were grade 3 tumors. For those reasons, according to the European breast cancer treatment guidelines, the vast majority of the low-RS patients would not be recommended chemotherapy anyway[33]. However, even in this low-RS patient population, 22% of the patients were categorized as clinically high-risk patients, and for these patients, the RS score might be useful. After a 9-year follow-up, there was no difference between the mid-range RS patients randomized to the endocrine therapy alone group and the group treated with both chemotherapy and endocrine therapy [invasive disease-free survival (IDFS) 83.3% vs. 84.3%, respectively]. Therefore, endocrine therapy was noninferior to chemo- and endocrine therapy for patients with node-negative, HER2-negative, hormone receptor-positive patients with RS between 11 and 25. However, a subset of patients younger than 50 and with RS 16-25 had some benefit from chemotherapy (p=0.03)[54]. Prospective trials are focusing on the Oncotype DX 21-gene RS assay in patients with node-positive tumors but the results are not yet available.

The MammaPrint gene profiling assay was created by analyzing genes related to disease recurrence in patients with lymph node-negative breast cancer. The researchers chose 70 genes that were most strongly associated with a short interval to distant metastasis, i.e., the poor-prognosis signature. The poor-prognosis signature included genes regulating invasion, cell cycle, metastasis and angiogenesis.[56] This 70-gene MammaPrint signature was prospectively evaluated in the MINDACT study[57]. The MINDACT study patients had ≤ 5 cm primary tumors and ≤ 3 axillary lymph node metastases. MammaPrint was applied to categorize the patients to either low or high genomic risk, and additionally, the patients were divided into low or high clinical risk. The patients with both low genomic and low clinical risk were omitted from chemotherapy, but they had an excellent 5-year distant metastasis-free survival rate of 94.7% (95% CI 92.5-96.2). The patients with discordant results of genomic and clinical risk assessment were randomized to either

chemotherapy or no-chemotherapy. The patients with high clinical risk and low genomic risk had an absolute benefit of 1.9% in distant metastasis-free survival [Hazard ratio (HR) 0.65, 95% CI 0.38-1.10, p=0.11] and a 3% benefit in DFS (HR 0.64, 95% CI 0.43-0.95, p=0.03) if they had received chemotherapy compared to the patients with no chemotherapy after 5-year follow-up, but the study was not powered to assess the statistical significance of these differences. The patients with low clinical risk and high genomic risk had smaller differences in distant metastasis-free survival depending on whether they had received chemotherapy or not (96.1% vs. 93.9%, respectively, HR 0.90, 95% CI 0.40-2.01, p=0.80).[57]

The TAILORx and MINDACT studies only enrolled patients with node-negative breast cancer. The PlanB trial focused on a more high-risk patient population with node-positive patients included[58]. Chemotherapy was omitted for patients with Oncotype DX RS ≤ 11, and three-year DFS was excellent, at 98%, with endocrine therapy alone[58]. According to the PlanB trial, the RS score is an independent prognostic marker for early breast cancer, with the multivariate HR for DFS being at about same level as for tumor grade[58].

National Comprehensive Cancer Network (NCCN) clinical practice guidelines for breast cancer recommend the use of Oncotype DX for adjuvant chemotherapy considerations. Oncotype DX is both predictive and prognostic with category level 1 evidence for node-negative patients according to the NCCN guidelines[41]. For node-positive patients, Oncotype DX adds only prognostic information, and its predictive value will be evaluated in the future RxPONDER study. Evidence for MammaPrint is also considered level 1 for breast cancer prognosis, but so far, its predictive value is undetermined.[41] The St Gallen treatment consensus guidelines for early breast cancer considered that gene expression profiles might be most valuable for chemotherapy decision making in patients with tumors between 1 and 3 cm, 0-3 positive axillary lymph nodes and intermediate tumor proliferation[38]. However, the Finnish breast cancer group does not yet recommend the use of the gene expression profiles for adjuvant chemotherapy treatment decisions[32].

2.1.5   Adjuvant chemotherapy treatment of luminal A, luminal B and triple-negative breast cancer

General recommendations for adjuvant systemic therapy for HER2-negative early breast cancer are presented in Table 2. For suitable patients, the adjuvant chemotherapy schedule should include an anthracycline, a taxane and an alkylating

agent[59]. Adjuvant capecitabine may be added to triple-negative patients based on the reduced recurrence rates in the triple-negative subgroup in the FinXX trial[60]. For patients with a high risk of cardiac complications who are not suitable for anthracycline therapy, docetaxel with cyclophosphamide can be considered[61]. Docetaxel-cyclophosphamide is also feasible for elderly patients and is superior to the doxorubicin-cyclophosphamide combination[61]. Adjuvant chemotherapy is usually administered for four to eight chemotherapy cycles[33].

Table 2. Systemic treatment recommendations for HER2-negative breast cancer subtypes[33,38]

	Recommended therapy	Comments
Luminal A	Endocrine therapy	Consider chemotherapy for patients with multiple lymph node metastases, high tumor grade or at least 2-5 cm tumors
Luminal B	Endocrine therapy + chemotherapy	Chemotherapy can be omitted for stage ≤ T1bN0
Triple-negative	Chemotherapy	Chemotherapy is recommended for patients with stage T1bN0 or higher

Neoadjuvant chemotherapy is recommended for patients with locally advanced, inoperable breast cancer or inflammatory breast cancer[33]. Patients with a large primary tumor and a hope for breast conservation may also be considered for neoadjuvant systemic treatment depending on the tumor biology[33,38]. However, the neoadjuvant chemotherapy approach is the preferred choice for stage II to III TNBC[38]. Adding platinum salt to the standard anthracycline-taxane neoadjuvant treatment can be considered for triple-negative patients since pathological complete response (pCR) is more frequent[62,63]. Inconsistent results from the use of nab-paclitaxel instead of standard paclitaxel as neoadjuvant treatment were published[64,65]. The GeparSepto trial reported a clinically significant difference in pCR in favor of nab-paclitaxel for triple-negative patients[64]. However, there was no significant difference in pCR in triple-negative patients between standard solvent-based paclitaxel and nab-paclitaxel in the ETNA trial[65]. TNBC patients who do not achieve pCR with neoadjuvant chemotherapy treatment may be further treated with capecitabine for eight cycles postoperatively[66]. Capecitabine, in this setting, reduced the risk of disease recurrence by 40% and the risk of death by approximately 50%[66].

2.1.6 Adjuvant treatment of HER2-positive breast cancer

For HER2-positive breast cancer, the backbone of the adjuvant treatment is HER2-targeted antibody trastuzumab. HER2-positive breast cancer is always considered a disease with high risk for recurrence, with the exception of very small tumors (≤ 5 mm); therefore, adjuvant chemotherapy with trastuzumab is recommended[38]. Trastuzumab reduces breast cancer recurrence or death by approximately 40% in patients with HER2-positive early breast cancer[67–70]. Trastuzumab is administered either sequentially or concurrently with standard chemotherapy therapy containing taxane and anthracycline[38]. After completion of the chemotherapy, trastuzumab is continued up to one year. A less toxic option for stage I HER2-positive disease with a shorter treatment duration is weekly paclitaxel and trastuzumab for 12 weeks followed by trastuzumab for up to one year. This treatment option results in an excellent IDFS rate of 98.7% at three years[71].

Neratinib is an irreversible tyrosine kinase inhibitor (TKI) targeting HER1, HER2 and HER4. In the ExteNET trial, neratinib was given for one year after standard trastuzumab-based adjuvant treatment. The five-year IDFS was 2.5% higher in the neratinib group compared to the placebo group (HR 0.73, 95% CI 0.57-0.92, p=0.0083). Nevertheless, severe grade 3 diarrhea was common, although it was manageable with loperamid, in the patients receiving neratinib (40% of the patients).[72]

The optimal duration of adjuvant trastuzumab therapy has been extensively studied, but no other duration has found to be superior compared to one year, although cardiac adverse events were less common with shorter treatment duration[73–76]. Additionally, extended trastuzumab treatment up to two years did not result in a survival benefit in the HERA trial[77].

Other anti-HER-2 therapies were also investigated in the neoadjuvant or adjuvant setting as a dual HER2 inhibition with trastuzumab. Pertuzumab, another HER2 receptor-targeting antibody, is currently the standard first-line treatment of metastatic HER2-positive breast cancer in combination with trastuzumab and docetaxel, based on the CLEOPATRA trial[78]. Pertuzumab was also investigated in the treatment of early breast cancer. As a neoadjuvant treatment, pertuzumab increased the pCR rate up to 45.8% when combined with trastuzumab and docetaxel[79]. However, in the adjuvant setting, pertuzumab resulted in only a modest improvement in the survival of the HER2-positive patients, with an absolute increase in the IDFS of 0.9% at three years when combined with the standard trastuzumab-containing chemotherapy[80].

Lapatinib, a TKI targeting HER1 and HER2, was also investigated in several trials in adjuvant and neoadjuvant settings[81–87]. As a neoadjuvant treatment, a dual blockade with lapatinib and trastuzumab increased the pCR rate, but no survival benefit was demonstrated with lapatinib-containing treatments[81–87]. Therefore, considering the extra toxicity and cost, lapatinib cannot be recommended as a neoadjuvant or adjuvant treatment.

Patients who attain a pCR due to neoadjuvant treatment have improved survival[88,89]. For that reason, it is rational to focus research efforts on patients with residual disease after neoadjuvant therapy and therefore a higher risk of disease recurrence. Trastuzumab emtansine (T-DM1), an antibody-drug conjugate of trastuzumab and cytotoxic agent emtansine, was studied in HER2-positive patients with residual disease after neoadjuvant treatment, and the results of this study (the KATHERINE trial) were recently published. Patients who had received T-DM1 up to one year following trastuzumab-containing neoadjuvant treatment and standard breast cancer surgery had a 3-year IDFS of 88.3%, compared to 77.0% in the standard trastuzumab treatment arm ($p<0.001$)[90]. Additionally, T-DM1 as a single-agent neoadjuvant treatment for HER2-positive breast cancer was less effective than dual blockade with trastuzumab and pertuzumab plus chemotherapy but was significantly less toxic[91].

2.1.7 Adjuvant endocrine treatment

Adjuvant endocrine therapy is recommended for breast cancer patients with estrogen receptor-positive disease[33,38]. The available endocrine therapy options are the estrogen receptor modulator tamoxifen, the nonsteroidal aromatase inhibitors (AIs) letrozole and anastrozole, the steroidal AI exemestane and the selective estrogen receptor downregulator fulvestrant[92,93]. Tamoxifen and AIs are taken orally once daily, and fulvestrant is injected subcutaneously on days 0, 14, and 28 and every 28 days thereafter. The daily doses of tamoxifen and AIs are fixed (20 mg, 2.5 mg, 1 mg and 25 mg, respectively). Adjuvant tamoxifen for five years reduced the risk of disease recurrence by approximately 40% and breast cancer mortality by approximately one-third in hormone receptor-positive patients[5,94,95]. Tamoxifen is the standard endocrine adjuvant treatment for premenopausal patients[33,38]. For postmenopausal breast cancer patients, five years of AI therapy is a more efficient endocrine therapy choice, since it reduced recurrence rates by approximately 30% compared to tamoxifen and reduced breast cancer mortality by approximately 15%[96].

However, the absolute benefit of AI treatment compared to tamoxifen was modest (10-year gain 3.6% in breast cancer recurrence and 2.1% in breast cancer mortality)[96]. Therefore, tamoxifen is still an appropriate choice for some patients based on differences in side-effect profiles between tamoxifen and AIs and the breast cancer prognosis of the individual patient[33,38].

Ovarian function suppression (OFS) using luteinizing hormone-releasing-hormone (LHRH) analogues may be considered for high-risk premenopausal patients[33,38]. The high-risk premenopausal patients who also received adjuvant chemotherapy had an increased 8-year DFS rate of 5.3% with the tamoxifen-OFS combination compared to tamoxifen alone (HR 0.76, 95% CI 0.60-0.97)[97]. Similarly, the 8-year DFS rate was increased by 9.0% with the exemestane-OFS combination compared to tamoxifen alone (HR 0.68, 95% CI 0.53-0.88)[97]. The St Gallen 2017 early breast cancer treatment guidelines recommend pairing OFS with either tamoxifen or exemestane for premenopausal patients with at least N2 nodal involvement and/or for patients aged ≤ 35 years[38].

Five-year adjuvant endocrine treatment has been a standard for decades, but multiple phase III trials investigated the benefit of extended adjuvant endocrine therapy up to ten years[98–108]. For high-risk premenopausal patients, adjuvant tamoxifen can be continued up to ten years, based on the results of the ATLAS and aTTOM trials[105,106]. Both of these studies reported a significant decrease in disease recurrence, by approximately 15%, and the ATLAS trial also demonstrated an overall survival (OS) benefit (HR for OS 0.87, 95% CI 0.78-0.97, p=0.01)[105,106]. Treatment with an AI after five years of tamoxifen increased 5-year DFS by 34% (p=0.01) in the MA.17 trial[98]. The other studies (NSABP, IDEAL and DATA) investigating extended AI treatment (2-5 years after initial tamoxifen therapy) demonstrated a trend towards higher DFS[99,102,103]. According to a meta-analysis of these studies, a nonsignificantly greater DFS benefit was observed in patients with larger tumors (≥ 2 cm, HR for DFS 0.77 vs. 0.88, p for difference = 0.44), nodal metastases (HR 0.72 vs. 0.83, p for difference = 0.31) and both hormone receptors positive (HR 0.68 vs. 1.01, p for difference = 0.31)[109]. Treatment guidelines for early breast cancer recommend considering extended endocrine therapy for patients with a high risk of relapse but also highlight the importance of taking into account the side effects and tolerability of endocrine therapy[33,38,110].

2.1.8   Adjuvant radiotherapy

Radiotherapy to the chest wall and regional lymph nodes after mastectomy reduced disease recurrence by 25% ($p<0.00001$) and breast cancer mortality by 16% ($p=0.001$) in the patients with axillary lymph node metastases[111]. In addition to lymph node-positive patients, post mastectomy radiation therapy is also recommended for patients with large (at least 5 cm) tumors independent of the nodal status and for patients with positive resection margins[33].

Adjuvant radiotherapy is recommended for all patients after breast-conserving surgery. After breast-conserving surgery, whole-breast irradiation (WBI) reduced the 10-year risk of disease recurrence by 48% and the 15-year risk of breast cancer mortality by 18%[112]. Traditionally, radiotherapy for the breast is given in 2-gray (Gy) fractions five days a week for a total of 25 times. However, hypofractionated WBI is considered effective and safe for patients of all ages and tumor characteristics[113–115]. The American Society for Radiation Oncology (ASTRO) guidelines recommend the hypofractionation scheme of either 40 Gy in 15 fractions or 42.5 Gy in 16 fractions[113]. Current hypofractionation schemes truncate the treatment duration to three weeks. Boost irradiation can be considered for patients with risk factors for local relapse: age < 50 years, grade 3 tumors, extensive DCIS or vascular invasion[116,117]. In a randomized phase III study, a 16 Gy boost was given to breast cancer patients who had undergone breast-conserving surgery. In the younger age groups, a clear reduction in ipsilateral local relapses was observed: 44% reduction in patients aged ≤ 40 years in the boost irradiation group compared to the no-boost group ($p=0.003$) and 34% reduction for patients aged 41-50 years ($p=0.007$). However, for patients older than 50 years, the reduction in local relapses was nonsignificant (for age 51-60 years $p=0.02$, for age >60 years $p=0.019$, statistical significance level in this study was 0.01)[116]. Without prolongation of the radiotherapy treatment duration, the boost can also be delivered concomitantly with the WBI, a technique called simultaneously integrated boost (SIB)[118,119].

Nodal irradiation is recommended for patients with lymph node metastases[33,111,112]. Recently, at the San Antonio Breast Cancer Symposium 2018, a new EBCTCG meta-analysis of 13 500 women was presented, focusing on regional node irradiation. In this meta-analysis, the patients treated with more contemporary radiotherapy techniques had better breast cancer survival and fewer recurrences than patients who had not received radiotherapy. The patients with at least four lymph node metastases were the group with the most pronounced radiotherapy benefit, with an 8% decrease in breast cancer mortality. In contrast, the patients with no

lymph node metastases did not gain a breast cancer survival benefit from radiotherapy.[120]

Accelerated partial breast irradiation (APBI) can further shorten the radiotherapy period. It can be considered for breast cancer patients with a low risk of recurrence: age at least 50 years, unifocal tumor with a diameter ≤ 3 cm, no lymph node metastases, nonlobular histology, no extensive intraductal component or lympho-vascular invasion and surgical margins ≥ 2 mm[121,122]. The update of an ASTRO Consensus statement considered low-risk DCIS patients also eligible for APBI: screen-detected, low or intermediate grade, size ≤ 2.5 cm and surgical margins ≥ 3 mm[122]. Several trials have compared APBI to WBI[123–130]. The radiation treatment schemes in the studies varied from a one-dose 21 Gy intraoperative radiotherapy session directed to the tumor bed with electrons to an accelerated external-beam radiation dose delivered twice daily at 3.85 Gy for a week for a total dose of 38.5 Gy[125,128]. No survival difference between APBI and WBI has been demonstrated, but the rates of local recurrence were variable between the studies[125–130]. In the RAPID trial, the cosmetic outcome of the breast was worse in the APBI group compared to the WBI group, but the cosmetic results in the Italian APBI trial were opposite[124,125]. In conclusion, APBI can be considered for patients with a low risk of local recurrence who prefer a shorter treatment duration over potentially worse cosmetic outcomes.

Adjuvant radiotherapy, particularly left-sided, increases the incidence of cardiac morbidities in long-term follow-up[131]. Cardiac side effects can be decreased using modern three-dimensional treatment planning and techniques such as deep-inspiration breast hold (DIBH)[132]. Cardiac toxicity can be further reduced by avoiding unnecessary concomitant use of adjuvant AI during adjuvant breast cancer radiotherapy[133]. In addition to the cardiac side effects of adjuvant radiotherapy, regional nodal irradiation increases the risk of peripheral lymphedema and pneumonitis[134].

2.1.9   Bevacizumab as a treatment of early breast cancer

Bevacizumab was also studied in the treatment of early breast cancer. Most bevacizumab studies in early breast cancer focused on neoadjuvant treatment. All of these neoadjuvant studies demonstrated a significant increase in the proportion of patients achieving a pCR with bevacizumab combined with various chemotherapy options[62,135–138]. In most studies recruiting both hormone receptor-positive and -

negative patients, the bevacizumab pCR benefit was more pronounced in triple-negative patients[135–137]. However, the pCR rates were higher in hormone receptor-positive patients than in triple-negative patients in the NSABP B-40 study[138]. In contrast to the other bevacizumab neoadjuvant studies, in the NSAB B-40 trial, the patients received adjuvant bevacizumab for 10 cycles in addition to the neoadjuvant bevacizumab. The chemotherapy backbone in the neoadjuvant treatment was either docetaxel alone, docetaxel + capecitabine or docetaxel + gemcitabine. This is the only bevacizumab neoadjuvant trial with significantly improved OS in patients treated with bevacizumab (HR 0.65, 95% CI 0.49-0.88, p=0.004). Interestingly, the OS improvement was more clear in hormone receptor-positive patients. However, the results of the study were somewhat conflicting since no DFS benefit was observed (HR 0.80, 95% CI 0.63-1.01, p=0.06)[139]. None of the other neoadjuvant trials reported was able to demonstrate a DFS or OS benefit[135,140,141].

The role of bevacizumab as an adjuvant therapy of HER2-negative breast cancer was evaluated in two large randomized phase III trials. Both studies reported no bevacizumab benefit. In the BEATRICE trial, only patients with triple-negative disease were included, and these 2591 patients with operable breast cancer received standard adjuvant chemotherapy with or without bevacizumab (10 mg/kg every two weeks or 15 mg/kg every three weeks) for one year. Adding bevacizumab to the adjuvant chemotherapy did not result in an IDFS (HR 0.87, 95% CI 0.73-1.03, p=0.11) or OS benefit (HR 0.93, 95% CI 0.74-1.17, p=0.52)[142]. Patients with high-risk HER2-negative breast cancer were enrolled in another adjuvant trial, E5103[143]. In this study, most patients had ER-positive disease (64%). The study had three treatment arms. In arm A, patients were treated with placebo combined with doxorubicin and cyclophosphamide (AC) followed by weekly paclitaxel. In arm B, patients received bevacizumab only during AC and paclitaxel. In arm C, patients received bevacizumab during AC and paclitaxel and then bevacizumab monotherapy for 10 cycles. There were no significant differences in the primary endpoint IDFS between the treatment arms [Arm C vs. arm A: HR 0.87 (95% CI 0.71-1.06, p=0.17), arm B vs. arm A: HR 0.93 (95% CI 0.78-1.16, p=0.62)] or in the OS [Arm C vs. arm A: HR 0.89 (95% CI 0.68-1.17, p=0.41), arm B vs. arm A: HR 1.01 (95% CI 0.77-1.33, p=0.92)].[143]

Taking into account the above-mentioned bevacizumab trials in early breast cancer, the role of bevacizumab remains unclear, and therefore, it is not recommended to be used as neoadjuvant or adjuvant treatment outside clinical trials[33,38].

2.2   Metastatic breast cancer (mBC)

The median (m) OS of mBC is approximately two years, and only one quarter of the patients is still alive five years after the diagnosis of metastatic disease[144]. The prognosis varies depending on the breast cancer subtype. In a Dutch retrospective analysis of mBC patients diagnosed between 2007 and 2009, the patients with hormone receptor-positive, HER2-positive mBC had the longest survival (mOS 34.4 months). In the same study, median survival for hormone receptor-positive, HER2-negative breast cancer patients was 24.8 months, hormone receptor-negative, HER2-positive patients 19.9 months, and triple-negative patients only 8.8 months (p<0.001).[145] In addition, the localization of the metastases at the initial advanced disease diagnosis was associated with significant differences in patient survival. The patients with only lymph node metastases or bone metastases had the longest survival (47 months and 43 months, respectively)[146]. The patients with visceral metastases had a median survival reduced by almost half (26 months), and for patients with central nervous system (CNS) metastases, the median survival was only 11 months (p<0.01)[146].

2.2.1   Diagnostics of advanced breast cancer

The diagnostic work-up for mBC includes imaging of the chest, abdomen and bone; medical history documentation; physical examination; and laboratory assesment[16]. A core biopsy should be performed, if accessible, of a metastatic lesion, especially in the de novo metastasis situation[16,147]. For patients with a history of early breast cancer, a biopsy should nevertheless be performed since the hormone and HER2 receptor status of the breast cancer may change over time. In a meta-analysis of 39 studies accessing receptor conversion and paired samples of primary tumors and metastatic lesions, ER receptor conversion was observed in 19% of patients, PR conversion in 31% of patients and HER2 conversion in 10% of patients[148]. However, since no prospective studies have been conducted on discordant receptor status patients, endocrine therapy and/or antiHER2 therapy should be considered when receptor status has been positive in at least one biopsy[16].

Metastatic TNBC is an aggressive subtype with the most treatment challenges in the field of breast cancer, since there are not yet any targeted therapy options available. TNBC has the highest risk of metastatic disease of the breast cancer subtypes and the poorest survival[149]. The risk of TNBC recurrence is high during

the first five years after primary breast cancer diagnosis, and after that, recurrences of TNBC are rare[150]. TNBC metastasizes more often to the visceral organs and the CNS than other breast cancer subtypes, while bone metastases are less frequently observed[151].

2.2.2 Circulating tumor markers for detection of breast cancer recurrence

CA15-3 and carcinoembryonic antigen (CEA) have been investigated and used as circulating tumor markers in breast cancer for decades. CA15-3 assays detect the shed or soluble form of the transmembrane protein Mucin-1 (MUC-1). Altered MUC-1 expression is associated with cancer pathogenesis and metastasis[152]. MUC-1 expression is observed at some levels in all invasive breast carcinomas[153]. CA27.29 is another MUC-1-associated antigen with comparable results to those of CA15-3[154,155].

CEA is a group of glycoproteins involved in cell adhesion. During fetal development, CEA is produced in the gastrointestinal tract, but the production ends before birth. However, elevated CEA levels are frequently observed in multiple malignancies, especially adenocarcinomas[153,156–159].

In a large retrospective patient cohort of over 1000 patients with local breast cancer diagnosis, an elevated preoperative level of CA15-3 or CEA was associated with breast cancer death ($p=0.0001$), and additionally, elevated CA15-3 was associated with breast cancer recurrence ($p=0.0003$)[160]. Monitoring serum CA15-3 and CEA at six-week intervals after primary breast cancer treatment, a 100% increase in the individual baseline value in each patient resulted in 38.3% sensitivity for CA15-3 to detect disease recurrence, 21.3% sensitivity for CEA and 55.3% sensitivity for the combination of both markers[161]. Another study retrospectively analyzed CA15-3 and CEA levels in the follow-up of breast cancer patients and demonstrated a specificity for both tumor markers >98% using an increase of 100% in the marker level as a cut-off. The sensitivity using the same cut-off levels was 55.6% for CA15-3, 40.6% for CEA and 66.3% for the combination of both markers[162]. The sensitivity for CA15-3 was even lower for detecting local recurrences[163,164]. However, another study found CEA monitoring to provide no additional value to CA15-3 monitoring[165]. In a prospective study of 1023 patients, CA15-3 and CEA were analyzed every 3-6 months during breast cancer surveillance. CA15-3 and CEA levels were elevated prior to the diagnosis of mBC in 41% and 40% of the patients, respectively, with a lead time of 4-5 months.[166] Retrospective analysis of a French

breast cancer patient cohort reported that half of the breast cancer relapses were diagnosed due to the symptoms of the patients, but 19% of the relapses were found due to elevated CA15-3 levels[167]. Monitoring the CA15-3 or CEA level has not demonstrated any survival benefit; therefore, routine monitoring of these markers during breast cancer follow-up is not currently recommended[33,168,169].

Other circulating markers have also been evaluated for prognosis or recurrence of breast cancer (Table 3). MicroRNAs are a class of small, single-stranded, noncoding RNA molecules that can be detected in liquid biopsies[170]. MicroRNAs 21, 23b, 126, 155, 190, 200b and 200c have all been associated with carcinogenesis and were therefore investigated as markers for breast cancer recurrence and prognosis (Table 3)[170,171]. Increased levels of alkaline phosphatase (ALP) were observed in 4% of breast cancer patients during follow-up, and elevated ALP level alone was not associated with a significantly higher risk of breast cancer recurrence (Table 3)[172]. Tissue polypeptide antigen (TPA), a marker for proliferation, had a sensitivity comparable to CA15-3, but lower specificity for breast cancer[173]. Using a combined analysis of CA15-3, CEA and TPA levels increased the sensitivity of these tumor markers during breast cancer follow-up[174]. Tissue polypeptide-specific antigen (TPS) detects cytokeratins 18 and 19, which are highly expressed in breast carcinomas[175]. TPS alone had lower sensitivity in detecting breast cancer recurrence, but combined with CA15-3, the sensitivity increased to 88%[176]. In another study, TPS was less sensitive than CA15-3 to detect breast cancer relapses, and the combination of CA15-3, CEA and/or TPS did not increase the sensitivity compared to CA15-3 alone for visceral recurrences. In addition, false-positive elevations were more common with the TPS assay (12%).[177]

NCCN guidelines do not recommend screening for metastases with laboratory markers in the absence of clinical signs of recurrent disease[41]. Similarly, the European guidelines state that monitoring tumor markers, such as CA15-3 and CEA, does not produce survival benefit and is therefore not recommended[33].

Table 3. Prognostic and predictive tumor markers investigated for breast cancer recurrence or diagnosis

Author, year	Circulating marker	Study design	Result
Swellam et al. 2019[170]	CEA CA15-3 miRNA-21 miRNA-126 miRNA-155	n=96 stage I-III BC patients n=47 patients with BBL	CEA: ≥ 5 ng/ml: BC 49%, BBL 19%, *p*<0.0001 CA15-3, ≥ 30 ng/ml: BC 52%, BBL 23%, *p*<0.0001 miRNA-21, ≥228.6-fold change: BC 64%, BBL 13%, *p*<0.0001 miRNA-126, ≤40-fold change: BC 89%, BBL 0%, *p*<0.0001 miRNA-155, ≥124-fold change: BC 96%, BBL 6%, *p*<0.0001
Papadaki et al. 2018[171]	miRNA-21 miRNA-23b miRNA 190 miRNA-200b miRNA-200c	n=155 consecutive patients with early breast cancer, treated with surgery and chemotherapy	miRNA-21, miRNA-23b, miRNA-200c higher in patients with BC relapse (*p*<0.001, *p*=0.028, *p*<0.001, respectively) miRNA-190 lower in patients with BC relapse (*p*=0.013) High miRNA-21 expression related to shorter DFS (*p*<0.001) High miRNA-200c expression related to shorted DFS (*p*=0.005)
Keshaviah et al. 2007[172]	CA15-3 ALP	Combined analysis of 7 trials n=3953	Elevated CA15-3: risk of recurrence HR 1.30, *p*=0.0005 Elevated ALP: risk of recurrence HR 1.04, *p*=0.82 Elevated CA15-3 and ALP: risk of recurrence HR 4.69, *p*<0.0001
Vizcarra et al. 1996[173]	CA15-3 CEA TPA	n=80 healthy controls n=421 local BC n=117 mBC	Specificities: CA15-3 95.7%, CEA 95.5%, TPA 81.9% Sensitivities: CA15-3 64.1%, CEA 44.4%, TPA 67.5%
Soletormos et al. 1993[178]	CA15-3 CEA TPA	n=90 TM monitoring during BC follow-up	Sensitivity for mBC diagnosis: CA15-3 48%, CEA 10%, TPA 19% Negative predictive value for CA15-3 86%
Nicolini et al. 1991[174]	CA15-3 CEA TPA	n=285 TM monitoring during BC follow-up	Sensitivity for mBC diagnosis: CA15-3 46%, CEA 7%, TPA 63% CA15-3+CEA+TPA 87% CA15-3+TPA 83%, CEA+TPA 70%
D'Alessandro et al. 2001[176]	CA15-3 TPS	n=349 TM monitoring during BC follow-up for 5 years	Sensitivity for mBC diagnosis: CA15-3 72%, TPS 66% CA15-3+TPS 88%
Given et al. 2000[177]	CA15-3 CEA TPS	n=1082 TM monitoring during BC follow-up	Sensitivity for mBC diagnosis: CA15-3: 68% for visceral, 69% for bone recurrence CEA 27% and 47%, respectively TPS 64% and 51%%, respectively

n: number of patients; miRNA: microRNA; BC: breast cancer; BBL: benign breast lesion; DFS: disease-free survival; ALP: alkaline phosphatase; mBC: metastatic breast cancer; TM: tumor marker

The presence of circulating tumor cells (CTCs) in the peripheral blood was associated with a higher risk of breast cancer recurrence. The CTC count was analyzed in 547 patients who participated in the E5103 phase III adjuvant trial[179]. The CTCs were positive in 26 patients (5%). The recurrence rates per person-year

of follow-up were 21% in the CTC-positive group and 2% in the CTC-negative group[179]. HER2 status may also be analyzed using immunofluorescence from CTCs. In a meta-analysis including approximately 3000 patients with early breast cancer, the presence of CTCs was associated with increased risk for breast cancer recurrence (HR 2.86, 95% CI 2.19-3.75, $p<0.01$) and worse OS (HR 2.78, 95% CI 2.22-3.48, $p<0.01$)[180]. In a retrospective analysis of 107 patients, 37 patients had HER2-positive CTCs. Of these 37 patients, only 10 patients (27%) had HER2-positivity in primary breast cancer tissue specimen, as determined by immunohistochemistry.[181] However, the development of other liquid biopsy methods to analyze circulating tumor DNA (ctDNA) will presumably lessen the use of CTCs in the future.

Liquid biopsies are a promising method for detecting early tumor relapses and have been evaluated in several trials. Fragmented DNA is released into the circulation by apoptotic and necrotic cancer cells, and ctDNA can be detected in peripheral blood by using polymerase chain reaction (PCR) or genome sequencing techniques[182]. In a study of 640 patients with various malignant diseases, ctDNA was detectable in 50% of patients with localized breast cancer and in > 80% of patients with mBC[183]. A meta-analysis of 69 trials and 5700 patients with breast cancer was published in 2018. In this pooled analysis, only 3 studies evaluated the association between breast cancer recurrence and ctDNA mutations. CtDNA mutations detected in peripheral blood were significantly associated with breast cancer recurrence (OR 3.79, 95% CI 1.80-8.00, $p<0.001$)[184]. A very recent paper was published in April 2019 focusing on prospective ctDNA surveillance of 49 breast cancer patients after adjuvant therapy. Plasma samples were collected every 6 months for up to 4 years. Plasma was analyzed for 16 gene variants selected from primary tumors. During follow-up, 18 patients faced breast cancer recurrence, and 16 of these relapses were predicted by ctDNA up to 2 years before clinical manifestation of the metastatic cancer (median time 9 months, range 0.5-24 months). Therefore, the sensitivity of the patient-specific ctDNA assay to detect relapses was 89%. CtDNA positivity was not observed in any of the 31 nonrelapsing patients.[185]

2.3   Treatment of metastatic breast cancer

The treatment recommendations for mBC patients depend on the hormone receptor status, HER2 status, prior (neo)adjuvant treatment, performance status of the patient, comorbidities, patient preference and presence of visceral crisis[16]. The age of the patient should not be a reason to withhold effective therapy, and careful

assessment for patient performance status and comorbidities should be done, preferably with the help of comprehensive geriatric assessment[16,186] .

For several decades, mBC was treated with either endocrine therapy or chemotherapy. HER2-targeted therapy has also been used for the treatment of advanced disease for patients with HER2-positive breast cancer. The efficacy of bevacizumab was investigated as a treatment of mBC in combination with either chemotherapy or endocrine therapy. Additionally, several new treatment options have emerged during the last decade: the mammalian target of rapamycin (mTOR) inhibitor everolimus, cyclin-dependent kinase 4 and -6 (CDK4/6) inhibitors, poly ADP ribose polymerase (PARP) inhibitors, and phosphatidylinositol 3-kinase (PI3K) inhibitors[187]. The estrogen receptor signaling pathway interacts with the signaling pathways related to mTOR inhibitors, PI3K inhibitors and CDK4/6 inhibitors, and the mechanisms of action of these compounds in the treatment of hormone receptor-positive mBC are presented in Figure 1.
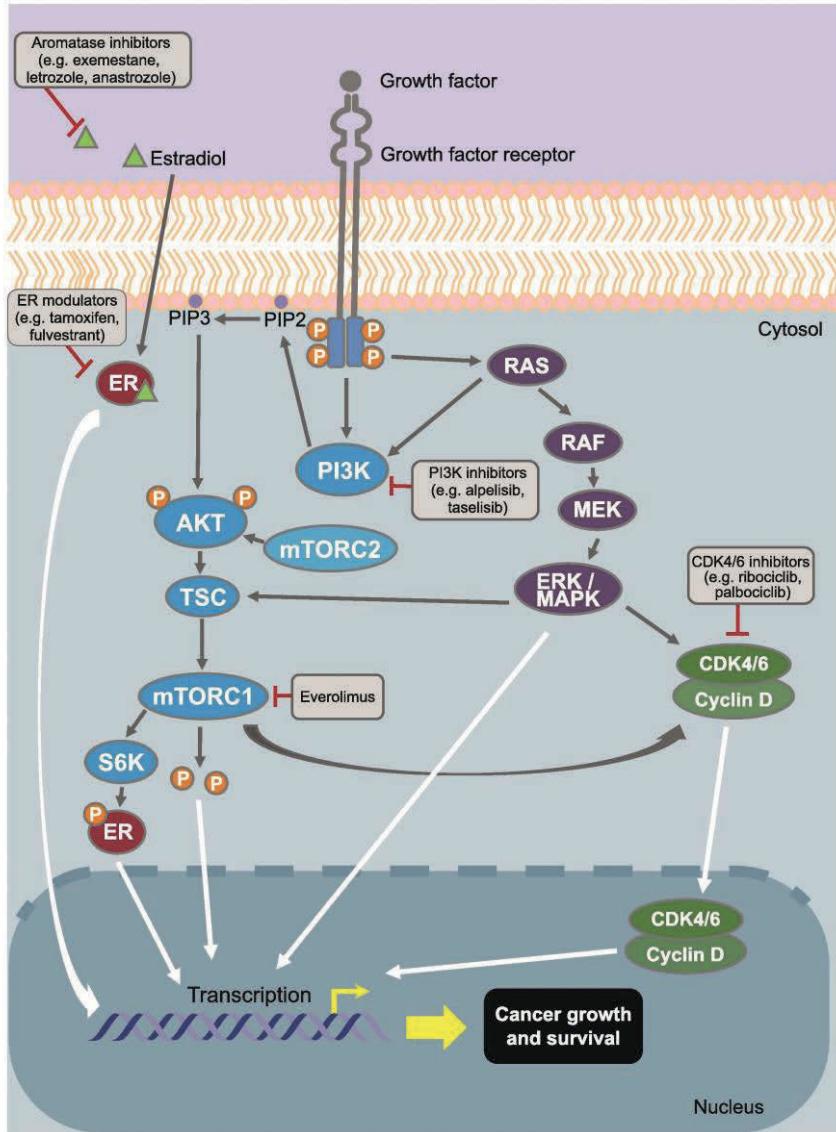
Figure 1. Nonchemotherapeutic treatment options for metastatic hormone receptor-positive breast cancer. Mechanisms of actions of endocrine therapies: aromatase inhibitors, tamoxifen and fulvestrant; mTOR inhibitor everolimus; PI3K inhibitors; and CDK4/6 inhibitors in cancer cells. Each of these is discussed in detail in the following chapters. AKT: protein kinase B; CDK: cyclin-dependent kinase; ER: estrogen receptor; ERK/MAPK: extracellular signal-regulated kinase/mitogen-activated protein kinase; MEK: mitogen-activated protein kinase; MTORC1: mammalian target of rapamycin complex 1; MTORC2: mammalian target of rapamycin complex 2; P: phosphorylation; PIP2: phosphatidylinositol 3,4-bisphosphinate; PIP3: phosphatidylinositol 3,4,5-bisphosphinate; PI3K: phosphatidylinositol 3-kinase; RAF: rapidly accelerated fibrosarcoma kinase; RAS: rat sarcoma kinase; S6: S6 kinase; TSC: tuberous sclerosis protein. Figure from O'Shaughnessy et al. 2018[187].

2.3.1 Endocrine therapy for hormone receptor-positive mBC

Endocrine therapy is recommended as the first-line treatment of advanced hormone receptor-positive disease in the absence of visceral crisis[16,41,147]. A meta-analysis of 4 phase III trials of first-line endocrine therapy and in 1400 patients revealed that response rates were higher in nonvisceral mBC than in visceral mBC (34% vs. 30%, respectively, p=0.038), but the duration of responses was equal in patients with vs. without visceral involvement[188]. Endocrine therapy trials in mBC have mainly enrolled postmenopausal patients. However, consensus guidelines recommend premenopausal patients be treated under the same principles as postmenopausal patients, but they should be offered OFS or ovarian ablation in combination with endocrine therapy[16,147].

The first trial of the antitumor activity of tamoxifen as a treatment of mBC was published in 1971[189]. It was followed two years later by another trial focusing on different dosage levels of tamoxifen, 10 and 20 mg, and 20 mg was found to be more effective[190]. The pharmaceutical company that ran the trial, ICI Pharmaceutical Division, abandoned further investigation of tamoxifen for financial reasons. Therefore, the next trial had to wait until the 1980's and showed improved survival with tamoxifen for patients with early breast cancer[191]. Since the 1970's tamoxifen has been the gold standard treatment for the first-line endocrine therapy of mBC[192].

AIs were compared to tamoxifen as a treatment of mBC. Letrozole was superior to tamoxifen in terms of response rates (32% vs. 21%, p=0.0002) and median progression-free survival (mPFS 9 vs. 6 months, p<0.0001)[193]. Two phase III trials compared the efficacy of anastrozole to tamoxifen as a first-line treatment in postmenopausal women[194,195]. The first one demonstrated similar efficacy results for anastrozole and tamoxifen, and in the second trial, anastrozole was superior to tamoxifen (median time to progression, mTTP, 11 months vs. 6 months, respectively, p=0.005)[194,195]. EORTC conducted a phase III study comparing the efficacy of exemestane and tamoxifen as first-line endocrine therapies in postmenopausal women. PFS was longer in the exemestane-treated patients than in the tamoxifen-treated patients (mPFS 10 months vs. 6 months, p=0.03)[196]. However, after longer follow-up, the difference in PFS between exemestane- and tamoxifen-treated patients disappeared (HR 0.87, 95% CI 0.70-1.08, p=0.121)[196].

Fulvestrant was compared to AIs and tamoxifen for the treatment of mBC cancer. The recommended dose for fulvestrant is currently 500 mg per injection, which was found to be superior to the dose of 250 mg per injection in the CONFIRM trial[197–199]. Initially, fulvestrant 250 mg was found to be noninferior to

tamoxifen, anastrozole and exemestane[200–202]. The phase II FIRST trial compared fulvestrant 500 mg to anastrozole as a first-line treatment for advanced breast cancer. The study failed to demonstrate a significant improvement in its primary end-point, clinical benefit rate (73% vs. 67%, respectively, p=0.386). However, the secondary end-points, OS and TTP, were superior with fulvestrant compared to anastrozole (HR for OS 0.70, p=0.04, 6-month improvement in mOS, HR for TTP 0.66, p=0.01, 10-month improvement in TTP)[203,204]. A larger phase III trial, FALCON, was conducted to confirm the findings of the FIRST trial[205]. Postmenopausal patients with metastatic hormone receptor-positive mBC were randomly assigned to receive either fulvestrant 500 mg or anastrozole as a first-line treatment. There were no differences in response rate (p=0.73) or clinical benefit rate (p=0.30) between the treatment arms. However, patients treated with fulvestrant had significantly longer mPFS compared to patients treated with anastrozole (mPFS 17 vs. 14 months, HR 0.80, p=0.049)[205]. OS results have not yet been published.

Fulvestrant was also investigated in combination with an AI, but the results of the three phase III studies were conflicting[206–208]. All three studies enrolled patients with hormone receptor-positive breast cancer with no previous therapy for advanced disease. The patients in the S0226 and FACT trials were randomized to receive anastrozole with or without fulvestrant. The SoFEA trial had 3 randomized treatment arms: fulvestrant + anastrozole, fulvestrant + anastrozole-placebo and exemestane. The S0226 and SoFEA trials enrolled only postmenopausal patients, but the FACT trial also enrolled premenopausal patients with the requirement to use LHRH analogue. In the FACT and SoFEA trials, no improvement was observed in PFS (FACT: HR 0.99, p=0.91, SoFEA: HR 1.0, p=0.98) or in OS (FACT: HR 1.0, p=1.00, SoFEA: HR 0.95, p=0.61) for the patients treated with the combination of fulvestrant and anastrozole compared to single-endocrine agent treatment. However, the S0226 trial, with a similar study design, reported a significant improvement in PFS and OS in fulvestrant plus anastrozole-treated patients compared to anastrozole alone (HR for PFS 0.68, p=0.007, HR for OS 0.82, p=0.03)[206,209]. The subgroup analysis of this study suggested that patients with no prior adjuvant endocrine therapy and a disease-free interval of more than 10 years had the greatest benefit from dual endocrine therapy[206]. The mOS for patients with no prior adjuvant endocrine therapy was 52 months with the anastrozole-fulvestrant combination compared to 40 months for patients treated with anastrozole alone[209]. Most of the patients enrolled in the FACT and SoFEA trials had a disease relapse during adjuvant endocrine therapy and therefore had more endocrine-resistant disease, which may explain the lack of survival benefit[207,208]. However, due to conflicting results, the use

of combination endocrine therapy for postmenopausal patients is not strongly recommended in the treatment guidelines of mBC[16,41].

2.3.2   mTOR inhibitors in mBC

Hormone receptor-positive mBC may be treated with a combination of the mTOR inhibitor everolimus and an AI. The PI3K/AKT/mTOR pathway becomes activated during secondary endocrine resistance, providing the rationale for combining mTOR inhibitors with endocrine therapy[210]. The everolimus and exemestane combination was studied in postmenopausal patients who had experienced disease progression during or shortly after treatment with a nonsteroidal AI, letrozole or anastrozole. Everolimus in combination with exemestane improved mPFS by 4 months compared to placebo plus exemestane (mPFS 7 months vs. 3 months, $p<0.001$)[211]. However, the response to everolimus plus exemestane was rare (9.5% vs. 0.4%, respectively, $p<0.001$). In addition, everolimus added treatment toxicity: 55% of study patients in the BOLERO-2 trial experienced ≥ grade 3 adverse events, e.g., stomatitis (8%), anemia (5%), hyperglycemia (4%) and pneumonitis (3%)[211,212]. Moreover, no OS benefit was observed with this combination (everolimus+exemestane: mOS 31 months; placebo+exemestane: mOS 27 months; HR 0.89, 95% CI 0.73-1.10, $p=0.14$)[212].

mTOR inhibitors were investigated as a first-line treatment for mBC. Phase II BOLERO-4 trial enrolled postmenopausal patients with no prior treatment for metastatic disease, and they were all treated with the combination of everolimus and letrozole[213]. The mPFS for the first-line treatment with everolimus and letrozole was 22 months (95% CI 18-25 months), and mOS was not yet reached[213]. The phase III HORIZON trial compared the efficacy of another mTOR inhibitor, temsirolimus, combined with letrozole vs. placebo combined with letrozole as a first-line treatment of advanced breast cancer[214]. No improvement in the primary end point PFS was reported (HR for PFS 0.90, 95% CI 0.76-1.07, $p=0.25$)[214].

Since everolimus and exemestane are both orally available and their toxicity is mostly manageable, the combination is considered a treatment option for postmenopausal patients with secondary endocrine-resistant mBC, according to the positive results of the BOLERO-2 trial[16,211,212].

2.3.3 CDK4/6-inhibitors in mBC

Recently, three CDK4/6-inhibitors, palbociclib, ribociclib and abemaciclib, demonstrated substantial PFS benefit in combination with endocrine therapy as a treatment of advanced hormone receptor-positive breast cancer. These compounds prevent the cell cycle from progressing from G1 to S phase[215]. As a first-line treatment for postmenopausal patients, palbociclib, ribociclib or abemaciclib combined with an AI provided a significant improvement in mPFS by 9.3-13.4 months compared to a placebo with an AI (palbociclib: HR 0.58, 95% CI 0.46-0.72, $p<0.001$; ribociclib: HR 0.57, 95% CI 0.46-0.70, $p<0.001$; abemaciclib: HR 0.54, 95% CI 0.42-0.70, $p<0.001$)[216–218]. CDK4/6 inhibitors were also investigated for the treatment of more hormone-resistant advanced breast cancer either with disease that had progressed on adjuvant endocrine therapy or while receiving first-line endocrine therapy for metastatic disease. In this setting, palbociclib, ribociclib or abemaciclib combined with fulvestrant demonstrated a mPFS benefit of 4.9-7.7 months compared to placebo with fulvestrant (palbociclib: HR 0.46, 95% CI 0.36-0.59, $p>0.001$; ribociclib: HR 0.59, 95% CI 0.48-0.73, $p<0.001$; abemaciclib: HR 0.55, 95% CI 0.45-0.68, $p<0.001$)[219–221]. Of the above-mentioned trials, PALOMA-3 (palbociclib) and MONARCH-2 (abemaciclib) also enrolled premenopausal patients with the requirement that they use a LHRH analogue alongside the study treatment[219,221]. For ribociclib, a separate phase III first-line endocrine therapy study for premenopausal patients was conducted[222]. In this trial (MONALEESA-7), the premenopausal patients received either ribociclib or a placebo with tamoxifen or nonsteroidal AI plus a LHRH analogue. The mPFS benefit (10.8 months) in the ribociclib treatment arm was similar to the benefit in studies focusing on postmenopausal patients (HR 0.55, 95% CI 0.44-0.69, $p<0.001$)[222].

CDK4/6 inhibitors were quite well tolerated. The most common side effect from these treatments was neutropenia, which was the most pronounced with palbociclib[216,217,223]. The specific side effects from ribociclib were the elevation of liver enzymes and the prolongation of the QTc interval, which results in a need of ECG monitoring[217]. Finally, abemaciclib caused less neutropenia, but diarrhea was common, although it was manageable with lopestrogenamid[223].

The PALOMA-3 and MONALEESA-7 trials are the only CDK4/6 trials with OS results already published[224,225]. PALOMA-3 compared palbociclib and fulvestrant to placebo and fulvestrant, but the study was not powered to demonstrate significant OS differences between the treatment groups. The numerical gain in mOS with the palbociclib vs. placebo remained the same as the previously reported mPFS

difference (34.9 months vs. 28.0 months, HR 0.81, 95% CI 0.64-1.03, *p*=0.09)[224]. The OS results of premenopausal patients in the MONALEESA-7 trial were recently reported in ASCO 2019, and it is the first CDK4/6 inhibitor study with OS results for a treatment-naïve patient population in advanced breast cancer. The OS was significantly longer in patients treated with ribociclib and endocrine therapy compared to placebo and endocrine therapy, although the mOS was not yet reached (HR 0.71, 95% CI 0.54-0.95, *p*=0.0098)[225].

The OS results of the other first-line CDK4/6 trials for postmenopausal patients are eagerly awaited. However, based on the PFS and preliminary OS results, CDK4/6 inhibitors are recommended for the treatment of hormone receptor-positive mBC for either first-line treatment in combination with an AI or for a later line combined with fulvestrant if the patient has already been treated with another endocrine therapy[16].

2.3.4   Chemotherapy of mBC

Chemotherapy is recommended for mBC patients with either triple-negative disease, HER2-positive disease (see section 2.4.1), hormone receptor-positive disease with visceral crisis or endocrine-resistant disease[16]. The ESO-ESMO Advanced Breast Cancer consensus guideline defined visceral crisis as severe organ dysfunction, as assessed by signs and symptoms, laboratory studies and rapid progression of disease[16].

The era of more modern chemotherapy regimens began in 1976 when an Italian oncologist, Gianni Bonadonna, published his results on the efficacy of the chemotherapy regimen of cyclophosphamide, methotrexate and fluorouracil (CMF) as a treatment of early breast cancer[226]. The same Italian group was the first to describe the use of anthracyclines in mBC in 1969[227]. However, it was not until the 1990's that the first anthracycline-containing regimen of doxorubicin combined with cyclophosphamide (AC) became the gold-standard adjuvant treatment of early breast cancer[228]. Epirubicin was later introduced as a less cardiotoxic anthracycline than doxorubicin[229]. Paclitaxel's antitumor activity was described in 1971, and it was the first taxane to be introduced[230]. Paclitaxel was originally isolated from the bark of the Pacific yew tree *Taxus brevifolia,* and like other taxane-group compounds, it binds to microtubules and induces their stabilization by inhibiting their depolymerization, thereby leading to apoptosis[231]. However, the development of paclitaxel was slow due to poor availability of raw material and poor solubility, and only in 1995 did the

United States Food and Drug Administration (FDA) approve paclitaxel for the treatment of mBC. Paclitaxel was compared to doxorubicin and to their combination as first-line chemotherapy of mBC in a phase III trial published in 2003[232]. Response rate, median time to treatment failure (TTF) and OS were equivalent between paclitaxel and doxorubicin monotherapies. However, the combination of doxorubicin and paclitaxel had a higher response rate and longer mTTF than the monotherapies, but no survival benefit was observed[232]. Docetaxel is a semisynthetic taxane compound derived from needles of the European yew tree *Taxus baccata,* with a similar mechanism of action to paclitaxel[233]. Phase III trial results demonstrated improved survival and TTP with docetaxel compared to paclitaxel for treatment of mBC that had progressed after anthracycline-containing chemotherapy (mOS 15 vs. 13 months, $p=0.03$)[234]. However, hematologic and nonhematologic toxicities were more common for docetaxel 100 mg/m² every 3 weeks compared to paclitaxel 175 mg/m² every 3 weeks[234].

2.3.4.1   Taxanes as the treatment of mBC

Taxanes were explored under different dosages and treatment cycles for their efficacy and tolerability as treatment of mBC. Weekly paclitaxel 80 mg/m² was compared with paclitaxel 175 mg/m² every three weeks in a phase III trial[235]. This study also enrolled HER2-positive patients, and they were all treated with trastuzumab. Weekly paclitaxel was superior to every three weeks, with response rates of 42% and 29%, respectively ($p=0.004$). Additionally, TTP and OS were also longer for weekly paclitaxel compared to paclitaxel every three weeks (mTTP 9 vs 5 months, $p<0.001$, mOS 24 vs 12 months, $p=0.0092$, respectively)[235]. In a meta-analysis of approximately 1500 mBC patients, weekly paclitaxel administration resulted in lower response rates [risk ratio (RR) 1.20, $p<0.001$], similar PFS (HR 1.02, $p=0.860$) and longer OS (HR 0.78, $p=0.001$) compared to paclitaxel administration every three weeks[236]. Additionally, the incidence of severe neutropenia, febrile neutropenia and peripheral neuropathy were lower with weekly paclitaxel administration compared to paclitaxel every three weeks[236]. Higher doses (210 mg/m² or 250 mg/m²) of paclitaxel every three weeks did not improve patient survival or response rates and caused more neurotoxicity and hematologic toxicity compared to the standard paclitaxel dose of 175 mg/m² every 3 weeks[237]. Weekly docetaxel administration has been less extensively investigated than the administration of paclitaxel. One phase III trial randomized 118 patients patients to receive either docetaxel 35 mg/m² weekly for 3 consecutive weeks followed by one

week of rest or docetaxel 75 mg/m² every 3 weeks[238]. The response rates were higher with the 3-weekly regimen than with weekly docetaxel (36% vs 20%, respectively, p-value was not reported), but no difference was observed in PFS or OS between the groups (p=0.46 or p=0.34, respectively)[238]. In addition, the weekly docetaxel regimen was better tolerated[238].

Both weekly paclitaxel and weekly docetaxel are also treatment options for elderly or frail patients with mBC. They were effective for these patients, and the toxicity was mostly manageable[239]. The toxicity profiles of these compounds differed in that anorexia, stomatitis and edema were more common with docetaxel and neurotoxicity and myalgia with paclitaxel[239].

Nab-paclitaxel was also studied for the treatment of mBC, but its position in the treatment of mBC is still uncertain[16]. Nab-paclitaxel is an albumin-bound paclitaxel administered as a colloidal suspension of 130 nm particles. This formula allows the infusion of significantly higher doses of paclitaxel than the standard solvent-based paclitaxel, shorter infusion schedule and no need for premedication[240]. In a phase III trial with mostly chemotherapy-pretreated mBC patients, response rates were higher and mTTP longer with nab-paclitaxel compared to standard paclitaxel (response rates 33% vs. 19%, p=0.001; mTTP 23 vs 17 weeks, p=0.006)[241]. The incidence of grade 4 neutropenia was significantly lower with nab-paclitaxel than with standard paclitaxel (9% vs 22%, p<0.001), but grade 3 neuropathy was more common with nab-paclitaxel (10% vs 2%, respectively, p<0.001)[241]. The same study group consequently conducted a trial with nab-paclitaxel compared to docetaxel as a first-line treatment of mBC. The study patients were randomized to receive either nab-paclitaxel 300 mg/m² every 3 weeks, nab-paclitaxel 100 mg/m² weekly, nab-paclitaxel 150 mg/m² weekly or docetaxel 100 mg/m² every 3 weeks. The group with the highest nab-paclitaxel dose of 150 mg/m² had significantly longer PFS than the docetaxel-treated patients (15 vs 8 months, p=0.012). However, no significant differences between response rates or the PFSs of other nab-paclitaxel dosages and docetaxel were observed.[242] Another phase III trial compared weekly nab-paclitaxel 150 mg/m² to weekly paclitaxel or ixabepilone as first-line treatment of mBC, and additionally, all study patients received bevacizumab. Ixabepilone was inferior to weekly paclitaxel, and this treatment arm was closed for futility at the first interim analysis. No significant efficacy differences were observed between weekly paclitaxel and weekly nab-paclitaxel, with a trend favoring weekly paclitaxel (HR for PFS 1.20, p=0.054). Additionally, weekly paclitaxel was better tolerated than nab-paclitaxel, with a hematologic toxicity grade ≥3 in 22% of patients compared to 55% in the nab-paclitaxel group.[243]

2.3.4.2 Second or later line chemotherapy for mBC

Patients with disease progression after taxane and anthracycline-containing cytotoxic therapy can be considered for additional chemotherapy, and multiple regimens exist for later-line chemotherapy options of mBC. Capecitabine, vinorelbine, gemcitabine, and platinum salts, either alone or in multiple combinations, demonstrated efficacy for mBC patients with prior taxane and anthracycline treatment[16,41]. With single capecitabine, the response rate ranged from 14 to 29% and PFS from 3 to 6 months in phase II-III studies in the anthracycline- and taxane-pretreated mBC population[244]. The efficacy of vinorelbine as a monotherapy was quite similar to that of capecitabine, with response rate ranging between 12 and 26% and PFS between 3 and 4 months in the pretreated mBC population[244]. Additionally, vinorelbine with capecitabine enabled an all-oral combination with neutropenia as the dose-limiting toxicity and similar efficacy to intravenous vinorelbine with capecitabine[245,246]. Furthermore, alopecia was not a side effect for capecitabine or vinorelbine, which might be particularly important to some patients[244].

Eribulin, an inhibitor of microtubule dynamics, also offers a valid treatment option for mBC patients[247]. Eribulin-treated patients had significantly longer OS than the patients who were treated with chemotherapy by the investigator's choice in a heavily pretreated mBC population (mOS 13 vs 10 months, p=0.041). In this phase III trial (EMBRACE), all study patients were previously treated with taxane and anthracycline, and 70% of the patients were previously treated with capecitabine[247]. In a subsequent phase III trial, patients with prior taxane and anthracycline therapy were randomized to receive either eribulin or capecitabine, but no significant OS or PFS benefit was observed with eribulin (mOS 16 vs 15 months, p=0.056, mPFS 4 vs 4 months, p=0.30, respectively)[248].

Gemcitabine was studied in combination with variable chemotherapeutic regimens and in a meta-analysis of more than 8000 mBC patients. Adding gemcitabine to various chemotherapy options improved the response rate, PFS and OS at the price of mainly increased hematologic toxicity[249]. However, the improvement in survival was limited to first-line combinations, and no PFS or OS improvement was observed with later-line gemcitabine-containing regimens[249].

Ixabepilone is an FDA-approved antineoplastic agent that stabilizes microtubule dynamics, leading to cell apoptosis[250]. Ixabepilone in combination with capecitabine had superior efficacy compared to capecitabine alone in the treatment of mBC patients with prior taxane and anthracycline therapy (RR for OS 0.91, 95% CI 0.84-0.99, p=0.03; RR for PFS 0.79, 95% CI 0.74-0.85, p<0.001)[251]. However, as first-line

therapy, ixabepilone was less effective than paclitaxel, and as later-line treatment, no significant efficacy differences between ixabepilone and eribulin were observed[251].

2.3.4.3 Special considerations for the chemotherapy treatment of triple-negative mBC

Chemotherapy for TNBC is recommended by the same principles as for other subtypes of mBC[16,41]. However, platinum salts, cisplatin and carboplatin can offer additional efficacy for patients with TNBC. BRCA1 mutation causes impairment in DNA double-strand repair, and DNA repair may be altered through other mechanisms, e.g., somatic or germline mutations in other genes, DNA methylation or attenuated mRNA expression[252]. Platinum compounds cause the formation of DNA-platinum adducts and intra- and interstrand DNA crosslinks; therefore, the cells with underlying deficiencies in the DNA repair system may be exceptionally sensitive to platinum salts[253].

As first-line therapy for metastatic TNBC, cisplatin combined with gemcitabine was superior to paclitaxel combined with gemcitabine in a phase III trial (HR for PFS 0.69, 95% CI 0.52-0.92, p=0.009). However, the difference between their mPFSs was minimal and not clinically significant (mPFS 7.7 vs. 6.5 months)[254]. Carboplatin area under curve (AUC) 6 every 3 weeks as a single agent was also compared to docetaxel 100 mg/m² every 3 weeks in a first-line phase III trial (TNT) for mBC enrolling mainly TNBC patients. The overall response rates (ORRs) between carboplatin- and docetaxel-treated patients were similar (31% vs 34%, p=0.66), as were the PFSs and OSs (mPFS 3 vs 4 months, p=0.40, mOS 13 vs. 12 months, p=0.96, respectively)[255]. The TNT trial also enrolled 43 patients with germline BRCA1/2 mutations. The BRCA-mutated patients had significantly higher response rates with carboplatin compared to docetaxel (68% vs. 33%, p=0.03) and longer PFS (mPFS 6.8 vs 4.4 months, p=0.002), but no OS benefit could be demonstrated with this small patient population[255]. The efficacy of platinum salts as a treatment for mBC has been recently explored in a meta-analysis, which suggested a significant PFS benefit with platinum-containing regimens for patients with metastatic TNBC[256]. However, the meta-analysis also reported a significant improvement in the frequency of response and in OS with cisplatin or carboplatin, but this result was not related to the hormone receptor status[256].

2.3.5   PARP inhibitors for mBC patients with germline BRCA1/2-mutation

Investigation of PARP inhibitors for the treatment of mBC has focused on BRCA1/2-mutated patients. PARP is a nuclear enzyme that regulates cell survival through DNA repair (Figure 2)[257]. The PARP inhibitors olaparib, veliparib and talazoparib trap PARP proteins on damaged DNA, resulting in cytotoxic PARP-DNA complexes[258].
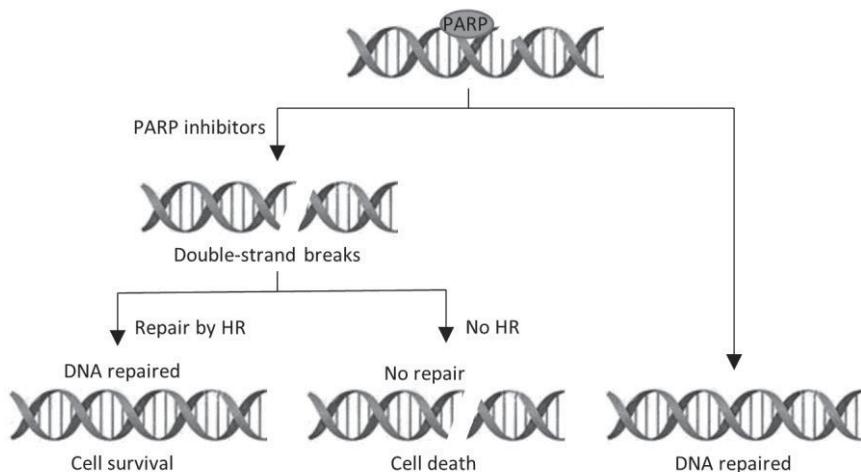
Figure 2.   Mechanism of action of PARP inhibitors in patients with BRCA-mutated tumors. Tumors with BRCA mutations have deficient homologous recombination repair pathways. HR: homologous recombination. Figure modified from Sonnenblick et al. 2015[259]

The phase III OlympiAD trial randomized mBC patients with germline BRCA mutations to receive either oral olaparib 300 mg twice daily or single-agent chemotherapy (capecitabine, vinorelbine or eribulin). PFS was significantly longer (mPFS 7 vs 4 months, $p<0.001$) and the response rate higher (60% vs. 29%) in patients treated with olaparib compared to chemotherapy[260]. In the entire patient population, no significant OS benefit was observed in olaparib-treated patients compared to patients treated with single-agent chemotherapy (HR for OS 0.90, p=0.513). However, the patients treated with olaparib as a first-line treatment had a significant 8-month improvement in mOS (23 vs 15 months, HR 0.51, p=0.02, respectively)[261].

The phase III EMBRACA trial had a similar treatment design as the OlympiAD trial. The patients with mBC and a germline BRCA1/2 mutation were randomized to receive talazoparib 1 mg orally once daily or single-agent chemotherapy

(capecitabine, eribulin, gemcitabine or vinorelbine). PFS was longer in patients treated with talazoparib compared to chemotherapy (9 vs 6 months, HR 0.54, $p<0.001$), and the response rate was higher (63% vs. 27%), but no OS benefit was demonstrated (HR 0.76, 95% CI 0.55-1.06, $p=0.11$)[262].

Veliparib was investigated for BRCA germline-mutated mBC patients in a phase II trial with three randomized treatment arms: veliparib + carboplatin/paclitaxel, veliparib + temozolomide, placebo + carboplatin/paclitaxel. No significant PFS or OS benefit was demonstrated (p-value for PFS 0.2, p-value for OS 0.16). However, the ORR was higher for patients treated with veliparib+carboplatin/paclitaxel (78%) compared to patients treated with placebo+carboplatin/paclitaxel (61%, $p=0.03$)[263].

PARP inhibitors were generally well tolerated, with grade 3-4 toxicity rates ranging from 26 to 37%, and treatment was discontinued less often than single-agent chemotherapy (5-6% vs. 8-9%, respectively)[260,262]. The most common grade ≥3 adverse events were hematologic: anemia, neutropenia and leukocytopenia. Additional side effects of all grades included nausea, diarrhea, vomiting, fatigue and headache.[260,262] In conclusion, taking into account the oral availability, good tolerability and the efficacy observed in phase III trials, olaparib and talazoparib are compelling treatment options for mBC patients with a germline BRCA1/2 mutation once reimbursed.

2.3.6   PI3K inhibitors

Phase III trial results of the efficacy of PI3K inhibitors were recently published. Approximately 40% of mBC patients with hormone receptor-positive and HER2-negative disease have activating PIK3CA mutations [252,264]. One of the mechanisms of resistance to endocrine therapy is the aberrant activation of PI3K signaling[210].

SOLAR-1 was a phase III study of the efficacy of an oral PI3K inhibitor, alpelisib, in hormone receptor-positive mBC patients with prior AI treatment[265]. The study patients were randomized to receive either alpelisib 300 mg daily with fulvestrant or placebo with fulvestrant. The primary end-point, PFS, in the cohort of PIK3CA-mutated cancer was significantly improved in patients treated with the combination of alpelisib and fulvestrant compared to placebo with fulvestrant (mPFS 11 vs. 6 months, HR 0.65, 95% CI 0.50-0.85, p<0.001). The most common adverse events related to alpelisib were hyperglycemia, gastrointestinal side effects and rash.[265]

Buparlisib is another oral PI3K inhibitor that was evaluated in the phase III BELLE-2 and BELLE-3 trials. BELLE-2 enrolled hormone receptor-positive mBC

patients with prior AI therapy, and the patients were randomized to receive fulvestrant with either buparlisib 100 mg daily or placebo. In the entire study population, mPFS was significantly longer in buparlisib-treated patients than in the placebo group (mPFS 7 vs 5 months, HR 0.80, 95% CI 0.68-0.94, p=0.0033). In the ctDNA PIK3CA mutant cohort, mPFS was also significantly longer in the buparlisib group than in the placebo group (mPFS 7 vs. 3 months, HR 0.58, 95% CI 0.41-0.82, p=0.001). However, 23% of the patients treated with buparlisib combined with fulvestrant had serious adverse events: increased liver enzymes, hyperglycemia and rash. The high toxicity rates led to the decision for no further studies to be continued with this combination.[266] On the other hand, BELLE-3 enrolled hormone receptor-positive, HER2-negative mBC patients with disease progression and who were treated or had been treated with an mTOR inhibitor. The mPFS improvement in patients treated with buparlisib and fulvestrant was modest compared to the mPFS in patients treated with placebo and fulvestrant (mPFS 4 vs. 2 months, HR 0.69, 95% CI 0.53-0.84, p=0.00030). Similarly, in the BELLE-3 trial, the frequency of serious adverse events was high (22%), and the most common grade $\geq$3 side-effects were increased liver enzymes (19%) and hyperglycemia (12%).[267]

The phase III SANDPIPER trial enrolled patients with hormone receptor-positive, HER2-negative advanced breast cancer and a PIK3CA mutation. The study patients were all previously treated with an AI. Patients were randomized to receive fulvestrant with the PI3K inhibitor taselisib 4 mg orally per day or with a placebo. The mPFS was longer in the taselisib group than in the placebo group (mPFS 7 vs. 5 months, HR 0.70, p=0.0037). The most common $\geq$ grade 3 adverse events were gastrointestinal toxicities and hyperglycemia. Dose discontinuations and reductions were more common in patients treated with taselisib and fulvestrant than placebo and fulvestrant (17% vs 2% and 37% vs 2%, respectively)[268].

Although alpelisib, buparlisib and taselisib are all PI3K inhibitors, their specificity to target receptors varies. PI3Kα, PI3Kβ and PI3Kγ are enzyme isoforms of PI3K[269]. Buparlisib is a pan-PI3K inhibitor, taselisib is a β-sparing PI3K inhibitor, and alpelisib targets specifically PI3Kα[265–268]. The specificity differences between the agents may explain the differences in the adverse events (grade 3 hyperglycemia: alpelisib 37%, buparlisib 15% and taselisib 10%). In addition, the numerical PFS improvement was longer with alpelisib (+5 months) than with buparlisib and taselisib (+2 months)[265–268]. Therefore, alpelisib is the most promising PI3K inhibitor for the future treatment of secondary hormone-resistant mBC with a PIK3CA mutation.

2.3.7   Immunotherapeutic treatment options for mBC

As for many other malignancies, immunotherapy has also been investigated for the treatment of mBC. Tasuku Honjo's group at Kyoto University identified and cloned the programmed death-1 (PD-1) receptor in the 1990's, and for this discovery, the Nobel prize in physiology and medicine was awarded to Tasuku Honjo in 2018[270,271]. Since the discovery of the PD-1 receptor, multiple PD-1 receptor- and programmed death ligand-1 (PD-L1) receptor-targeting antibodies have been investigated, e.g., for the treatment of melanoma, non-small cell lung cancer and renal cell carcinoma[272–274]. The interaction of PD-1 receptor with its ligand PD-L1 inhibits T-cell activation. This tumor-related immune suppression can be prevented by using PD-1 or PD-L1 inhibitors.[270,275–277]

Atezolizumab is a PD-L1 inhibitor that was investigated in the phase III IMpassion130 trial in combination with nab-paclitaxel as a treatment for advanced treatment naïve TNBC. The trial was randomized and placebo-controlled. The most significant result of the IMpassion130 trial was the long OS improvement in patients with PD-L1-positive tumors treated with the combination of atezolizumab and nab-paclitaxel (mPFS 25 vs 15.5 months, HR 0.62, 95% CI 0.45-0.86). Additionally, PFS was longer in patients treated with the combination of atezolizumab and nab-paclitaxel compared to patients treated with placebo and nab-paclitaxel (mPFS 7.2 vs 5.5 months, HR 0.80, 95% CI 0.69-0.92, $p=0.002$; PD-L1-positive patients: mPFS 7.5 vs. 5.0 months, respectively, HR 0.62, 95% CI 0.49-0.78, $p<0.001$).[278] The OS improvement by 9.5 months led to the FDA approval of atezolizumab for the treatment of advanced TNBC with tumor tissue PD-L1 expression ≥ 1%[279].

IMpassion130 is the only phase III immunotherapy trial on mBC with published results. Otherwise, only phase I-II data exist from PD-1/PD-L1 inhibitors for mBC treatment. Pembrolizumab, avelumab and atezolizumab were all investigated as single-agents for mBC treatment. In the Keynote-086 trial, 170 patients with advanced TNBC and prior taxane and anthracycline treatment were enrolled in the study. The ORR was only 5.3%, and the mOS was 9 months. However, the responses to pembrolizumab were durable: the median duration of response was not reached, and 63% of responses continued ≥ 12 months.[280] Atezolizumab as a single agent for advanced TNBC patients demonstrated similarly long responses to immunotherapy: the median duration of response was 21 months in the 10% of the study patients who responded to atezolizumab[281]. The phase Ib JAVELIN study enrolled heavily pretreated HER2-negative patients regardless of the hormone receptor status and evaluated the single-agent activity of the PD-L1 antibody

avelumab. The ORR in the triple-negative subgroup was 5.2%. However, only 2 responses (2%) were observed in 110 hormone receptor-positive patients in the study.[282]

2.3.8 HER2-positive mBC

2.3.8.1 First-line treatment of HER2-positive advanced breast cancer

HER2-targeted therapy is recommended for patients with HER2-positive mBC[16]. The CLEOPATRA trial demonstrated a substantial OS benefit with the pertuzumab-trastuzumab-docetaxel combination as first-line therapy compared to the previous standard therapy, trastuzumab-docetaxel (mOS 56.5 months vs. 40.8 months, HR 0.68; 95% CI 0.56 to 0.84, $p<0.001$)[283]. This has become the new standard first-line therapy for patients able to tolerate taxane[16,41]. The ORR was 80% and the mPFS 19 months in patients who received pertuzumab-trastuzumab-docetaxel combination therapy in the CLEOPATRA trial. Pertuzumab did not increase cardiac toxicity compared with trastuzumab-docetaxel. However, diarrhea and rash were more common in pertuzumab-treated patients compared to the control group.[283]

Other chemotherapy regimens were also investigated as first-line therapies in combination with pertuzumab and trastuzumab. Phase II studies with weekly paclitaxel combined with pertuzumab and trastuzumab and vinorelbine intravenously (i.v.) combined with pertuzumab and trastuzumab resulted in ORRs of 59% and 74%, respectively[284,285]. The mPFS of the weekly paclitaxel triplet combination was 19.5 months (95% CI 14-26 months) and the vinorelbine triplet combination 14.3 months (95% CI 11.2-17.5 months)[284,285]. These combinations may be an option for patients unable to tolerate docetaxel, since the side effects were more tolerable with weekly paclitaxel and vinorelbine triplet therapies[284,285].

The results of the phase IIIb PERUSE trial focusing on the safety of the pertuzumab-trastuzumab-taxane combination were published in February 2019[286]. Altogether, 1436 study patients received pertuzumab and trastuzumab at standard doses with either docetaxel (n=775), paclitaxel (n=589) or nab-paclitaxel (n=65) by the investigator's choice. The adverse events were consistent with the results of the CLEOPATRA trial. Some differences in the incidence of specific adverse events existed, according to the taxane compound: all-grade neuropathy was more common with paclitaxel than with docetaxel (31% vs 16%), febrile neutropenia and mucositis

were less common with paclitaxel compared to docetaxel (1% vs. 11% and 14% vs. 25%, respectively). The ORRs for different taxanes were similar: 79% for pertuzumab-trastuzumab-docetaxel, 83% for pertuzumab-trastuzumab-paclitaxel and 77% for pertuzumab-trastuzumab-nab-paclitaxel. The same was true of the mPFSs (20 months, 23 months and 18 months, respectively).[286]

T-DM1 was also investigated as first-line therapy of HER2-positive mBC. The phase III MARIANNE randomized patients to receive either the trastuzumab-taxane combination, T-DM1 or pertuzumab combined with T-DM1[287]. There were no statistically significant differences in ORR or PFS between the three treatment groups, although T-DM1 was better tolerated[287].

2.3.8.2 Endocrine therapy for HER2-positive mBC

Endocrine therapies can be used in combination with HER2-targeted therapies in patients with hormone receptor-positive and HER2-positive breast cancer. In the subgroup analysis of the CLEOPATRA trial, the patients with nonvisceral disease did not have a statistically significant improvement in PFS or OS[283]. According to the treatment guidelines of breast cancer, patients with hormone receptor-positive HER2-positive mBC can be considered for first-line endocrine therapy with HER2-targeted therapy if the tumor burden is minimal and there are no signs of rapid disease progression[16,41].

The PERTAIN trial randomized postmenopausal patients to trastuzumab and an AI with or without pertuzumab as first-line treatment of hormone receptor- and HER2-positive mBC. The mPFS was longer in patients treated with the triplet combination compared to the duplet combination (mPFS 18.9 months vs. 15.8 months, p=0.007)[288]. However, half of the PERTAIN study patients had received induction chemotherapy with a taxane for 18 to 24 weeks before the initiation of endocrine therapy[288]. The PERTAIN study reinforces the current practice of adding endocrine therapy to pertuzumab and trastuzumab maintenance therapy after discontinuation of the first-line taxane treatment[16].

2.3.8.3 Second and later-line treatment options for HER2-positive mBC

The phase III EMILIA trial enrolled patients who had previously received trastuzumab and a taxane. Patients were randomized to either T-DM1 or lapatinib-capecitabine. The ORR was 10% higher in the T-DM1 group than in the lapatinib-

capecitabine group (44% vs. 31%, respectively). The mPFS was 9.6 months in T-DM1-treated patients compared to 6.4 months in patients treated with lapatinib and capecitabine. The mOS was approximately five months longer in the T-DM1 group (HR 0.68, 95% CI 0.55-0.85, $p<0.001$)[289]. EMILIA also enrolled patients with brain metastases, and survival data for these patients were retrospectively analyzed[290]. The mPFS was quite similar between the T-DM1 and lapatinib-capecitabine groups (5.9 months vs. 5.7 months). However, the T-DM1-treated patients had a significantly longer mOS of 26.8 compared to 12.9 months in lapatinib-capecitabine-treated patients (HR 0.38, 95% CI 0.18-0.80, $p=0.0081$)[290]. More heavily pretreated patients were enrolled in the TH3RESA trial: they had at least two previous HER2-targeted treatment lines, including trastuzumab, lapatinib and taxane[291]. In this pretreated patient population, T-DM1-treated patients had a 7-month-longer mOS compared to the physician's choice chemotherapy group (HR 0.68, 95% CI 0.54-0.85, $p=0.0007$). No T-DM1 trials have been conducted in pertuzumab-trastuzumab dual blockade-treated patients. However, since the OS benefit with T-DM1 in second- and later-line trials is clear, T-DM1 is considered a standard second-line treatment for metastatic HER2-positive breast cancer[16,41].

Lapatinib was also investigated as a treatment of metastatic HER2-positive breast cancer. As first-line therapy, mPFS was shorter with the lapatinib-taxane combination than with the trastuzumab-taxane combination (mPFS 9.1 months vs. 13.6 months, $p<0.001$)[292]. For patients previously treated with anthracycline, taxane and trastuzumab, adding lapatinib to capecitabine resulted in a modest two-month improvement in mPFS compared to capecitabine alone (mPFS 6.2 months vs. 4.3 months, $p<0.001$), and no OS benefit was observed[293]. However, the ORR for patients with brain metastases was 29.2% with the lapatinib-capecitabine combination according to a meta-analysis of 800 patients, suggesting that patients with CNS involvement might benefit from this combination[294]. Dual blockade with trastuzumab and lapatinib for patients previously treated with a trastuzumab-containing regimen resulted in modest but statistically significant improvement in PFS (by three weeks) compared to lapatinib alone (mPFS 11.1 weeks vs. 8.1 weeks, $p=0.011$). However, the combination of the two HER2-targeted therapies demonstrated a survival benefit (mOS 14 months vs. 9.5 months, HR 0.74, 95% CI 0.57-0.97, $p=0.026$)[295]. The combination of trastuzumab and lapatinib was also studied in the phase III ALTERNATIVE trial combined with an AI in postmenopausal patients previously treated with endocrine therapy, trastuzumab and a chemotherapy[296]. The mPFS was superior with the trastuzumab-lapatinib-AI combination compared to trastuzumab-AI (mPFS 11 months vs. 5.7 months,

p=0.0064). Additionally, patients treated with the lapatinib-AI combination had a longer mPFS than patients treated with the trastuzumab-AI combination (mPFS 8.3 months vs. 5.7 months, p=0.0361)[296]. To conclude, lapatinib can be used as a treatment of mBC in combination with capecitabine or trastuzumab. However, the improvement in patient survival with T-DM1 has moved lapatinib combinations to the role of later-line treatments of advanced HER2-positive breast cancer[16].

2.3.9   Bevacizumab as a treatment of advanced breast cancer

2.3.9.1   First-line treatment of mBC with bevacizumab

Several phase III first-line trials were conducted for bevacizumab-chemotherapy combinations as treatments of mBC (Table 4). The E2100, AVADO, RIBBON-1 and TURANDOT trials evaluated the efficacy of bevacizumab-taxane combinations. In the E2100 trial, the patients treated with the bevacizumab-paclitaxel combination had a higher ORR and longer mPFS than the patients treated with paclitaxel alone[297]. In the AVADO trial, the patients treated with a higher bevacizumab dose had a significantly longer mPFS than the patients treated with the placebo-docetaxel combination. However, there were no statistically significant differences in PFS between bevacizumab 7.5 mg/kg combined with docetaxel and placebo-docetaxel.[298] The ORR were higher in bevacizumab-containing treatment arms. The RIBBON-1 trial randomized patients to either placebo with chemotherapy or bevacizumab with chemotherapy. The chemotherapy was chosen by the investigator, and the available options were capecitabine, a taxane or an anthracycline. As above, bevacizumab-containing treatment arms had a longer mPFS than the placebo groups.[299] The TURANDOT trial compared two bevacizumab-containing treatment arms: bevacizumab-paclitaxel and bevacizumab-capecitabine. The objective of the study was to demonstrate noninferior OS with the bevacizumab-capecitabine combination versus the bevacizumab-paclitaxel combination. The bevacizumab-capecitabine-treated patients had a noninferior mOS compared to bevacizumab-paclitaxel-treated patients. However, the mPFS was significantly shorter in bevacizumab-capecitabine-treated patients than in bevacizumab-paclitaxel-treated patients.[300]

No OS benefit was demonstrated in any of these phase III first-line bevacizumab trials (Table 4)[297–301]. The first-line bevacizumab treatment improved the mPFS by 2-6 months, and response to treatment was 11-18% more frequent[297–299]. However, because of the lack of OS benefit, bevacizumab plus chemotherapy is recommended

only for the treatment of selected patients with high tumor burden and therefore a higher need for tumor response[16,41].

Table 4.   Phase III trials with bevacizumab as first-line treatment of HER2-negative metastatic breast cancer

Trial Author, year	n	Treatment arms	RR	Median PFS	Median OS
E2100 Miller et al. 2007[297]	722	BEV 10 mg/kg d1, d15 + PAC90 Q4W PAC90 Q4W	37% 21%	11.8 months 5.9 months **$p<0.001$**	26.7 months 25.2 months $p=0.16$
AVADO Miles et al. 2010[298]	736	BEV 7.5 mg/kg + DOC100 Q3W BEV 15 mg/kg + DOC100 Q3W Placebo + DOC100 Q3W	55% 64% 46%	9.0 months 10.1 months 8.2 months $p^1=0.12$, **$p^2=0.006$**	30.8 months 30.2 months 31.9 months $p^1=0.72$, $p^2=0.85$
RIBBON-1 Robert el al. 2011[299]	1237	BEV 15 mg/kg Q3W + CAP Placebo + CAP BEV 15 mg/kg Q3W + TX/ANT Placebo + TX/ANT	35% 24% 51% 38%	8.6 months 5.7 months 9.2 months 8.0 months **$p^3<0.001$, $p^4<0.001$**	NR NR NR NR $p^3=0.27$, $p^4=0.83$
TURANDOT Zielinski et al. 2016[300]	564	BEV 10 mg/kg d1, d15 + PAC90 Q4W BEV 15 mg/kg + CAP Q3W	44% 27%	10.9 months 8.1 months **$p=0.0066$**	30.2 months 26.1 months $p=0.007$
MERiDiAN Miles et al. 2017[301,302]	481	BEV 10 mg/kg d1, d15 + PAC90 Q4W Placebo + PAC 90 Q4W	54% 33%	11.0 months 8.8 months **$p=0.0007$**	28.8 months 25.8 months $p=0.59$

n: number of patients; BEV10: bevacizumab 10 mg/kg; d: day; PAC90: paclitaxel 90 mg/m² on days 1,8 and 15; Q2W: every two weeks; Q3W: every three weeks; Q4W: every 4 weeks; m: months; HR: hazard ratio; DOC100: docetaxel 100 mg/m²; CAP: capecitabine; TX: taxane; ANT: Anthracycline; RR: response rate; PFS: progression-free survival; p^1: BEV7.5 vs. placebo; p^2: BEV15 vs. placebo; p^3: BEV+CAP vs. placebo+CAP; p^4: BEV+TX/ANT vs. placebo+TX/ANT; OS: overall survival; NR: not reported

MERiDiAN had a similar study design as E2100 trial, and the efficacy results were similar (Table 4)[302]. One objective of the MERiDiAN trial was to evaluate baseline plasma VEGF-A level as a predictive biomarker. However, the baseline plasma VEGF-A level did not identify the patients with the most benefit from bevacizumab (plasma VEGF-A high vs. low, interaction p-value 0.46)[302].

The ATHENA trial was an open-label single-arm study that evaluated the efficacy and tolerability of bevacizumab-based regimens as first-line treatments of mBC. Altogether, 2251 patients were enrolled in the study, and most study patients were treated with a bevacizumab-taxane combination. The mTTP in the ATHENA trial

was 9.5 months, which was quite similar to those of phase III trials with bevacizumab, and no new toxicity concerns were raised[303].

Taxanes and capecitabine were the most extensively investigated cytotoxic regimens in combination with bevacizumab, but other chemotherapy combinations were also studied. Nab-paclitaxel was not superior to bevacizumab-paclitaxel, with mPFSs of 9.3 months and 11 months, respectively (HR 1.20, 95% CI 1.00-1.45, p=0.054)[243]. Adding intravenous vinorelbine to bevacizumab-capecitabine did not prolong PFS compared to the bevacizumab-capecitabine combination (HR for PFS 0.84, 95% CI 0.70-1.01, p=0.058)[304].

Bevacizumab for the treatment of HER2-positive mBC was studied in the randomized phase III AVEREL trial[305]. The study patients were treated with trastuzumab-docetaxel with or without bevacizumab as first-line therapy[305]. Bevacizumab did not improve patient survival (mPFS 17 months in the bevacizumab group and 14 months in the non-bevacizumab group, HR 0.82, 95% CI 0.65-1.02, p=0.775) or response rate (74% vs. 70%, respectively, p=0.35)[305].

2.3.9.2   Bevacizumab as second-line therapy for mBC

Bevacizumab was also studied as a second- or later-line therapy. RIBBON-2 included patients with one previous cytotoxic treatment for mBC. Patients received chemotherapy with capecitabine, gemcitabine, vinorelbine or a taxane and were randomized to receive either bevacizumab or placebo. Median PFS improvement by two months was observed in patients in the bevacizumab-chemotherapy arm (HR 0.78. 95% CI 0.64-0.93, p=0.007).[306] RIBBON-2 included 112 patients with TNBC, and the efficacy data for these patients were retrospectively analyzed. In this triple-negative subgroup, the PFS improvement was more pronounced with bevacizumab (mPFS 6 months vs. 3 months, HR 0.50, p=0.0006).[307]

Another phase III trial (AVG2119g) evaluated capecitabine with or without bevacizumab in mBC patients previously treated with 1-2 chemotherapy regimens for metastatic disease. The ORR for patients treated with the bevacizumab-capecitabine combination was 20%, and the ORR for the capecitabine group was 9% (p=0.001). However, no significant differences in PFS or OS were observed.[308] The phase III TANIA trial enrolled patients previously already treated with first-line bevacizumab-containing chemotherapy. The study patients were randomized to either continue bevacizumab with second-line chemotherapy or receive chemotherapy treatment alone. Continuing bevacizumab resulted in a statistically significant but modest PFS improvement (mPFS 6.3 months vs. 4.2 months, HR

0.75, 95% CI 0.61-0.93, p=0.007).[309] Additionally, the continuation of bevacizumab was possible with third-line chemotherapy, but this did not improve survival[310]. As in other later-than-first-line studies, no OS benefit was demonstrated[310].

2.3.9.3　Maintenance therapy with bevacizumab

Bevacizumab as a maintenance therapy after first-line chemotherapy was also studied[311,312]. The HER2-negative hormone receptor-positive patients in the phase III randomized AROBASE trial were previously treated with first-line bevacizumab-taxane for 16-24 weeks. The patients with nonprogressive disease were randomized to either continuation with bevacizumab-taxane or maintenance bevacizumab with exemestane. The Independent Data and Monitoring Committee recommended terminating the patient enrollment because the probability of reaching a statistically significant improvement in PFS by the end of the study was only 7% at that point[311]. The HR for PFS was 1.0 for bevacizumab-taxane compared to bevacizumab-exemestane (95% CI 0.7-1.5, p=0.998).[311] Maintenance bevacizumab was compared to bevacizumab-capecitabine for nonprogressive patients previously treated with first-line bevacizumab-docetaxel for 3-6 cycles in the phase III IMELDA trial. Bevacizumab-capecitabine improved PFS and OS compared to bevacizumab alone (mPFS 12 months vs. 4 months, HR 0.38, 95% CI 0.27-0.55, p<0.001, mOS 39 months vs. 24 months, HR 0.43, 95% CI 0.26-0.69, p=0.003).[312] In conclusion, the IMELDA trial suggested that bevacizumab maintenance therapy without endocrine therapy or chemotherapy was not considered as an effective therapy choice.

2.3.9.4　Bevacizumab combined with endocrine therapy

Postmenopausal patients with hormone receptor-positive advanced breast cancer were enrolled in two phase III studies focusing on first-line endocrine therapy with bevacizumab. The LEA trial evaluated endocrine therapy (letrozole or fulvestrant) with or without bevacizumab as first-line treatment of hormone receptor-positive mBC. Neither mPFS nor mOS was superior in the bevacizumab-containing treatment arm.[313] Similarly, the CALGB 40503 trial enrolled postmenopausal patients with hormone receptor-positive advanced breast cancer, and they were treated with letrozole with or without bevacizumab. Bevacizumab with letrozole prolonged PFS compared to letrozole alone (mPFS 20 months vs. 16 months, HR 0.75, 95% CI 0.59-0.96, p=0.016). No significant OS difference was observed.[314]

2.3.9.5   Adverse events related to bevacizumab

Bevacizumab increases treatment toxicity, but adverse events are mostly grade 1-2 and are manageable. The specific adverse events related to bevacizumab include hypertension, proteinuria, bleeding and thromboembolic events[297–299,306].

Hypertension of at least grade 3 was observed in 4-15% of the mBC patients treated with bevacizumab, and it was the most common bevacizumab-related toxicity[297–299,306,315]. Early hypertension during bevacizumab treatment was also linked to longer PFS in some studies, but overall, it did not predict clinical benefit[316,317].

Proteinuria was also quite common, but grade ≥3 proteinuria was reported in only 2.0-3.6% of the patients in the phase III trials focusing on the treatment of mBC[297–299,306]. Bleeding, primarily epistaxis, of all grades was observed in up to half of the bevacizumab-treated patients, but severe bleeding was rare[297–299,306]. Febrile neutropenia and sensory neuropathy were also more common in bevacizumab-treated patients[318]. However, thromboembolic events and gastrointestinal perforations were not significantly more common in phase III breast cancer trials[319]. Additionally, grade 3-4 cardiovascular side effects, mainly left ventricular systolic function, were reported more often in bevacizumab-treated patients, with a frequency of 1-6% of the bevacizumab-treated patients[297–299,306,319].

A meta-analysis of 20 000 patients treated with bevacizumab focusing on cardiovascular toxicities demonstrated an increased risk for cerebral ischemia (RR 3.11, p=0.003), arterial adverse events (e.g., myocardial ischemia, RR 1.37, p=0.004) and venous adverse events (e.g., venous thromboembolism, RR 1.29, p<0.001)[320]. Fatal adverse events related to bevacizumab were also reported. However, fatal events were not more common for mBC patients treated with bevacizumab than for patients treated with chemotherapy alone[321]. Hypertension and proteinuria were more commonly reported in older breast cancer patients ≥ 70 years old treated with bevacizumab-chemotherapy combination than in younger patients[322]. For patients treated with bevacizumab plus endocrine therapy, the treatment toxicity was more common in older patients (> 65 years) and in patients with other comorbidities: impaired vision or lower physical function[323].

2.4 Prognostic circulating markers for advanced breast cancer

2.4.1 VEGF-system and biomarkers for bevacizumab

Oxygen supply to the tumor is essential for the malignant tissue to proliferate. Therefore, angiogenesis plays a key role in tumorigenesis. A schematic illustration of the tumor angiogenesis pathways under hypoxemic conditions is presented in Figure 3. Under hypoxemic conditions in the tumor tissue, hypoxia-inducible factor-1 alpha (HIF-1α) is upregulated, leading to an increase in the transcription of numerous genes involved in angiogenic processes, e.g., VEGFs, fibroblast growth factor 1 (FGF-1), platelet-derived growth factor (PDGF), MMPs, angiopoietins 1 and 2 (Ang1 and Ang2) and Tie2[324,325]. HIF-1α translocates to the nucleus from the cytoplasm during hypoxia and forms a complex with HIF-1β. The complex binds to hypoxia-response elements (HREs), which are located in regulatory sites of HIF target genes[326]. Overexpression of HIF-1α is common in primary tumors and in metastases, and it is associated with poor patient outcome in multiple malignancies[326,327]. As hypoxia is the inducer of tumor angiogenesis, hypoxic or anoxic conditions are noted in 50-60% of solid tumors[328].
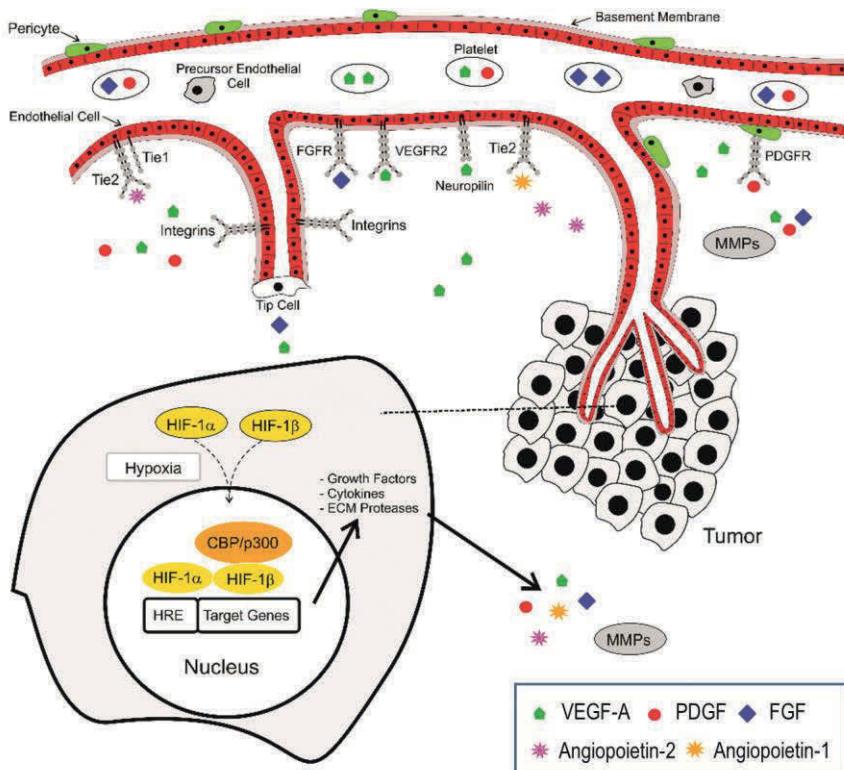
Figure 3. Angiogenic signaling in the hypoxemic tumor environment. Tumor cells react to tissue hypoxia and secrete angiogenetic growth factors and cytokines to promote blood vessel sprouting. ECM: Extracellular matrix; FGF: Fibroblast growth factor; FGFR: Fibroblast growth factor receptor; HIF-1: Hypoxia-inducible factor-1; HRE: Hypoxia-response element; MMPs: Matrix metalloproteinases; PDGF: Platelet-derived growth factor; PDGFR: Platelet-derived growth factor receptor; VEGF-A: Vascular endothelial growth factor; VEGFR2: Vascular endothelial growth factor receptor 2. Figure created by Jukka Lehtiniemi, Tampere University

As a consequence of the release of proangiogenic growth factors, a process known as 'angiogenic switch' is triggered[20]. Growth factors induce chemotaxis of endothelial cells and vessel sprouting towards the hypoxemic tumor, resulting in an atypical morphology and poor functioning of the vasculature[20,329]. During tumor angiogenesis, proangiogenic pathways are upregulated compared to physiological conditions[330]. Tip cells are the cells that lead the sprouting vessels, and they do not proliferate themselves. Tip cells direct the migration and form the connections between the sprouting vessels. Proliferating endothelial stalk cells are the adjacent cells behind the tip cells.[331]

The VEGF family has a key role in tumor angiogenesis. The family consists of five growth factors (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF)) and their receptors (VEGFR-1, VEGFR-2, VEGFR-3, neuropilin-1 and neuropilin-2)[332]. VEGFs differ in their physiological functions due to differences in the binding specificities to VEGF receptors[333]. VEGF-A is the most widely investigated and best-characterized member of the VEGF family. High intratumoral VEGF-A expression is observed in many tumor types, including breast cancer, gastrointestinal tract malignancies, kidney cancer, ovarian cancer and lung cancer, among others[334]. In response to hypoxia via HIF-1 signaling, VEGF-A is synthetized and secreted by multiple cells in the tumor microenvironment: fibroblasts, inflammatory cells including tumor associated macrophages (TAMs). It is also secreted by autocrine secretion by tumor cells themselves[328,335]. The main biological functions of VEGF-A include vascular endothelial cell proliferation and survival, activation of enzymes that are involved in extracellular matrix degradation, increased vascular permeability, chemotaxis for macrophages and mobilization of endothelial precursor cells from bone marrow[336]. VEGF-A binds with higher affinity to the tyrosine kinase receptor VEGFR-1 than to VEGFR-2. However, the kinase activity of VEGFR-1 is less than that of VEGFR-2, and therefore, the biological effects of VEGF-A are mediated mainly by VEGR-2[332]. On some cell types, VEGF-A also interacts with neuropilin-1, which can enhance the interaction of VEGF-A and VEGFR-2[332,337].

Other pathways significantly influence tumor angiogenesis. The delta-like 4 (Dll-4)-Notch pathway regulates tumor angiogenesis and is upregulated in tumor vasculature[338]. Additionally, hypoxia can cause MET receptor overexpression. The MET pathway can increase tumor proliferation through multiple pathways, including RAS, PI3K, and STAT3. VEGF-A stimulation through VEGFR-2 regulates MET signaling. Additionally, the interaction of neuropilin-1 and the MET receptor increases the proliferation and survival of tumor cells. [326]

Multiple prognostic and predictive circulating markers were evaluated with the aim of identifying the patients who would most benefit from bevacizumab (Table 5). In the AVADO trial, high VEGF-A was associated with shorter PFS among patients given bevacizumab 7.5 mg/kg (p=0.01)[339]. Therefore, the phase III MERiDiAN trial was conducted to evaluate high VEGF-A level as a prospective biomarker for bevacizumab benefit. However, MERiDiAN failed to prove a high baseline plasma VEGF-A level was applicable in selecting patients who would benefit most from bevacizumab (Table 5).[301,302] CTCs ≥ 3/7.5 ml were prognostic for shorter TTP (p<0.05). However, the study group explored different cut-off levels

for CTCs, and none of the other thresholds was predictive of TTP. Circulating endothelial cells (CECs) were also evaluated as prognostic markers, but CECs were not prognostic for a bevacizumab benefit at any threshold.[340] VEGF-A gene polymorphisms were analyzed from a baseline blood sample by PCR in the MO19391 trial. Of the several polymorphisms tested, only 926 C > T was associated with shorter TTP (p=0.02, Table 5).[341]

Extensive effort has been devoted to the search for tissue and genetic biomarkers for bevacizumab-treated patients, but none of these demonstrated its potential as a predictive biomarker for clinical use[342]. High expression of VEGFR-3 was associated with more frequent responses (p=0.038), and high expression of VEGFR-1 was associated with poor survival (p=0.025) in mBC patients treated with bevacizumab-containing chemotherapy in a small retrospective analysis[343]. However, the above-mentioned results were not validated in a larger patient cohort. VEGF-A single-nucleotide polymorphisms (SNPs) as prognostic markers were evaluated in 363 tumor blocks of patients who participated in the E2100 trial[344]. The VEGF-2578 AA and VEGF-1154 A genotype were associated with improved OS compared with the alternate genotype combination (p=0.023 and p=0.001, respectively)[344]. The same study group also reported that VEGF-A amplification was associated with worse OS (p=0.08).

From the extensive retrospective biomarker evaluation, plasma VEGF-A levels were considered the most promising prognostic marker for bevacizumab efficacy. This hypothesis was tested prospectively in the MERiDiAN trial as reported above (Table 5)[302]. As baseline plasma VEGF-A level did not help identify the patients with the most benefit from bevacizumab according to the MERiDiAN trial, no suitable prognostic marker for clinical use currently exists.

Table 5.   Circulating prognostic and predictive markers for bevacizumab benefit in breast cancer patients

Marker	Author, year	Study design	n	Result
VEGF-A high	Miles 2013[339]	AVADO, phase III, mBC BEV + DOC	396	BEV 7.5 mg/kg: HR for PFS 0.52 (95% CI 0.33-0.81) **Interaction *p*=0.01** BEV 15 mg/kg: HR for PFS 0.49 (95% CI 0.31-0.76) Interaction *p*=0.08
	Gianni 2013[305]	AVEREL, phase III, mBC BEV + DOC + trastuzumab	162	HR for PFS 0.70 (95% CI 0.43-1.14) Interaction *p*=0.80
	Cameron 2013[345]	BEATRICE, phase III Stage I-III TNBC Adjuvant BEV	1178	Third quartile cut-off: HR for DFS 0.64 (0.35-1.16) Interaction *p*=0.36
	Miles 2017[302] Miles 2018[301]	MERiDiAN, phase III, mBC BEV + PAC	481	HR for PFS 0.64 (95% CI 0.47-0.88) Interaction *p*=0.46 HR for OS 0.85 (95% CI 0.63-1.14), *p*=0.27
VEGFR-2 high	Miles 2013[339]	AVADO, phase III, mBC BEV + DOC	396	BEV 7.5 mg/kg: HR for PFS 0.46 (95% CI 0.28-0.74) **Interaction *p*=0.03** BEV 15 mg/kg: HR for PFS 0.54 (95% CI 0.35-0.85) Interaction *p*=0.25
	Cameron 2013[345]	BEATRICE, phase III Stage I-III TNBC Adjuvant BEV	1178	HR for DFS 0.61 (0.39-0.97) **Interaction *p*=0.03**
CTCs	Bidard 2010[340]	MO19391, mBC BEV + taxane	67	Baseline CTC ≥ 3/7.5 ml: shorter TTP (*p*<0.05)
VEGF-A polymorphism	Etienne-Grimaldi 2011[341]	MO19391, mBC BEV + taxane	137	936CT or 936TT: median TTP 11.5 months (95% CI 10.2-25.8) 936CC: median TTP 9.7 months (95% CI 7.8-12), *p*=0.02

n: number of patients; VEGF-A: vascular endothelial growth factor-A; mBC: metastatic breast cancer; BEV: bevacizumab; DOC: docetaxel; HR: Hazard ratio; PFS: progression-free survival; TNBC: triple-negative breast cancer; DFS: disease-free survival; PAC: paclitaxel; VEGFR-2: vascular endothelial growth factor receptor-2; CTC: circulating tumor cells

2.4.2   Angiopoietin-Tie pathway

The endothelial angiopoietin/Tie (Ang/Tie) system is a second receptor tyrosine kinase signaling pathway in addition to VEGF and its receptors. The Ang/Tie system is important during fetal angiogenesis, lymphatic vessel development and homeostasis of the mature vasculature[346,347], as well as in tumor angiogenesis [348,349]. The Ang/Tie system includes human angiopoietin growth factors 1, 2 and 4 (Ang1, Ang2, Ang4), which are ligands for Tie2 receptor[350–352]. In contrast, Tie1 is an orphan receptor with no known ligand.

Significant interest has emerged in targeting the Ang/Tie system for the treatment of malignant diseases. Ang1 and Ang2 have opposing actions on the Tie2 receptor. Ang1 stabilizes the vasculature and activates Tie2 more strongly than Ang2. On the other hand, Ang2 can act as an agonist or antagonist to the Tie2 receptor depending on the autocrine circumstances.[350] In normal homeostasis, the Ang2 level is low, but the Ang2/Ang1 ratio is increased in diseases with inflammation, such as sepsis and malignancies including breast cancer[353,354]. HIF-1-mediated signaling in hypoxic malignant tissues also induces the secretion of angiopoietins[355]. High Ang2 level correlates with poor patient survival in multiple malignancies[356–358]. Trebananib, a peptibody that inhibits the binding of Ang1 and Ang2 to Tie2, was already investigated in a phase III trial for the treatment of ovarian cancer, and other Ang/Tie system-targeting antibodies or TKIs are currently being evaluated in clinical trials[359–361].

The significance of the Tie1 receptor in the tumor environment is less well understood. All angiopoietins bind to Tie2, and the Tie1 receptor has no known ligands[346,362]. However, Tie1 participates in the Ang-Tie2 signal transduction complex[346,362–364]. Additionally, Tie1 expression in tumor vessel endothelium is increased during tumor angiogenesis[365]. Tie1 deletion in mice led to a decrease in microvessel density, intratumoral necrosis and growth delay in tumors at late stages[366]. Additionally, Tie1 loss in mice reduced the extravasation of tumor cells and decreased metastases[366]. In another preclinical study, Tie1 deletion reduced tumor growth and angiogenesis[348]. Increased concentrations of Tie1 were observed in association with acute or chronic inflammation due to ectodomain cleavage of Tie1[367,368]. However, circulating Tie1 levels in breast cancer have not been explored.

2.4.3   Cytokines and other circulating proteins

Inflammatory processes are heavily involved in tumorigenesis, angiogenesis and metastasis. Lifestyle-related cancer risk factors, such as tobacco smoking, sun exposure and obesity, are linked to cancer through chronic inflammation[369]. Similarly, some chronic viral and bacterial infections also increase cancer risk via inflammation[370]. Additionally, in malignant diseases, the tumor itself triggers inflammatory mechanisms that will engender a protumorigenic microenvironment[371]. Infiltrating immune cells and fibroblasts secrete cytokines, chemokines and growth factors that mediate inflammatory signals and promote tumor growth[372,373]. Chemokines are a group of cytokines that promote chemotaxis of leukocytes at the source of chemokine secretion[374]. Chemokines are further classified into four subfamilies depending on whether there are cysteines at the amino terminus: CXC, CC, CX3X and C[375]. Only selected cytokines, chemokines and growth factors relevant to this thesis are discussed in more detail below.

2.4.3.1   Interleukins 8, 6 and 18

IL-8 (also known as CXCL8) is a proinflammatory cytokine[376,377]. Other chemokines in the same CXC subfamily are epithelial neutrophil-activating protein (CXCL5), granulocyte chemotactic peptide-2 (CXCL6), neutrophil-activating protein (CXCL7 and melanoma growth stimulatory activity proteins (CXCL1, CXCL2 and CXCL3)[375]. A common feature of CXC chemokines is their role in neutrophil migration. These chemokines can be secreted by both immune cells and non-leukocytes, e.g., epithelial, endothelial and tumor cells[378]. IL-8 signaling is mediated by the G-protein-coupled receptors cysteine-X-cysteine chemokine receptor 1 (CXCR1) and CXCR2[379]. These receptors have different chemokine-binding specificity: IL-8 and CXCL6 are ligands for CXCR1, whereas CXCR2 has multiple ligands: IL-8, CXCL1-3, CXCL5 and CXCL7[380]. The effects of IL-8 secretion in a tissue are complex. It recruits and activates neutrophils and attracts macrophages, dendritic cells, mast cells and myeloid-derived suppressor cells[381–385]. Cancer stem cell survival and migration are potentiated by IL-8 action via CXCR1[386]. IL-8 itself promotes further IL-8 production and release in macrophages, mast cells and keratinocytes[387–389]. IL-8 also stimulates the invasiveness of tumor cells[390,391].

IL-8 also plays a role in angiogenesis, increasing the proliferation and survival of endothelial cells[392]. IL-8 induces endothelial cell VEGF-A production, and by an

autocrine mechanism, it increases the expression of VEGFR2[393]. Indeed, two studies reported IL-8 mediated resistance to antiangiogenic therapies[394,395].

High circulating IL-8 level and tumor expression are associated with poor prognosis in cancer patients[396–398]. Even in local breast cancer, a high serum IL-8 concentration is associated with a shorter DFS[399,400]. IL-8 level correlates with the tumor burden in many malignant diseases[401]. Additionally, high IL-8 level is associated with chemoresistance in malignant diseases[402,403]. Interestingly, changes in circulating IL-8 level reflected and predicted the response to the PD-1 inhibitor nivolumab or pembrolizumab in non-small cell lung cancer or melanoma patients[404].

Multiple IL-8-targeting antibodies or CXCR1/2 inhibitors have been evaluated in clinical trials for the treatment of inflammatory and infectious diseases and for the treatment of cancer[405,406]. Reparixin, an inhibitor of CXCR1 and CXCR2, was already evaluated for the treatment of HER2-negative mBC in a phase Ib study. Serious toxicities related to reparixin in combination with paclitaxel were rare (3%), and no dose-limiting toxicities were observed.[407] A phase II trial investigating the efficacy of reparixin with paclitaxel for the treatment of metastatic TNBC is underway (clinicaltrials.gov, NCT02370238). An anti-IL-8 antibody, HuMax-IL-8, demonstrated promising *in vitro* activity for TNBC[408].

Interleukin-6 (IL-6) is another proinflammatory cytokine in the tumor microenvironment that is present with increased levels in the serum and at the tumor site in breast cancer patients[409]. IL-6 participates in the proliferation and differentiation of malignant cells[410,411]. IL-6 signaling is mediated through the interleukin-6 receptor (IL-6R)-glycoprotein 130 (gp130) complex. Binding of IL-6 with its receptor can activate multiple proliferation pathways in cancer cells, including JAK/STAT3, PI3K/AKT and Ras/MAPK.[412] Increased serum IL-6 is associated with poor survival in breast cancer[413–415]. IL-6, IL-6R and gp130 are also targeted by multiple monoclonal antibodies and antagonists in anticancer drug development efforts[412].

The importance of interleukin-18 (IL-18) in malignancies is less well understood than the roles of IL-8 and IL-6. IL-18 increases the expression of adhesion molecules, nitric oxide synthesis and chemokine production[416]. IL-18 expression was significantly higher in breast cancer tissue compared to the surrounding tissue of the same patient. However, IL-18 expression was similar in breast cancer tissue compared to IL-18 expression in benign breast diseases.[417] High serum IL-18 level correlated with poor prognosis in patients with early TNBC[418]. Accordingly, high IL-18 expression in tumor stroma is associated with better clinical responses to neoadjuvant chemotherapy in locally advanced or inflammatory breast cancer[419]. *In*

vitro, high expression of IL-18 was noted in doxorubicin-resistant cell lines[420]. Interestingly, in the era of immuno-oncologic therapies, IL-18 was found to increase immunosuppression by upregulating PD-1 expression[421]. IL-18 was also investigated in combination with PD-1 and CTLA-4 inhibitors for augmenting immunotherapy activity[422]. In addition, dose-escalation studies were conducted with recombinant human IL-18 as a part of a combination treatment of lymphoma[423,424].

2.4.3.2   Matrix metalloproteinases

MMPs are a family of membrane-bound and secreted proteinases that take part in intravasation and extravasation processes in the extracellular matrix[425]. In addition, MMPs have a role in multiple functions that modify the tumor microenvironment: proliferation, invasion of tumor cells, cell survival, angiogenesis, adipogenesis and inflammatory processes[426]. During angiogenesis, MMPs degrade extracellular matrix to facilitate endothelial cell invasion for sprouting blood vessels[427]. MMPs also target tumor cell receptors and thereby activate proliferation pathways and inhibit apoptosis[428]. The secretion of MMPs, including MMP-9 and MMP-2, is induced by the HIF-1 pathway[426]. MMP-9 has an essential role in tumor angiogenesis by triggering the angiogenic switch[20,429]. TAMs are the major source of MMP-9, and once it is released into the tumor microenvironment, VEGF-A and fibroblast growth factor-2 (FGF-2) are secreted[430,431]. MMP-9 is also expressed in multiple inflammatory cells, mesenchymal cells, endothelial cells and pericytes, but to a lesser extent in tumor cells[432]. MMP-2 also contributes to tumor growth since tumor angiogenesis and proliferation are downregulated in MMP-2 knockout mice, and stromal MMP-2 and MMP-9 may act synergistically[427,433].

Studies of MMP-9 as a prognostic marker in breast cancer have yielded conflicting results. Most studies have reported that high MMP-9 expression in tumor tissue or in plasma/serum was associated with a higher risk of breast cancer relapse and/or shorter OS[434–439]. However, opposite results have also been published, with high MMP-9 tissue expression being a potential indicator for favorable prognosis[440,441]. In meta-analyses, MMP-9 and MMP-2 expression were both associated with poor prognosis in localized breast cancer[442–444]. However, the prognostic role of MMP-9 and MMP-2 has been investigated mostly in localized breast cancer and, its prognostic value in mBC is still unexplored.

2.4.3.3 YKL-40

YKL-40 (also known as chitinase-3-like protein 1) plays a role in the proliferation of fibroblasts and chondrocytes, differentiation of macrophages, inflammation, remodeling of extracellular matrix and organization and migration of endothelial cells[445–447]. Patients with malignant pleural effusions had higher levels of YKL-40 in serum and pleural fluid than patients with nonmalignant pleural effusions[448]. High circulating YKL-40 level was associated with poor survival in patients with renal cell carcinoma, lung cancer, gastric cancer and hepatocellular cancer patients[449–453]. In addition, higher serum YKL-40 level was observed in melanoma patients compared to healthy controls[454]. The clinical significance of YKL-40 has also been investigated in breast cancer patients, but the results of these studies have been inconsistent[455–462]. According to a meta-analysis, high YKL-40 was associated with unfavorable OS (HR 1.48, 95% CI 1.11-1.97) and shorter DFS (HR 1.51, 95% CI 1.10-2.07) in breast cancer patients[463].

2.4.3.4 Resistin

Obesity is a risk factor for breast cancer in postmenopausal women[464,465]. Therefore, adipocytokines, including resistin, among others, may be related to breast cancer development and prognosis. Resistin is involved in the regulation of insulin resistance[466]. In malignancies, resistin may promote the metastatic potential of breast cancer cells by inducing epithelial-to-mesenchymal transition[467]. The expression of resistin receptor CAP1 was associated with more unfavorable breast tumor characteristics – estrogen receptor negativity and higher tumor grade[468]. Circulating resistin level was also elevated in breast cancer patients compared to healthy controls[469–471]. Additionally, compared with low resistin expression, high resistin expression in the primary breast cancer tissue was associated with poorer patient survival and more unfavorable clinicopathological features of the primary cancer[472]. Interestingly, resistin attenuated doxorubicin-induced apoptosis in a preclinical breast cancer cell line study. Therefore, the researchers suggested resistin antagonism should be investigated to overcome chemotherapy resistance in mBC patients.[473]

In premenopausal breast cancer patients, however, high serum resistin was associated with longer DFS and was correlated with node-negativity[474]. Similarly, in another study of premenopausal breast cancer patients, patients with invasive ductal breast carcinoma had lower serum resistin than the patients with DCIS or healthy controls[475].

2.4.3.5 HMGB1

High-mobility group box 1 (HMGB1) is a ubiquitous nuclear protein that participates in DNA repair, transcription, replication and the stabilization of nuclear homeostasis[476–478]. HMGB1 can also translocate from the nucleus into the cytoplasm and may be secreted from the cell, where it can trigger inflammatory responses and autophagy processes[479,480]. HMGB1 is expressed at higher levels in many tumor types compared with healthy tissue, and its expression is associated with many diseases, including cancer[481,482]. In a meta-analysis on multiple tumor types, high HMGB1 expression was associated with shorter OS and PFS [HR 1.99 (95% CI 1.71-2.31) and HR 2.26 (95% CI 1.65-3.10), respectively].[483] HMGB1 was considered a promising biomarker for breast cancer since circulating HMGB1 level increased significantly in responding breast cancer patients after a single dose of neoadjuvant chemotherapy, whereas no significant change was observed in nonresponders (p=0.002 and p=0.17, respectively)[484]. In TNBC patients, cytoplasmic expression of HMGB1 was significantly associated with higher tumor-infiltrating lymphocyte (TIL) levels and higher nuclear grade[485]. Furthermore, HMGB1 was investigated as a drug target in multiple trials, and recently, a metabolite of acetylsalicylic acid was also found to inhibit HMGB1[486].

2.5 Circulating tumor markers for disease monitoring

Liquid biopsies are considered a promising method for monitoring mBC and for detecting resistance mutations. In a series of 30 women undergoing therapy for mBC, ctDNA, CA15-3 and CTCs were compared to standard imaging for monitoring metastatic cancer. In this comparison, ctDNA was more strongly correlated with changes in tumor burden than CA15-3 or CTCs[487]. At disease progression, the ctDNA level increased in 17 of the 19 women (89%) with confirmed progression on CT. CTCs increased in 7 of 19 progressing patients (37%) and CA15-3 level in 9 of 18 progressing women (50%).[487]

Estrogen receptor ESR1 mutations are a common mechanism of secondary endocrine resistance[488–491]. In a secondary analysis of the BOLERO-2 trial, ESR1 mutations (Y537S and D538G) were analyzed from cell-free DNA (cfDNA) in baseline plasma samples. ESR1 mutations were detected in 41% of the study patients, and OS in these patients was significantly shorter than for patients without ESR1 mutation (mOS 20.7 vs 32.1 months, $p<0.001$).[492] Therefore, the presence of

ESR1 mutations is associated with poor prognosis, but its predictive value remains unexplored.

A potential resistance mechanism to CDK4/6 inhibitors was also identified by ctDNA analysis. Next-generation sequencing (NGS) of ctDNA was performed for the samples of 34 mBC patients with disease progression during CDK4/6 inhibitor treatment. FGFR-1/2 amplification or an activating mutation was observed in 14/34 patients (41%). Moreover, baseline samples of MONALEESA-2 patients were analyzed for FGFR1 mutation status, and the patients with FGFR-1 amplification had shorter PFS compared to wild-type FGFR-1 patients. In the same study, FGFR-1 TKI erdafitinib demonstrated promising activity in combination with palbociclib and fulvestrant in FGFR-1-amplified xenografts.[493]

PI3K inhibitors are promising new agents for the treatment of hormone receptor-positive HER2-negative mBC with prior AI therapy. The SOLAR-1 trial reported positive PFS results for alpelisib in the mBC patients with a PIK3CA-mutation as described in section 2.3.6[265]. The PIK3CA-mutation could be reliably analyzed in ctDNA: the patients with plasma PIK3CA-mutation positivity had a mPFS of 10.9 months, and the patients with tissue PIK3CA positivity had a similar mPFS, 11.0 months.[494] Therefore, the possibility of analyzing PIK3CA mutations by liquid biopsies facilitates the introduction of PI3K inhibitors to the clinic.

HER2 resistance mechanisms can be detected by ctDNA. A study of 18 HER2-positive patients included 6 patients with progression of mBC. Analysis of ctDNA detected resistance mechanisms to anti-HER therapy: HER2 amplification (3/6), mutations in the gene TP53 (3/6) and mutations in genes related to mTOR/PI3K pathway (3/6).[495]

A high CTC count in patients with mBC is associated with an unfavorable prognosis. The mCTC count in patients with mBC was 3/7.5 ml of blood (IQR 0-25) in a retrospective analysis including 20 studies and 1944 patients[496]. A high CTC count ($\geq$5 CTC/7.5 ml) was observed in 47% of the mBC patients. The patients with high CTC counts had significantly shorter PFS and OS (HR for PFS 1.92, 95% CI 1.73-2.14; HR for OS 2.78, 95% CI 2.42-3.19).[496] In another study, mBC patients with detected CTCs $\geq$ 1 per 7.5 ml had significantly shorter mOS compared to the patients with no detectable CTCs (mOS 0.7 vs 1.8 years, $p<0.001$)[497]. CTCs were prospectively evaluated in one trial that aimed to evaluate CTCs for predicting response, PFS and OS in mBC patients[498]. A high CTC count ($\geq$ 5 per 7.5 ml of whole blood) predicted poor survival (mPFS 2.7 months for CTC count $\geq$ 5 vs. 7.0 months for CTC count < 5, $p<0.001$). However, the usefulness of CTCs for disease monitoring was limited because many patients had very low CTC counts or no

detectable CTCs (< 2 CTCs/7.5 ml in 66% of the mBC study patients).[498] The SWOG S0500 trial randomly assigned patients with rising CTC counts after 21 days of chemotherapy to either continue initial therapy or to change to another chemotherapy. The patients had similar survival in both treatment arms. Thus, changing the chemotherapy regimen due to rising CTC count after one cycle of chemotherapy did not improve either OS or PFS. However, the patients with rising CTC counts after 21 days of chemotherapy had significantly shorter OS than the patients with decreasing CTCs after 21 days of chemotherapy or no CTCs at baseline (mOS 13 vs 23 vs 35 months, respectively, $p<0.001$).[499] In conclusion, a high CTC count is strongly associated with poor prognosis, but no data support its use as a predictive marker for therapy response.

2.5.1   CA15-3

CA15-3 monitoring in mBC patients is recommended only for patients with nonmeasurable metastatic disease[16,41]. Approximately 80% of mBC patients have elevated CA15-3 levels[500]. Tampellini et al. conducted a trial with 790 mBC patients with the aim of studying whether serial CA15-3 measurements would provide additional prognostic information in addition to standard clinicopathological factors. The changes in CA15-3 level were mostly related to tumor responses, but individual discrepancies existed.[501] Therefore, serial CA15-3 level measurements cannot be used alone for monitoring responses in mBC.

One clinical problem associated with CA15-3 monitoring is the possibility of a spiking phenomenon. In a study of approximately 600 patients, spikes in CA15-3 level were observed in 5% of the mBC patients, but the survival of these patients was similar to that in patients without CA15-3 spikes at the beginning of the treatment. The peak of the spike occurred at a median of 6 weeks, with decreasing CA15-3 levels thereafter.[502] The spike in the beginning of the treatment might have been due to necrosis and apoptosis of tumor cells. In another study, CA15-3 levels at 8 and 12 weeks of therapy correlated with the treatment response, but not the marker level at 4 weeks, a time frame matching the spike phenomenon[503].

3 AIMS OF THE STUDY

This study was designed to evaluate the benefit from adding bevacizumab to standard first-line taxane chemotherapy in patients with advanced or metastatic breast cancer. Another aim was to evaluate the feasibility of the bevacizumab-taxane combination considering the known additional toxicity related to bevacizumab. In our study, bevacizumab was also continued after taxane discontinuation as a maintenance therapy. For hormone receptor-positive patients, endocrine therapy was administered with bevacizumab maintenance treatment. Therefore, an additional goal was to evaluate whether endocrine treatment and bevacizumab would have a synergistic benefit. The primary end-point of the study was progression-free survival. The secondary end-points included safety, response rate and overall survival.

The aim of the exploratory biomarker study was to evaluate the prognostic value of plasma cytokines and other circulating proteins. The specific markers explored were Tie1, Ang2, IL-8, IL-6, IL-18, MMP-2, MMP-9, YKL-40, resistin and HMGB1.

An additional aim was to evaluate if the new lectin-based CA15-3 assays would be more specific than conventional, widely used CA15-3 for monitoring the response by patients with mBC.

4 PATIENTS AND METHODS

4.1 Patients

Altogether, 65 patients were enrolled in this academic, prospective, nonrandomized phase II study in three Finnish University hospitals: Tampere, Turku and Oulu. Enrollment took place between May 2009 and October 2013. The data collection was closed in April 2015 after all the primary and secondary end-points of the study were met. The median follow-up time was 24.1 months (range 1.6-66.3 months).

The study patients were diagnosed with histologically or cytologically confirmed HER2-negative mBC and had not received previous chemotherapy for the metastatic disease. Previous endocrine treatment was also allowed for advanced disease. Both pre- and postmenopausal hormonal status and measurable and nonmeasurable disease were allowed. The additional inclusion criteria included good performance status [Eastern Cooperative Oncology Group (ECOG) performance status 0-2] and adequate hematological, renal and hepatic function. The patients were suitable for taxane chemotherapy treatment, and they were allowed to have received chemotherapy as neoadjuvant or adjuvant treatment. The disease-free interval had to be at least 6 months after the completion of taxane-containing (neo)adjuvant chemotherapy. The patients were excluded in the case of CNS metastases, pre-existing peripheral neuropathy of at least grade 2 by the National Cancer Institute Common Toxicity Criteria (NCI-CTC), version 3.0[504]. Additionally, patients with recent surgeries, a need for anticoagulants or thrombolytic agents, a history of coagulopathies, uncontrolled hypertension or clinically significant cardiovascular disease were excluded. Patients with a history of abdominal fistula, abscess or gastrointestinal perforation were not allowed to enter the study. Finally, the study patients were not allowed to have a history of other malignancies.

4.2 Treatment

In part I of the study, the patients received taxane chemotherapy combined with the VEGF-A antibody bevacizumab. Both regimens were administered intravenously.

Either paclitaxel or docetaxel was used as the taxane. The simplified study scheme is presented in Figure 4. Chemotherapy treatment was continued until maximal response, disease progression, unacceptable toxicity or patient refusal. Maximal response was defined as an achieved response with no change between two consecutive response evaluations. The study was initiated with docetaxel combined with bevacizumab, and this combination was continued until the AVADO trial results were published in 2010 showing negative results from the docetaxel-bevacizumab combination[298]. Therefore, an amendment was made, and the study was continued with paclitaxel-bevacizumab.

If docetaxel or paclitaxel was discontinued for reasons other than disease progression, the patients continued to receive bevacizumab every three weeks in part II of the study (Figure 4). In addition, the patients with hormone receptor-positive breast cancer received endocrine therapy according to the investigator's choice. The part II treatment was continued until disease progression, unacceptable toxicity or withdrawal of patient consent. After disease progression, the continuation of bevacizumab was optional, and the second-line chemotherapy was the investigator's choice. The recommended chemotherapy option for the second-line treatment was capecitabine.
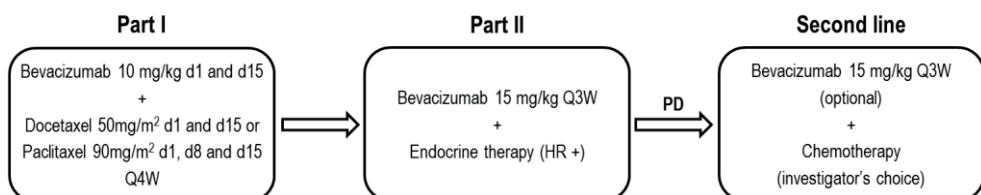
Figure 4. Study scheme. The patients without disease progression entered study part II, when the taxane (docetaxel or paclitaxel) was discontinued. In part II, only hormone receptor-positive patients received endocrine therapy in combination with bevacizumab. d: day; Q4W: every 4 weeks; HR: hormone receptor; PD: progressive disease; Q3W: every 3 weeks

4.3   Toxicity and response evaluation

Toxic side effects were monitored and graded according to the NCI-CTCAE, version 3.0[504]. Tumor evaluations by CT were performed every 12 weeks until disease progression according to the Response Evaluation Criteria in Solid Tumors

(RECIST), version 1.1[505]. After trial discontinuation, study patients were followed up every 6 months for their survival by patient records. OS was estimated from the date of randomization to the death of the patient, patient refusal or the last follow-up date.

4.4   Plasma samples

Plasma samples were gathered for investigational purposes. The samples were obtained at study baseline, every sixth week during study part I and at taxane discontinuation. During study part II, the plasma samples were obtained every three weeks during the first two months and thereafter every 12 weeks and at the final study visit.

Additionally, plasma samples were analyzed from women who participated in a breast cancer primary prevention study currently in progress at Tampere University. These women served as healthy control samples.

4.5   Measurement of Tie1, Ang2, IL-8, IL-6, IL-18, MMP-2, MMP-9, YKL-40, resistin and HMGB1

The plasma analyses were performed on the samples gathered at baseline, after six weeks of treatment, after six months of treatment and at the final study visit. Additionally, Tie1 was measured in 10 female breast cancer prevention study participants as healthy controls. Tie1 and Ang2 levels were measured in patient plasma samples using a modified hTie1 and hAng2 enzyme-linked immunosorbent assay (ELISA) protocol. Plasma IL-8, IL-6, IL-18, MMP-2, MMP-9, YKL-40, resistin and HMGB1 concentrations were measured by ELISAs. ELISAs were carried out according to a standard protocol. The detailed laboratory protocols can be found in the original publications (publications II-III).

4.6   Measurement of CA15-3 and the glycovariant forms of CA15-3

CA15-3 analyses were performed using three different assays: conventional CA15-3 and two glycovariant forms, CA15-3[MGL] and CA15-3[WGA]. They were analyzed at

baseline, at week six of treatment, at month six of treatment and at final study visit. In addition, samples were analyzed from 20 healthy control subjects who participated in the breast cancer primary prevention study. In addition, the conventional CA15-3 concentrations were analyzed with a CA15-3 enzyme immunoassay (Fujirebio Diagnostics Inc., Malvern, PA, USA) according to the manufacturer's instructions. The glycovariant forms of CA15-3 were analyzed using an in-house protocol described in original publication IV.

4.7 Statistical analysis

The median PFSs and OSs were calculated according to the Kaplan-Meier method. Adverse events and treatment responses were displayed in standard frequency tables. The statistical plan for the biomarker analysis was exploratory. Tie1, Ang2, IL-8, IL-6, IL-18, MMP-9, MMP-2, YKL-40, resistin and HMGB1 were dichotomized as low or high for each patient using the median value as the cutoff. In addition, IL-8, IL-6, IL-18, MMP-9, MMP-2, YKL-40, resistin and HMGB1 levels were divided into four groups using the baseline quartile ranges as the cutoff values. The Mann-Whitney U test was used to compare differences in the baseline Tie1, Ang2 and IL-8 levels between groups with different baseline characteristics and between IL-8 trajectory groups. The Wilcoxon signed rank test was used to compare Tie1, Ang2 and IL-8 levels at different time points. The Wilcoxon signed rank test was also used to compare baseline and week-six CA15-3 levels in relation to the treatment response. Spearman's correlation was used to analyze the correlation between the conventional CA15-3 level and CA15-3^{MGL} or CA15-3^{WGA} level. Hazard ratios (HRs) with 95% CIs were calculated using Cox proportional hazard regression analysis. Multivariable analyses were adjusted for age (continuous), menopausal status (premenopausal/postmenopausal), hormone receptor status (negative/positive), the presence of visceral metastasis (yes/no), the number of metastatic lesions (cutoff of three metastatic lesions) and the extent of the disease (cutoff of three metastatic sites). Interleukin-8 values were clustered using trajectory analysis[506]. Trajectory analysis divided patients into groups with similar change patterns of interleukin-8 level during chemotherapy treatment. *P*-values under 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 23 (SPSS Inc., Chicago, IL, USA) and IL-8 trajectory analysis by R version 3.3.0.

4.8   Ethics

The study protocol was approved by the Ethics Committee of Tampere University Hospital (R08142M). The trial was also registered at clinicaltrials.gov with trial identifier NCT00979641. Written informed consent was obtained from all patients enrolled in the study.

Additionally, plasma samples were analyzed from women who participated in a breast cancer primary prevention study currently in progress at Tampere University. All participants in this breast cancer prevention study gave their written informed consent, and the Ethics Committee of Tampere University Hospital approved the study (R15023).

5 SUMMARY OF RESULTS

5.1 Patient characteristics

All 65 patients enrolled in the study were included in the survival analysis. The baseline characteristics of the patients are presented in Table 6. Plasma samples for the biomarker analysis were available from 58 patients, and the characteristics of these patients can also be found in Table 6. Baseline plasma samples were available from 53 patients.

Table 6. The baseline characteristics of patients

	Overall study population (n=65)	Plasma biomarker population (n=58)
Median age (range), years	57 (32-75)	58 (32-75)
Menopausal status, n (%)		
Premenopausal	10 (15.4)	9 (15.5)
Postmenopausal	55 (84.6)	49 (84.5)
History of early stage disease, n (%)	57 (87.7)	52 (89.7)
Disease free interval, n (%)		
≤ 24 months	11 (19.3)	10 (19.2)
> 24 months	46 (80.7)	42 (80.8)
Hormone receptor status, n (%)		
ER+ and/or PR+	53 (81.5)	47 (81.0)
ER- and PR-	12 (18.5)	11 (19.0)
Prior endocrine therapy for metastatic disease, n (%)	18 (27.7)	15 (25.9)
Number of metastatic lesions, n (%)		
≤ 3	14 (21.5)	11 (19.0)
> 3	51 (78.5)	47 (81.0)
Extent of disease		
< 3 sites	39 (60.0)	36 (62.1)
≥ 3 sites	26 (40.0)	22 (37.9)
Site of metastatic disease, n (%)		
Visceral disease	53 (81.5)	46 (79.3)
Nonvisceral disease	12 (18.5)	12 (20.7)

Abbreviations: n: number of patients; ER: estrogen receptor; PR: progesterone receptor

5.2 Clinical efficacy results (Publications I-III)

In treatment part I, 32 patients were treated with the docetaxel-bevacizumab combination and 33 patients with the paclitaxel-bevacizumab combination. The patients with no disease progression at taxane discontinuation entered treatment part II. Of the 38 patients in part II, the majority had hormone receptor-positive disease (87%), and only five patients (13%) had hormone receptor-negative breast cancer. In part II, the hormone receptor-positive patients also received endocrine therapy with maintenance bevacizumab. Letrozole was the most common drug choice for these patients (n=19). Other endocrine therapy choices included anastrozole (n=4), exemestane (n=4), tamoxifen (n=3) and fulvestrant (n=3).

The mPFS of all study patients was 11.3 months (95% CI 9.7-16.0), and the mOS was 35.1 months (95% CI 22.2-50.3). The ORR was 61.5% (n=40), with one complete response included. Stable disease was observed in 15 patients (23.1%). Only three patients (4.6%) did not respond to the treatment, and the best response for these three patients was a progressive disease. The mOS for patients with hormone receptor-positive disease was 45.0 months (95% CI 30.2-51.3). The mOS for the patients with triple-negative mBC was significantly shorter, as expected, at 17.9 months (95% CI 8.5-26.9, p=0.011).

The plasma biomarker population had similar efficacy results as the entire study population. The mPFS for the plasma biomarker population was 11.3 months (95% CI 8.3-14.4), and the OS was 37.5 months (95% CI 25.4-49.6). The ORR for the plasma biomarker population was 71.7%.

After disease progression, the study patients were allowed to receive bevacizumab in combination with second-line chemotherapy, and 17 study patients were treated in this setting. The investigator's choice as the chemotherapy regimen was capecitabine for 15 patients. One patient each received paclitaxel and vinorelbine. The mPFS for second-line therapy was 5.1 months (95% CI 4.4-16.1 months), and the OS was 33.8 months (95% CI 24.7-not reached). The ORR for the second-line treatment was 41%.

5.3 Toxicity (Publication I)

In treatment part I, the bevacizumab-taxane combination was generally well tolerated, and the reported toxicities were mostly grade 1-2. The grade 3-4 toxicities of the chemotherapy in part I are reported in Table 7. One patient died from

gastrointestinal perforation during the part I treatment. This patient had pre-existing diverticulosis and developed diverticulitis early during bevacizumab-taxane combination treatment. This resulted in gastrointestinal perforation and peritonitis.

Table 7.   Grade 3-4 adverse events during treatment part I

	Patients (n=65)
Adverse events	Grade 3-4
Neutropenia	25 (38%)
Leukocytopenia	13 (20%)
Infection	9 (14%)
Neutropenic infection	4 (6%)
Pain	1 (2%)
Fatigue	2 (3%)
Diarrhea	1 (2%)
Elevated lives enzymes	2 (3%)
Nausea	1 (2%)
Peripheral neuropathy	1 (2%)
Cardiac disorders	1 (2%)
Osteonecrosis of the jaw	1 (2%)
Drug hypersensitivity	1 (2%)
Gastrointestinal perforation	1* (2%)

*Patient died, grade 5 adverse event

Similarly, the toxicities were mostly grade 1-2 in treatment part II. Grade 3-4 adverse events during part II included two infections, two elevated liver enzymes, two hyponatremia and one each of leukocytopenia, peripheral neuropathy, anorexia, congestive heart failure and coronary artery thrombosis.

Bevacizumab-related toxicities were monitored closely. One patient died during treatment part I, as mentioned above. This patient with pre-existing diverticulosis had diverticulitis and consequently a gastrointestinal perforation. The grade 5 adverse event was suspected to be related to bevacizumab. Additionally, one patient had grade 4 proteinuria. Otherwise, bevacizumab-related toxicities were grade 1-2, including hypertension, proteinuria, hemorrhage, epistaxis and gastrointestinal fistula or abscess.

During second-line bevacizumab-chemotherapy treatment, the side effects were mostly related to capecitabine. The reported grade 3-4 side effects were three cases of hand-and-foot syndrome and a single case of grade 4 diarrhea.

5.4 Results of the circulating prognostic markers

5.4.1 IL-8 (Publication II)

The patients were dichotomized into two groups using the median baseline IL-8 level of 9.4 pg/ml as the cut-off value. The high-IL-8 group had significantly shorter OS by age-adjusted Cox regression (HR 2.14, 95% CI 1.10-4.12, p=0.023). However, in multivariate analysis, the difference between the low- and high-level groups was no longer statistically significant (p=0.159).

Trajectory analysis resulted in three trajectory groups (Figure 5). The patients in trajectory group 1 had a significantly lower circulating IL-8 concentration than the patients in groups 2 and 3 at baseline (p<0.001 and p=0.002, respectively), at week six (p<0.001, p=0.002), at month six (p=0.006, p<0.001) and at the final visit (p=0.001, p<0.001). The OS was significantly shorter for the trajectory group 2 and 3 patients compared to trajectory group 1 (Figure 5, p=0.004 and p=0.001, respectively).
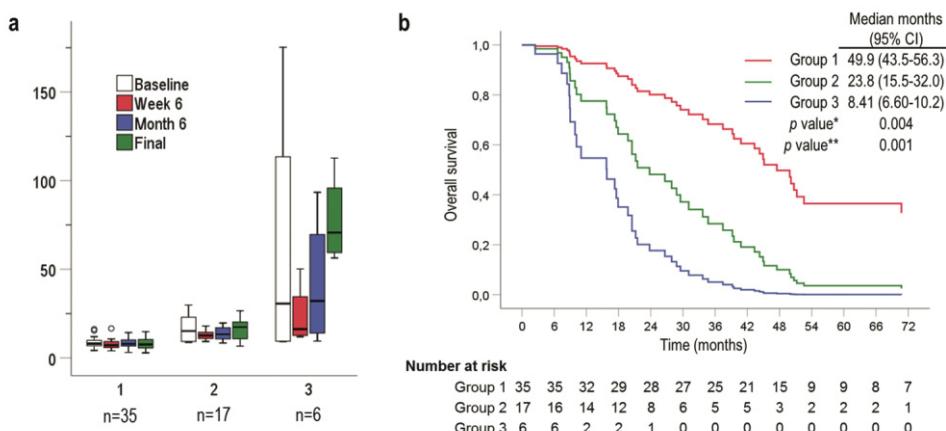
Figure 5. **a.** The trajectory groups. The trajectory group 1 patients had a significantly lower plasma IL-8 level than the patients in trajectory groups 2 and 3 during the entire first-line treatment period. **b.** Overall survival of the three trajectory groups. Patients with constantly low plasma IL-8 during the entire treatment period in trajectory group 1 had an exceptionally long median OS. * p-value group 2 vs. group 1, ** p-value group 3 vs. group 1

For identifying patients with a favorable prognosis, a cut-off value of 16.6 pg/ml was found to be useful. All plasma IL-8 levels in trajectory group 1 patients remained below 16.6 pg/ml before and during the entire chemotherapy treatment period. The OS was significantly shorter for patients with plasma IL-8 levels higher than 16.6 pg/ml before or during the treatment (multivariate HR 3.90, 95% CI 1.88-8.12, $p<0.001$).

Additionally, the patients with baseline plasma IL-8 levels in the highest quartile (> 18.8 pg/ml) had poor survival regardless of the IL-8 levels during the treatment. The multivariate HR for PFS was 6.52 (95% CI 2.60-27.0, $p<0.001$) for the highest IL-8 quartile, and the multivariate HR for OS was 8.38 (95% CI 2.60-26.9, $p=0.010$).

5.4.2 IL-6, IL-18, MMP-2, MMP-9, YKL-40 and HMGB1 (Publication II)

Cox regression analyses were also performed for plasma IL-6, IL-18, MMP-2, MMP-9, YKL-40 and HMGB1 concentrations. Using the median as the cut-off value, a baseline plasma MMP-9 level >76.4 ng/ml was borderline significant for longer OS (multivariate HR 0.52, 95% CI 0.26-1.03, $p=0.063$). In addition, the highest baseline plasma quartile level of YKL-40 was a sign of poor prognosis in age-adjusted Cox regression (HR 3.08, 95% CI 1.10-8.61, $p=0.031$). However, the highest YKL-40 plasma quartile level was no longer significant in multivariate analysis ($p=0.211$). The median and quartile levels were used as cut-off values, and no significant differences were observed in terms of PFS. For IL-6, IL-18, MMP-2, resistin and HMGB1, there were no significant OS differences, either.

5.4.3 Tie1 and Ang2 (Publication III)

The median plasma Tie1 concentration at baseline was 21.0 ng/ml (95% CI 17.8-23.3), and the median Ang2 concentration at baseline was 1.29 ng/ml (95% CI 1.03-1.52). The Tie1 concentration of the healthy controls was significantly lower than the baseline plasma level of the mBC patients ($p<0.001$). The median concentrations were used as cut-off levels for the high- and low-Tie1 and -Ang2 groups.

The mPFS and mOS were significantly longer in the low-baseline-Tie1 group compared to the high-Tie1 group (Figure 6). The multivariate HR for OS for the high-Tie1 group was 3.07 (95% CI 1.39-6.79, $p=0.005$). In contrast, the baseline Ang2 level was not prognostic for PFS or OS in multivariate Cox regression analysis. However, the longest mOS was observed in patients with both Tie1 and Ang2 levels

low at baseline. The multivariate HR for the patients with both Tie1 and Ang2 high at baseline was 4.32 (95% CI 1.44-12.94, p=0.009). The mOS for patients with low baseline levels of both Tie1 and Ang2 was 46.8 months (95% CI 23.8-79.8). However, the mOS for patients with high baseline levels of both Tie1 and Ang2 was only 21.5 months (95% CI 8.8.-34.7).
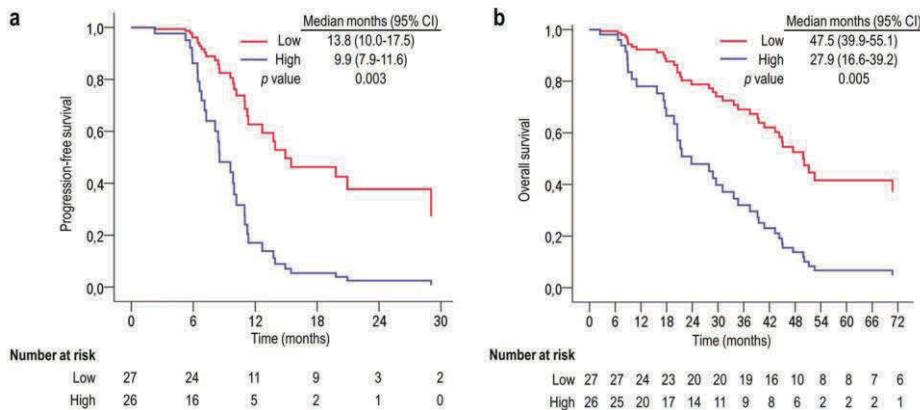
Figure 6. **a.** Progression-free survival and **b.** Overall survival of the plasma biomarker population grouped by baseline plasma Tie1 level. Cut-off value 21.0 ng/ml[507].

5.5 Glycovariant CA15-3 assays compared to the conventional CA15-3 (Publication IV)

CA15-3[MGL] and CA15-3[WGA] lectin-based assays were compared to the conventional CA15-3 assay on the baseline plasma samples of 53 mBC patients. Receiver operating characteristic (ROC) curves were plotted, and AUCs were calculated. The clinical sensitivities were 81.1% for CA15-3[WGA], 67.9% for CA15-3[MGL] and 66.0% for conventional CA15-3 at 90% specificity. The difference in the AUC was significant for CA15-3[WGA] (p=0.007) but not for CA15-3[MGL] (p=0.655) compared to the conventional CA15-3. The mBC patients had a significantly higher pretreatment CA15-3 concentration than the healthy controls by all three CA15-3 assays: conventional CA15-3 (p<0.001), CA15-3[MGL] (p=0.013) and CA15-3[WGA] (p<0.001).

A strong correlation was observed between the baseline conventional CA15-3 and CA15-3[WGA] concentrations (r=0.90, p<0.001). The correlation between baseline

conventional CA15-3 and CA15-3^{MGL} levels was weaker (r=0.68, p<0.001). Additionally, we studied plasma samples from 19 patients who had confirmed disease progression at the final study visit. A clinically meaningful 30% increase in CA15-3 concentration was observed in 8 patients (42%) with conventional CA15-3, 9 patients (47%) with CA15-3^{MGL} and 5 patients (26%) with CA15-3^{WGA}. The patients with rising CA15-3 levels were not all the same individuals by the different CA15-3 assays.

6   DISCUSSION

In this study, we conducted a prospective, nonrandomized study to evaluate the feasibility of bevacizumab treatment in combination with the standard taxane regimen as a first-line chemotherapy treatment of mBC. We explored the prognostic value of several plasma proteins, including Tie1, Ang2, IL-8, IL-6, IL-18, MMP-2, MMP-9, YKL-40, resistin and HMGB1. Furthermore, we studied whether lectin-based CA15-3[MGL] and CA15-3[WGA] assays would be more sensitive than the conventional CA15-3 assay in monitoring the responses to treatment in patients with mBC.

6.1   Patients

All 65 patients who entered the study were included in the efficacy and toxicity results. The median age of our study patients was 57 (range 32-75). The median age of study patients in other studies with first-line bevacizumab-taxane treatment ranges between 54-59[297–299,315]. Therefore, in this respect, our study population matches those of other studies. However, in a real-life setting, patients with mBC are older at the time of metastatic cancer diagnosis, with a median age ranging from 61 to 64[12,508]. This suggests that patients entering our and other clinical trials are younger and therefore more fit than the general mBC population.

Most of our study patients had hormone receptor-positive disease (82%). In phase III first-line trials of bevacizumab for mBC, hormone receptor-positive tumors were observed in 60-84% of the patients, which is about the same level as in our study[297–299,302].

Many of our study patients had poor prognostic features of mBC at study initiation. Visceral disease was common (82%), and 80% of the patients had more than 3 metastatic lesions. In the phase III trials with bevacizumab, visceral disease was as common as in our study (68-87%)[297,299]. However, 40% of our patients had already received taxane as adjuvant therapy. This was more common than in the E2100 and AVADO trials (15-17%) and might be related to a more chemoresistant study population[297,298].

We had plasma samples from only 58 patients and baseline samples from 53 patients. However, the patients with plasma samples available were representative of the entire study population. The baseline characteristics of the plasma biomarker population were similar to those in the entire study population. Additionally, the PFS and OS were similar for these two patient populations.

6.2 Bevacizumab as a treatment for metastatic breast cancer

The mOS in our study reached almost three years (35.1 months), which can be considered an excellent result in mBC patients. The mOS ranged between 27 and 31 months in the first-line phase III mBC trials with bevacizumab treatment[297,298,300,302]. The PFS of our study (11.3 months) was similar to those in first-line phase III trials (8.6-11.8 months)[297–300,302].

There are several possible reasons for the long OS in our study. In contrast to phase III first-line mBC studies of bevacizumab, we continued bevacizumab as a maintenance therapy after discontinuation. In addition, patients with hormone receptor-positive disease received endocrine therapy with bevacizumab maintenance. Maintenance treatment was given in 58% of our study patients, and most of these patients (87%) had hormone receptor-positive disease. VEGF and estrogen signaling pathways have several interaction points, and synergistic effects might be possible[509–512]. Additionally, 17 patients (26% of our study patients) received bevacizumab with second-line chemotherapy. In the treatment of colorectal carcinoma, continuation of bevacizumab with second-line chemotherapy has resulted in OS improvement[513]. On the other hand, accelerated tumor progression has been reported after short-term angiogenesis inhibition[514]. Therefore, prolonged VEGF inhibition might be a reason for the long OS in our study. Hypothetically, the accelerated tumor progression after discontinuation of bevacizumab might be the reason several phase III studies with bevacizumab were unable to demonstrate an OS benefit despite a statistically significant PFS improvement[297–299].

6.2.1 The clinical benefit for adding bevacizumab to standard chemotherapy treatment

As discussed above, all of the phase III trials evaluating bevacizumab for the treatment of mBC have been unable to demonstrate an OS benefit[297–299,301,306].

However, consistently higher response rates are reported with bevacizumab compared to standard chemotherapy[297–299,302,306]. The PFS improvement with bevacizumab is rather modest, 2-6 months, but there is a subset of patients who would benefit from bevacizumab[297–299,302]. Extensive biomarker research has been carried out with the aim of recognizing these patients, but no means exist to select these individuals in standard clinical care. Thus, the treatment decisions will be based on the clinical features of mBC. Current guidelines recommend the use of bevacizumab only in selected cases[16]. Similarly, in the NCCN breast cancer guidelines, bevacizumab-paclitaxel is categorized as a useful combination in certain circumstances and not as a preferred regimen[41]. Based on the clinical trial results, the most suitable mBC patient for bevacizumab treatment would be the one with a high need for tumor response. The need for the response would be related to high tumor burden and visceral disease. Therefore, the high frequency of responses achieved with bevacizumab-chemotherapy combinations would be a strong argument for the use of this combination for these few patients.

6.3   Methodological considerations and study limitations

Our study was a prospective, nonrandomized, academic phase II study with one treatment arm, and we treated 65 patients. Because the study had no comparator arm, it is difficult to draw strong conclusions from the results. For example, the reasons for the long OS that we found in our patients are partially speculative. The prospective, academic study design can definitely be considered a strength of the study.

In 2017, breast cancer was the cause of death for 923 women and 5 men in Finland. Between 2012 and 2016, 54% of all the patients who died of breast cancer were over 70 years old in Finland.[1] Finland is a sparsely populated country with long distances between cities, and malignant diseases are treated in five university hospitals and in several central hospitals[515]. Due to the limited number of women suffering from mBC and the scattered health care system, enrolling a large number of patients in a mBC clinical trial is challenging in Finland. Additionally, many patients with mBC might have impaired treatment-limiting performance status related to age, other comorbidities and metastatic disease itself, which is another limitation for enrolling patients in clinical studies. We succeeded in enrolling 65 patients with mBC in an academic prospective trial in Tampere, Turku and Oulu University Hospitals. For the above-mentioned enrollment challenges, the number

of patients recruited to the mBC trial in Finland is significant. Furthermore, our study met its primary enrollment goal, which is another strength of our trial.

The adverse events were monitored prospectively according to the study protocol and were graded by CTCAE terminology; therefore, the toxicity in our study can be compared to toxicity of other clinical trials. The responses were evaluated using RECIST criteria. The above- mentioned study protocols are strengths of our study.

Plasma samples were not available from all study patients, although obtaining plasma samples was designed in the study protocol. The reasons for missing plasma samples are not known. Additionally, the reasons for the long OS of our study patients remain partly speculative since our study did not include a comparator arm.

6.4 Circulating IL-8 levels during chemotherapy of advanced disease

Low plasma IL-8 levels before and during first-line bevacizumab-taxane treatment are associated with excellent long-term prognosis. The mOS of our trajectory group 1 patients, with constantly low IL-8 levels, was 50 months. The majority of our patients (60%) belonged to trajectory group 1.

High IL-8 levels can be a sign of a chemoresistant form of breast cancer[402,403]. The trajectory group 3 patients, with the highest IL-8 levels in our study, had extremely poor survival, although only 6 patients belonged to this subgroup (mOS 8 months). The patients with intermediate IL-8 levels, in trajectory group 2, also had significantly worse OS than the trajectory group 1 patients (mOS 24 months vs. 50 months, respectively, $p=0.004$). In addition, the hypothesis of chemoresistance is supported by our finding of poor survival of the patients with the highest quartile of IL-8 level before initiation of first-line chemotherapy (HR 8.38, $p>0.001$). Therefore, IL-8 levels should be prospectively analyzed in clinical trials in the future to confirm our hypothesis of chemoresistance and possibly to guide treatment decisions for these poor-prognosis patients. Interestingly, an association of IL-8 level with treatment response was demonstrated in melanoma and non-small cell lung cancer patients receiving anti-PD-1 treatment, and this treatment approach could be worthwhile to evaluate in mBC patients with high IL-8 levels[404].

6.5 Plasma IL-6, IL-18, MMP-2, MMP-9, YKL-40 and HMGB1 levels as prognostic markers

We explored additional cytokines and circulating markers with the aim of evaluating their prognostic significance. IL-6, IL-18, MMP-2, MMP-9, YKL-40 and HMGB1 were not significantly prognostic in our patient population. The median IL-6 concentration was 4 pg/ml in one study that reported high IL-6 was associated with poor prognosis[414]. In our study, the highest quartile cut-off level for plasma IL-6 was 3.8 pg/ml. Therefore, we had very few patients with high IL-6 levels and presumably a poor prognosis on this basis. Additionally, the limited number of patients in our study was possibly one explanation for the other markers not demonstrating their weaker prognostic value.

6.6 Tie1 and Ang2 as prognostic markers for metastatic breast cancer

In our study, a baseline plasma Tie1 concentration higher than the baseline cut-off level of 21.0 ng/ml was strongly associated with both OS and PFS (HR 3.07, p=0.005 and HR 3.78, p=0.003, respectively). High Tie1 expression was demonstrated in malignant tissue in previous studies[365,516–518]. However, our finding of the prognostic role of circulating Tie1 level in mBC patients is a novel and interesting result. The role of plasma Tie1 level as a prognostic marker should be validated prospectively in a larger mBC patient cohort. Furthermore, plasma samples as liquid biopsies are easily accessible compared to tissue samples, considering the possible need for surgical procedures for their acquisition.

Plasma Tie1 in mBC patients was significantly higher than in healthy controls (p<0.001). This finding suggests Tie1 also plays a role in malignant processes. In a preclinical study, additive tumor growth inhibition was observed in Tie1-deficient mice, caused by the blockage of angiopoietin activity[348]. Ang2 impacts tumor growth at a different phase than Tie1, and this might be the reason for the possible synergism[366]. TKIs and antibodies targeting the Ang2/Tie system are being investigated in clinical trials, and in the future, tyrosine kinase receptors targeting the intracellular part of Tie1 would be worthwhile to explore[360].

The patients with high circulating levels of both Tie1 and Ang2 had a mOS of 47 months, compared to the mOS of 22 months in patients with low plasma concentrations of both Tie1 and Ang2 (p=0.009). Circulating Ang2 level was

associated with poor prognosis in cancer patients in previous studies, but the combination of Tie1 and Ang2 levels was not previously explored[357,358,519,520]. The possible synergism of Tie1 and Ang2 might explain the combined effect on patient prognosis in our study[366].

6.7 Lectin glycovariants of CA15-3 for monitoring advanced breast cancer

The aim of this study was to increase the sensitivity and specificity of CA15-3 in breast cancer monitoring by using new lectin-based glycovariants in the CA15-3 assay. The use of a nanoparticle-lectin immunoassay was successful in the improving the CA12-5 assay[521]. In our study, the clinical sensitivity was significantly higher for the lectin-based CA15-3[WGA] assay (81%) than for the conventional CA15-3 assay (66%) at 90% specificity (p=0.007). In addition, the baseline CA15-3[WGA] level correlated with baseline CA15-3 using the conventional assay (r=0.90, p<0.001). On the other hand, the sensitivity of CA15-3[MGL] was not significantly higher than the sensitivity for the conventional CA15-3 assay (p=0.655), and the correlation between these assays was weaker (r=0.68, p<0.001).

CA15-3 monitoring for treatment response is recommended particularly for patients with nonmeasurable disease as an aid to other clinical parameters[16,522]. If CA15-3 is used for disease monitoring, at least a 20-30% increase is required before considering treatment discontinuation, also taking into account the clinical evidence[522]. Clinical problems related to CA15-3 monitoring include the possibility of individual discrepancies in CA15-3 levels in contrast to the clinical situation[501]. In addition, only 80% of mBC patients have elevated levels of CA15-3[500]. In our study, the sensitivity of CA15-3[WGA] was significantly higher than the sensitivity of the conventional CA15-3 assay. Taking into account the above-mentioned clinical challenges in CA15-3 monitoring, the new CA15-3[WGA] assay should be further evaluated in prospective clinical trials.

6.8 Future studies

We have analyzed plasma levels of many other growth factors and receptors belonging to the VEGF family, and the results of these analyses will be published in the future. In addition, we have plasma available for additional exploratory analyses

of mBC patients. Tissue samples from the primary tumors and metastases of our study patients were gathered and will be analyzed in the future. We will continue to collaborate with our research partners to further improve and evaluate the lectin-based tumor marker method. Furthermore, we will hopefully find collaborators to evaluate the prognostic significance of the Tie1 receptor in mBC patients with a prospective study design.

7 SUMMARY AND CONCLUSIONS

The present study aimed to evaluate the feasibility of bevacizumab in combination with standard docetaxel or paclitaxel treatment as the first-line treatment of mBC. In publications II-III, IL-8, Tie1, Ang2, IL-6, IL-18, MMP-2, MMP-9, YKL-40, resistin and HMGB1 were evaluated for their prognostic value in mBC. In paper IV, new lectin-based CA15-3 methods were compared to the conventional CA15-3 assay with the aim of improving the sensitivity and specificity of the CA15-3 assay.

The main findings were the following:

1. Bevacizumab therapy in combination with standard taxane treatment is feasible, and the additional toxicity related to bevacizumab is mostly manageable. Our study scheme also included bevacizumab as maintenance therapy and optional bevacizumab with the second-line chemotherapy, and this approach resulted in a long overall survival for our patients. However, bevacizumab is currently indicated only for the first-line treatment of mBC based on the phase III trial results; therefore, similar treatment designs as in our trial cannot be used in standard clinical care.

2. Low plasma IL-8 levels before and during first-line chemotherapy in patients with mBC are prognostic for excellent long-term survival. These patients might be suitable for less frequent follow-up visits once our result is validated in a larger prospective mBC cohort.

3. Patients with high IL-8 concentrations have a poor prognosis. These patients may have more chemoresistant disease and therefore could be referred to clinical trials with other treatment approaches than traditional chemotherapy, e.g., immunotherapy.

4. The mBC patients with high circulating Tie1 levels at the baseline of their first-line chemotherapy treatment have a poor prognosis. However, the prognosis for their survival is even worse if the plasma Ang2 concentration is also high.

5. The new lectin-nanoparticle immunoassay CA15-3^{WGA} is more sensitive than the conventional CA15-3 assay and should be further prospectively evaluated.

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Supplementary Table 1. Breast cancer staging by AJCC 7th edition

Primary tumor (T)

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
Tis (Paget)	Paget disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget disease are categorized based on the size and characteristics of the parenchymal disease, although the presence of Paget disease should still be noted
T1	Tumor ≤ 20 mm in greatest dimension
T1mi	Tumor ≤ 1 mm in greatest dimension
T1a	Tumor > 1 mm but ≤ 5 mm in greatest dimension
T1b	Tumor > 5 mm but ≤ 10 mm in greatest dimension
T1c	Tumor > 10 mm but ≤ 20 mm in greatest dimension
T2	Tumor > 20 mm but ≤ 50 mm in greatest dimension
T3	Tumor > 50 mm in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)
T4a	Extension to chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma

Regional lymph nodes (N)

Clinical

Nx	Regional lymph nodes cannot be assessed (eg, previously removed)
N0	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted or in clinically detected* ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected* ipsilateral internal mammary nodes and in the absence of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s), with or without level I, II axillary node involvement, or in clinically detected * ipsilateral internal mammary lymph node(s) and in the presence of clinically evident level I, II axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s), with or without axillary or internal mammary lymph node involvement
N3a	Metastasis in ipsilateral infraclavicular lymph node(s)

N3b	Metastasis in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)

N3c	Metastasis in ipsilateral supraclavicular lymph node(s)

*"Clinically detected" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics highly suspicious for malignancy or a presumed pathologic macrometastasis on the basis of fine-needle aspiration (FNA) biopsy with cytologic examination.

Pathologic (pN)*

pNx	Regional lymph nodes cannot be assessed (for example, previously removed, or not removed for pathologic study)
pN0	No regional lymph node metastasis identified histologically. Note: Isolated tumor cell clusters (ITCs) are defined as small clusters of cells ≤ 0.2 mm, or single tumor cells, or a cluster of < 200 cells in a single histologic cross-section; ITCs may be detected by routine histology or by immunohistochemical (IHC) methods; nodes containing only ITCs are excluded from the total positive node count for purposes of N classification but should be included in the total number of nodes evaluated
pN0(i-)	No regional lymph node metastases histologically, negative IHC
pN0(i+)	Malignant cells in regional lymph node(s) ≤ 0.2 mm (detected by hematoxylin-eosin [H&E] stain or IHC, including ITC)
pN0(mol-)	No regional lymph node metastases histologically, negative molecular findings (reverse transcriptase polymerase chain reaction [RT-PCR])
pN0(mol+)	Positive molecular findings (RT-PCR) but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in 1-3 axillary lymph nodes and/or in internal mammary nodes, with metastases detected by sentinel lymph node biopsy but not clinically detected**
pN1mi	Micrometastases (> 0.2 mm and/or > 200 cells, but none > 2.0 mm)
pN1a	Metastases in 1-3 axillary lymph nodes (at least 1 metastasis > 2.0 mm)
pN1b	Metastases in internal mammary nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected†
pN1c	Metastases in 1-3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected**
pN2	Metastases in 4-9 axillary lymph nodes or in clinically detected‡ internal mammary lymph nodes in the absence of axillary lymph node metastases
pN2a	Metastases in 4-9 axillary lymph nodes (at least 1 tumor deposit > 2.0 mm)
pN2b	Metastases in clinically detected‡ internal mammary lymph nodes in the absence of axillary lymph node metastases
pN3	Metastases in ≥ 10 axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected*** ipsilateral internal mammary lymph nodes in the presence of ≥ 1 positive level I, II axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected†; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in ≥ 10 axillary lymph nodes (at least 1 tumor deposit > 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes
pN3b	Metastases in clinically detected‡ ipsilateral internal mammary lymph nodes in the presence of ≥ 1 positive axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected**
pN3c	Metastases in ipsilateral supraclavicular lymph nodes

*Classification is based on axillary lymph node dissection, with or without sentinel lymph node biopsy. Classification based solely on sentinel lymph node biopsy without subsequent axillary lymph node dissection is designated (sn) for "sentinel node"—for example, pN0 (sn).

** "Not clinically detected" is defined as not detected by imaging studies (excluding lymphoscintigraphy) or not detected by clinical examination.

*** "Clinically detected" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics highly suspicious for malignancy or a presumed pathologic macrometastasis on the basis of FNA biopsy with cytologic examination.

Distant metastasis (M)

M0	No clinical or radiographic evidence of distant metastasis
cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven > 0.2 mm

Stage	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0-1	N1mi	M0
IIA	T0-1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0-2	N2	M0
	T3	N1-2	M0
IIIB	T4	N0-2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

PUBLICATIONS

PUBLICATION
I

Bevacizumab Combined with Docetaxel or Paclitaxel as First-line Treatment of HER2-negative Metastatic Breast Cancer

Tiainen Leena, Tanner Minna, Lahdenperä Outi, Vihinen Pia, Jukkola Arja, Karihtala Peeter, Paunu Niina, Huttunen Teppo, Kellokumpu-Lehtinen Pirkko-Liisa

Bevacizumab Combined with Docetaxel or Paclitaxel as First-line Treatment of HER2-negative Metastatic Breast Cancer

LEENA TIAINEN[1,2], MINNA TANNER[1,2], OUTI LAHDENPERÄ[3], PIA VIHINEN[3],
ARJA JUKKOLA[4], PEETER KARIHTALA[4], NIINA PAUNU[2],
TEPPO HUTTUNEN[5] and PIRKKO-LIISA KELLOKUMPU-LEHTINEN[1,2]

[1]Department of Oncology, School of Medicine, University of Tampere, Tampere, Finland;
[2]Department of Oncology, Tampere University Hospital, Tampere, Finland;
[3]Department of Oncology, Turku University Central Hospital, Turku, Finland,
[4]Department of Oncology and Radiotherapy, Medical Research Center Oulu,
Oulu University Hospital and University of Oulu, Oulu, Finland;
[5]Oy 4Pharma Ltd, Turku, Finland

Abstract. *Aim: The study evaluated the efficacy of bevacizumab combined with a taxane-based treatment for advanced breast cancer. Patients and Methods: In this non-randomized phase II study 65 patients received 10 mg/kg bevacizumab i.v. (days 1 and 15, q4w) plus either 50 mg/m^2 docetaxel (days 1 and 15, q4w) or 90 mg/m^2 paclitaxel (days 1,8 and 15, q4w) i.v. until disease progression, maximal response, unacceptable toxicity or the withdrawal of consent. Patients without progression continued bevacizumab at 15 mg/kg i.v. (q3w) alone, or with endocrine therapy. (NCT00979641). Results: Progression-free survival was 11.3 months (95% confidence interval=9.7-16.0 months) and overall survival was 35.1 months (95% confidence interval=22.2-50.3 months). More than half of the patients (62%) responded at least partially. Bevacizumab-related serious adverse events occurred in 10.8% patients and one patient died because of gastrointestinal perforation. Conclusion: Treating advanced breast cancer with a bevacizumab-containing regimen as the first-line cytotoxic treatment resulted in excellent response rates and long survival.*

Metastatic breast cancer remains an incurable disease (1, 2). In Finland, nearly 5,000 patients are diagnosed with invasive

Correspondence to: Leena Tiainen, Department of Oncology, Tampere University Hospital, Teiskontie 35, 33521 Tampere, Finland. Tel: +358 331163130, Fax: +358 331163019, e-mail: leena.tiainen@pshp.fi

breast cancer every year, and the incidence has increased steadily over the past decades. The Finnish cancer registry data from 2014 shows that 815 women died of metastatic breast cancer, which was the most common cause of cancer death in women (3). In the CONCORD-2 study, a central analysis of population-based registry data worldwide for cancer survival was conducted, and the results were published in The Lancet in November 2014. The study reported that the treatment results of breast cancer in Finland are among the best in the world. The 5-year-survival rate of patients with breast cancer in Finland was 86.8% [95% confidence interval (CI)=85.9-87.7%) from 2005-2009, and was the highest in Northern Europe (4). However, new treatment options for advanced human epidermal growth factor receptor 2 (HER2)-negative disease are rare, and the overall survival benefit observed in these patients is modest (5, 6). For this reason, advanced HER2-negative breast cancer is a treatment challenge worldwide.

Bevacizumab is a recombinant humanized monoclonal antibody that inhibits vascular endothelial cell proliferation by blocking the binding of vascular endothelial growth factor A (VEGFA) to its receptor, therefore inhibiting tumor angiogenesis (7). Bevacizumab improves the outcomes of cytotoxic treatment in many metastatic malignancies, including colorectal, kidney, lung and ovarian cancer (8-11). There has been much debate about the status of bevacizumab treatment in metastatic breast cancer. Currently, the European Medicines Agency has only approved bevacizumab when combined with paclitaxel or capecitabine in a first or second-line setting (http://www.ema.europa.eu/ema/). In 2011, the US Food and Drug Administration revoked its accelerated approval of a breast cancer indication for bevacizumab due to the lack of a benefit in breast cancer overall survival and, in addition, due to the potentially life-threatening side-effects (http://www.fda.gov/).

In locally advanced and metastatic breast cancer, taxane-based treatment (docetaxel or paclitaxel), either in combination with another agent or as single-agent, therapy is considered one of the most effective choices for first-line treatment (5, 12), when cytotoxic treatment is needed. Combining bevacizumab with chemotherapy has been studied in certain phase III studies (13-18). Most of these studies investigated the benefit of bevacizumab combined with a taxane. Furthermore, other chemotherapy regimens have been explored, including capecitabine, anthracycline, vinorelbine and gemcitabine. Adding bevacizumab has led to higher response rates and longer progression-free survival (PFS) throughout the trials, but no significant differences in overall survival (OS) have yet been observed.

In addition to chemotherapy options, bevacizumab can also be combined with endocrine therapy, and the effect may be synergistic. Intracellular VEGF and estrogen signaling pathways cross at several points, and it can be hypothesized that adding bevacizumab to hormonal treatment might delay the development of endocrine therapy resistance (19, 20). In hormone receptor-positive advanced breast cancer, endocrine treatment with either an anti-estrogen or an aromatase inhibitor is a keystone of the treatment (5). It is used in metastatic breast cancer in biologically non-aggressive forms of the disease and in more aggressive forms after a maximal chemotherapy response has been achieved (5). For the first-line therapy of advanced breast cancer, an aromatase inhibitor combined with bevacizumab was investigated in a phase III LEA trial (21). Similarly, as reported in chemotherapy trials, the endocrine therapy–bevacizumab combination resulted in higher response rates but failed to demonstrate statistically significant improvements in both PFS and OS compared to endocrine-therapy alone.

This study aimed to investigate whether bevacizumab combined with either docetaxel or paclitaxel is a feasible choice for first-line therapy in metastatic breast cancer. The study also evaluated if using bevacizumab maintenance therapy with an endocrine therapy would have synergistic effects.

Patients and Methods

Patients. We screened and treated 65 patients at three study centers in Finland: Tampere, Oulu and Turku University hospitals. The study was initiated in May 2009 and data closure took place in April 2015. The median follow-up time was 24.1 months (range=1.6-66.3 months). Pre- and postmenopausal women were eligible if they had histologically or cytologically confirmed HER2-negative metastatic adenocarcinoma of the breast and were considered as candidates for taxane treatment. Patients were not allowed any prior chemotherapy for advanced disease but could have been treated with (neo)adjuvant chemotherapy if the disease-free interval was at least 6 months. Previous endocrine therapy for advanced disease was allowed. Both measurable and non-measurable (bone-only) diseases were eligible. Good performance status was required [Eastern Cooperative Oncology Group (ECOG) performance status 0-2]. Additional inclusion criteria included adequate hematological, renal and hepatic functions.

Patients were excluded if they had history of central nervous system metastases or pre-existing peripheral neuropathy at least grade 2 by National Cancer Institute Common Toxicity Criteria (NCI-CTC), version 3.0 (22). Additionally, circumstances that could increase the serious adverse events associated with bevacizumab were excluded, such as major surgery within the previous month, minor surgery within the last 24 hours prior to bevacizumab initiation, the use of anticoagulants or thrombolytic agents, a history of bleeding diathesis or coagulopathy, uncontrolled hypertension, clinically significant cardiovascular disease, a non-healing wound, an active peptic ulcer or bone fracture, a history of abdominal fistula, and a gastrointestinal perforation or intra-abdominal abscess within 6 months of enrollment. Furthermore, patients with a history of other malignancies were excluded.

The study protocol was approved by the Ethics Committee of Tampere University Hospital (R08142M) and the trial identifier is NCT00979641. Written informed consent was obtained from all patients included in the study.

Treatment. In part I of the treatment, the patients received taxane therapy intravenously (*i.v.*; 50 mg/m^2 docetaxel on days 1 and 15 or 90 mg/m^2 paclitaxel on days 1, 8 and 15) and 10 mg/kg bevacizumab *i.v.* on days 1 and 15 on a treatment cycle of 28 days. Treatment was continued until the maximal response, progressive disease, unacceptable toxicities necessitating the termination of taxane treatment or the patient's refusal. The maximal response was defined as an achieved response (a complete response (CR) or a partial response PR) that was the same between two response evaluations, or stable disease (SD) for more than 6 months. The study was initiated with the docetaxel-bevacizumab combination. After the negative results from the AVADO trial (14) were published, an amendment to the study protocol was made and the following enrolled patients were treated with a combination of paclitaxel and bevacizumab. In part II of treatment, after taxane treatment was discontinued, the responding patients continued to receive 15 mg/kg bevacizumab intravenously on day 1 q 21 days. In hormone receptor-positive patients, an endocrine therapy according to the investigator's choice was added to bevacizumab. This second part of the treatment was given until disease progression, unacceptable treatment-related toxicities or the withdrawal of the patient's consent. The study scheme is presented in Figure 1.

After disease progression, the continuation of bevacizumab with a second-line therapy was optional. The preferred chemotherapy option was capecitabine or the investigator's choice. Capecitabine was administered at a dose 1000 mg/m^2 twice-daily per os given on days 1-14 of a 3-week cycle.

Dose modifications, toxicity and response evaluations. The dosing of bevacizumab was not modified during the study. In case of grade 3-4 bevacizumab-related toxicity, bevacizumab was either temporarily or permanently suspended. If bevacizumab was permanently discontinued but chemotherapy not interrupted, the patient entered the follow-up phase of the study. The bevacizumab-related toxicities were monitored closely and specific treatment algorithms were made for hypertension, proteinuria, thromboembolic events, hemorrhage, gastrointestinal perforations and impaired wound-healing. The dose of the taxane was allowed to be reduced according to each clinic's standards of care in the case of taxane-related toxicity. Toxic effects
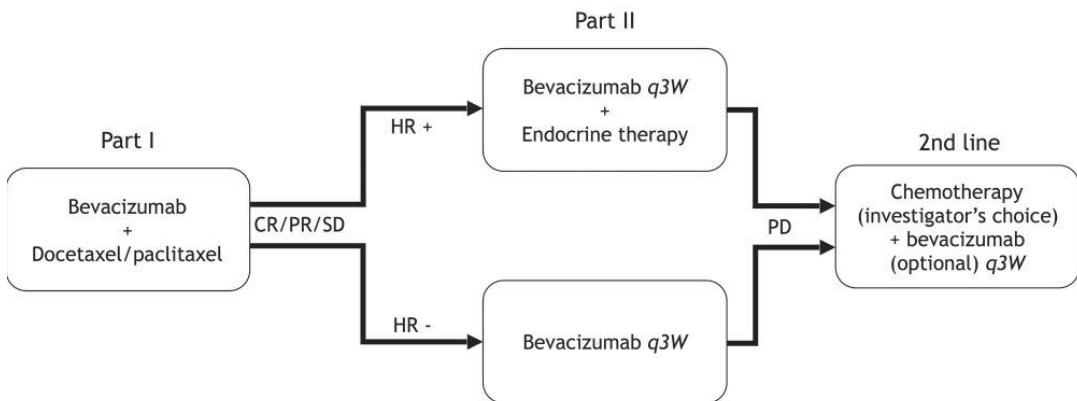
Figure 1. *Study scheme. CR: Complete response, PR: partial response, SD: stable disease, HR: hormone receptor, q3W: every 3 weeks, PD: progressive disease.*

were graded according to the NCI-CTC, version 3.0 (22). For second-line capecitabine, dose modifications were made according to the investigator's assessment. In patients with moderate renal impairment, the dose of capecitabine was reduced by 25%.

Tumor assessment was performed every 12 weeks until progression, according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 (23). All patients were followed-up every 6 months for an evaluation of their status and for survival by following the patient records.

Statistical analysis. The primary endpoint of the study was PFS in the first-line treatment setting and it was calculated from the date of treatment initiation to the date of investigator-assessed disease progression according to the RECIST criteria (23) or to the date of patient death. Secondary end-points were safety, the response rate and OS. Adverse events are displayed in standard frequency tables. The proportions of patients with CR, PR, SD and progressive disease (PD) as the best response were tabulated for each part of the treatment. OS was calculated from the date of treatment initiation to the date of death due to any cause. The median PFS and OS were calculated according to the Kaplan–Meier method. The analysis of PFS and OS included the stratification variable taxane choice (docetaxel–bevacizumab or paclitaxel–bevacizumab) and hormone receptor status. The Kaplan–Meier estimates obtained from the model were compared with the historical control group (14).

A total of 65 patients were expected to enter the study. This number would provide a probability of 80% for detecting a difference corresponding to a ratio of 1.34 between this study group and historical control group (equal to PFS of 10.7 months *versus* 8 months). The basis of the assumptions was that the accrual period was 18 months, the follow-up period was 36 months and the median PFS of the historical control group was 8.2 months in a series of 241 patients (14).

Results

Patient baseline characteristics. Between May 2009 and October 2013, 65 patients were enrolled. The baseline characteristics are shown in Table I. The majority of patients were post-menopausal with hormone receptor-positive disease. Additionally, most patients had received different combinations of adjuvant therapy and the vast majority of patients had received adjuvant chemotherapy. Furthermore, 34% of the patients with either estrogen or progesterone receptor-positive disease had received endocrine therapy for advanced disease.

Most patients had a heavy disease burden: visceral disease was common (82%) and liver metastases occurred in 51% of patients. Two-fifths of the patients had more than three metastatic sites. In addition, bone-only disease was observed only in five patients (Table I).

Efficacy. All 65 patients were evaluated for treatment efficacy and the PFS and OS results are shown in Figures 2 and 3. In part I of treatment, 32 patients were treated with docetaxel and 33 patients with paclitaxel. A total of 38 patients (58%) entered part II of treatment. Of these patients, the majority had hormone receptor-positive disease (87%) and only five patients had hormone receptor-negative disease. All hormone receptor-positive patients received endocrine therapy in part II in addition to bevacizumab according to the physician's choice, with letrozole being the most common drug (n=19). Other hormonal drugs that were used included anastrozole (n=4), exemestane (n=4), tamoxifen (n=3) and fulvestrant (n=3).

The median PFS for the first-line treatment was 11.3 months (95% CI=9.7-16.0, Figure 2) and the median OS was 35.1 months (95% CI=22.2-50.3; Figure 3). The overall response rate was high. One patient (1.5%) had a CR and 39 had PR (60.0%) in part I. SD was observed in 15 patients (23.1%). Thus, the clinical benefit rate (CR+PR+SD) for part I was 84.6%. Only three patients (4.6%) had PD as the best response

Table I. *Demographic and baseline characteristics of patients (n=65).*

Characteristic	Value
Median age (range), years	57 (32-75)
Menopausal status, n (%)	
Pre-menopausal	10 (15.4)
Post-menopausal	55 (84.6)
History of early-stage disease, n (%)	
Total	57 (87.7)
Disease-free interval, n (%)	
≤24 Months	11 (16.9)
>24 Months	46 (70.8)
Hormone receptor status, n (%)	
ER+PR+/ER+PR−	53 (81.5)
ER−PR−	12 (18.5)
Estrogen receptor status, n (%)	
Positive	51 (78.5)
Negative	14 (21.5)
Progesterone receptor status, n (%)	
Positive	46 (70.8)
Negative	19 (29.2)
Prior adjuvant chemotherapy, n (%)	
Total	46 (70.8)
Taxane	26 (40.0)
Anthracycline	38 (58.5)
Prior hormonal therapy, n (%)	
Total	44 (67.7)
(Neo)adjuvant	38 (58.5)
Metastatic/advanced disease	18 (27.7)
Current stage of disease, n (%)	
IV	65 (100.0)
Hormonal therapies used in metastatic setting, n (%)	
Anastrozole	4 (10.5)
Exemestane	7 (18.4)
Fulvestrant	5 (13.2)
Letrozole	12 (31.6)
GnRH analogs	3 (7.9)
Tamoxifen	4 (10.5)
Number of metastatic lesions, n (%)	
≤3	14 (21.5)
>3	51 (78.5)
Extent of disease	
<3 Sites	39 (60.0)
≥ 3 Sites	26 (40.0)
Site of metastatic disease, n (%)	
Visceral	53 (81.5)
Non-visceral	12 (18.5)

ER: Estrogen receptor; PR: progesterone receptor; GnRH: gonadotrophin-releasing hormone.

in part I. Docetaxel- and paclitaxel-based regimens led to similar median survival values: median PFS 11.3 months (95% CI=9.1-16.8) for docetaxel *vs*. 11.3 months (95% CI 7.4-30.7, p=0.47) for paclitaxel, median OS 38 months (95% CI=19.8-50.4) *vs*. 34.2 months (95% CI=18.1-not reached, p=0.77) respectively. The median OS for patients with hormone receptor-positive disease was 45.0 months (95% CI=30.2-51.3)

and for patients with triple-negative disease, it was 17.9 months (95% CI=8.5-26.9, p=0.011).

Subsequent therapy. Patients were allowed to receive bevacizumab together with a second-line chemotherapy according to investigators' choice. A total of 17 patients began second-line bevacizumab–chemotherapy combination. The preferred chemotherapy in the protocol was capecitabine (n=15) but patients also received paclitaxel and vinorelbine. The median PFS for second-line therapy was 5.1 months (95% CI=4.4-16.1 months) and the OS was 33.8 months (95% CI=24.7 months-NR). With the second-line bevacizumab–chemotherapy, seven patients responded partially (41%) and six patients had SD as the best response to the treatment (35%). No CRs were observed. Disease progression occurred in three patients (18%). For one patient, the response could not be defined because at data closure, the first response evaluation had not yet been performed.

Safety. During part I of the treatment, the bevacizumab–chemotherapy combination was generally well tolerated and most toxicity was mild (grade 1-2). The worst grade of a side-effect per patient is presented. The adverse events of all grades (1-4) that were most frequently reported were neutropenia (n=45, 69%), musculoskeletal pain (n=45, 69%), alopecia (n=44, 68%), leukocytopenia (n=41, 63%), fatigue (n=35, 54%), mucositis (n=35, 54%), anemia (n=35, 54%), epistaxis (n=34, 52%), constipation (n=27, 42%), nail disorders (n=23, 35%), proteinuria (n=22, 34%), diarrhea (n=22, 34%), elevated liver enzymes (n=20, 31%), nausea (n=20, 31%) and peripheral neuropathy (n=18, 28%). Serious adverse events during part I chemotherapy treatment are presented in Table II. The most common serious adverse event was neutropenia but febrile neutropenia was rare. One patient had a grade 5 toxicity due to the treatment and died during part I of the study. This patient had pre-existing diverticulosis and then developed diverticulitis, which resulted in gastrointestinal perforation and peritonitis. During bevacizumab maintenance, grade 3-4 adverse events were rare. The serious adverse events from part II treatment are presented in Table III.

Bevacizumab treatment-related adverse events according to the investigators' judgment are summarized in Table IV. The gastrointestinal perforation, mentioned above, was suspected to be related to bevacizumab. Hypertension and proteinuria were frequently reported but were usually of low grade. However, one patient suffered from grade 4 proteinuria and renal failure. In addition, over half of the patients had low-grade epistaxis.

In the second-line setting, the expected side-effects for capecitabine occurred in 17 patients treated in this part of the trial. The serious adverse events reported were grade 3 hand and foot syndrome (n=3) and a single case of grade 4 diarrhea.
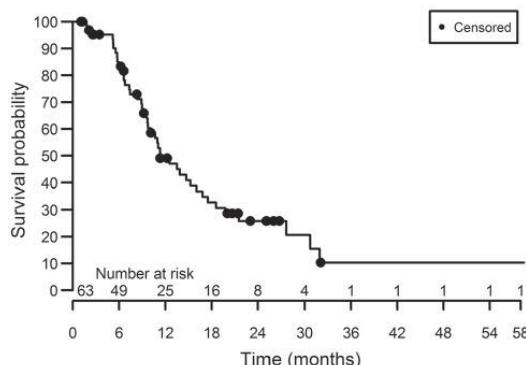
Figure 2. *Progression-free survival for the whole patient cohort.*
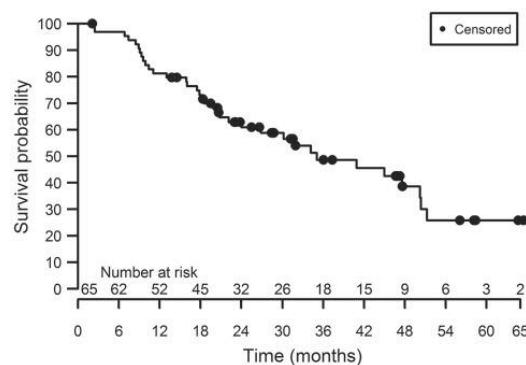
Figure 3. *Overall survival for the whole patient cohort.*

Discussion

This study resulted in excellent OS of almost 3 years (35.1 months) in patients with advanced breast cancer with poor prognostic features at the beginning of the trial. Visceral metastases were common (80%), and most patients had multiple metastases. Prior taxane treatment as adjuvant chemotherapy was given to 40% of these patients. The most favorable results towards a benefit from adding bevacizumab to chemotherapy are reported in the E2100 trial (13). In that study, the number of patients with visceral disease was similar to that observed in our study (79.5-87.1% depending on the treatment arm). Additionally, the extent of disease (42.0-46.3% of patients had more than three metastatic sites) was quite similar in both studies. Only approximately 15% of E2100 patients were pre-treated with taxanes in an adjuvant setting compared to 40% of our patients. PFS was reported

Table II. *Grade 3-4 adverse events experienced by patients in part I of the treatment.*

	Patients (n=65)	
Adverse event	Grade 3	Grade 4
Fatigue	2	
Neutropenia	9	16
Leukocytopenia	11	2
Elevated liver enzymes	1	1
Infection	9	
Febrile neutropenia or neutropenic sepsis	3	1
Peripheral neuropathy	1	
Pain	3	1
Diarrhea	1	
Nausea	1	
Cardiac disorders	1*	
Osteonecrosis of the jaw	1	
Drug hypersensitivity	1	
Gastrointestinal perforation		1**

*Supraventricular tachycardia; **patient died, grade 5 adverse event.

Table III. *Grade 3-4 adverse events experienced by patients in part II of the treatment.*

	Grade 3-4/patients	
Adverse event	HR+ (n=33)	HR− (n=5)
Infection	2	
Leukocytopenia	1	
Elevated liver enzymes	2	
Peripheral neuropathy	1	
Anorexia	1	
Cardiac disorders	2*	
Hyponatremia	1	1

HR+: Hormone receptor-positive (estrogen receptor+ or progesterone receptor+); HR−: hormone receptor-negative. *Congestive heart failure, coronary artery thrombosis.

Table IV. *Bevacizumab-related events experienced by patients in this study.*

	Patients (n=65)			
Adverse event	Grade 1-2	Grade 3	Grade 4	Grade 5
Hypertension	16	2		
Proteinuria	18	3	1	
Bleeding/hemorrhage	9			
Epistaxis	34			
Gastrointestinal fistula/abscess	2			
Gastrointestinal perforation				1

to be very similar between the E2100 study and our study (11.8 months in E2100 and 11.3 months in our trial). Nevertheless, the OS was remarkably longer in our trial: 35.1 months compared to 26.7 months observed in the E2100 trial.

There are some possible explanations for the long OS observed in this study. The main difference in our study when compared to other studies of first-line chemotherapy combining bevacizumab with taxanes (13, 14, 16, 18) is that after a maximal response was reached in our study, bevacizumab was continued as a maintenance treatment with endocrine therapy in patients with hormone receptor-positive disease. Bevacizumab maintenance was given to 38 patients (58%) and the majority of these patients (87%) had hormone receptor-positive disease and received endocrine therapy with bevacizumab. Intracellular estrogen signaling pathways and VEGF pathways have several interactions (19, 20, 24, 25); therefore, endocrine treatment may add a substantial benefit to bevacizumab monotherapy, as also recently shown with androgen signaling pathways and VEGF in prostate cancer (26). In addition, using biweekly instead of triweekly docetaxel infusions might have led to lower treatment toxicity and, therefore, to prolonged survival. This was previously demonstrated in our randomized phase III Prosty trial where triweekly and biweekly docetaxel dosing were compared in advanced castration-resistant prostate cancer (27). Weekly paclitaxel compared to triweekly infusions has also demonstrated survival benefit in advanced breast cancer (28).

This trial has many differences compared to the LEA trial (21). In the LEA trial, patients with advanced disease were endocrine treatment-naïve. In our study, one-third of the patients with hormone receptor-positive disease had received hormonal therapies for advanced disease, meaning that the patients seemed to have less hormone treatment-sensitive disease. Half of the patients in our trial also had liver metastasis compared to only 20% in the LEA trial. Thus, our patients had less favorable prognoses. The OS for this patient population is, as expected, shorter with less favorable prognostic features. In the LEA trial, the OS was 52.1 months in patients treated with the first-line bevacizumab-endocrine therapy combination. This exceeds that of the patients with hormone receptor-positive disease of our study by only 7.1 months, which is less than expected considering the poor prognostic features of the disease at the beginning of our patients' treatments. Both these studies favor the hypothesis of an interaction between hormonal and angiogenetic cellular pathways in breast cancer.

In preclinical studies, it has been reported that tumor progression may be accelerated after short-term angiogenesis inhibition (29). On the other hand, treating colorectal cancer with second-line bevacizumab-chemotherapy combination after disease progression with first-line therapy including bevacizumab was shown to have survival benefits (30). Therefore, some patients with metastatic adenocarcinoma

may benefit from prolonged VEGF inhibition in terms of survival. This is one possible explanation for the long OS seen in our study.

High response rates have been reported in all of the trials with bevacizumab combined with a first-line chemotherapeutic. The response rates previously reported with the bevacizumab–taxane combination range from 36.9% to 64.1% compared to 21.2-46.4% with single taxane therapy (13, 14, 31). Similarly, good responses were achieved in this study. The clinical benefit rate was 84.6% and 62% of patients responded at least with PR according to the RECIST criteria, which is in line with previously published data.

In this small series of patients, no difference in PFS or OS was observed between the two taxane-treated groups. Half of the patients in the study were treated with paclitaxel and the other half with docetaxel. Thus, there is an indication that docetaxel and paclitaxel are similarly effective with bevacizumab.

Bevacizumab adds treatment toxicity compared to single taxane chemotherapy. In this study, bevacizumab-related serious events were rare (10.8%). However, one patient died because of bevacizumab-related toxicity, which in this case was a fatal peritonitis. The contributing factor was underlying diverticulosis in our patient. Additionally, high-grade proteinuria and hypertension were observed, which are known side-effects of bevacizumab (13-17, 31). Caution should be exercised when treating patients with known risk factors for the use of bevacizumab, namely a history of thromboembolic events, cardiovascular disease or risk factors for abdominal infection and fistula, among others. The other grade 3-4 toxicities observed were related to chemotherapy or to the metastatic disease itself and were reported at the anticipated rates. In the AVADO trial, 75-78% of the patients, depending on the treatment arm, treated with a bevacizumab–docetaxel combination had at least one grade 3 toxicity due to the treatment (14), whereas a minimum of grade 3 toxicity was observed in 71% of our patients. Only 24% of the patients had grade 3-4 toxicity during bevacizumab–capecitabine treatment in our study. No unexpected new side-effects were reported in our study. In conclusion, combining bevacizumab with paclitaxel or docetaxel or to second-line capecitabine has an acceptable side-effect profile.

The small sample size does not allow us to draw any conclusions about the efficacy of second-line chemotherapy.

Although our patients presented many poor prognostic features at baseline, the OS achieved of nearly 3 years is remarkable. This study intended to determine whether bevacizumab adds an advantage to taxane treatment followed by a bevacizumab maintenance therapy with an endocrine therapy. With an OS of 17.9 months in patients with triple-negative disease and 45.0 months in a hormone receptor-positive study population, it can be concluded that combining bevacizumab with a conventional taxane

treatment is a treatment option. This is especially true in patients with a heavy disease burden and needing rapid tumor shrinkage. We have gathered a comprehensive serum, plasma and tumor biopsy collection from the study population and we aim to explore markers predictive for the long response to bevacizumab combination therapies.

Acknowledgements

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PUBLICATION
II

Low Plasma IL-8 Levels During Chemotherapy Are Predictive of Excellent Long-Term Survival in Metastatic Breast Cancer

Tiainen Leena, Hämäläinen Mari, Luukkaala Tiina, Tanner Minna, Lahdenperä Outi, Vihinen Pia, Jukkola Arja, Karihtala Peeter, Moilanen Eeva, Kellokumpu-Lehtinen Pirkko-Liisa

Original Study

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Low Plasma IL-8 Levels During Chemotherapy Are Predictive of Excellent Long-Term Survival in Metastatic Breast Cancer

Leena Tiainen,[1,3] Mari Hämäläinen,[4] Tiina Luukkaala,[2] Minna Tanner,[1,3] Outi Lahdenperä,[5] Pia Vihinen,[5] Arja Jukkola,[1,3] Peeter Karihtala,[6] Eeva Moilanen,[4] Pirkko-Liisa Kellokumpu-Lehtinen[1,3]

Abstract

Plasma interleukin (IL)-8 levels were monitored in 58 patients with metastatic breast cancer before and during first-line chemotherapy, and changes in the IL-8 levels were correlated with patient survival data. Monitoring plasma IL-8 levels before and during chemotherapy identifies patients with excellent prognosis whose IL-8 levels stay constantly below 16.6 pg/mL.

Background: Interleukin (IL)-8 is a proinflammatory cytokine, and high levels of IL-8 are associated with poor prognosis in many malignancies. The objective of this study was to explore the clinical benefit of monitoring plasma IL-8 levels during breast cancer chemotherapy. **Patients and Methods:** We conducted an exploratory analysis of several circulating proteins, including IL-8, in the plasma. Plasma samples were obtained from 58 metastatic breast cancer patients who took part in a prospective phase 2 first-line bevacizumab chemotherapy trial. Samples were analyzed before therapy, after 6 weeks and 6 months of treatment, and at the final study visit. On the basis of a trajectory analysis of the plasma IL-8 levels, the patients were divided into 3 trajectory groups. **Results:** Plasma IL-8, IL-6, IL-18, matrix metalloproteinase (MMP)-2, MMP-9, YKL-40, resistin, and high-mobility group box 1 (HMGB1) concentrations were measured, and the most pronounced predictor of patient survival was IL-8. On the basis of the trajectory analysis of the IL-8 levels, the majority of patients (n = 35, 60%) belonged to trajectory group 1, and these patients had significantly lower IL-8 levels before and during the entire chemotherapy treatment period than did the patients in the other groups. Trajectory group 1 patients had significantly better overall survival compared to patients in trajectory group 2 (n = 17; age-adjusted HR = 2.45; 95% confidence interval, 1.21-5.97; P = .012) and 3 (n = 6; age-adjusted HR = 8.65; 95% confidence interval, 3.16-23.7; P < .001). **Conclusion:** Low IL-8 levels during chemotherapy treatment might help identify patients with prolonged survival.

Introduction

Breast cancer is the most common cause of cancer-related death in women.[1] Currently, patients with human epidermal growth factor 2 (HER2)-negative advanced breast cancer will survive for approximately 2 to 3 years after diagnosis of advanced cancer.[2-5]

The disease of most patients will respond to chemotherapy and endocrine therapy, but the cancer will eventually progress. More investigational effort should be expended to find patients with disease that will not respond to current therapies and who are in need of novel investigational treatment options. Furthermore, early

[1]Department of Oncology, Faculty of Medicine and Health Technology
[2]Research, Development and Innovation Center, Tampere University Hospital and Health Sciences, Faculty of Social Sciences, Tampere University, Tampere, Finland
[3]Department of Oncology, Tampere University Hospital, Tampere, Finland
[4]The Immunopharmacology Research Group, Faculty of Medicine and Health Technology, Tampere University and Tampere University Hospital, Tampere, Finland
[5]Department of Oncology and Radiotherapy and FICAN West Cancer Center, Turku University Central Hospital, Turku, Finland

[6]Department of Oncology and Radiotherapy, Medical Research Center Oulu, Oulu University Hospital and University of Oulu, Oulu, Finland

palliative care improves patient quality of life, symptom management, and even treatment outcomes.[6-8] In particular, patients with chemoresistant cancer might benefit from earlier palliative symptom management if these patients could be better identified.

Interleukin (IL)-8 (alternatively known as CXCL8) is a proinflammatory cytokine.[9] Its complex effects on the tumor microenvironment may result in tumor proliferation, survival, and chemoresistance in malignant disease.[10-14] High IL-8 serum levels and tumor expression are known to be associated with poor patient prognosis in many malignant diseases, including breast cancer.[13,15,16] Even in localized breast cancer, patients with high circulating IL-8 levels have a poorer prognosis than patients with low IL-8 levels.[17,18]

In addition to IL-8, many other cytokines and circulating regulatory factors are associated with breast cancer and are considered to be potential biomarkers for cancer prognosis.[19-34] Serum concentrations of IL-6 and IL-18 are elevated in breast cancer patients,[19,20] and high circulating IL-6 levels are linked to shorter survival in metastatic breast cancer patients than are low circulating IL-6 levels.[21,22] Additionally, IL-6 and IL-18 are associated with chemotherapy resistance.[23,24] Matrix metalloproteinase (MMP)-2 and MMP-9 serum levels are associated with poor overall survival (OS), even in patients with localized breast cancer.[25] YKL-40 (also known as chitinase-3−like protein 1) has been suggested to play a role in cell proliferation, differentiation, inflammation, and tissue remodeling, and has been associated with malignancies with poor survival.[35-37] In patients with either local or advanced breast cancer, high serum YKL-40 levels predict a poor prognosis.[26-28]

Obesity is a known risk factor for breast cancer.[38] Therefore, adipocytokines, including resistin, may be related to breast cancer development and prognosis. Serum resistin levels are known to be elevated in breast cancer patients compared to healthy controls.[29,30] Additionally, compared to low resistin expression, high resistin expression in the primary breast cancer tumor tissue is associated with poorer patient survival and more unfavorable clinicopathologic features of the primary cancer.[31] High-mobility group box 1 (HMGB1) is a ubiquitous nuclear protein that contributes to DNA repair and the stabilization of nuclear homeostasis.[32] HMGB1 is expressed at higher levels in many tumor types compared to healthy tissue,[33] and its expression is associated with many diseases, including cancer.[34]

We conducted an exploratory analysis of multiple plasma cytokines and other circulating proteins. The aim of the study was to identify prognostic markers for metastatic breast cancer. IL-8 levels, a promising biomarker, were explored before and during chemotherapy treatment for their value in predicting patient prognosis. We also measured plasma levels of IL-6, IL-18, MMP-9, MMP-2, YKL-40, resistin, and HMGB1, and investigated their prognostic significance.

Patients and Methods

We conducted a prospective phase 2 trial for metastatic breast cancer patients. The study patients had histologically verified HER2-negative advanced breast cancer and had not received previous chemotherapy in a metastatic setting. A total of 65 patients were enrolled onto the trial at 3 Finnish university oncology clinics between 2009 and 2013 (NCT00979641). The study inclusion criteria, trial design, and clinical results have been published previously.[2] In brief, study patients were treated with a bevacizumab and taxane (paclitaxel or docetaxel) combination as the first-line treatment for metastatic breast cancer. Patients without disease progression continued bevacizumab treatment after the taxane chemotherapy was discontinued. Patients with estrogen receptor−positive breast cancer also received endocrine therapy with bevacizumab maintenance therapy. For second-line therapy after disease progression, the continuation of bevacizumab was optional with chemotherapy. All patients provided written informed consent, and the regional ethics committee of Tampere University Hospital approved the study protocol (R08142M).

Plasma samples were gathered before the initiation of chemotherapy (baseline), after 6 weeks of treatment, after 6 months of treatment, and at the final study visit.

Measurement of Plasma Cytokines

Plasma IL-8, IL-6, IL-18, MMP-2, MMP-9, YKL-40, resistin, and HMGB1 concentrations were measured by enzyme-linked immunosorbent assays (ELISAs) using reagents from BD Biosciences (Erembodegem, Belgium; IL-8), eBioscience (San Diego, CA; IL-6 and IL-18), R&D Systems Europe (Abingdon, UK; MMP-2, MMP-9, YKL-40, resistin), and IBL International (Hamburg, Germany; HMGB1). ELISAs were carried out according to a standard protocol. In brief, for MMP-2, MMP-9, YKL-40, and resistin, a 96-well plate was coated with capture antibody and incubated overnight at 4°C. The wells were washed with phosphate-buffered saline−0.05% Tween 20 and blocked with 1% bovine serum albumin in phosphate-buffered saline, 250 μL per well, for 1 hour at room temperature (RT). The wells were washed, and the standards, and samples diluted in reagent diluent (1% bovine serum albumin in phosphate-buffered saline) were added to the wells and incubated for 2 hours at RT. The wells were washed. Detection antibodies diluted in reagent diluent (with normal goat serum for MMP-9) were added and incubated for 1.5 h at RT. Streptavidin-conjugated to horseradish peroxidase was added after the wash step and incubated for 15 minutes at RT. The wells were washed, and BioFX TMB substrate solution (SurModics, Eden Prairie, MN) was added and incubated for 15 minutes in the dark at RT. After adding 50 μL of stop solution (1 N H_2SO_4), the absorbance of each well was measured at 450 nm with a correction wavelength at 540 nm within 20 minutes with a Victor3 Multilabel Counter (Perkin Elmer, Turku, Finland), and the results were calculated from a standard curve using the smoothed spline method with MultiCalc software (Perkin Elmer). For IL-8, IL-6, IL-18, and HMGB1, ELISAs were performed according to the manufacturer's protocols and then measured and calculated as stated above.

Patient Characteristics

Plasma samples were available from 58 patients (89%). Patient characteristics are listed in Table 1. After taxane discontinuation, patients without disease progression and with hormone receptor−positive disease received endocrine therapy in combination with bevacizumab. Letrozole was the most common endocrine therapy choice (n = 19). The other endocrine therapies included anastrozole (n = 4), exemestane (n = 4), tamoxifen (n = 3), and fulvestrant (n = 3).

Table 1	Baseline Characteristics and Efficacy Results in Plasma Biomarker Population and of Patients With Baseline Samples Available Compared to Overall Study Population	

Characteristic	Plasma Biomarker Population (N = 58)	Overall Study Population (N = 65)
Age (y), median (range)	58 (32-75)	57 (32-75)
Menopausal Status		
Premenopausal	9 (15.5)	10 (15.4)
Postmenopausal	49 (84.5)	55 (84.6)
History of early stage disease	52 (89.7)	57 (87.7)
Disease-Free Interval, mo		
≤24	10 (19.2)	11 (16.9)
>24	42 (80.8)	46 (70.8)
Hormone Receptor Status		
ER+ and/or PR+	47 (81.0)	53 (81.5)
ER− and PR−	11 (19.0)	12 (18.5)
No. of Metastatic Lesions		
≤3	11 (19.0)	14 (21.5)
>3	47 (81.0)	51 (78.5)
Extent of Disease		
<3 sites	36 (62.1)	39 (60.0)
≥3 sites	22 (37.9)	26 (40.0)
Site of Metastatic Disease		
Visceral disease	46 (79.3)	53 (81.5)
Nonvisceral disease	12 (20.7)	12 (18.5)
Overall survival, median (95% CI)	37.5 (25.4-49.6)	35.1 (22.2-50.3)
Progression-free survival, median (95% CI)	11.3 (8.3-14.4)	11.3 (9.7-16.0)
Best Response to Treatment		
Complete response/partial response	38 (71.7)	40 (61.5)
Stable disease	13 (24.5)	15 (23.1)
Progressive disease	2 (3.8)	3 (4.6)

Data are presented as n (%) unless otherwise indicated.
Abbreviations: CI = confidence interval; ER = estrogen receptor; PR = progesterone receptor.

Baseline samples were available from 53 patients. Breast cancer progression was the reason for study discontinuation for most patients (n = 36, 55%). Final plasma samples were available from 50 patients, of whom 24 had disease progression as the reason for treatment discontinuation (48%). The remaining 26 patients discontinued the study treatment as a result of treatment side effects. Plasma samples at week 6 and month 6 were available only from patients who were following the study treatment plan at that time point.

Statistical Analysis

The statistical plan for the analysis was exploratory. IL-8, IL-6, IL-18, MMP-9, MMP-2, YKL-40, resistin, and HMGB1 levels were dichotomized as low or high for each patient using the median value for each molecule as the cutoff value. Additionally, IL-8, IL-6, IL-18, MMP-9, MMP-2, YKL-40, resistin, and HMGB1 levels were divided into 4 groups using the baseline quartile ranges as the cutoff values.

IL-8 values were clustered by the trajectory analysis originally presented by Nagin.[39] Trajectory groups are clusters of individuals following similar trajectories to an outcome over time.[40] The trajectories were created according to all measurements of IL-8 levels in each patient as a continuous outcome measure. These trajectories are presented in Figure 1. The analyses undertaken were latent class mixture models of quadratic trajectories including a random intercept and concomitant variables. Models were fitted by the FlexMix package[41] of the statistical program R 3.3.0.[42] Relative goodness of fit was assessed using the Bayesian information criteria.

Because of the nonparametric distribution of the IL-8 levels, medians with the confidence interval (CI) of the median are reported. The Mann-Whitney U test was used to compare the median IL-8 levels of different baseline characteristics and trajectory groups. Hazard ratios (HR) with 95% CIs were calculated by Cox proportional hazard regression analysis. Multivariable analyses were adjusted for age (continuous), menopause status (premenopausal/postmenopausal), hormone receptor status (negative/positive), presence of visceral metastasis (yes/no), number of metastatic lesions (cutoff of 3 metastatic lesions), and extent of disease (cutoff of 3 metastatic sites). Median OS, median progression-free survival (PFS), and their CIs were calculated by the Kaplan-Meier method. The Wilcoxon signed-rank test was used to compare the baseline, week 6, month 6, and final plasma IL-8 levels between the different trajectory groups. $P < .05$ was considered statistically significant. Statistical analyses were performed by SPSS 23 software (IBM, Armonk, NY).

Results

IL-8 Levels and Patient Baseline Characteristics

There were no statistically significant differences in the baseline IL-8 levels between groups with different baseline characteristics, including menopause status ($P = .104$), hormone receptor status ($P = .152$), number of metastatic lesions ($P = .539$), and presence of visceral disease ($P = .941$). Borderline significantly lower baseline IL-8 levels were observed in patients with <3 metastatic sites compared to the patients with ≥3 metastatic sites (<3 metastatic sites median baseline IL-8: 8.9 pg/mL; 95% CI, 7.8-9.9 pg/mL vs. ≥3 metastatic sites median IL-8: 12.5 pg/mL; 95% CI, 8.0-25.4 pg/mL; $P = .057$).

Prognostic Significance of Baseline IL-8 Levels

The patients were divided into two groups (low and high baseline plasma IL-8 level) using a median value of 9.4 pg/mL as the cutoff point. The PFS and OS of these IL-8 groups are listed in Table 2. The high baseline IL-8 group had a significantly shorter OS ($P = .023$).

Trajectory Analysis of IL-8 Levels

The distributions of the 3 trajectory groups are presented in Figure 1 and Table 3. Trajectory group 1 patients had constantly low IL-8 concentrations; the range of IL-8 levels in trajectory group

Figure 1 Interleukin 8 Trajectory Groups. Shown Are Trajectory Groups 1 (n = 35), 2 (n = 17), and 3 (n = 6)
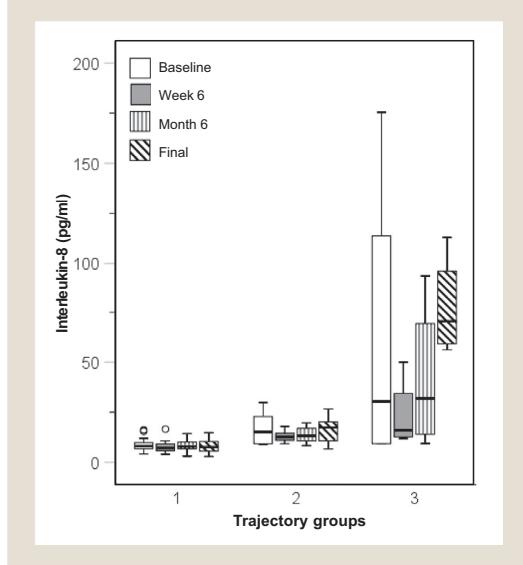
1 was 2.6 to 16.6 pg/mL during the entire treatment period. Trajectory groups 2 and 3 had significantly higher IL-8 levels at baseline, at week 6, at month 6, and at the final study visit compared to trajectory group 1 (Table 3). The final IL-8 levels of trajectory group 3 patients were significantly higher than their month 6 IL-8 plasma levels ($P = .043$). In trajectory group 3, there were no significant changes in the IL-8 levels between the baseline and week 6 and between week 6 and month 6. The changes in the IL-8 levels in trajectory groups 1 and 2 over time were not statistically significant.

The patients belonging to trajectory group 3 with very high IL-8 levels had significantly shorter PFS than the patients belonging to the other groups (Table 4, Figure 2A). No significant differences in PFS were detected between the patients in trajectory groups 1 and

2. In addition, the patients in trajectory groups 2 and 3 had significantly shorter OS than the patients in trajectory group 1 using both an age-adjusted HR and a multivariable Cox model adjusted for age, menopause status, hormone receptor status, presence of visceral metastases, number of metastatic lesions, and extent of the disease (Table 4, Figure 2B).

To further examine the clinical utility of IL-8 levels, a cutoff value of 16.6 pg/mL was found to be useful for finding patients with a significantly more favorable long-term prognosis. All the IL-8 levels in trajectory group 1 remained below 16.6 pg/mL before and during the entire chemotherapy treatment period. A cutoff value of 16.6 pg/mL could identify all of the 35 patients who were categorized into trajectory group 1. In contrast, only one trajectory group 2 patient (1/17, 5.9%) had IL-8 levels constantly below 16.6 pg/mL, and all of the patients in trajectory group 3 had IL-8 levels higher than 16.6 pg/mL before or during chemotherapy treatment. For PFS, the age-adjusted HR was borderline significant for the patients with IL-8 levels higher than 16.6 pg/mL before or during chemotherapy treatment (age-adjusted HR 2.00; 95% CI, 0.97-4.14; $P = .060$), while the multivariable HR was not statistically significant (multivariable HR = 1.91; 95% CI, 0.89-4.09; $P = .094$; Figure 3A). However, the HR for OS was strongly significant for both the age-adjusted and multivariable Cox models for the patients with IL-8 levels higher than 16.6 pg/mL before or during chemotherapy treatment (age-adjusted HR 3.02; 95% CI, 1.60-5.71; $P = .001$, multivariable HR = 3.90; 95% CI, 1.88-8.12; $P < .001$; Figure 3B).

Highest Baseline IL-8 Quartile Level and Prognosis

A very high baseline plasma IL-8 level was also a strong sign of poor prognosis without knowledge of IL-8 levels during treatment. The highest (>18.8 pg/mL) baseline IL-8 level quartile patients had the poorest prognosis in terms of median PFS and OS, at 9.6 months (95% CI, 5.47-13.7 months) and 19.7 months (95% CI, 8.60-30.9 months), respectively (Supplemental Table 1 in the online version). The multivariable HR for PFS was 6.52 (95% CI, 1.58-26.9; $P = .010$) for the highest plasma IL-8 quartile, and the multivariable HR for OS was 8.38 (95% CI, 2.60-27.0; $P < .001$). All of the patients in the highest quartile belonged to trajectory groups 2 (n = 9) and 3 (n = 4). Altogether, a high baseline IL-8 level > 18.8 pg/mL (the highest quartile) could identify 62%

Table 2 Cox Regression Analysis for PFS and OS Grouped by Low or High Baseline IL-8 Levels Using Median as Cutoff Value

Baseline IL-8	pg/mL	No. Patients	No. Events	Adjusted HR 1[a]			Adjusted HR 2[b]		
				HR	95% CI	*P*	HR	95% CI	*P*
PFS									
Low	≤9.4	27	15	1			1		
High	>9.4	26	16	1.44	0.70-2.93	.316	1.32	0.58-3.00	.493
OS									
Low	≤9.4	27	16	1			1		
High	>9.4	26	23	2.14	1.10-4.12	.023[c]	1.65	0.82-3.34	.159

Abbreviations: CI = confidence interval; HR = hazard ratio; IL = interleukin; OS = overall survival; PFS = progression-free survival.
[a]HR adjusted for age.
[b]HR adjusted for age, menopause status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions, and extent of disease.
[c]Statistically significant.

Plasma IL-8 Levels During Chemotherapy

Table 3	Median IL-8 Levels in 3 Trajectory Groups								
Trajectory Group	No. Patients	Baseline IL-8 (pg/mL)		Week 6 IL-8 (pg/mL)		Month 6 IL-8 (pg/mL)		Final IL-8 (pg/mL)	
		Median	95% CI	Median	95% CI	Median	95% CI	Median	95% CI
1	35	8.05	7.40-9.40	7.30	5.90-9.00	7.60	6.50-8.90	7.90	6.30-9.90
2	17	21.7	12.4-27.5	13.4	11.3-18.0	11.6	8.40-16.1	15.1	7.30-20.2
3	6	38.9	9.30-175	16.2	11.9-50.2	39.4	9.50-93.4	78.9	56.4-113
1 vs. 2 P		<.001[a]		<.001[a]		.006[a]		.001[a]	
1 vs. 3 P		.002[a]		.002[a]		<.001[a]		<.001[a]	
2 vs. 3 P		.199		.332		.009[a]		.001[a]	

Abbreviations: CI = confidence interval of median; IL = interleukin.
[a]Statistically significant.

(13/21) of the patients in the poorer prognosis trajectory groups 2 and 3 with the baseline plasma samples available.

IL-6, IL-18, MMP-9, MMP-2, YKL-40, Resistin, and HMGB1 Baseline Levels as Prognostic Markers for Survival

A Cox regression analysis was also performed for all other measured markers: IL-6, IL-18, MMP-9, MMP-2, YKL-40, resistin, and HMGB1. Using the median and quartile levels as cutoff values, there were no statistically significant differences in PFS using all individual markers (Supplemental Tables 2 and 3 in the online version). Using the median as a cutoff value, a high baseline MMP-9 level was borderline significant for longer OS (multivariable HR = 0.52; 95% CI, 0.26-1.03; P = .063). Using the baseline quartile levels as cutoff values, the baseline quartile level of 50% to 75% for MMP-9 was prognostic for OS (multivariable HR = 0.37; 95% CI, 0.13-1.01; P = .054), as was the highest baseline quartile MMP-9 level (multivariable HR for OS 0.22; 95% CI, 0.07-0.68; P = .009). The highest baseline quartile level of YKL-40 was a sign of poor prognosis in an age-adjusted Cox regression (HR 3.08; 95% CI, 1.10-8.61; P = .031). However, in multivariable analysis, the highest baseline level of YKL-40 lost its prognostic significance (multivariable HR = 2.13; 95% CI, 0.65-6.97; P = .211). For IL-6, IL-18, MMP-2, resistin, and HMGB1, the median and

quartile cutoff level groups revealed no significant OS differences (Supplemental Tables 4 and 5 in the online version).

Discussion

IL-8 level monitoring during chemotherapy for metastatic breast cancer is a promising approach for identifying patients with good prognosis. High baseline plasma IL-8 levels are known to be a poor prognostic marker in breast cancer.[15] However, to our knowledge, our study is novel in its monitoring of plasma IL-8 levels in metastatic breast cancer patients during chemotherapy. We identified a large group of patients belonging to trajectory group 1 (35/58, 60.3%) who had a substantially better prognosis than the rest of the patients. The median OS (50 months) for trajectory group 1 patients was exceptionally good (95% CI, 43.5-56.3 months) in patients with metastatic HER2-negative breast cancer. In contrast, the median OS for trajectory group 2 patients (median OS 24 months; 95% CI, 15.5-32.0 months) was less than half of the OS in the group 1 patients. Interestingly, the remaining 6 patients belonging to trajectory group 3 had exceptionally high IL-8 levels during the entire chemotherapy period, and these patients had a short median OS of 8 months. High IL-8 levels are known to be a sign of chemoresistance.[12,43] The poor survival of our trajectory group 3 patients is a confirmatory finding for the previously reported chemoresistant nature of metastatic cancer with high IL-8 levels.

| Table 4	PFS and OS of Patients of 3 Trajectory Groups							
Trajectory Group	No. Patients	No. Events	HR 1[a]			HR 2[b]		
			HR	95% CI	P	HR	95% CI	P
PFS								
1	35	19	1			1		
2	17	9	1.27	0.52-3.08	.589	0.94	0.35-2.54	.917
3	6	6	4.56	1.65-12.6	.003[c]	4.01	1.24-12.9	.020[c]
OS								
1	35	22	1			1		
2	17	15	2.45	1.21-5.97	.012[c]	3.29	1.45-7.45	.004[c]
3	6	6	8.65	3.16-23.7	<.001[c]	7.82	2.27-26.9	.001[c]

Abbreviations: CI = confidence interval; HR = hazard ratio; IL = interleukin; OS = overall survival; PFS = progression-free survival.
[a]HR adjusted for age.
[b]HR adjusted for age, menopause status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions, and extent of disease.
[c]Statistically significant.

PFS and OS by Multivariable Cox Regression. (A) PFS and (B) OS of 3 Trajectory Groups Using Multivariable Cox Regression Adjusted for Age, Menopause Status, Hormone Receptor Status, Presence of Visceral Metastasis, Number of Metastatic Lesions, and Extent of Disease. Median Survivals and Their Confidence Intervals Were Calculated by Kaplan-Meier Method. *Log-rank *P* Value Between Trajectory Groups 1 and 2. **Log-rank *P* Value Between Trajectory Groups 1 and 3
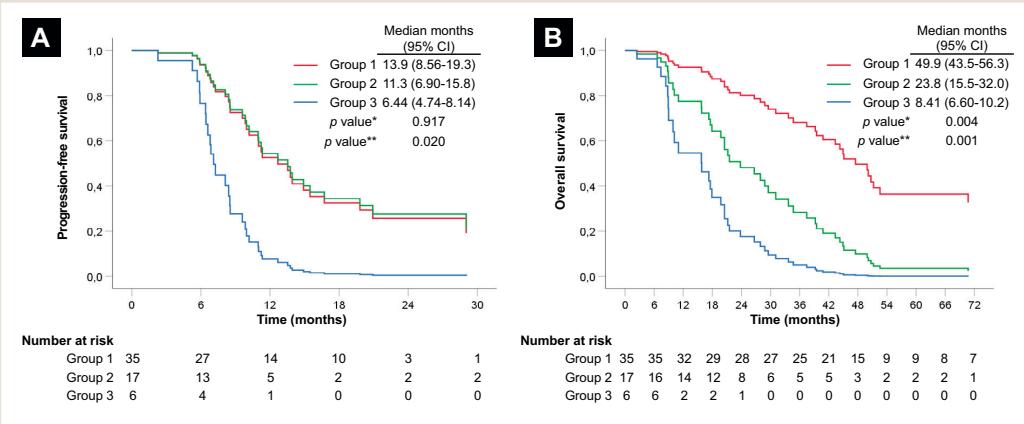

Abbreviations: OS = overall survival; PFS = progression-free survival.

In addition to the high IL-8 levels during chemotherapy treatment, exceptionally high baseline IL-8 levels were a strong sign of poor prognosis, even without knowledge of IL-8 levels during treatment. In our study, the IL-8 levels in the highest baseline quartile were above 18.8 pg/mL, and the PFS (multivariable HR = 6.52; 95% CI, 1.58-26.9; $P = .010$) and OS (multivariable HR = 8.38; 95% CI, 2.60-27.0; $P < .001$) of these patients were significantly shorter than those of the patients in the lowest IL-8 quartile group. This result was similar to a previous report that showed that patients with baseline plasma IL-8 levels higher than

Figure 3 **PFS and OS Based on Trajectory Analysis. (A) PFS and (B) OS in Patient Population Dichotomized Based on Trajectory Analysis Using All Plasma IL-8 Levels Before and During Chemotherapy Treatment. Red Line Indicates That Plasma IL-8 Levels at Baseline, Week 6, Month 6, and at Study Discontinuation Are Below 16.6 pg/mL. Blue Line Indicates That One or Several Measurements of Plasma IL-8 Levels Are Above 16.6 pg/mL Before or During Chemotherapy Treatment. Multivariable Cox Regression Adjusted for Age, Menopause Status, Hormone Receptor Status, Presence of Visceral Metastasis, Number of Metastatic Lesions, and Extent of Disease. Median Survivals and Their Confidence Intervals Were Calculated by Kaplan-Meier Method**
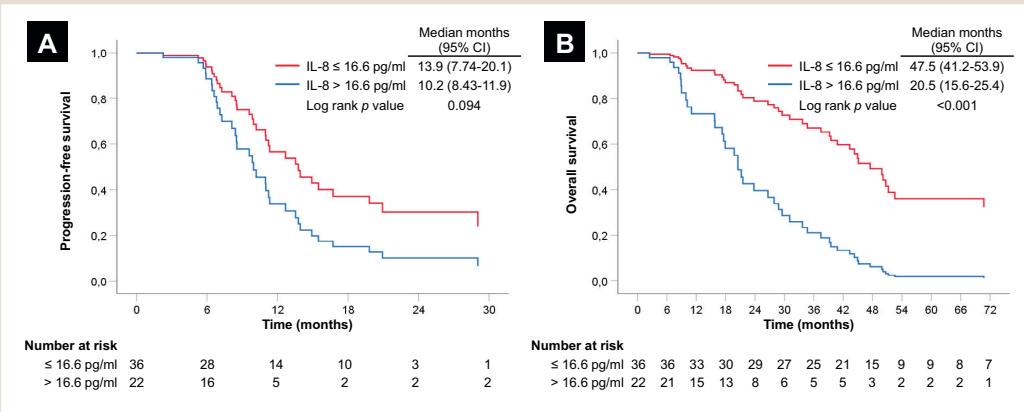
Abbreviations: IL = interleukin; OS = overall survival; PFS = progression-free survival.

the median value of 17.2 pg/mL had shorter survival than patients with lower IL-8 levels ($P = .0045$).[15]

Several studies have been conducted to find a clinically useful biomarker to select patients who might benefit from the addition of the vascular endothelial growth factor A (VEGF-A) antibody bevacizumab to standard chemotherapy for the treatment of metastatic breast cancer.[44-48] In our study, the patients were treated with bevacizumab combined with either paclitaxel or docetaxel chemotherapy as first-line treatment for metastatic breast cancer. It has been shown that IL-8 can promote angiogenesis and may activate vascular endothelial growth factor receptor 2 (VEGFR2).[49] VEGF-A is a ligand for VEGFR2. In our study, very high baseline IL-8 levels were a sign of poor prognosis. Accordingly, in our study, the patients with the highest plasma IL-8 levels at baseline had the shortest treatment benefit. The high baseline plasma levels of proangiogenic IL-8 might be one reason for the lack of benefit from bevacizumab-based therapy. However, because our study did not have a placebo control arm as a comparator, this hypothesis should be tested prospectively in future studies.

The other markers analyzed in our study failed to demonstrate any clear prognostic significance. Zhang and Adachi[21] reported that patients with circulating IL-6 levels higher than the median concentration of 4 pg/mL in their study exhibited poor survival. However, the highest quartile plasma IL-6 cutoff value for our study patients was 3.8 pg/mL, suggesting that most of our study patients had low plasma IL-6 concentrations. This is in accordance with the finding that plasma IL-6 levels were not prognostic in our hands. In addition, the limited patient population in our study might partly explain why the other tested circulating markers had no prognostic value.

The plasma analyses in our study were exploratory and were performed retrospectively. In the future, it would be useful to monitor plasma IL-8 levels prospectively in clinical trials involving metastatic breast cancer patients. IL-8 levels are known to correlate with the tumor burden in many malignant diseases.[50] Rising IL-8 levels during treatment could be a sign of chemoresistance, and it therefore might be beneficial to refer patients with rising IL-8 levels to new treatment modalities. It might be worthwhile to study whether patients with high plasma concentrations of the proinflammatory cytokine IL-8 would benefit from novel immunotherapies. In an unselected metastatic breast cancer population, the response rates to immunotherapies have been low.[51] However, in a report of novel immunotherapies, a clear association was seen between the treatment response and IL-8 levels in melanoma and non—small-cell lung cancer patients.[52] Nevertheless, the correlation between high IL-8 levels and the response rates to immunotherapies in metastatic breast cancer remains unexplored.

Conclusion

Low plasma IL-8 levels during chemotherapy in metastatic breast cancer patients are a clear sign for excellent long-term prognosis. We found that patients with constantly low plasma IL-8 levels had a better prognosis than the patients with plasma IL-8 levels higher than 16.6 pg/mL. Plasma IL-8 levels might therefore be useful for the selection of patients with excellent prognosis and those who might be suitable for less intensive radiologic imaging and follow-up visits.

Clinical Practice Points

- High circulating IL-8 levels are associated with poor prognosis in patients with advanced breast cancer and are related to chemoresistance.
- Metastatic breast cancer patients with constantly low plasma IL-8 levels during first-line chemotherapy have an excellent long-term prognosis.
- Very high baseline plasma IL-8 levels are associated with significantly shorter PFS and OS.
- Monitoring circulating IL-8 levels during first-line chemotherapy might be beneficial to distinguish good-prognosis patients who might be suited to less intensive treatment and follow-up schedules.
- Patients with very high plasma IL-8 levels either at the beginning of chemotherapy treatment or during therapy for metastatic breast cancer should be followed more intensively because of the chemoresistant nature of their disease.
- In the future, whether patients with high plasma IL-8 levels and therefore poor prognosis might benefit from novel treatment modalities, ie, immunologic therapy, should be prospectively explored.

Disclosure

The authors have stated that they have no conflict of interest.

Supplemental Data

Supplemental tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.clbc.2019.03.006.

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Plasma IL-8 Levels During Chemotherapy

Supplemental Table 1	PFS and OS for Study Patients Grouped by Baseline IL-8 Quartile									
				HR 1[a]			**HR 2[b]**			
Baseline IL-8	**pg/mL**	**No. Patients**	**No. Events**	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**	
PFS										
<Q25	<7.7	13	5	1			1			
Q25-Q50	7.7-9.4	14	10	2.12	0.70-6.39	.180	2.15	0.53-8.65	.279	
Q50-Q75	9.4-18.8	13	7	1.34	0.42-4.27	.618	0.99	0.20-4.76	.995	
>Q75	>18.8	13	9	5.22	1.62-16.8	.006[c]	6.52	1.58-26.9	.010[c]	
OS										
<Q25	<7.7	13	6	1			1			
Q25-Q50	7.7-9.4	14	10	2.70	0.95-7.69	.062	3.46	1.08-11.0	.035[c]	
Q50-Q75	9.4-18.8	13	10	2.29	0.81-6.46	.115	1.64	0.51-5.28	.406	
>Q75	>18.8	13	13	7.44	2.62-21.1	<.001[c]	8.38	2.60-27.0	<.001[c]	

Abbreviations: CI = confidence interval; HR = hazard ratio; IL = interleukin; OS = overall survival; PFS = progression-free survival; Q = quartile.
[a]HR adjusted for age.
[b]HR adjusted for age, menopause status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions, and extent of disease.
[c]Statistically significant.

Supplemental Table 2	Cox Regression Analysis for PFS Grouped by Low or High Baseline IL-6, IL-18, MMP-9, MMP-2, YKL-40, Resistin, and HMGB1 Levels Using Median as Cutoff Value								
				HR 1[a]			**HR 2[b]**		
Baseline	**Value**	**No. Patients**	**No. Events**	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
IL-6	pg/mL								
Low	≤1.8	27	15	1			1		
High	>1.8	26	16	0.84	0.40-1.73	.637	0.44	0.18-1.07	.071
IL-18	pg/mL								
Low	≤99.2	27	17	1			1		
High	>99.2	26	14	0.60	0.29-1.25	.176	0.71	0.31-1.60	.411
MMP-9	ng/mL								
Low	≤76.4	27	18	1			1		
High	>76.4	26	13	0.72	0.35-1.47	.370	0.56	0.25-1.29	.177
MMP-2	ng/mL								
Low	≤244.5	27	15	1			1		
High	>244.5	26	16	0.91	0.42-1.95	.810	0.80	0.37-1.74	.585
YKL-40	ng/mL								
Low	≤60.3	27	16	1			1		
High	>60.3	26	15	1.26	0.60-2.65	.536	0.951	0.40-2.22	.909
Resistin	ng/mL								
Low	≤13.4	27	15	1			1		
High	>13.4	26	16	1.44	0.69-2.99	.325	1.13	0.53-2.39	.749
HMGB1	ng/mL								
Low	≤7.1	27	15	1			1		
High	>7.1	26	16	1.28	0.60-2.71	.512	1.27	0.59-2.71	.535

Abbreviations: CI = confidence interval; HMGB1 = high-mobility group box 1; HR = hazard ratio; IL = interleukin; MMP = matrix metalloproteinase; PFS = progression-free survival.
[a]HR adjusted for age.
[b]HR adjusted for age, menopause status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions, and extent of disease.

Supplemental Table 3	PFS Analysis by Cox Regression for Study Patients Using Baseline IL-6, IL-18, MMP-9, MMP-2, YKL-40, Resistin, and HMGB1 Quartile Levels as Cutoff Values							
			HR 1[a]			HR 2[b]		
Baseline	Value	No. Patients	HR	95% CI	*P*	HR	95% CI	*P*
IL-6	pg/mL							
<Q25	<0.7	15	1			1		
Q25-Q50	0.7-1.8	12	0.85	0.30-2.40	.767	1.38	0.33-5.65	.652
Q50-Q75	1.8-3.8	14	0.57	0.21-1.57	.286	0.31	0.09-1.05	.060
>Q75	>3.8	12	1.23	0.43-3.55	.692	1.02	0.27-3.80	.973
IL-18	pg/mL							
<Q25	<53.5	13	1			1		
Q25-Q50	53.5-99.2	14	1.23	0.45-3.36	.676	1.11	0.40-3.13	.831
Q50-Q75	99.2-264.3	14	0.65	0.24-1.75	.401	0.70	0.24-2.01	.516
>Q75	>264.3	12	0.65	0.23-1.87	.432	0.81	0.24-2.69	.733
MMP-9	ng/mL							
<Q25	<49.6	13	1			1		
Q25-Q50	49.6-76.4	14	1.02	0.40-2.60	.963	0.84	0.28-2.47	.756
Q50-Q75	76.4-129.6	13	0.82	0.31-2.18	.700	0.68	0.24-1.92	.474
>Q75	>129.6	13	0.61	0.20-1.89	.396	0.34	0.09-1.30	.118
MMP-2	ng/mL							
<Q25	<218.8	13	1			1		
Q25-Q50	218.8-244.5	14	1.35	0.46-3.90	.579	1.43	0.44-4.70	.548
Q50-Q75	244.5-284.0	13	1.05	0.34-3.18	.927	0.96	0.27-3.36	.959
>Q75	>284.0	13	1.15	0.35-3.75	.807	1.05	0.31-3.46	.935
YKL-40	ng/mL							
<Q25	<38.3	13	1			1		
Q25-Q50	38.3-60.3	14	0.99	0.35-2.78	.995	1.68	0.54-5.28	.368
Q50-Q75	60.3-113.3	13	1.30	0.42-3.98	.640	1.24	0.42-3.70	.688
>Q75	>113.3	13	1.23	0.43-3.49	.698	1.03	0.27-3.89	.962
Resistin	ng/mL							
<Q25	<11.4	13	1			1		
Q25-Q50	11.4-13.4	14	1.04	0.36-3.00	.931	1.43	0.40-5.06	.572
Q50-Q75	13.4-15.6	13	1.69	0.60-4.73	.315	2.06	0.61-6.99	.242
>Q75	>15.6	13	1.30	0.46-3.67	.609	1.01	0.33-3.06	.982
HMGB1	ng/mL							
<Q25	<5.1	13	1			1		
Q25-Q50	5.1-7.1	14	1.27	0.45-3.61	.643	0.74	0.24-2.30	.610
Q50-Q75	7.1-9.7	13	1.87	0.60-5.83	.281	2.29	0.62-8.45	.213
>Q75	>9.7	13	1.24	0.42-3.68	.688	0.76	0.24-2.35	.640

Abbreviations: CI = confidence interval; HMGB1 = high-mobility group box 1; HR = hazard ratio; IL = interleukin; MMP = matrix metalloproteinase; PFS = progression-free survival; Q = quartile.
[a]HR adjusted for age.
[b]HR adjusted for age, menopause status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions, and extent of disease.

Plasma IL-8 Levels During Chemotherapy

| Supplemental Table 4 | Cox Regression Analysis for OS Grouped by Low or High Baseline IL-6, IL-18, MMP-9, MMP-2, YKL-40, Resistin, and HMGB1 Levels Using Median as Cutoff Value | | | | | | | | |

Baseline	Value	No. Patients	No. Events	HR 1[a]			HR 2[b]		
				HR	95% CI	P	HR	95% CI	P
IL-6	pg/mL								
Low	≤1.8	27	19	1			1		
High	>1.8	26	20	1.10	0.57-2.12	.771	1.12	0.52-2.39	.771
IL-18	pg/mL								
Low	≤99.2	27	18	1			1		
High	>99.2	26	21	1.38	0.72-2.63	.319	1.21	0.60-2.43	.588
MMP-9	ng/mL								
Low	≤76.4	27	21	1			1		
High	>76.4	26	18	0.73	0.38-1.37	.330	0.52	0.26-1.03	.063
MMP-2	ng/mL								
Low	≤244.5	27	20	1			1		
High	>244.5	26	19	0.96	0.47-1.93	.910	1.42	0.66-3.05	.362
YKL-40	ng/mL								
Low	≤60.3	27	18	1			1		
High	>60.3	26	21	1.87	0.94-3.73	.071	1.41	0.66-2.99	.370
Resistin	ng/mL								
Low	≤13.4	27	19	1			1		
High	>13.4	26	20	1.13	0.60-2.12	.701	1.09	0.56-2.13	.784
HMGB1	ng/mL								
Low	≤7.1	27	20	1			1		
High	>7.1	26	19	0.93	0.47-1.86	.850	1.19	0.57-2.48	.626

Abbreviations: CI = confidence interval; HMGB1 = high-mobility group box 1; HR = hazard ratio; IL = interleukin; MMP = matrix metalloproteinase; PFS = progression-free survival.
[a]HR adjusted for age.
[b]HR adjusted for age, menopause status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions, and extent of disease.

Supplemental Table 5	OS Analysis by Cox Regression for Study Patients Using Baseline IL-6, IL-18, MMP-9, MMP-2, YKL-40, Resistin, and HMGB1 Quartile Levels as Cutoff Values

			HR 1[a]			HR 2[b]		
Baseline	Value	No. Patients	HR	95% CI	P	HR	95% CI	P
IL-6	pg/mL							
<Q25	<0.7	15	1			1		
Q25-Q50	0.7-1.8	12	2.66	1.04-6.77	.039[c]	1.64	0.48-5.60	.426
Q50-Q75	1.8-3.8	14	1.51	0.59-3.88	.386	0.92	0.31-2.65	.878
>Q75	>3.8	12	2.29	0.82-6.39	.111	2.23	0.76-6.54	.142
IL-18	pg/mL							
<Q25	<53.5	13	1			1		
Q25-Q50	53.5-99.2	14	0.67	0.26-1.73	.415	0.52	0.19-1.43	.205
Q50-Q75	99.2-264.3	14	0.80	0.31-2.00	.635	0.65	0.25-1.66	.369
>Q75	>264.3	12	1.62	0.66-3.96	.290	1.20	0.42-3.39	.729
MMP-9	ng/mL							
<Q25	<49.6	13	1			1		
Q25-Q50	49.6-76.4	14	0.50	0.20-1.21	.128	0.39	0.14-1.06	.067
Q50-Q75	76.4-129.6	13	0.50	0.20-1.23	.133	0.37	0.13-1.01	.054[c]
>Q75	>129.6	13	0.48	0.19-1.22	.128	0.22	0.07-0.68	.009[c]
MMP-2	ng/mL							
<Q25	<218.8	13	1			1		
Q25-Q50	218.8-244.5	14	1.93	0.75-4.99	.170	1.81	0.59-5.52	.295
Q50-Q75	244.5-284.0	13	1.21	0.41-3.56	.728	1.77	0.52-5.97	.356
>Q75	>284.0	13	1.73	0.61-4.91	.300	2.16	0.74-6.29	.158
YKL-40	ng/mL							
<Q25	<38.3	13	1			1		
Q25-Q50	38.3-60.3	14	1.56	0.56-4.32	.386	2.00	0.70-5.74	.195
Q50-Q75	60.3-113.3	13	1.97	0.63-6.06	.238	2.10	0.68-6.41	.191
>Q75	>113.3	13	3.08	1.10-8.61	.031[c]	2.13	0.65-6.97	.211
Resistin	ng/mL							
<Q25	<11.4	13	1			1		
Q25-Q50	11.4-13.4	14	1.10	4.31-2.83	.835	0.97	0.30-3.08	.966
Q50-Q75	13.4-15.6	13	1.22	0.47-3.13	.673	1.26	0.46-3.45	.645
>Q75	>15.6	13	1.17	0.45-3.03	.744	0.95	0.35-2.55	.919
HMGB1	ng/mL							
<Q25	<5.1	13	1			1		
Q25-Q50	5.1-7.1	14	1.81	0.73-4.44	.195	1.65	0.65-4.21	.288
Q50-Q75	7.1-9.7	13	1.22	0.46-3.24	.679	1.95	0.67-5.63	.217
>Q75	>9.7	13	1.35	0.49-3.68	.550	1.34	0.48-3.72	.568

Abbreviations: CI = confidence interval; HMGB1 = high-mobility group box 1; HR = hazard ratio; IL = interleukin; MMP = matrix metalloproteinase; PFS = progression-free survival; Q = quartile.
[a]HR adjusted for age.
[b]HR adjusted for age, menopause status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions, and extent of disease.
[c]Statistically significant.

PUBLICATION
III

High baseline Tie1 level predicts poor survival in metastatic breast cancer

Tiainen Leena, Korhonen Anne E, Leppänen Veli-Matti, Luukkaala Tiina, Hämäläinen Mari, Tanner Minna, Lahdenperä Outi, Vihinen Pia, Jukkola Arja, Karihtala Peeter, Aho Sonja, Moilanen Eeva, Alitalo Kari, Kellokumpu-Lehtinen Pirkko-Liisa

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RESEARCH ARTICLE

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High baseline Tie1 level predicts poor survival in metastatic breast cancer

Leena Tiainen[1,2*], Emilia A. Korhonen[3], Veli-Matti Leppänen[3], Tiina Luukkaala[4], Mari Hämäläinen[5], Minna Tanner[1,2], Outi Lahdenperä[6], Pia Vihinen[6], Arja Jukkola[7], Peeter Karihtala[7], Sonja Aho[1,2], Eeva Moilanen[5], Kari Alitalo[3] and Pirkko-Liisa Kellokumpu-Lehtinen[1,2]

Abstract

Background: Angiopoietin growth factors (Angs) regulate angiogenesis and lymphangiogenesis by binding to the endothelial Tie2 receptor. Ang2 expression is elevated in tissue hypoxia and inflammation, which also induce cleavage of the extracellular domain of the orphan Tie1 receptor. Here we have examined if the concentrations of Ang2 and the soluble extracellular domain of Tie1 in patient plasma are associated with the prognosis of patients with metastatic breast cancer.

Methods: Plasma Tie1 and Ang2 levels were measured in metastatic breast cancer patients treated in a phase II trial with a taxane-bevacizumab combination chemotherapy in the first-line treatment setting. They were analyzed before treatment, after 6 weeks and 6 months of treatment, and at the final study visit. Using the median concentrations as cutoffs, Tie1 and Ang2 data were dichotomized into low and high concentration groups. Additionally, we analyzed Tie1 concentrations in plasma from 10 healthy women participating in a breast cancer primary prevention study.

Results: Plasma samples were available from 58 (89%) of the 65 patients treated in the trial. The baseline Tie1 levels of the healthy controls were significantly lower than those of the metastatic patients ($p < 0.001$). The overall survival of the patients with a high baseline Tie1 level was significantly shorter (multivariate HR 3.07, 95% CI 1.39–6.79, $p = 0.005$). Additionally, the progression-free survival was shorter for patients with a high baseline Tie1 level (multivariate HR 3.78, 95% CI 1.57–9.09, $p = 0.003$). In contrast, the baseline Ang2 levels had no prognostic impact in a multivariate Cox proportional hazard regression analysis. The combined analysis of baseline Tie1 and Ang2 levels revealed that patients with both high Tie1 and high Ang2 baseline levels had a significantly shorter overall survival than the patients with low baseline levels of both markers (multivariate HR for overall survival 4.32, 95% CI 1.44–12.94, $p = 0.009$).

Conclusions: This is the first study to demonstrate the prognostic value of baseline Tie1 plasma concentration in patients with metastatic breast cancer. Combined with the results of the Ang2 analyses, the patients with both high Tie1 and Ang2 levels before treatment had the poorest survival.

Trial registration: Clinicaltrials.gov: NCT00979641, registration date 19-DEC-2008. The regional Ethics Committee: R08142M, registration date 18-NOV-2008.

Keywords: Tie1, Angiopoietin-2, Angiogenesis, Metastatic breast cancer, Prognostic marker

* Correspondence: leena.tiainen@pshp.fi
[1]Department of Oncology, Faculty of Medicine and Health Technology, Tampere University and Tays Cancer Centre, P.O. Box 100, FI-33014 Tampere, Finland
[2]Department of Oncology, Tampere University Hospital, P.O. Box 2000, FI-33521 Tampere, Finland
Full list of author information is available at the end of the article

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Background

Several drugs targeting the vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFR) are currently used as treatment of various cancer types in clinics [1]. VEGF targeting antibodies alleviate age-related macular degeneration, but in cancer therapy, they provide only limited benefits. For this reason, a significant interest has emerged in the targeting of the more recently character-ized Ang/Tie endothelial growth factor system, which has essential functions in embryonic development, the regen-eration of the mature vasculature, tissue inflammation and tumor angiogenesis [2–6]. Angiopoietin growth fac-tors (Ang1–4) bind to the Tie2 receptor. The homologous Tie1 protein does not bind angiopoietins directly, al-though it participates in the Ang-Tie2 signal transduction complex [2, 6–8].

Ang1 stabilizes the vasculature after angiogenesis and is a more potent Tie2 agonist than Ang2, which can act as an agonist or antagonist of the Tie2 receptor, depend-ing on a number of other factors [9–11]. In normal homeostasis, Ang2 levels are low, but the Ang2/Ang1 ratio is increased in inflamed tissues, e.g. in sepsis and in malignancies, including breast cancer [12, 13]. High Ang2 levels are associated with poor patient survival in multiple malignancies, breast cancer among others [14–19]. Some of the Ang/Tie system targeted antibodies have already been evaluated in clinical trials, but so far, the effects of anti-Ang2 monotherapy have been modest [20–22]. A better understanding of Ang function is clearly needed for the rational development of effective Ang-pathway tar-geted therapies. Although Tie1 expression in endothelial cells is increased in tumor vessels and deletion of the Tie1 gene in tumor-bearing mice decreased tumor growth and angiogenesis in preclinical experiments [4, 23], the signifi-cance of Tie1 in tumor progression is also unclear. Tie1 ectodomain cleavage occurs in vivo in association with acute [11] and chronic inflammation [24], leading to in-creased concentration of the soluble extracellular domain in the serum of patients with severe viral infections [11]. Furthermore, Tie1 deletion in a murine metastasis model tightened endothelial barrier and therefore, reduced meta-static foci [25].

In the present study, we investigated the prognostic value of the circulating levels of Tie1 and Ang2 in pa-tients who received first-line taxane-bevacizumab com-bination -chemotherapy combination for the treatment of metastatic breast cancer. Additionally, we explored if a combined analysis of Tie1 and Ang2 levels would help to identify the patients with poor prognosis in need of novel treatment approaches.

Methods

All together 65 patients with histologically verified HER2-negative advanced breast cancer were enrolled

into the single-arm, prospective, phase 2 study in three Finnish university cancer clinics between May 2009 and October 2013 (NCT00979641). The method of patient recruitment, the study design and the clinical trial results were previously published [26]. Briefly, patients included in the study received a taxane (paclitaxel 90 mg/m^2 on days 1, 8 and 15 or docetaxel 50 mg/m^2 on days 1 and 15) with bevacizumab (10 mg/kg on days 1 and 15) on a treat-ment cycle of 4 weeks as the first-line chemotherapy for metastatic breast cancer. Docetaxel was given to 32 pa-tients and 33 patients received paclitaxel.

Bevacizumab 15 mg/kg every three weeks was continued as maintenance therapy for those patients with non-pro-gressive disease after taxane discontinuation. In addition to bevacizumab, patients with hormone receptor-positive disease received endocrine therapy. Furthermore, bevaci-zumab could be continued with second-line chemother-apy. All patients provided written informed consent and the regional Ethics Committee approved the study proto-col (R08142M).

Blood samples were obtained from the patients during treatment. EDTA samples for plasma analysis were obtained at the baseline, every 6 weeks during the bev-acizumab-taxane combination, at the discontinuation of taxane treatment, during the bevacizumab maintenance therapy, first every three weeks for the first two months and thereafter every 12 weeks, and at the final study visit.

Healthy control samples were obtained from 10 women participating in a mammography screening program at the Hatanpää Breast Clinic in Tampere. These women vol-untarily participated in a breast cancer primary prevention study currently in progress at the University of Tampere and, as a part of the accepted protocol, blood samples were drawn for scientific purposes. All participants gave their written informed consent and the regional Ethics Committee approved the study (R15023).

Measurement of plasma Tie1 and Ang2 levels by ELISA assay

Tie1 and Ang2 levels were measured in patient plasma samples using a modified hTie1 and hAng2 ELISA protocol (R&D Systems Europe Ltd., Abingdon, UK, Duoset, DY5907 and DY623, respectively). Briefly, a 96-well plate was coated with 100 μl of diluted capture anti-body (1:180 in PBS) per well and incubated o/n at room temperature (RT). The wells were washed three times with PBS-0.05% Tween 20, followed by blocking with 300 μl/well of the Reagent Diluent 2 (R&D, Y995) for 1.5 h at RT on an orbital shaker for Tie1 or with 250 μl/well of 1% BSA in PBS for 1 h at RT for Ang2. The wells were washed 3 x with PBS-0.05% Tween 20. For Tie1, 50 μl/well of the reagent RD1–89 (R&D, DILUENT08) was added. Standards and samples diluted in the RD5–17 reagent (R&D, RD508) were pipetted into the wells at

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100 μl/well and incubated for 2 h at RT on an orbital shaker. For Ang2, 100 μl of a sample or standards in diluent reagent (1% BSA in PBS) was added to the wells, and incubated for 2 h at RT. The wells were washed 3 x with PBS-0.05% Tween 20 and 100 μl of detection antibody diluted 1:180 in diluent reagent (Reagent Diluent 2, R&D, for Tie1, 1% BSA in PBS for Ang2) was added and incubated for 2 h at RT on an orbital shaker for Tie1 and for 1.5 h at RT for Ang2. After washing 3 x with PBS-0.05% Tween 20, 100 ul of SA-HRP solution per well (in Reagent Diluent 2 for Tie1, and in 1% BSA in PBS for Ang2) was added before incubating for 20 min at RT. The wells were washed 3 x with PBS-0.05% Tween 20. Then a mixture of Color Reagent A and Color Reagent B for Tie1 (R&D, DY999) and BioFX®TMB substrate solution for Ang2 (SurModics, Eden Prairie, MN, USA) was added at 100 μl/well and incubated for 20 min in the dark at RT. Stop solution (50 μl of 1 M HCl) was added, and the absorbance of each well was measured within 20–30 min using a microplate reader with the filter set to 450 nm and the correction wavelength set to 540 nm. The interassay coefficients of variation for Tie1 and Ang2 were 11.4 and 7.1%, respectively.

Patient characteristics

The patient population and the analyzed plasma samples were identical to our previous paper focusing on plasma interleukin-8 levels as a prognostic marker [27]. At the baseline, plasma samples were available from 53 patients (82%). Overall, plasma samples were available from 58 (89%) of the 65 patients treated in the study. Key characteristics of the study population and the main efficacy outcomes are presented in Table 1. Plasma samples for Tie1 and Ang2 were analyzed at four time points: at the baseline, six weeks after the treatment initiation, six months after the treatment initiation and at the final visit. The number of patients that had plasma samples analyzed and the reasons for exclusions are presented in a flow chart (Fig. 1). Six weeks' and six months' samples were available only for those patients that were still on study treatment at that time point.

The median progression-free survival (PFS) and overall survival (OS) were similar for patients treated with docetaxel or paclitaxel (PFS: $p = 0.47$, OS: $p = 0.77$). The median OS for patients with triple-negative breast cancer was 17.9 months (95% CI 8.5–26.9). Furthermore, the median OS for patients with hormone receptor positive metastatic breast cancer was 45.0 months (95% CI 30.2–51.3).

The mean age of the ten healthy controls was 57.8 years (range 54–67).

Statistical analysis

The statistical plan for the biomarker analysis was exploratory. Tie1 and Ang2 were dichotomized as low or

Table 1 Baseline characteristics and the efficacy outcomes of the plasma biomarker population compared to the overall study population

	Plasma biomarker population ($n = 58$)	Overall study population ($n = 65$)
Age, years		
Median (range)	58 (32–75)	57 (32–75)
Menopausal status, n (%)		
Pre-menopausal	9 (15.5)	10 (15.4)
Post-menopausal	49 (84.5)	55 (84.6)
History of early stage disease, n (%)	52 (89.7)	57 (87.7)
Disease free interval, n (%)		
≤ 24 months	10 (19.2)	11 (19.3)
> 24 months	42 (80.8)	46 (80.7)
Hormone receptor status, n (%)		
ER+ and/or PR+	47 (81.0)	53 (81.5)
ER- and PR-	11 (19.0)	12 (18.5)
Number of metastatic lesions, n (%)		
≤ 3	11 (19.0)	14 (21.5)
> 3	47 (81.0)	51 (78.5)
Extent of disease		
< 3 sites	36 (62.1)	39 (60.0)
≥ 3 sites	22 (37.9)	26 (40.0)
Site of metastatic disease, n (%)		
Visceral disease	46 (79.3)	53 (81.5)
Non-visceral disease	12 (20.7)	12 (18.5)
Median overall survival, months (95% CI)	37.5 (25.4–49.6)	35.1 (22.2–50.3)
Median progression-free survival, months (95% CI)	11.3 (8.3–14.4)	11.3 (9.7–16.0)
Response to treatment		
Complete response/partial response	38 (71.7)	40 (61.5)
Stable disease	13 (24.5)	15 (23.1)
Progressive disease	2 (3.8)	3 (4.6)

high for each patient using the median value as the cut-off. Sensitivity, specificity and area under curve (AUC) for plasma Tie1 concentration were determined using receiver operator characteristic (ROC) analysis. Baseline Tie1 or Ang2 levels as independent prognostic factors (below/above median) were evaluated using Cox proportional hazard regression analysis. Multivariate analysis was performed using the Cox model, and it was adjusted by age (continuous), menopausal status (yes/no), hormone receptor status (negative/positive), presence of visceral metastasis (yes/no), number of metastatic lesions (cut-off of three metastatic lesions) and extent of the disease (cut-off of three metastatic sites). The Mann-Whitney U test

Fig. 1 Flow diagram of study patient plasma samples analyzed at baseline, after six weeks' treatment, after six months' treatment and at final visit. Final plasma samples were taken at the final study visit and the reasons for discontinuation are presented in the chart
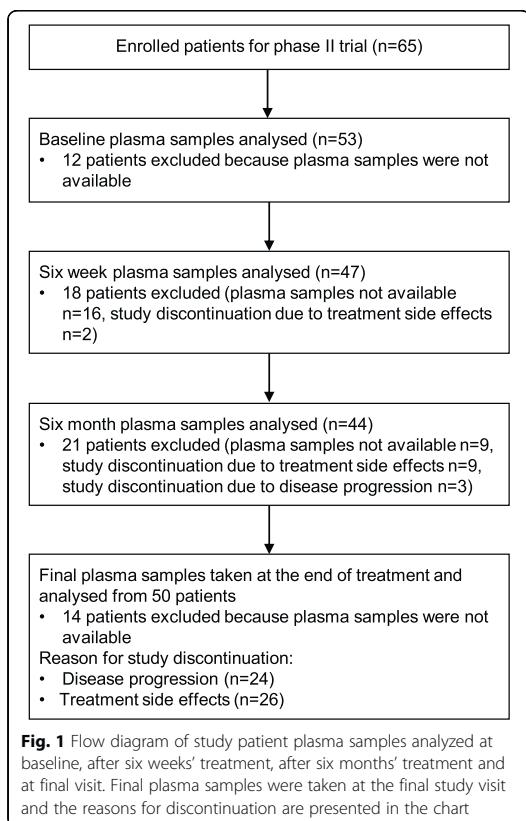
was used to compare differences in the baseline Tie1 and Ang2 levels between groups with different baseline characteristics. The Wilcoxon signed rank test was used to compare between baseline and week 6 plasma Tie1 and Ang2 levels. *P*-values under 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 23 statistical software package (SPSS Inc., Chicago, IL, USA).

Results

Tie1 and Ang2 plasma levels

For the study population, the median Tie1 level at baseline was 21.0 ng/ml (95% CI 17.8–23.3, Fig. 2a), and the median Ang2 level at baseline was 1.29 ng/ml (95% CI 1.03–1.52, Fig. 2b). The baseline Tie1 levels were significantly lower in the healthy controls than in the metastatic breast cancer patients (Fig. 2a). The median Tie1 level for healthy controls was 12.8 ng/ml (95% CI 10.4–16.5, Fig. 2a). The most optimal cut-off value (16.0 ng/ml) for plasma Tie1 concentration had a sensitivity of 77.4%, but a specificity of only 30.0%, for distinguishing metastatic

breast cancer patients from healthy controls with an AUC 0.917 (95% CI 0.839–0.995, *p* < 0.001).

There were no statistically significant differences in baseline Tie1 or Ang2 levels between groups with different baseline characteristics, including menopausal status (*p* = 0.09 for Tie1, *p* = 0.13 for Ang2), hormone receptor status (*p* = 0.80 for Tie1, *p* = 0.14 for Ang2), number of metastatic lesions (*p* = 0.69 for Tie1, *p* = 0.37 for Ang2) or visceral disease (*p* = 0.92 for Tie1, *p* = 0.15 for Ang2). Only the patients with more than three metastatic sites had significantly higher baseline Tie1 levels than the patients with fewer metastatic sites (median Tie1 23.7 ng/ml, 95% CI 21.0–29.0 vs. 17.8 ng/ml, 95% CI 16.0–21.1, *p* = 0.002). Similarly, the patients with more than three metastatic sites had significantly higher baseline Ang2 levels (median Ang2 1.08 ng/ml, 95% CI 0.66–1.36 vs. 1.54 ng/ml, 95% CI 1.23–2.29, *p* = 0.008).

Differences in Tie1 and Ang2 concentrations between baseline and week six samples were analyzed to evaluate the treatment effect. The median baseline Tie1 level was 21.0 ng/ml (95% CI 17.8–23.3), which was significantly higher than the median Tie1 level at six weeks (15.4 ng/ml [95% CI 14.1–17.1], *p* < 0.001, Fig. 2a). The median decrease in the Tie1 level between these two time points was 22.9% (95% CI 20.9–27.4). The median baseline Ang2 level was 1.29 ng/ml (95% CI 1.03–1.52) and the median Ang2 level at six weeks was 0.62 ng/ml (95% CI 0.57–0.84). The median decrease in the levels of Ang2 from the baseline to six weeks, 47.0% (95% CI 34.5–52.9), was also statistically significant (*p* < 0.001, Fig. 2b).

Effect of Tie1 or Ang2 levels on survival

Median progression-free survival was longer for patients in the low baseline Tie1 level group than for the patients in the high baseline Tie1 group (Fig. 3a, Table 2). No difference was observed in progression-free survival in relation to baseline Ang2 levels (Fig. 3b, Table 2).

The overall survival was significantly shorter for patients with a high baseline Tie1 concentration (Fig. 3c, Table 3). Additionally, patients with high baseline Ang2 levels had shorter overall survival when analyzed by the age-adjusted Cox hazard regression model (Table 3). However, in a multivariate Cox model adjusted by age, menopausal status, hormone receptor status, presence of visceral metastases, number of metastatic lesions and extent of disease, a high baseline levels of Ang2 alone was not a significant factor for poor prognosis (Fig. 3d, Table 3).

Effect of combined analysis of Tie1 and Ang2 levels on survival

For progression-free survival, the combined analysis of baseline Tie1 and Ang2 levels did not add any value compared to the Tie1 analysis on its own (Fig. 4a, Table 2). However, the combined analysis for high or low baseline

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Fig. 2 Plasma Tie1 and Ang2 levels. **a** Tie1 levels of healthy controls compared to the Tie1 levels of patients with metastatic breast cancer at baseline and at week six after treatment. **b** Ang2 levels of patients with metastatic breast cancer at baseline and at week six after treatment. mBCa = metastatic breast cancer
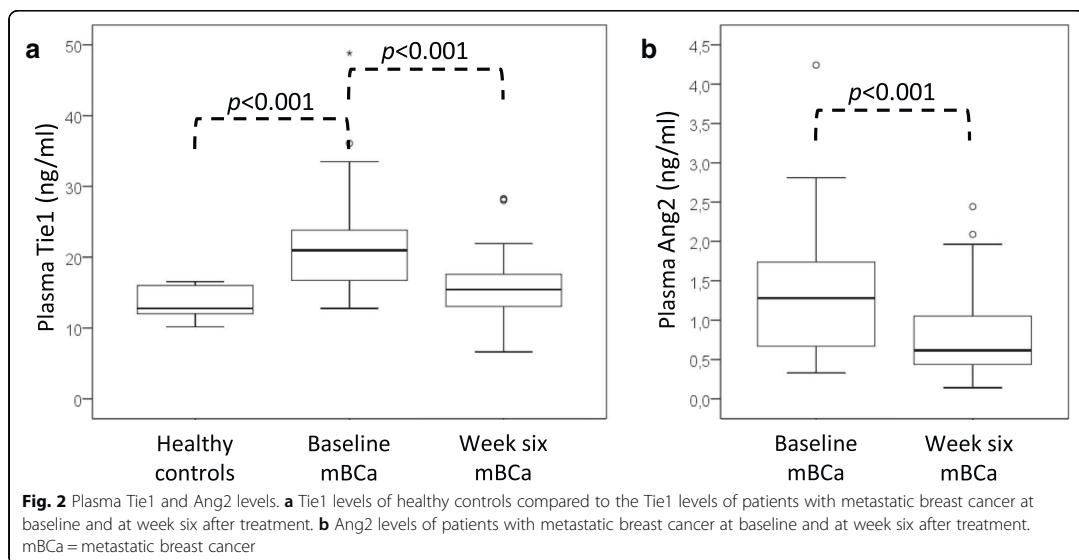
Tie1 and Ang2 levels was more effective in the selection of patients with better overall survival (Fig. 4b, Table 3). The median overall survival for patients with low baseline levels of both Tie1 and Ang2 was 46.8 months (95% CI 23.8–79.8). In contrast, the median overall survival for patients with high baseline levels of both Tie1 and Ang2 was only 21.5 months (95% CI 8.8–34.7).

Changes in plasma tie 1 or Ang2 levels and prognosis
The median decline in Ang2 levels between baseline and week six was 47.0%. The patients with Ang2 level decline higher than the median value had significantly worse prognoses. Multivariate Hazard Ratio (HR) for overall survival was 4.53 (95% CI 1.82–11.27, $p = 0.001$). In contrast, a high Tie1 decline during the first six weeks of treatment was not prognostic. The median Tie1 decline during this time period was 22.9%. The patients had similar survival whether they had Tie1 decline higher or lower than median value between baseline and week six (multivariate HR for overall survival 1.04, 95% CI 0.46–2.33, $p = 0.921$).

Only seven patients, i.e. 14% of the patients whose final samples were available, had at least 30% increased Tie1 plasma concentrations at their final visits, when compared to the previous measurements in each patient. For all these patients, the reason for study discontinuation was disease progression. Nevertheless, these patients had a similar overall survival as the patients with stable or declining Tie1 levels (multivariate HR 2.30, 95% CI 0.90–5.85, $p = 0.078$). At least 30% increased Ang2 concentration was observed in 24 patients at their final visits (48% of the patients whose final samples were

available). The overall survival of these patients was significantly worse than in the patients with stable or declining Ang2 values (multivariate HR 2.17, 95% CI 1.09–4.31, $p = 0.027$).

Discussion
The baseline concentration of the extracellular fragment of the orphan Tie1 receptor in bevacizumab plus taxane-treated breast carcinoma patients was found to be associated with both their overall survival and their progression-free survival (multivariate HR for overall survival 3.07, 95% CI 1.39–6.79, $p = 0.005$, multivariate HR for progression-free survival 3.78, 95% CI 1.57–9.09, $p = 0.003$). Previous studies have reported strong Tie1 expression in malignant tissues, including breast cancer [23, 28–30]. In gastric cancer, patients with Tie1 expression in their formalin-embedded tissue specimens had worse survival than the patients without Tie1 expression [30]. However, the prognostic value of circulating Tie1 levels has not been previously studied in malignant diseases.

Metastatic breast cancer patients had significantly higher baseline plasma Tie1 levels than the healthy controls ($p < 0.001$). However, circulating Ang2 levels are known to be higher on cancer patients [31] and therefore, we did not analyze plasma Ang2 levels on healthy controls.

Previous studies have indicated that the high concentration of the circulating Tie2 ligand Ang2 is associated with poor patient prognosis [16–19], and Ang2/Tie system-targeting antibodies and tyrosine kinase inhibitors are currently in clinical trials, including in those focused on breast cancer [20, 22]. In our study however, the

Fig. 3 Progression-free survivals and overall survivals grouped by baseline plasma Tie1 or Ang2 levels **a** Progression-free survival for patients with high or low plasma Tie1 at baseline. **b** Progression-free survival for patients with high or low plasma Ang2 at baseline. **c** Overall survival for patients with high or low plasma Tie1 at baseline. **d** Overall survival for patients with high or low plasma Ang2 at baseline. Cox regression analysis adjusted by age, menopausal status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions and extent of the disease

baseline Ang2 level was not a significant prognostic marker for either progression-free survival or overall survival. However, it has been reported that an increase in serum Ang2 concentration during anti-VEGF treatment contributes to acquired drug resistance [32]. In our study, for the final plasma samples, a cut-off point of 30% was chosen because it was considered as a clinically meaningful change. In half of the patients of our study, the Ang2 plasma concentration was the highest at their final visit, and these patients had poor overall survival (multivariate HR 2.17, 95% CI 1.09–4.31, p = 0.027), perhaps because of increased acquired tumor chemoresistance [32].

Targeting of both Tie1 and Ang2 would be an interesting trial approach in the future for the treatment of breast cancer. In our study, high baseline Tie1 and Ang2 concentrations were associated with median overall survival of only 21.5 months (95% CI 8.8–34.7). This was significantly less than in the patients who had low plasma concentrations of both Tie1 and Ang2 (46.8 months, 95% CI 23.8–79.8, p = 0.009). Interestingly, additive inhibition of tumor growth was observed when angiopoietin activity was blocked in Tie1-deficient mice [4]. The possible synergistic effect of dual inhibition of Tie1 and Ang2 might be due to Ang2 influencing earlier phase in tumor growth than Tie1 [25].

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Table 2 Cox regression analysis for progression-free survival

	N	n	Progression-free survival			
			Age-adjusted HR [95% CI]	p value	multivariate HR[a] [95% CI]	p value
Baseline Tie1						
Low	27	16	1		1	
High	26	15	**2.13 [1.02–4.46]**	**0.043**	**3.78 [1.57–9.09]**	**0.003**
Baseline Ang2						
Low	27	14	1		1	
High	26	17	1.21 [0.59–2.47]	0.597	1.22 [0.54–2.77]	0.620
Combined analysis						
Tie1 and Ang2 low	18	9	1		1	
Tie1 low, Ang2 high	9	7	1.27 [0.47–3.43]	0.632	1.16 [0.39–3.39]	0.783
Tie1 high, Ang2 low	9	5	**3.86 [1.18–12.57]**	**0.025**	**4.45 [1.25–15.79]**	**0.021**
Tie1 and Ang2 high	17	10	2.02 [0.80–5.07]	0.133	**3.88 [1.25–12.06]**	**0.019**

Abbreviations: *N* number of patients, *n* number of events, *HR* hazard ratio, *CI* confidence interval. Statistically significant results are highlighted in bold
[a]Hazard ratio adjusted by age, menopausal status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions and extent of the disease

According to our study, high baseline Tie1 level appears to be the best way to find the patients with short progression-free survival. In fact, the baseline Ang2 level and the combined analysis of Tie1 and Ang2 baseline levels do not provide additional information in terms of progression-free survival compared to Tie1 levels alone.

The Tie1 levels in healthy individuals were lower than in patients with metastatic disease before chemotherapy. During the bevacizumab and taxane therapy, the Tie1 levels declined substantially. However, the decline in Tie1 concentration was not related to the patient survival. Only the decrease in Ang2 concentration was prognostic, with a multivariate hazard ratio of 4.53 (95% CI 1.82–11.27, $p = 0.001$).

Bevacizumab has been investigated in several phase III trials as treatment of metastatic breast cancer. However, none of the trials has proven overall survival advantage for patients treated with bevacizumab [33]. Therefore, bevacizumab in only recommended for the treatment of highly selected patients with a need of a tumor response more commonly achieved with bevacizumab [33, 34]. All of our study patients were treated with bevacizumab. However, the effect of bevacizumab to Tie1 levels remains unexplored in this study. However, the main finding of our study was the prognostic value of pretreatment circulating Tie1 levels and bevacizumab did not confound this analysis.

Although, to our knowledge, this is the first study to evaluate the prognostic role of plasma Tie1 levels in

Table 3 Cox regression analysis for overall survival

	N	n	Overall survival			
			Age-adjusted HR [95% CI]	p value	multivariate HR[a] [95% CI]	p value
Baseline Tie1						
Low	27	15	1		1	
High	26	24	**2.82 [1.41–5.66]**	**0.003**	**3.07 [1.39–6.79]**	**0.005**
Baseline Ang2						
Low	27	15	1		1	
High	26	24	**2.33 [1.20–4.54]**	**0.012**	1.58 [0.72–3.46]	0.246
Combined analysis						
Tie1 and Ang2 low	18	8	1		1	
Tie1 low, Ang2 high	9	7	2.21 [0.78–6.25]	0.135	1.34 [0.42–4.22]	0.612
Tie1 high, Ang2 low	9	7	2.77 [0.95–8.09]	0.062	2.73 [0.88–8.46]	0.080
Tie1 and Ang2 high	17	17	**4.79 [1.93–11.90]**	**0.001**	**4.32 [1.44–12.94]**	**0.009**

Abbreviations: *N* number of patients, *n* number of events, *HR* hazard ratio, *CI* confidence interval. Statistically significant results are highlighted in bold
[a]Hazard ratio adjusted by age, menopausal status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions and extent of the disease

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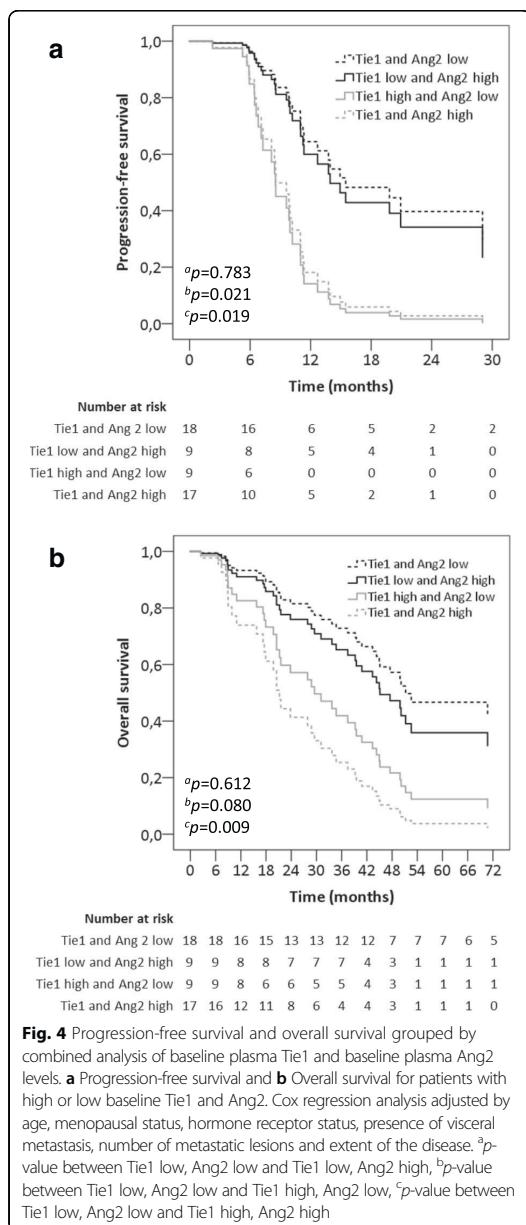
Fig. 4 Progression-free survival and overall survival grouped by combined analysis of baseline plasma Tie1 and baseline plasma Ang2 levels. **a** Progression-free survival and **b** Overall survival for patients with high or low baseline Tie1 and Ang2. Cox regression analysis adjusted by age, menopausal status, hormone receptor status, presence of visceral metastasis, number of metastatic lesions and extent of the disease. [a]*p*-value between Tie1 low, Ang2 low and Tie1 low, Ang2 high, [b]*p*-value between Tie1 low, Ang2 low and Tie1 high, Ang2 low, [c]*p*-value between Tie1 low, Ang2 low and Tie1 high, Ang2 high

breast cancer patients, the study has some limitations. Our study is a single-arm study with no control arm, and thus, the impact of bevacizumab on patient survival and the Tie1 and Ang2 concentrations during therapy cannot be evaluated. Furthermore, the study population size is limited, and therefore, our findings must be validated in a larger patient cohort.

Although immunohistochemical staining of Tie1 in tumor samples is associated with poor patient survival in breast cancer [35], the availability of tissue samples from metastatic tumors varies depending on tumor location, tumor load and the clinical need to accept the complication risks and discomfort related to needle aspirations. Circulating prognostic markers are more useful, and thus, high baseline circulating Tie1 and Ang2 levels before and during the treatment can be an additional way to identify patients with poor prognoses in this patient population, regardless of standard clinical characteristics. Most such patients do not derive a long-term benefit from the current chemotherapy treatment options. Novel treatment approaches, for example immunotherapies, are entering the clinics for many malignant diseases, and patients with poor prognoses should increasingly be referred to clinical trials. In preclinical studies, anti-angiogenic drugs and immune checkpoint inhibitors have demonstrated synergistic benefits [36], and they should be further studied in prospective clinical trials.

Conclusions

High baseline plasma Tie1 level is a promising prognostic marker for both poor progression-free survival and for poor overall survival in metastatic breast patients treated with bevacizumab-taxane combination. The predictive value of circulating Tie1 levels should be evaluated in prospective clinical trials.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
The Ethics Committee of Tampere University Hospital approved the study protocol (R08142M). The trial identifier is NCT00979641. All study patients gave their written informed consent. In addition, the participants in the breast cancer primary prevention study gave their written informed consent for their blood samples to be used for scientific purposes, and this study was also approved by the Ethics Committee of Tampere University Hospital (R15023).

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Oncology, Faculty of Medicine and Health Technology, Tampere University and Tays Cancer Centre, P.O. Box 100, FI-33014 Tampere, Finland. [2]Department of Oncology, Tampere University Hospital, P.O. Box 2000, FI-33521 Tampere, Finland. [3]Wihuri Research Institute and Translational Cancer Biology Program, University of Helsinki, Biomedicum Helsinki, P.O. Box 63, FI-00014 Helsinki, Finland. [4]Research, Development and Innovation Centre, Tampere University Hospital and Faculty of Medicine and Health Technology, Tampere University, P.O. Box 2000, FI-33521 Tampere, Finland. [5]The Immunopharmacology Research Group, Faculty of Medicine and Health Technology, Tampere University and Tampere University Hospital, P.O. Box 100, FI-33014 Tampere, Finland. [6]Department of Oncology and Radiotherapy, Turku University Central Hospital, P.O. Box 52, 20521 Turku, Finland. [7]Department of Oncology and Radiotherapy, Medical Research Center Oulu, Oulu University Hospital and University of Oulu, P.O. Box 10, 90029 OYS Oulu, Finland.

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IV

Lectin nanoparticle assays for detecting breast cancer-associated glycovariants of cancer antigen 15-3 (CA15-3) in human plasma

Terävä Joona, Tiainen Leena, Lamminmäki Urpo, Kellokumpu-Lehtinen Pirkko-Liisa, Pettersson Kim, Gidwani Kamlesh

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Lectin nanoparticle assays for detecting breast cancer-associated glycovariants of cancer antigen 15-3 (CA15-3) in human plasma

Joonas Terävä [1☺‡], Leena Tiainen[2☺‡], Urpo Lamminmäki[1], Pirkko-Liisa Kellokumpu-Lehtinen[2], Kim Pettersson[1], Kamlesh Gidwani [1]*

1 Department of Biochemistry/Biotechnology, University of Turku, Turku, Finland, **2** Faculty of Medicine and Health Technology, Tampere University and Department of Oncology, Tampere University Hospital, Tampere, Finland

☺ These authors contributed equally to this work.
‡ These authors are co-first authors on this work.
* kamgid@utu.fi

Abstract

Cancer antigen 15–3 (CA15-3) is widely utilized for monitoring metastatic breast cancer (BC). However, its utility for early detection of breast cancer is severely limited due to poor clinical sensitivity and specificity. The glycosylation of CA15-3 is known to be affected by BC, and therefore it might offer a way to construct CA15-3 glycovariant assays with improved cancer specificity. To this end, we performed lectin-based glycoprofiling of BC-associated CA15-3. CA15-3 expressed by a BC cell line was immobilized on microtitration wells using an anti-CA15-3 antibody. The glycosylation of the immobilized CA15-3 was then detected by using lectins coated onto europium (III)-doped nanoparticles (Eu^{+3}-NPs) and measuring the time-resolved fluorescence of Eu. Out of multiple lectin-Eu^{+3}-NP preparations, wheat germ agglutinin (WGA) and macrophage galactose-type lectin (MGL) -Eu^{3+}-NPs bound to the BC cell line-dericed CA15-3 glycovariants (CA15-3^{Lectin}). To evaluate the clinical performance of these two lectin-based assays, plasma samples from metastatic BC patients (n = 53) and healthy age-matched women (n = 20).Plasma CA15-3^{Lectin} measurements better distinguished metastatic BC patients from healthy controls than the conventional CA15-3 immunoassay. At 90% specificity, the clinical sensitivity of the assays was 66.0, 67.9 and 81.1% for the conventional CA15-3, CA15-3^{MGL} and CA15-3^{WGA} assays, respectively. Baseline CA15-3^{MGL} and CA15-3^{WGA} were correlated to conventional baseline CA15-3 levels (r = 0.68, p<0.001, r = 0.90, p>0.001, respectively). However, very low baseline CA15-3^{MGL} levels ≤ 5 U/mL were common in this metastatic breast cancer patient population.In conclusion, the new CA15-3^{Lectin} concept could considerably improve the clinical sensitivity of BC detection compared to the conventional CA15-3 immunoassays and should be validated further on a larger series of subjects with different cancer subtypes and stages.

Introduction

Breast cancer (BC) is the most common cancer type and the second leading cause of cancer death in women worldwide [1]. Cancer antigen 15–3 (CA15-3 also known as MUC1) is shed from tumor cells and is a well-known serological marker for monitoring the clinical course of BC patients. A persistent increase in circulating concentration of this marker may suggest an inadequate response to cancer therapy in patients with metastatic BC. However, it has poor sensitivity, especially at early stages of the disease.[2] CA15-3 can also be elevated in healthy individuals and in patients with benign conditions, and it lacks the specificity needed for cancer screening, diagnosis, staging, and/or sole use in monitoring of post-therapy recurrence [3]. A study on retrospective samples found the sensitivity of the commercial Elecsys CA 15–3 immunoassay to be 7, 11, 39 and 78% on stage I, II, III and IV BC patients, respectively [4]. Recently an ultrasensitive, simple and reliable electrochemical immunosensor was developed to detect the lowest alteration of CA 15–3 and CA125, biomarker of breast and ovarian cancer patients respectively [5,6].

For monitoring metastatic breast cancer, international recommendations for the treatment of metastatic BC only recommend the monitoring of CA15-3 levels for patients with non-evaluable metastases, mainly bone-dominant disease [7,8]. Transient increases in plasma CA15-3 levels are possible without tumor progression [9]. This phenomenon is observed especially in the beginning of chemotherapy due to necrosis and apoptosis of tumor cells. Additionally, consensus about clinically meaningful increase in plasma CA15-3 levels to predict disease progression or clinically meaningful decrease to reflect a treatment response do not exist today. Nevertheless, in general plasma CA15-3 levels correlate with the response to chemotherapy in patients with metastatic breast cancer [10,11].

Protein glycosylation plays an important role in a wide variety of normal and disease-related biological processes. The phenomenon of aberrant glycosylation associated with malignant transformation, tumor progression and metastasis is well documented [12] and occurs in essentially all types of human cancers. A large number of altered glycosyl epitopes are classified as tumor-associated carbohydrate antigens. [13,14] Among these, the aberrant expression of Tn and sialyl-Tn antigens, L-fucose and terminal *N*-acetylglucosamine (GlcNAc) have been widely detected in breast cancers [15,16]. Especially, abnormal *O*-glycans, such as Tn antigen, are found in over 90% of breast cancers[17]. Overall, changes in glycosylation result in the production of various cancer-associated glycoproteins with cancer-associated glycoforms, which are antigenically distinct from the corresponding molecules of the normal tissue. Taking into account these modifications, the specificity of diagnostic cancer markers can be expected to be improved by using the aberrant glycoforms as targets.

CA15-3 is upregulated and aberrantly glycosylated in breast and other carcinomas [18].The CA15-3, derived from a large transmembrane protein Mucin 1 with molecular weight ranging from 500 to 1000 kDa, contains multiple O- and N-linked glycosylation sites. The *O*-glycans of CA15-3 produced by the normal breast tissue are core 2-based and can be complex, while the *O*-glycans added to the BC mucin are mainly core 1-based [19]. The resulting truncated glycans carried on BC-associated CA15-3 include Tn and T antigens and their sialylated forms [14]. CA15-3 purified from the culture medium of human BC YMB-S cells contains 3-O-sulfated or 3-sialylated core 1 and extended core 1 glycans. [20]

Glycans participate in early stages of tumorigenesis [12] and it has been reported that the expression level of an enzyme responsible for mucin-type glycosylation, N-acetylgalactosaminyltransferase-14, declines with breast cancer progression [21]. Thus, it is reasonable to assume that the cancerous glycovariants of glycoprotein tumor markers appear early and differ throughout the course of the disease. Therefore, glycovariant markers may be useful for early detection as well as for monitoring cancer progression.

Various lectins, members of a carbohydrate binding protein family, have previously been used to investigate the differences in glycosylation between soluble glycoproteins expressed by cancerous and benign tissues. A recent study described the use of a 3-sulfated core 1 -specific galectin-4 (Gal-4) to establish an assay exhibiting superior clinical performance compared to the conventional CA15-3 immunoassay for BC detection [22]. Also, C-type lectin receptors (CLR) such as macrophage galactose-type lectin (MGL) have been demonstrated to show increased binding to CA15-3 from lysates of colon cancer tissue compared to the healthy lysed colon tissues of the same patients [23]. The *Lens culinaris* agglutinin, a lectin found in lentil, in turn binds specifically to hepatocellular carcinoma -associated glycovariant of α-fetoprotein (AFP) and is the only lectin used in a commercial application to detect a biomarker glycovariant [24]. While showing these promising binding specificities, lectins unfortunately tend to have weak binding affinity, which apparently limits their exploitation in practical assay applications.

We previously reported a novel lectin-based approach for the detection of cancer-associated glycosylation of CA125, a well-known mucin 16 -derived cancer marker used e.g. for monitoring of epithelial ovarian cancer. The approach, relying on the use of highly fluorescent europium(III)-doped nanoparticles (Eu^{+3}-NPs) coated with the lectin MGL, enabled highly sensitive detection of CA125 produced by ovarian cancer cell line OVCAR-3. In the clinical evaluation, the resulting optimized assay (CA125^{MGL}) showed good discrimination between the samples of epithelial ovarian cancer patients and those with endometriosis, a condition that has decisively hampered the use of CA125 for early detection/screening of ovarian cancer. [25] In addition, we found that the new assay could alarm clinicians much earlier (4–6 months) than the conventional CA125 assay about disease relapse. These results motivated us to explore possibilities of the lectin nanoparticle assay concept for detecting the altered glycosylation of CA15-3 in the blood streams of BC patients.

In the present work, we utilized the lectin-Eu^{+3}-NP approach for the glycoprofiling of CA15-3 with a panel of 28 lectins in order to identify lectins recognizing BC related changes in carbohydrate structures of CA15-3. The discovered promising lectins were then validated with plasma from patients with metastatic BC and healthy female controls. Additionally, we explored new CA15-3^{lectin} assays in monitoring response of metastatic breast cancer.

Materials and methods

Clinical samples

Plasma samples from 53 metastatic breast cancer patients were analyzed. These patients participated in a first-line chemotherapy trial for metastatic breast cancer (NCT00979641). The samples were analyzed at baseline, after six weeks of chemotherapy treatment, after six months of study treatment and at the final study visit. The trial design and the patient demographics have been published previously [26]. In brief, the patients with metastatic HER2-negative BC were enrolled into the trial, if they had not received previous chemotherapy for the advanced disease. The mean age of the study patients was 58 years (range 32–75). Most of the patients had hormone receptor positive disease (81%) and visceral metastases (79%). The median time between six-month sample and the final plasma sample was 11.8 months (inter quartile range 3.5.-18.9 months). The Ethics Committee of Tampere University Hospital approved the study protocol (R08142M). Clinically meaningful change in CA15-3 levels was defined as 30% similarly as the partial response criterion in the response evaluation criteria in solid tumors [27]. The definition of clinically meaningful change in circulating tumor markers varies around 20–40% in previous studies [10,28]. Disease progression was defined as investigator-assessed radiological progression according to the RECIST criteria [27].

Control plasma samples were obtained from 20 healthy women participating in a mammography-screening program in Tampere City Breast Clinic. These women voluntarily took part in a breast cancer primary prevention study currently in progress at University of Tampere and as a part of the study, plasma samples were drawn for scientific purposes. The mean age of these healthy controls was 56 years (range 54–67). All participants gave written informed consent (Ethics approval R15023).

Reagents

CA15-3 isolated from the breast cancer cell line ZR-75-1 (ATCC CRL-1500) (BC-CA15-3), two monoclonal anti-CA15-3 antibodies; Ma552 and Ma695, that specifically recognize a PDTRPAPG region of the protein core and sialylated carbohydrate epitope expressed on the CA15-3 antigen respectively, were provided by Fujirebio Diagnostics (Göteborg, Sweden). Streptavidin-coated yellow 96-well plates, wash buffer and red assay buffer were purchased from Kaivogen (Turku, Finland). Europium(III)-doped Fluoro-Max polystyrene nanoparticles (97 nm in diameter) (Eu^{+3}-NP) were acquired from Seradyn (Indianapolis, IN, USA). A panel of plant lectins with different glycan binding specificities (Table A in S1 Dataset) was obtained from Vector laboratories (Burlingame, CA, USA). The recombinant human lectins were purchased from R&D Systems (Abingdon, United Kingdom).

Preparation of lectin-Eu^{3+}-NPs

The use of Eu^{+3}-NPs has been described before [29]. The coating of lectins on Eu^{+3}-NPs was performed essentially as described before [30]. The buffer used for storage of the lectin coated Eu^{+3}-NPs was 10 mM Tris-HCl, pH 7.8, supplemented with 0.1% BSA and 0.01% sodium azide at +4°C, covered from light. The particles were thoroughly vortexed and sonicated before every use to disperse aggregates.

Labelling of antibodies with biotin

Both solid-phase monoclonal antibodies (Ma552 and Ma695 mAb) were biotinylated with 40-fold molar excess of biotin isothiocyanate, for 4 h at room temperature (RT). The labelled antibodies were separated from the unconjugated biotin by using NAP-5 and NAP-10 gel-filtration columns (GE Healthcare, Schenectady, NY, USA) by using 50 mM Tris–HCl (pH 7.75), containing 150 mM NaCl and 0.5 g/L NaN3. The labelled antibodies were stabilized with 1 g/L BSA (Bioreba, Nyon, Switzerland) and stored at +4°C. [31]

In-house CA15-3 Lectin-NP assay

The assay principle is represented in Fig 1. Biotinylated Ma552 or Ma695 mAb (100 ng/30 μl/well) in buffer solution was incubated for 1 h at RT to immobilize them on streptavidin-coated yellow low-fluorescence microtiter wells. The wells were washed two times with wash buffer and 25 μl of CA15-3 standard/sample (diluted 1:40 in buffer) was added and incubated for 1 h at RT with slow shaking. The immobilized BC-CA15-3 was detected by lectin-Eu^{+3}-NPs as a tracer by using time-resolved fluorescence (TRF). Ten million lectin Eu^{+3}-NPs per well in 25 μl of assay buffer containing additional 6 mM $CaCl_2$ was added. The wells were incubated for two hours at RT in shaking and washed six times. To detect the lectin-Eu^{3+}-NPs bound to BC-CA15-3, the TRF of Eu (λ_{ex}: 340 nm; λ_{em}: 615 nm) was measured for 400 μs after a 400 μs delay using Victor^{3}V 1420 Multilabel counter (Wallac, Turku, Finland).
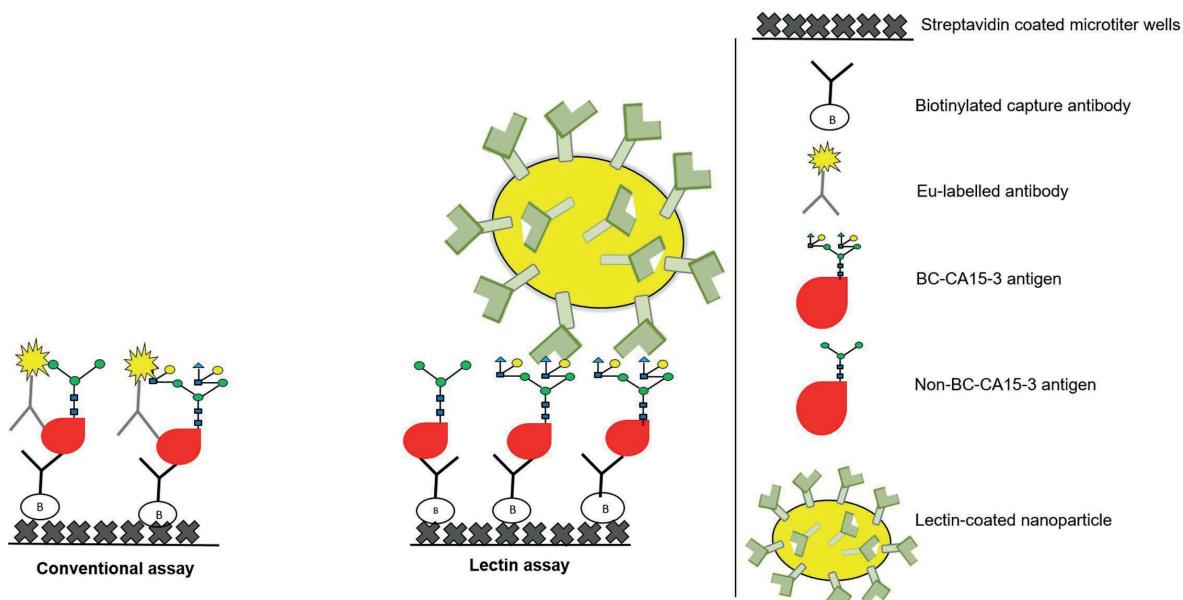
Fig 1. The principle of the conventional and in-house Eu^{+3}-NP-based CA15-3 lectin assays. In the conventional CA15-3 immunoassay, the capture and tracer mAbs bind to the protein and glycan epitopes of CA15-3. Alternatively, in the lectin assay, the CA15-3 is captured with mAbs and detected with lectins, which have been coated on the surface of Eu^{3+}-NPs. This method allows multivalent binding of the tracer to the glycan moieties of BC-CA15-3.

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Conventional CA15-3 immunoassay

CA15-3 concentrations were analysed in plasma samples with a CA15-3 enzyme immunoassay (Fujirebio Diagnostics Inc., Malvern, PA, USA) according to manufacturer's instructions.

Statistical analysis

Receiver operating characteristics (ROC) were determined and compared, and the areas under the curve (AUC) values calculated using R version 3.3. [32] with the pROC package [33]. The measured concentrations of each assay (Table B in S1 Dataset) were used as the classifier. The comparison of ROCs was done using the bootstrap method provided in the pROC package. Due to the nonparametric distribution of the CA15-3 levels, medians with the interquartile range (IQR) of the median were reported. CA15-3 levels of healthy controls were compared to CA15-3 levels of metastatic BC patients using the Mann Whitney U-test. Wilcoxon Rank test was used when comparing baseline and week six CA15-3 levels in relation to the treatment response. Spearman's correlation was used to study the correlation between conventional CA15-3 levels and CA15-3^{MGL} or CA15-3^{WGA} levels. The Wilcoxon signed-rank test and Spearman's correlation analyses were performed using SPSS version 23 statistical software package (SPSS Inc., Chicago, IL, USA). P value of less than 0.05 was considered significant in all statistical tests.

Results

Screening of lectins for binding to BC-CA15-3

Altogether 28 lectins with various carbohydrate-binding specificities (Table A in S1 Dataset) were tested to investigate the glycosylation patterns of the cancer cell line -derived BC-CA15-3

preparation. Fig 2 shows the signal-to-background ratios obtained with the corresponding lectin-NP tracers using two different monoclonal antibodies (Ma552 and Ma695) for capturing CA15-3. Four of the tested nanoparticle tracers; MGL- WGA-, Gal-4-, and DSL-NPs, recognized BC-CA15-3 and the trend was similar for both capture antibodies. WGA exhibited highest signal-to-background ratio followed by MGL, Gal-4 and DSL (Fig 2). WGA- and MGL-NPs displayed excellent recovery (93% to 98%) when BC-CA15-3 was spiked into pooled healthy plasma samples whereas Gal-4- and DSL-NPs scarcely bound to BC-CA15-3 spiked
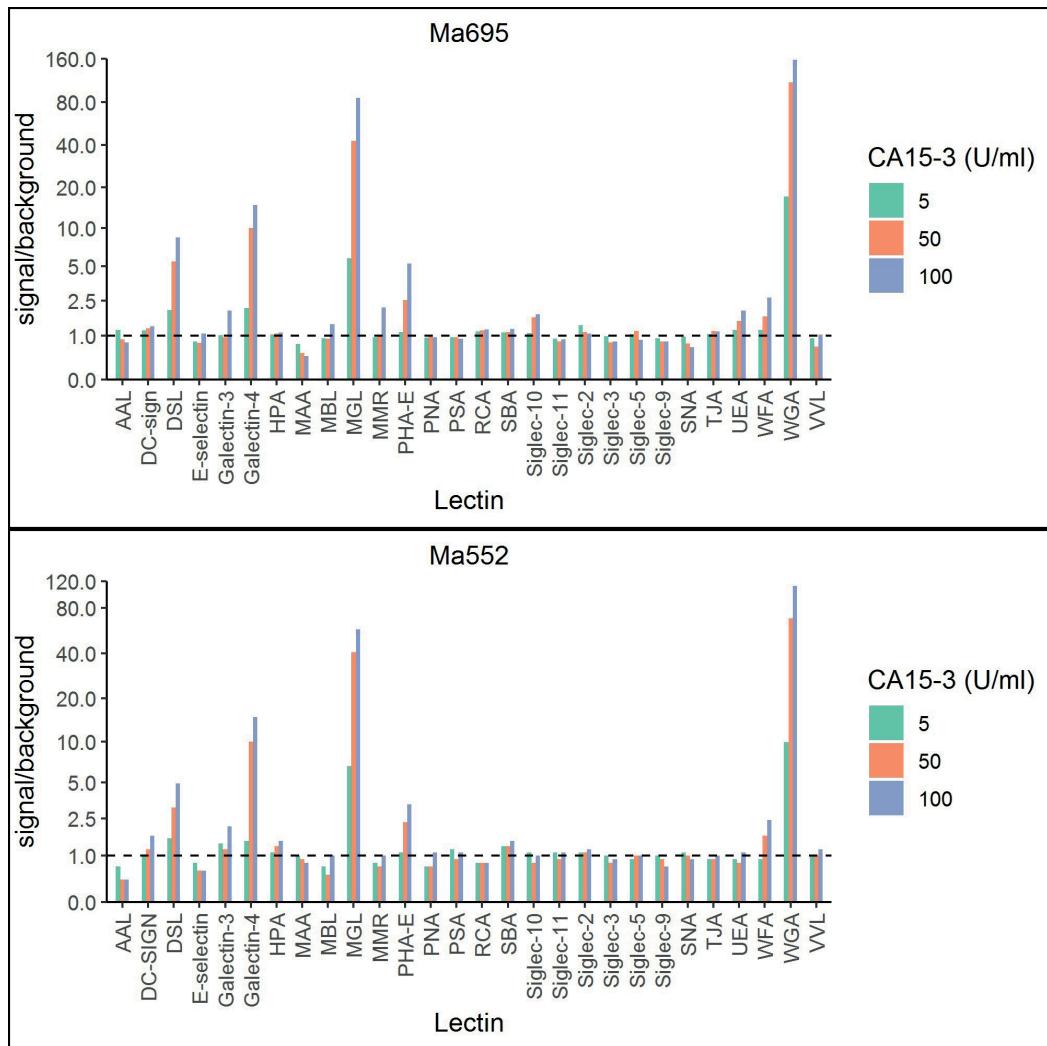

Fig 2. Lectin NPs binding to BC-associated CA15-3 from cell line ZR-75-1 (ATCC CRL-1500) using the lectin assay principle depicted in Fig 1. The different lectin Eu^{+3}-NPs used are shown on the *x-axis* and the *y-axis* displays the signal to background ratios using either biotinylated Ma695 (bioMa695) or biotinylated Ma552 (bioMa552) as the capture mAb.

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similarly in plasma. We selected MGL (here after, CA15-3^{MGL}) and WGA (CA15-3^{WGA}) for further evaluation using clinical samples.

Characteristics of CA15-3^{MGL} CA15-3^{WGA} assays

The analytical performance of the CA15-3^{MGL} and CA15-3^{WGA} assays were preliminarily tested using a BC-CA15-3 in a range of concentrations from 1 to 1000 U/mL. Saturation was not observed at the maximum used BC-CA15-3 of 1000 U/mL. The limit of detection, which was set to be the concentration of BC-CA15-3 required for a signal equivalent to the mean of blank calibrator (n = 20) plus three times the standard deviation, was less than 1 U/mL. Linear in response was observed at a maximum of 125 U/mL (S1 Fig). No cross-reactivity was observed towards two other glycoprotein cancer markers, CA125 and prostate specific antigen (S2 Fig).

Plasma CA15-3, CA15-3^{MGL}, and CA15-3^{WGA} concentrations in the study cohort

We next studied whether CA15-3 in the plasma of BC patients binds with MGL and WGA similar to CA15-3 of a breast cancer cell line. The baseline EDTA plasma samples from 53 patients with metastatic BC and 20 healthy individuals were measured for CA15-3^{MGL} and CA15-3^{WGA} and compared with the conventional CA15-3 immunoassay. To assess the diagnostic value of the tumor markers in metastatic BC, ROC curves were plotted and AUC was calculated. The highest AUC value was achieved with CA15-3^{WGA} (0.943) followed by CA15-3^{MGL} (0.852) while the conventional CA15-3 immunoassay yielded the lowest AUC of 0.827 (Fig 3). At 90% specificity the sensitivities of the assays were 81.1, 67.9 and 66.0% for the CA15-3^{WGA}, CA15-3^{MGL} and conventional CA15-3, respectively. The difference in the AUC compared to the conventional assay was significant for CA15-3^{WGA} (p = 0.007) but not for CA15-3^{MGL} (p = 0.655).

Metastatic BC patients had higher median baseline plasma levels of conventional CA15-3 as well as CA15-3^{MGL} and CA15-3^{WGA} levels than the healthy controls (Table 1). Plasma samples were available from 53 metastatic breast cancer patients. However, both baseline and week six samples were available only from 41 patients. Median CA15-3 levels were lower at week six than at baseline for all three CA15-3 assays in the entire study population (p-values 0.007, <0.001, <0.001 for CA15-3, CA15-3^{MGL} and CA15-3^{WGA}, respectively). The decline in CA15-3 levels was more pronounced in responding patients for all CA15-3 assays, especially CA15-3^{MGL} (Table 2). For all the three different CA15-3 assays, the responding patients had a significant decrease in all assays of CA15-3 between baseline and week six (p-values 0.003, <0.001 and <0.001 for CA 15–3, CA15-3^{MGL} and CA15-3^{WGA}, respectively, Table 2).

Baseline conventional CA15-3 and CA15-3^{MGL} levels correlated to each other (r = 0.68, p<0.001, Fig 4A and 4B). However, almost half of the metastatic BC patients had very low baseline CA15-3^{MGL} levels ($\leq$ 5 U/Ml, dashed vertical line in Fig 4B). A stronger correlation was observed between conventional CA15-3 and CA15-3^{WGA} (r = 0.90, p<0.001, Fig 4C and 4D).

Additionally, we studied CA15-3 levels at disease progression (Fig 5). We had plasma samples available from 19 patients who had a disease progression at final study visit. A clinically meaningful 30% increase in the final CA15-3 levels was observed in eight patients (42%) with the conventional CA15-3, nine patients (47%) with the CA15-3^{MGL} and six patients (32%) with the CA15-3^{WGA}. The patients with rising CA15-3 levels at disease progression were not entirely the same individuals for the different CA15-3 assays. Specifically, five patients had similar increase in final CA15-3^{MGL} levels and CA15-3 levels. However, four patients with
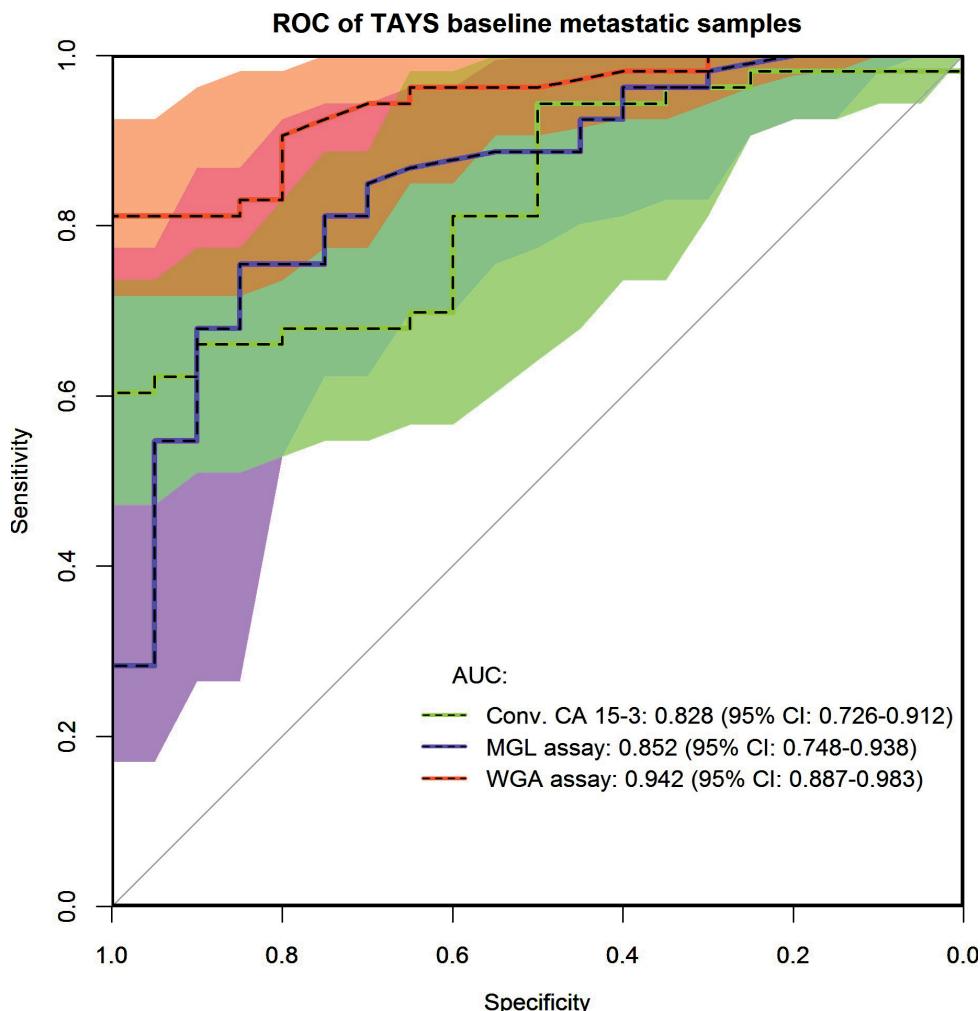
Fig 3. ROC plot displaying the AUC of conventional CA15-3 (green), CA15-3^{MGL} (purple) and CA15-3^{WGA} (red) from metastatic breast cancer patients (n = 53) and healthy control (n = 20). The 95% confidence intervals of the ROCs are depicted as shaded areas and displayed numerically in brackets. The color of shadings corresponds to the plotted lines and the overlap of conventional CA15-3 and CA15-3^{MGL} is dark green, the overlap of all assays is brown and the overlap of CA15-3^{MGL} and CA15-3^{WGA} is dark red.

rising CA15-3^{MGL} levels did not have an increase in conventional CA15-3 levels. Furthermore, a similar increase was observed in four patients in final CA15-3^{WGA} and conventional CA15-3 levels. However, two patients with rising CA15-3^{WGA} levels did not have an increase in conventional CA15-3 levels. Additionally, at least 30% decrease in the final CA15-3 levels at disease progression was observed in three patients (16%) with the conventional CA15-3, 3 patients (16%) with CA15-3^{MGL} and five patients (26%) with CA15-3^{WGA}.

Table 1. CA15-3 levels by conventional CA15-3, CA15-3^{MGL}, and CA15-3^{WGA} assay for healthy controls and for metastatic BC patients at study baseline.

	n	Conventional CA15-3	CA15-3^{MGL}	CA15-3^{WGA}
Healthy controls				
Median CA15-3 U/mL (IQR)	20	13.3 (7.9–23.1)	2.0 (0.2–3.6)	1.6 (0.5–2.7)
Metastastic BC patients, Baseline				
Median CA15-3 U/mL (IQR)	53	47.4 (18.9–99.9)	4.4 (1.3–16.5)	7.0 (3.1–41.0)
p-value[a]		<0.001	0.013	<0.001

Abbreviations: n = number of patients, IQR = interquartile range, BC = breast cancer

[a] Mann-Whitney *U*-test

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Discussion

The results suggest that the glycovariant specific assays provide advantages over the conventional CA15-3 immunoassay in monitoring of BC patients, and especially for the detection of metastatic disease and its recurrence.

CA15-3 is a tumor marker commonly used for monitoring patients with advanced BC. However, the currently employed sandwich immunoassays that target two protein epitopes have moderate clinical sensitivity and specificity. [34] While it has been established that abnormal glycosylation occurs in cancers and there has been investigations into multiple different approaches for their detection [35] the efforts to further develop the CA15-3 based diagnostic assay have been limited. The changes in glycosylation can lead to altered interactions of glycoproteins expressed by the tumor cell with different lectins. The development of glycoprofiling assays for blood-derived products has been made difficult by the fact that cancer specific glycovariants may only exist in small amounts in blood and are therefore problematic to detect. We have previously utilized the lectin-NP -based platform successfully to explore the

Table 2. CA15-3 levels with different assays depending on the best response to the chemotherapy treatment.

	n	Baseline Median CA15-3, U/mL (IQR)	Week 6 Median CA15-3 U/mL (IQR)	Change, median % (IQR)[a]	p[b]	Declining CA15-3 levels, n (%)[c]	Increase in CA15-3 levels, n (%)[d]
Conventional CA15-3							
PR	25	71.1 (29.4–228)	55.4 (28.8–103)	-23.8 [-52.7-(-14.0)]	**0.003**	10 (40.0)	2 (8.0)
SD	14	19.2 (12.6–81.4)	25.8 (14.9–71.0)	-0.4 (-37.1–60.5)	0.875	4 (28.6)	5 (35.7)
PD	2	24.1 (15.0–33.1)	29.2 (17.5–40.9)	+20.1 (16.7–23.6)	0.180	0	0
CA15-3^{MGL}							
PR	25	6.3 (2.1–45.1)	2.4 (0.9–4.8)	-75.0 [-86.4-(-41.0)]	**<0.001**	18 (78.2)	3 (13.0)
SD	14	3.2 (1.0–5.4)	2.2 (0.8–3.6)	-33.3 (-67.0–33.3)	**0.036**	7 (53.8)	4 (30.7)
PD	2	4.1 (3.2–5.0)	3.0 (2.2–3.9)	-17.0 (-56.0–21.9)	0.655	1 (50.0)	0
CA15-3^{WGA}							
PR	25	13.2 (5.3–76.5)	8.0 (3.5–33.2)	-27.2 [-55.9-(-19.4)]	**<0.001**	12 (48.0)	2 (8.0)
SD	14	3.2 (2.4–8.7)	5.0 (2.7–8.2)	+22.2 (-25.4–60.0)	0.851	3 (21.4)	7 (50.0)
PD	2	4.7 (2.3–7.0)	5.8 (2.3–9.3)	+16.4 (0–32.9)	0.317	0	1 (50.0)

Abbreviations: n = number of patients, CI = confidence interval, PR = partial response, SD = stable disease, PD = progressive disease

[a] Change in CA15-3 levels from baseline to week six in percentiles, median

[b] Wilcoxon Rank Test

c Patients with ≥ 30% decline in CA15-3 levels from baseline to week six

d Patients with ≥ 30% increase in CA15-3 levels from baseline to week six

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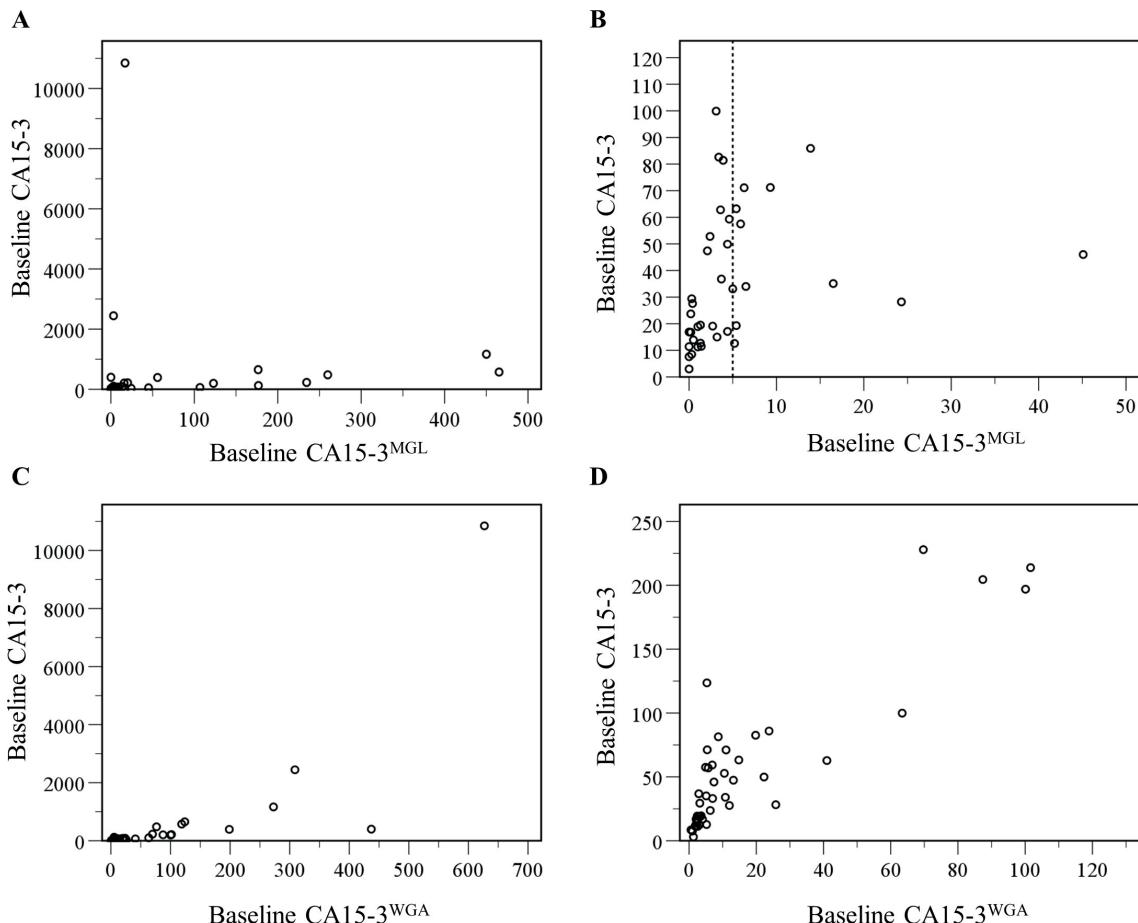
Fig 4. Correlation of the conventional CA15-3 and CA15-3^Lectin assays. A: Scatterplot of baseline conventional CA15-3 levels and baseline CA15-3^MGL levels in metastatic breast cancer patients. r = 0.68, p<0.001 **B:** Enlargement of the scatterplot for the patients with the lowest CA15-3 levels for both conventional CA15-3 and CA15-3^MGL. Very low baseline CA15-3MGL levels < 5 U/mL were observed in 29 patients (44.6%), dashed vertical line at x-axis **C:** Scatterplot of baseline conventional CA15-3 levels and baseline CA15-3^WGA levels in metastatic breast cancer patients. r = 0.90, p<0.001. **D:** Enlargement of the scatterplot for the patients with conventional CA15-3 < 250 U/mL and CA15-3WGA < 130 U/mL, 85% of the study patients.

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glycosylation of serum glycoprotein CA125 in ovarian cancer patients [25]. Two features in a combination enhance the analytical sensitivity of lectin-NPs; 1) signal amplification provided by the thousands of Eu chelates doped in a single particle and 2) the strengthening of the functional affinity (avidity) of the lectins to their target glycostructure epitopes enabled by the high-density immobilization of lectin on the particles.

The present study shows for the first time that a qualitative glycovariant assay to detect the changes of the CA15-3 glycoprotein can improve on the diagnostic utility of current assays. We developed an assay for sensitive and quantitative detection of aberrant glycosylation on BC-CA15-3 providing enhanced preference for the cancer-associated glycovariant of the tumor marker. An antibody recognizing the protein/glycan epitopes of CA15-3 was used for
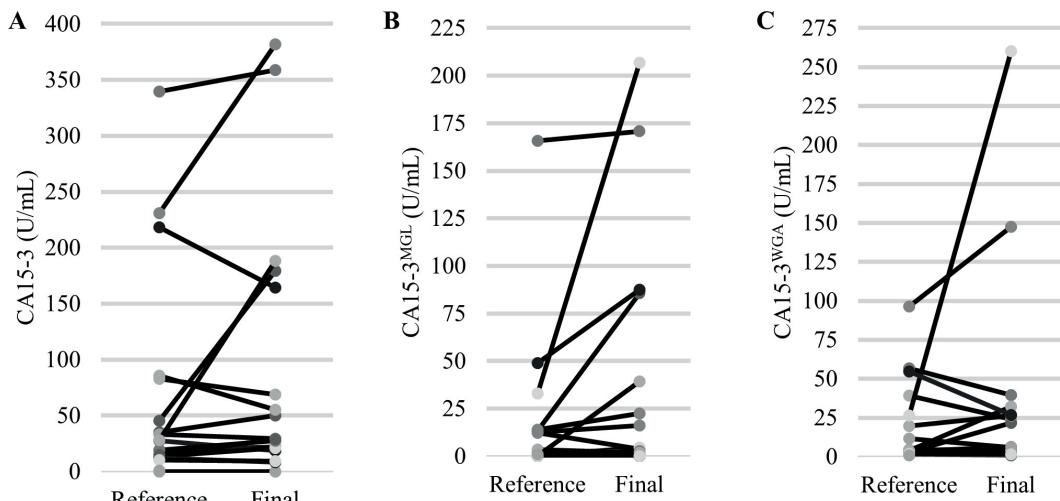
Fig 5. CA15-3 levels of 19 patients who had a disease progression at the time of final plasma sampling and a previous reference plasma available while on study treatment (Reference). A. Conventional CA15-3. Two patients with very high CA15-3 levels were excluded (Reference CA15-3 110.6 U/mL, Final 986.4 U/mL and Reference 1825.5 U/mL, Final 3909.7 U/mL) **B.** CA15-3^{MGL}. **C.** CA15-3^{WGA}. One patient with very high CA15-3^{WGA} level was excluded (Reference CA15-3^{WGA} 393.1 U/mL, Final CA15-3^{WGA} 430.9 U/mL).

the immobilization and a panel of lectins was tested for the ability to bind the immobilized CA15-3. The tested panel of 28 lectins covers a variety of common human glycans. Only two of the tested lectin-Eu^{+3}-NP preparations exhibited satisfactory binding to the BC-associated CA15-3. Those lectins were WGA and recombinant human MGL. WGA and MGL recognize the GlcNAc and GalNAc -containing epitopes respectively, frequently expressed on the surface of cancer cells [36–38]. Using CA15-3^{WGA} and CA15-3^{MGL} assays, in the plasma of metastatic BC patients are likely to serve as more cancer-specific than the conventional assay. In patients with metastatic BC, the newly developed CA15-3^{WGA} assay was able to detect 81% compared to 66% with conventional CA15-3 assay when only 10% of controls were misdiagnosed with both assays.

Consistent with these findings, Nollau P *et al.* describe the use of recombinant MGL (also known as CLEC10A), for the detection of ligands in sections from formalin-fixed, paraffin-embedded normal and cancerous mammary tissues. In comparison to normal mammary glands, a pronounced staining of tumour tissues was observed. [37] Beatson R *et al.* also observed that MUC1 carrying both Tn and STn epitopes can bind to the human lectin MGL, and using atomic force microscopy they showed that Tn and sTn bind to MGL with a similar de-adhesion force. [39] Our study reports the binding of that same human lectin MGL with plasma of BC patients and particularly with CA15-3. Blixt *et al.* reported that high levels of a subset of autoantibodies to the core 3-MUC1 (GlcNAcβ1-3GalNAc-MUC1) and STn-MUC1 glycoforms were significantly associated with reduced incidence and increased time to metastasis, which also supports our findings of MGL binding [18].

As far as we know, this study is the first to report WGA's specificity for BC-CA15-3. WGA is a plant lectin, which specifically recognizes the sugars NeuNAc and GlcNAc [39]. It has been reported that terminal GlcNAc is characteristic of a group of protein- and lipid-linked glycans overexpressed in many malignant tumor tissues including breast carcinoma [16].

Chandrasekaran EV *et al* studied the complex carbohydrate-lectin interactions by determining the effects of substituents in mucin core 2 tetrasaccharide Galβ1-4GlcNAcβ1-6(Galβ1–3) Gal-NAcα-OR and fetuin glycopeptides on their binding to agarose immobilized lectin WGA. Compounds with α2-3-sialyl T-hapten, α2-6-Sialyl LacdiNAc, α2-3-sialyl D-Fucβ1–3 GalNAc and Fucα-1-2 D-Fucβ-1-3GalNAc displayed regular binding and GalNAc, LewisX and Lacdi-NAc plus D-Fuc β-1-3 GalNAcα exhibited particularly tight binding.[40] A previous study by Bird-Liebermann EL *et al* identified GlcNAc as a biomarker for endoscopic visualization of Barrett's esophagus to detect dysplastic esophageal tissue [41]. Using a serum CA15-3 lectin assay based on antibody-capture, Ideo *et al*. showed that 3-sulfated core 1 specific Gal-4 can be used to measure CA15-3 that is present in BC[22]. We observed in our study that Gal-4 bound more poorly to BC-CA15-3 than MGL and WGA.

The specificity provided by the immobilizing antibody together with the glycan-recognition of the lectins, which is enhanced through the avidity made possible by the Eu+3-NPs, constitute the technical concept behind the novel CA15-3^{MGL} and CA15-3^{WGA} assays. These assay strongly prefer the cancer-associated glycovariants compared to conventional CA15-3 sandwich immunoassay. Based on previously published research on the nature of CA15-3 glycosylation in malignant and benign states, the WGA preference for cancer CA15-3 would have been difficult to predict. It is possible that the glycan eiptopes reactive with MGL and WGA are be present on several CA15-3 glycans, being a sizable 500–1000 kDa glycoprotein. The extracellular domain of CA15-3 consists mainly of 25–150 tandem repeats of 20 amino acids. Each repeat carries five *O*-linked glycosylation sites, thus glycans can potentially be repeated 125–750 times on each molecule allowing engagement of relevant lectins.[20] The high amount of glycans makes the presence of multiple binding sites for MGL and WGA Eu^{+3}-NPs plausible and may provide for high avidity even at low CA15-3 concentration. The low limit of detection and great linearity of the standard in the range of 1-100 U/ml of analyte agrees with this assessment.

The monitoring of conventional CA15-3 levels for therapy response of metastatic BC is currently recommended only as an adjunctive assessment to aid clinical decisions[42].This is mostly due to low sensitivity and specificity of the conventional CA15-3 assay. Additionally, the conventional CA15-3 levels may have discrepancies compared to clinical findings and radiological assessments[10]. Although for majority of patients, the tumor markers decline in responding patients and increase in progressing patients, misinterpretations are possible if tumor markers are evaluated alone for an individual patient. In our study, 10 patients (40%) with a partial response to study treatment had at least 30% decline in conventional CA15-3 levels between baseline and week six. However, declining plasma levels for responding patients were more common both with the CA15-3^{MGL} method (18 patients, 78%) and the CA15-3^{WGA} method (12 patients, 48%). Therefore, new CA15-3^{lectin} assays recognize the responding patients better than the conventional CA15-3. False positive increases in CA15-3 levels were observed for responding patients for all three assays between baseline and week six (two patients for conventional CA15-3 and CA15-3^{WGA}, three patients for CA15-3^{MGL}). At the time of progressive disease, only minority of patients had over 30% increase in their CA15-3 levels [conventional CA15-3 assay 8 patients (42%), CA15-3^{MGL} 9 patients (47%), CA15-3^{WGA} 5 patients (26%)].

Comparing the new lectin assays to one another, CA15-3^{WGA} seems to be more suitable for clinical use than CA15-3^{MGL}. The clinical utility for CA15-3^{MGL} levels is limited due to very low < 5 IU/mL baseline levels detected for almost half of our study patients. Additionally, the correlation between conventional CA15-3 and CA15-3^{WGA} was more pronounced (r = 0.90, p<0.001). Nevertheless, for this limited patient population, CA15-3^{MGL} and CA15-3^{WGA} seem not yet to be ideal assays for clinical utility and the possibilities for misinterpretations for an

individual patient remains as it does for the conventional CA15-3 assay. However, it would be worthwhile test these CA15-3[lectin] assays in a prospective trial involving metastatic breast cancer patients.

This study suggests that using CA15-3 mAb and WGA and MGL Eu3+NPs are more sensitive in distinguishing metastatic BC patients from healthy controls than conventional CA15-3 immunoassay.

Due to the limited amount of patient samples used in this proof of concept study report, studies for further validation, to establish the clinical performance of CA15-3[WGA] and CA15-3[MGL] assays for BC surveillance, and monitoring progression and therapeutic responses of metastatic disease, are now under investigation. The findings also warrant further investigation of this approach in other cancers.

Supporting information

S1 Fig. Calibration curves of (A) CA15-3 WGA and (B) CA15-3-MGL NPs-lectin assay. Both are linear in range of 1 to 125 U/ml with excellent analytical sensitivity. (PPTX)

S2 Fig. Assay cross-reactivity with other common tumor markers. No cross reactivity with CA15-3 lectin assay was observed with ovarian cancer cell line associated CA125 and prostate cancer associated LnCAp PSA (PPTX)

S1 Dataset. Table A. Lectins used. Table B. Concentrations measured from controls and metastatic cases (baseline samples). (DOCX)

S2 Dataset. Patient cohort data. (SAV)

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Author Contributions

Formal analysis: Joonas Terävä, Leena Tiainen.

Funding acquisition: Joonas Terävä, Pirkko-Liisa Kellokumpu-Lehtinen.

Investigation: Leena Tiainen.

Methodology: Kamlesh Gidwani.

Project administration: Kim Pettersson.

Resources: Leena Tiainen, Pirkko-Liisa Kellokumpu-Lehtinen.

Supervision: Urpo Lamminmäki, Pirkko-Liisa Kellokumpu-Lehtinen, Kim Pettersson, Kamlesh Gidwani.

Writing – original draft: Joonas Terävä, Leena Tiainen, Urpo Lamminmäki, Pirkko-Liisa Kellokumpu-Lehtinen, Kim Pettersson, Kamlesh Gidwani.

Writing – review & editing: Joonas Terävä, Leena Tiainen, Urpo Lamminmäki, Pirkko-Liisa Kellokumpu-Lehtinen, Kim Pettersson, Kamlesh Gidwani.

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