CHAPTER 2

Targeting breast cancer through its microenvironment: Current status of preclinical and clinical research in finding relevant targets

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ABSTRACT

It is increasingly evident that not only breast cancer cells, but also the tissue embedding these cells: the tumor microenvironment, plays an important role in tumor progression, metastasis formation and treatment sensitivity. This review focuses on the current knowledge of processes by which the microenvironment affects breast cancer, including formation of the metastatic niche, metabolic stimulation, stimulation of tumor cell migration, immune modulation, angiogenesis and matrix remodeling. The number of drugs targeting key factors in these processes is expanding, and the available clinical data is increasing. Therefore current strategies for intervention and prediction of treatment response are outlined. At present, targeting the formation of the metastatic niche and metabolic stimulation by the breast cancer microenvironment, are already showing clinical efficacy. Intervening in the stimulation of tumor cell migration and immune modulation by the microenvironment are upcoming fields of great research interest. In contrast, targeting microenvironmental angiogenesis or matrix remodeling appears to be of limited clinical relevance in breast cancer treatment so far. Further research is warranted to optimize intervention strategies and develop predictive tests for the relevance of targeting involved factors within the microenvironment in order to optimally personalize breast cancer treatment.

Abbreviations: 18F, Fluor-18; 89Zr, zirconium-89; 111In, Indium; CAF, Cancer associated fibroblast; cMET, C-mesenchymal-epithelial transition factor; CSF, Colony stimulating factor; CI, Confidence interval; CTLA, Cytotoxic T lymphocyte-associated antigen; CXCL, Chemokine (C-X-C motif) ligand; CXCR, C-X-C motif receptor; E2, Estradiol; ECM, Extracellular matrix; ER, Estrogen receptor; FES, Fluoroestradiol; HER, Human epidermal growth factor receptor; HGF, Hepatocyte growth factor; HR, Hazard ratio; IGF, Insulin-like growth factor; IGF-1R, Insulin-like growth factor 1 receptor; IL, Interleukin; IR, Insulin receptor; LOX, Lysyl oxidase; LOXL, Lysyl oxidase ligand; MAPK, Mitogen-activated protein kinase; MBC, Metastatic breast cancer; MDSC, Myeloid-derived suppressor cells; MMP, Matrix metalloprotease; OPG, Osteoprotegerin; PD, Programmed cell death; PD-L, Programmed cell death ligand; PET, Positron emission tomography; PI3K, Phosphoinositide 3-kinase; PTHrP, Parathyroid hormone-related protein; RANK, Receptor activator of nuclear factor κB; RANKL, Receptor activator of nuclear factor κ ligand; SDF, Stromal derived growth factor; SUV, Standardized uptake value; TAM, Tumor-associated macrophage; TGFβ, Transforming growth factor β; TGFβR, Transforming growth factor β receptor; TIL, Tumor infiltrating lymphocyte; TKI, Tyrosine kinase inhibitor; TNBC, Triple negative breast cancer; TNF-α, Tumor necrosis factor α; VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor.
INTRODUCTION

Breast cancer is the most common cause of cancer death among women worldwide (1). In 2010, 207,090 women were diagnosed with breast cancer in the United States (2). Approximately 6% of all breast cancer patients have metastatic disease at the time of diagnosis, and currently 20% will eventually develop metastatic breast cancer (MBC) (3). Once metastasized, breast cancer is generally incurable.

Recent treatment strategies focus on induction of tumor cell death using chemotherapeutic, anti-hormonal and targeted agents. However, it is increasingly recognized that not only the tumor cells, but also the tissue embedding the tumor cells; their microenvironment, plays an important role in tumor progression and metastasis. This role in the complexity of metastasis (4) can be assumed from the metastatic pattern of breast cancer to specific organs (5). The importance of the cancer microenvironment is underlined by the recent inclusion of the microenvironment in the so called “hallmarks of cancer” (6, 7). Furthermore, microenvironmental characteristics affect breast cancer prognosis and chemosensitivity, and as such are increasingly incorporated in gene expression profiles (8, 9). Novel drugs targeting key factors in the microenvironment are being developed.

The tumor microenvironment includes soluble factors, extracellular matrix (ECM) and stromal cells (10). Involved soluble factors comprise growth factors, hormones, immunoglobulins, cytokines and chemokines (10). The ECM contains proteoglycans, hyaluronic acid and fibrous proteins (collagen, fibronectin and laminin). Involved stromal cells include fibroblasts, (pre-)adipocytes, cells of the vascular system (endothelial cells) and immune cells (11, 12). Combinations of different cellular, extracellular and soluble factors can act to support multiple processes in the breast cancer microenvironment that promote progression and metastasis. This review focuses on the current knowledge of processes involved in the breast cancer microenvironment, and how they affect breast cancer progression and metastasis. These processes include: formation of the metastatic niche, metabolic stimulation, stimulation of tumor cell migration, immune modulation, angiogenesis and matrix remodeling. We will place them in the current order of importance as targets for breast cancer therapy, based on the clinical evidence with the available targeting agents (Table 1). Per factor involved in the processes, the mechanism of action and preclinical data is described, which is followed by the currently available clinical data. Thereafter, the present data regarding treatment response prediction is outlined per factor. Finally, we will describe potential future directions exploiting the microenvironment in breast cancer treatment.
SEARCH STRATEGIES AND SELECTION CRITERIA

Articles for this review were found by searches of PubMed, abstracts American association for cancer research (AACR) and American society of clinical oncology (ASCO) and the clinicaltrials.gov database by use of the terms ‘breast cancer’, ‘microenvironment’ combined with ‘metastasis’ ‘metabolic dysfunction’ ‘migration’ ‘immune cells’ ‘angiogenesis’ or ‘matrix remodeling’ and combinations of these terms with the selected soluble factors. In addition, relevant papers from the reference lists of selected papers were included. Only studies written in English were included.
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*Level of evidence:
* Indirect effect. Anti tumor effect of bisphosphonates not fully proven to be TGFβ dependent.

**Abbreviations:** TGFβ, Transforming growth factor β; TGFBR, Transforming growth factor β receptor; TKI, Tyrosine kinase inhibitor; PBMC, Peripheral blood mononuclear cell; RANK, Receptor activator of nuclear factor kB; RANKL, Receptor activator of nuclear factor kB ligand; OPG, Osteoprotegerin; NTX, N-terminal telopeptide; E2, Estradiol; ER, Estrogen receptor; 18F, Fluor-18; FES, Fluoroestradiol; PET, Position emission tomography; IGF, Insulin-like growth factor; IGF-1R, Insulin-like growth factor 1 receptor; 111In, Indium-111; SPECT, Single-photon emission computed tomography; PD, Programmed cell death; PD-L, Programmed cell death ligand; TIL, Tumor infiltrating lymphocyte; CTLA, Cytotoxic T lymphocyte-associated antigen; HGF, Hepatocyte growth factor; cMET, C-mesenchymal-epithelial transition factor; SDF, Stromal derived growth factor; CXCR, C-X-C motif receptor; VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor; 89Zr, Zirconium-89; MMP, Matrix metalloprotease; LOX, Lysyl oxidase
FORMATION OF THE METASTATIC NICHE

The importance of the interaction of the breast cancer cells with their microenvironment has long been suggested by the specificity of the metastatic pattern (13). In MBC patients, metastasis patterns even differ per breast cancer subtype (5). In general however, bone is by far the most common metastatic site involving 65% of patients with MBC (5, 14, 15). Crucial factors involved in the development of bone metastases are transforming growth factor (TGF)β and receptor activator of nuclear factor κB ligand (RANKL) (Figure 1A).

TGFβ – mechanism of action and preclinical data

The cytokine TGFβ has tumor suppressive properties in the physiological setting. However, during malignant progression, TGFβ signaling promotes growth, progression and invasion of the tumor (16). Both cancer and cancer associated fibroblasts (CAF)s excrete TGFβ by autocrine as well as paracrine secretion, giving rise to a tumor-promoting microenvironment (17, 18) (Figure 1A.1 (circle tumor cell) and 1A.2 (circle microenvironment)). Activated TGFβ binds to the TGFβI- and TGFβII-receptor (-R) which both induce Smad2 phosphorylation which in turn activates transcriptional factors (19). In human triple negative breast cancer (TNBC) metastatic models in mice, reducing TGFβ signaling, either pharmacologically (with pan-TGFβ antibody 1D11 or TGFβ receptor inhibitor Ki26894 or LY2109761 or molecularly (with a short hairpin against Smad4), reduced metastases (20-22) (Figure 1A.3 (circle targeting)). However, in a metastatic human luminal breast cancer mouse model, targeting TGFβ signaling with 1D11 did not influence metastases formation after intracardiac breast cancer cell injection (23). Moreover, deletion of the TgfβII receptor gene in mouse mammary epithelial cells increased tumor growth and pulmonary metastasis formation (24). This suggests not only that targeting of TGFβ in early phases of tumorigenesis has tumor promoting effects, but also that there is likely to be a breast cancer subtype specific aspect to this.

TGFβ is also described to be implicated in epithelial mesenchymal transition (EMT). This change in phenotype allows cancer cells to increase metastatic potential (25). Although debate about the clinical relevance and existence of EMT still exists (26, 27), preclinical evidence for a role of TGFβ in breast cancer EMT is present. TGFβ derived from CAFs, isolated from human breast cancer tissue, was shown to induce an EMT like phenotype of breast cancer cells MCF-7, MDA-MB-231 and T47D in vitro, characterized by increased vimentin, fibronectin, matrix metalloprotease (MMP) expression and increased migration (28). This phenotype was inhibited by adding a TGFβ neutralizing antibody. In a rat mammary cancer model MTLn3E, (transient) TGFβ signaling was active in single cell motility of breast cancer cells, which led to hematogenous spread and pulmonary metastases. Blocking TGFβ signaling genetically reduced hematogenous spread but did not affect local metastasis to lymph nodes (29). These data indicate that TGFβ signaling can phenotypically change breast cancer cells, inducing metastatic characteristics.

TGFβ signaling can be inhibited by bisphosphonates (Figure 1A.3). Bisphosphonates
are commonly used as supportive treatment in MBC patients with bone metastases. In a metastatic mouse model with human breast cancer cells, treatment with bisphosphonates reduced TGFβ signaling in breast cancer cells (20). *In vitro* bisphosphonate treated cells, showed no effect on cell survival, indicating that the anti-cancer effect *in vivo* is likely occurring via the microenvironment. This is presumably mediated by reduced osteoclast activity whereby less TGFβ is released from the bone matrix (30).

Preclinical studies suggest that the anti-cancer effect of bisphosphonates is estradiol (E2) level dependent. Lowered E2 levels promote bone turnover activity (31), this could lead to the release of bisphosphonate from the bone matrix (32). Also, in bone-trope xenograft mouse models, more bone metastases developed in oophorectomized mice compared to control mice. Zoledronic acid treatment reduced tumor growth only in the oophorectomized mice (33). These findings support the clinical findings and suggest that the development of bone metastasis and the effect of zoledronic acid are E2 dependent.

**TGFβ – clinical data**

TGFβ is highly expressed in the bone tissue surrounding bone metastases (34). High circulating plasma levels of TGFβ1, measured by enzyme-linked immunosorbent assay, reflected a worse prognosis in 117 and 439 (mainly early stage) primary breast cancer patients (35, 36). Three clinical trials studied the effect of the biphosphonate zoledronic acid in the adjuvant setting. In the ABCSG-12 trial involving 1,803 patients, disease free survival at 62 months was increased from 88% to 92% (hazard ratio (HR) 0.68; 95% confidence interval (CI) 0.51–0.91; P = 0.009) by the addition of the biphosphonate to endocrine therapy (37). The ZO-FAST study compared immediate with delayed (after fracture or high risk thereof) zoledronic acid administered with adjuvant endocrine therapy. The disease free survival increased by immediate zoledronic acid administration from 92% to 95%, (HR 0.588; 95% CI 0.361–0.959; P = 0.0314) at 36 months follow up (38). In the AZURE trial however, amongst 3,360 patients, disease free survival was 77% and no difference between zoledronic acid treatment and control was seen at a median follow-up of 59 months (adjusted HR in zoledronic acid group 0.98; 95% CI 0.85 - 1.13; P = 0.79) (39). In this study the majority of patients received chemotherapy rather than endocrine therapy alone. A subgroup analysis in patients being postmenopausal for more than 5 years showed an increase in disease free survival from 71% to 78.2% (adjusted HR in zoledronic acid group 0.98; 95% CI 0.85 - 1.13; P = 0.02) 5 years after randomization. In the NEO-ZOTAC study, amongst 250 human epidermal growth factor receptor (HER)2 negative breast cancer patients, no difference in pathologic response rate was seen with or without zoledronic acid, administered in the neo-adjuvant setting (40). A meta-analysis amongst 17,751 from 41 randomized clinical trials compared outcome of breast cancer patients with and without adjuvant bisphosphonate treatment and found reduction of breast cancer mortality and bone recurrence in postmenopausal patients (41).

Together, this suggests that bisphosphonates can increase disease survival of breast cancer patients with low systemic E2 levels. Treatment with bisphosphonates is already incorporated in standard treatment of breast cancer patient with bone metastases. But
despite the evidence of clinical efficacy, the working mechanism is not fully clarified. At osseous sites, bisphosphonates interfere with osteolysis, thereby presumably reducing TGFβ release. Reduction of bone resorption levels during bisphosphonate treatment support this working mechanism. However, no data is available regarding TGFβ levels during bisphosphonate treatment. Moreover, as this mechanism cannot explain the reduction in breast cancer recurrence at non osseous sites, future research has to elucidate whether more parameters play a role in this setting. Currently, several trials are ongoing to further study the anti-cancer effect of zoledronic acid (Table 1).

**TGFβ – prediction of treatment response**

With regard to biomarkers for effective TGFβ targeting, there are limited data available. In a syngeneic rat tumor model, *ex vivo* pSmad2 protein levels in peripheral blood mononuclear cells correlated with change in tumor pSmad2 protein levels in response to TGFβ receptor (TGFβR) tyrosine kinase inhibitor (TKI) LY2157299 (42). A TGFβ response gene signature retrieved from primary breast tumors comprising 153 genes was developed to identify tumors with high TGFβ signaling activity. In a cohort of 368 samples, tumors positive for this gene set did indeed show higher mRNA levels of TGFβ1 and TGFβ2 (43). In estrogen receptor (ER) negative tumors, this response signature correlated with recurrent disease in the lungs. A study in 12 glioblastoma patients using zirconium-89 (\(^{89}\text{Zr}\)) labeled GC1008, an antibody against active isoforms of TGFβ, for visualization TGFβ showed a 15 times higher median standardized uptake value (SUV\(_{\text{max}}\)) in tumor lesions than in normal brain tissue on positron emission tomography (PET) scans (44). There is one ongoing phase I/II trial in MBC patients with GC1008 in combination with local radiotherapy (Table 1) (Figure 1A.3).

**RANK/RANKL/OPG – mechanism of action and preclinical data**

As mentioned previously, another crucial factor involved in the development of bone metastases is RANKL. The role of the receptor activator of nuclear factor κB (RANK)/RANKL/osteoprotegerin (OPG) pathway in promoting and sustaining breast cancer bone metastases is supported by an increasing amount of preclinical and clinical data. The development of bone metastasis is caused by a vicious cycle involving interplay between cancer cells and their surroundings (Figure 1A.1 and 1A.2). Cancer cells secrete parathyroid hormone-related protein (PTHrP) (45). PTHrP subsequently stimulates microenvironmental osteoblasts to produce RANKL, which in turn stimulates osteolytic activity by osteoclasts. Enhanced osteolysis releases growth factors, such as TGFβ, from the bone matrix. This induces tumor growth, and thereby PTHrP excretion, completing the vicious cycle. Under physiological circumstances, excessive bone resorption is prevented by OPG. OPG is secreted by osteoblasts and competes with RANKL in binding to RANK (46) (Figure 1A.2). The RANK/RANKL/OPG axis also plays a role in primary breast cancer development. In mouse mammary tissue, progesterone can induce RANKL expression in epithelial cells (47), thereby exerting a mitogenic effect. A murine anti RANKL antibody reduced tumor formation in a spontaneous mouse mammary tumor model (48). RANKL treatment
of SKBR3 breast cancer cells stimulated proliferation and led to protection from cell death in response to irradiation and doxorubicin in vitro (49). In tumors, OPG can be down regulated via different mechanisms such as reduced synthesis (50). Treatment of mice with OPG shows that the RANK/RANKL/OPG axis is also of importance in bone metastases. In a MDA-MB-231 breast cancer metastatic mouse model, administration of OPG strongly reduced skeletal tumor burden and the number of osteoclasts present in the lesions (51).

**RANK/RANKL/OPG – clinical data**

High RANK and low OPG mRNA expression in 295 primary breast cancer tumors was correlated with worse overall survival (52). High RANK expression, measured by immunohistochemistry in 93 breast cancer samples, was associated with earlier onset of bone metastases development (52). Data from small clinical studies (56 patients) suggest that PTHrP levels, measured immunohistochemically, are higher in bone metastases compared to primary breast cancers (53, 54). The importance of RANKL in the development of skeletal related events has been proven with denosumab, a monoclonal antibody that binds human RANKL to inhibit bone destruction (55) (Figure 1A.3). A randomized double blind study in 2,046 MBC patients with at least one bone metastasis, showed superiority of denosumab compared to zoledronic acid in delaying time to first on-study skeletal-related event (56). Time to disease progression, overall survival and adverse events rates were similar between these groups. Denosumab is now part of standard clinical care to supplement the treatment of bone metastasis in MBC. Clinical trials are ongoing to study the anti-cancer effect of denosumab (Table 1).

**RANK/RANKL/OPG – prediction of treatment response**

Data on biomarkers for targeting RANKL are limited, and assessment is mostly based on clinical grounds: skeletal related events, recurrence and death. N-terminal telopeptide is a bone turnover marker which is released as a result of osteolysis (57). Denosumab treatment decreased urine N-terminal telopeptide levels in MBC patients with bone metastases (58). However, serum levels RANK/RANKL/OPG levels did not correlate with these endpoints in 30 MBC patients treated with bisphosphonates (59).

In conclusion, bone is clinically the most seductive environment for breast cancer. The formation of the metastatic niche by the microenvironment there, is affected by TGFβ and RANK/RANKL/OPG signaling. Standard treatment options in MBC that may at least in part exert their effect by influencing these factors are bisphosphonates and denosumab. TGFβ inhibitors are currently investigated in clinical trials.
Figure 1| Processes in breast cancer microenvironment that promote progression and metastasis: Factors related to 1) tumor cell or 2) microenvironment and 3) targeting options.
**METABOLIC STIMULATION**

The metabolic environment can profoundly affect breast cancer behavior. Microenvironmental factors contributing in the process of metabolic stimulation of breast cancer are obesity, inflammation and metabolic dysfunction. Soluble factors involved in this are E2, insulin and insulin-like growth factor (IGF)-1 (60) (Figure 1B).

**Obesity and inflammation – mechanism of action and preclinical data**

The mechanisms linking obesity and breast cancer development and outcome are multifactorial involving inflammation, hormonal imbalance and metabolic dysfunction. Obesity leads to inflammation of adipose tissue which is characterized by necrotic adipocytes surrounded by macrophages (61) and the level of breast inflammation is correlated with aromatase activity and BMI (62, 63) (Figure 1B.2 (circle microenvironment)). A preclinical study described a link between high fat diet and breast cancer growth (64). Administration of the cholesterol metabolite named 27-hydroxycholesterol, which mimics estrogen in certain tissues, resulted in faster tumor growth and more metastasis formation in MMTV-PyMT mice. On a high fat, high cholesterol diet these mice showed also more rapid tumor growth compared to mice on a normal diet.

**Obesity and inflammation – clinical data**

Obesity increases the risk of the occurrence of breast cancer. Multiple studies have found an increased risk of developing breast cancer for postmenopausal women with a high BMI. A study conducted amongst pooled data of seven prospective studies in which of 337,819 women were included, found an increased risk of breast cancer for postmenopausal women with obesity (65). The relative risk of developing breast cancer was 1.27 for women with a high BMI (≥ 33 kg/m²) compared to normal BMI. In a meta-analysis amongst 221 databases including 31,839 incident cases, an 5 kg/m² increase in BMI resulted in an relative risk of developing breast cancer of 1.12 (66). Similar results were obtained in a recent study amongst 5.24 million individuals and almost 35,000 breast cancer cases (67). Postmenopausal breast cancer risk was increased with a HR
of 1.05 per 5 kg/m² increase in BMI. In contrast, these three studies showed a reduced risk on breast cancer in premenopausal women with obesity. At this moment, no clear explanation exists to clarify this discrepancy. Adjusting for free E2 levels, the relative risk of postmenopausal breast cancer development was strongly reduced, suggesting that bioavailable estrogen plays an important role in the link between high BMI and breast cancer risk (68). For premenopausal women, data regarding E2 show contradictory results (69). Moreover, as obesity increases the risk on TNBC in premenopausal women (70), also factors other than E2 are likely to play a role. Clearly, numerous metabolic changes occur during the menopause, which could potentially explain this difference in breast cancer risk due to obesity in pre- and postmenopausal women. Future studies are needed to gain insight in the mechanisms explaining this phenomenon.

The risk of breast cancer related mortality is also increased in obese patients. In a prospective, population based study in almost 500,000 women, the relative risk of breast cancer death, was 2.1 in postmenopausal obese women (body mass index (BMI) (≥40 kg/m²) compared to normal weight women, independent of ER status, (71). Pre- and peri-menopausal women were excluded from this study. A meta-analysis amongst 80,000 breast cancer patients in adjuvant trials showed an increased breast cancer related mortality in obese (BMI ≥ 30 kg/m²) compared to normal weight (BMI 20-25 kg/m²) premenopausal patients with ER+ breast cancer (72). For yet unknown reasons, no clear effect of obesity was seen in postmenopausal women. This discrepancy might be caused by a tumor effect in which tumors of premenopausal women are more sensitive to obesity related effects. In other, smaller studies obesity was a risk factor for recurrence and development of metastases in the complete group of breast cancer patients regardless of menopausal and ER status (73, 74).

Together, these data implicate that risk of developing breast cancer is increased for postmenopausal women with obesity, whereas this risk is reduced in premenopausal women. However, the outcome of breast cancer seems to be worse for obese women regardless of menopausal status. However, although the described studies involved many patients, most of the studies were conducted retrospectively. Also, different cut-off values for BMI and different endpoints were used. This implicates that there is need for prospective cohort studies to clarify the effect of obesity on breast cancer risk and outcome.

Furthermore, dietary fat reduction seems to prolong disease free survival in women with resected breast cancers independently of ER presence (Figure 1B.3 (circle targeting)). In a group of 2,437 women with resected early stage breast cancer, patients were randomized between dietary intervention and control groups. In the dietary intervention group, 9.8% relapsed compared to 12.4% in the control group (P = 0.034) (75). In a prospective observational study, physical activity equivalent to 3-5 hours walking a week improved survival in 2,987 breast cancer patients (76). The exact mechanism behind this effect remains to be speculated about (77). Clinical trials are ongoing to study the anti-cancer effect of dietary fat reduction and physical exercise (Table 1). Chronic inflammation is related to the development of various cancer types (78). In two case-control studies with in total almost 2000 post-menopausal women, systemic levels of the aspecific inflammatory marker C-reactive protein or soluble tumor necrosis factor
receptor 2 were associated with overweight and increased breast cancer risk (79, 80). A smaller study in 97 overweight breast cancer survivors showed that weight loss resulted in a decrease of inflammatory and obesity markers as insulin, C-reactive protein, tumor necrosis factor (TNF)α, leptin, interleukin (IL)-6 (81).

There are no response prediction makers for obesity and inflammation known at this moment.

**E2 – mechanism of action and preclinical data**

Presumably the most powerful factor by which elevated body weight promotes breast cancer, is E2 (82). The conversion from testosterone by aromatase enzyme cytochrome p450 leads to the production of E2 (83). During the fertile phase E2 is primarily produced in the ovaries, while various cells including adipocytes in the breast, excrete E2 in postmenopausal women (84, 85). E2 binds to the nuclear ER present on breast cancer cells and CAFs (86-89) (Figure 1B.1 (circle tumor cell) and 1B.2), leading to cancer cell proliferation.

**E2 – clinical data**

Increased aromatase activity in fat tissue leads to elevated E2 levels in breast tumors compared to normal breast tissue (85). Interestingly, high BMI breast cancer patients is associated with higher aromatase activity (68) leading to high E2 levels and augmented breast cancer risk (82). Weight loss alone or in combination with exercise, on the other hand, reduced systemic E2 levels in overweight patients (90, 91). This phenomenon is proposed as a cause for the worse prognosis observed in women who experience weight gain after breast cancer treatment (92). The effect of physical exercise alone on E2 levels is inconsistent, although modest at most (90, 93). E2 signaling can be targeted using aromatase inhibitors or ER antagonists (such as fulvestrant or tamoxifen) (Figure 1B.3). Studies comparing treatment efficacy of estrogen targeting between obese and normal weight patients showed contradicting results (74, 94). As can be expected, ER positive tumor cells can indirectly be influenced by oophorectomy. Lowering circulating E2 by oophorectomy in unaffected BRCA1 mutation carriers also reduces the risk of breast cancer by 56% (95). This is very intriguing as the majority of BRCA1 associated breast cancers is ER negative (96). The discrepancy might be explained by E2 responsiveness of luminal progenitor cells of BRCA1 associated basal tumors (97). This may explain the reduced incidence of secondary breast cancers by tamoxifen in BRCA1 or BRCA2 carriers with breast cancer (OR= 0.50, 95% CI 0.28-0.89) (95). Another potential explanation is the fact that, in ER- human xenografts as well as syngeneic tumors, increased tumor growth is seen under treatment of E2 suggesting a stromal mediated effect (98-100).

**E2 – prediction of treatment response**

Data regarding therapy response prediction by using systemic E2 levels as biomarker are scarce. High E2 levels are associated with high patient BMI as result of incomplete aromatase inhibition (101). Studies evaluating patient outcome after aromatase inhibition suggest that a high BMI reduces therapy outcome in breast cancer patients (102-104). However, future studies with
higher power to discriminate between BMI categories are required to confirm this. Currently, no imaging techniques are available to visualize E2. In the future, aromatase imaging might be possible. An aromatase PET tracer is in preclinical development (105, 106), although not for the purpose of breast cancer research. ER expression can be determined by 18F-fluoroestradiol (18F-FES) PET. Studies indicated that low tumor FES uptake on baseline 18F-FES PET can predict failure of endocrine therapy, whereas its positive predictive value was relatively limited (107-110).

**Insulin – mechanism of action and preclinical data**

In addition to the endocrine importance of adipose tissue in the breast, obesity is related to metabolic dysfunction, which can also affect tumor progression (111, 112). In obesity, non-esterified fatty acids compete with glucose as a metabolic fuel, inducing insulin resistance leading to high glucose and insulin levels. Insulin is being produced by pancreatic β-cells and binds to the insulin receptor on the cell membrane of nearly all cell types (Figure 1B.1 and 1B.2). Insulin binding to the insulin receptor on breast cancer cells activates the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways and results in a cascade of proliferative and anti-apoptotic events. The PI3K pathway mediates the glucose regulatory effects of insulin but is inhibited in insulin resistance and, therefore, hyperinsulinemia, leading to increased signal transduction, is required to restore normal PI3K pathway activity. Since signaling via the MAPK pathway is preserved despite insulin resistance, high insulin levels in the microenvironment of breast cancer cells lead to hyperactivation of this pathway and enhanced cellular proliferation (113). In insulin resistance, insulin responsive tissues, such as skeletal muscle, become insulin resistant, stimulating insulin production. Epithelial cells including breast cancer cells probably remain relatively insulin sensitive and the consequent increased insulin-mediated signaling can lead to enhanced proliferation in cell line models (114). PyVmT mice, with inactive Insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor (IR) in skeletal muscles to induce insulin resistance, showed accelerated tumor growth compared to non insulin resistant mice. Tumors of these diabetic mice showed increased IR and IGF-1R phosphorylation while blockade of IR and IGF-1R with a small molecule inhibitor diminished the tumor growth inducing effects of insulin resistance (115). This tumor promoting effect of insulin seems to occur independently of IGF-1R, since treatment with an insulin analogue increased tumor growth without an increase in IGF-1R phosphorylation in two syngeneic mouse models (116).

Metformin belongs to the biguanide class of oral hypoglycemic agents. It reduces insulin resistance, and leads to lower insulin and glucose levels which may also reduce tumor cell growth (Figure 1B.3). Metformin indeed diminishes the growth of breast cancer cells in vitro (117). In obese rats in which mammary tumor growth was induced by 1-methyl-1-nitrosourea injection, metformin treatment reduced tumor burden (78). Insulin and hyperinsulinemia can also promote tumorigenesis indirectly by influencing the levels of other modulators, such as IGFs, sex hormones, inflammatory processes and adipokines (118). Insulin resistance and hyperinsulinemia suppress the production of sex hormone-binding globulin by the liver (119). This can lead to increased availability of free sex hormones favoring breast cancer development and progression (120).
**Insulin – clinical data**

Also in humans, insulin resistance as well as exogenous insulin injections have been associated with an increased risk of cancer and cancer recurrence (121). Metformin is prescribed to over 120 million type 2 diabetic patients worldwide. Retrospectively, patients with breast cancer who received neoadjuvant chemotherapy were studied. Of all patients, those with diabetes receiving metformin during their neoadjuvant treatment had a higher pathologic complete response rate compared to diabetic patients not receiving metformin (24% vs 8%; \( P = 0.007 \)) (122). Several trials are ongoing to further study the anti-cancer effect of metformin (Table 1). Next to a decrease in E2 levels (described above), a low caloric diet with or without physical exercise decreased insulin levels after 12 months, in overweight and obese primary breast cancer patients (90).

**IGF-1 – mechanism of action and preclinical data**

A related metabolic factor is IGF-1, which is produced by the liver as well as by CAFs (123) (Figure 1B.1 and 1B.2). IGF-1 activates, by binding to its receptor IGF-1R at the tumor cell membrane, the PI3K/AKT pathway. AKT is phosphorylated which leads to cell proliferation and inhibition of apoptosis of the tumor cell. Insulin resistance can result in high IGF-1 levels through various mechanisms (124). High IGF-1 levels in the microenvironment promote cancer cell growth. Transgenic overexpression of mammary IGF-1R in mice induced phosphorylation of IGF-1R and downstream proteins such as AKT (32). This was accompanied by increased tumor formation. By targeting IGF-1R on the tumor cells, the binding of IGF-1 to its receptor is blocked (Figure 1B.3). Inhibition of IGF-1 signaling with monoclonal antibody dalotuzumab reduced tumor growth in a MDA-MB-231 xenograft mouse model (125). An overwhelming amount of additional preclinical data is available regarding the role of IGF-1 in breast cancer (reviewed in (126)).

**IGF-1 – clinical data**

IGF-1R is overexpressed in numerous solid tumors including breast cancer (127), and is implicated (in both clinical and preclinical studies) in resistance to hormonal therapy and human HER2 targeting (127, 128). BRCA1 mutation carriers primarily develop TNBC (80%), and these tumors express elevated IGF-1R levels. Mutated BRCA1 fails to suppress IGF-1R, whereas tumors with wild-type BRCA1 are able to suppress IGF-1R (129). In effect, the large majority of TNBCs express cytoplasmic and membranous IGF-1R (127, 130), which is associated with a worse prognosis (127). Despite a strong rationale to intervene with IGF1-R, clinical trials in (breast) cancer with anti IGF-1R antibodies have until now failed to show significant clinical relevance (131). Clinical trials studying the effect of IGF-1R inhibition in breast cancer are ongoing (Table 1).

**IGF-1 – prediction of treatment response**

Studies investigating the role of IGF1-1R expression, measured on tumor samples or circulating tumor cells, as biomarker for response prediction are too small to be conclusive and are not conducted in breast cancer samples (132, 133). In BALB/c nude mice with an IGF-1R expressing human bone sarcoma xenograft, indium-111 (\(^{111}\)In)-labeled R1507 immuno-single-photon
emission computed tomography was performed before treatment with R1507. $^{111}$In-R1507 uptake correlated with tumor response (134).

In conclusion, metabolic stimulation of breast cancer is induced by obesity and inflammation, E2, insulin and IGF-1 in the breast cancer microenvironment. Intervention strategies, including weight and dietary fat reduction and metformin treatment, have proven to benefit breast cancer patients. No clinical benefit from IGF-1R inhibitors has been seen so far. Clinical trials studying inhibition of this factor are ongoing.

**IMMUNE RESPONSE MODULATION**

The immune system plays a major role in cancer development. Although the host immune system should act against tumor cells, various factors in the tumor microenvironment in fact act in favor of the cancer cells, by modulating the immune response. In breast cancer, key immunological players are T-cells, immune checkpoint receptors and tumor-associated macrophages (TAMs) (Figure 1C).

**T-cells** – mechanism of action and preclinical data

T-cells can recognize and destroy cancer cells. Several therapies have shown to modulate T-cell behavior by switching the balance from a tumor promoting to a tumor impeding environment. Myeloid-derived suppressor cells (MDSC) suppress T-cell activation in the microenvironment. IL-12 is excreted by antigen presenting cells and promotes antitumor immune response and blocks MDSC (135) (Figure 1C.2 (circle microenvironment)), thereby releasing the break from T-cell suppression. A subset of T cells, γδ T cells, show enhanced anti-tumor toxicity (136). Nitrogen containing bisphosphonates interfere with the mevalonate pathway, thereby stimulating the proliferation of Vγ9Vδ2 T-cells. Risedronate treatment of mice inoculated with T47D breast cancer and human PBMCs, reduced tumor growth compared to risedronate only treated controls (137). This was accompanied by a higher percentage of Vγ9Vδ2 T-cells and more Ki67 positive breast cancer cells.

Also standard therapies depend in their action on the immune system. Tumor cell death induced by cytotoxic therapies, such as anthracyclines, can activate of cytotoxic T-cells (138). And conventional drugs, such as trastuzumab, and newer drugs like IDO-inhibitors increase anti-tumor T-cell activity (139, 140).

**T-cells** – clinical data

Infiltration by memory T-cells seen in a large cohort of primary tumors, including breast cancer, was the strongest positive prognostic factor in favor of disease free survival and overall survival at all disease stages (141). In HER+ or TNBC breast cancer patients treated with neoadjuvant chemotherapy with or without trastuzumab, the presence of tumor infiltrating lymphocytes (TILs) and mRNA levels of immune related genes was associated with higher treatment
response (142, 143). The importance of targeting the immunological support of tumor cells by the microenvironment is increasingly supported by clinical data. In a phase I trial involving various HER2 positive metastatic cancers, including seven patients with MBC, patients received a combination of paclitaxel, trastuzumab and IL-12. Among the seven MBC patients, one experienced a complete response and two a partial response (144).

**T-cells – prediction of tumor response**

A study conducted in 1,282 breast cancer patients performed a genomic approach to identify trastuzumab sensitivity and found an immune enriched signature (including TNF receptor signaling, CD8+ T-cell receptor signaling and interferon gamma pathway signaling), occurring in 50% of the patients, to be predictive (145). The pre-treatment presence of serum MDSCs in breast cancer patients was predictive for worse outcome in a small study conducted in 106 breast cancer patients (146). In the clinical study described above, which studied paclitaxel, trastuzumab and IL-12 treatment, there was increased activation of extracellular signal-regulated kinases in peripheral blood mononuclear cells and increased levels of interferon γ and several chemokines in patients achieving a clinical benefit compared to patients with progressive disease (144).

**PD-1 – mechanism of action and preclinical data**

Programmed cell death (PD)-1 is present on T-cells and functions as an immune checkpoint receptor which plays a role in tumor progression (147) (Figure 1C.1 (circle tumor cell) and 1C.2). After binding to its ligand PD-L1, that is present on tumor cells, the T-cell is inactivated, enabling tumor cells to evade the host’s immune system (148). Blockade of the interaction between PD-1 and PD-L1 potentiates immune response in vitro and in vivo. PD-L1 expressing mammary tumor cells HBL-100 induced CD8+ T-cells apoptosis which could be diminished by adding a PD-L1 blocking antibody (147). In vivo, similar results were obtained in a syngeneic myeloma mouse model. Overexpression of PD-L1 resulted in enhanced tumor growth that was inhibited by a PD-L1 blocking antibody (149) (Figure 1C.3 (circle targeting). This mechanism has been found to be of importance in many other tumor types as well. Moreover, an anti-HER2 therapy enhancing effect of PD-1 blockade was studied a breast cancer mouse model. In immunocompetent MMTV-ErbB-2 transgenic mice PD-1 antibody treatment improved the therapeutic activity of anti-HER2 therapy (150).

**PD-1 – clinical data**

PD-L1 is electively expressed by many solid tumors and by isolated tumor cells within the microenvironment in response to inflammatory stimuli. Half of 44 human breast cancer specimens showed PD-L1 expression immunohistochemically. PD-L1 expression in these specimens correlated with a more aggressive tumor histology (151). The presence of PD-1 positive TILs, measured by immunohistochemistry in 660 breast cancer samples was correlated
with lower overall patient survival (152). Anti-PD-L1 antibody BMS-936559 was administered to 207 extensively pretreated patients, including four with breast cancer (Figure 1C.3). The overall objective response rate was 13% and 34% had prolonged disease stabilization (153). Several phase 1 trials with PD-1 and PD-L1 antibodies in solid cancer patients are ongoing (Table 1). High PD-L1 gene expression levels in patients treated with neoadjuvant trastuzumab, pertuzumab or both were associated with a lower complete response rate. These results provide a rationale for combining HER2-targeted treatments with immune-modulating agents and may allow the prediction of treatment benefit (154).

**PD-1 – prediction of treatment response**

PD-L1 expression, measured immunohistochemically, correlated to clinical activity of anti PD-1 as well as anti PD-L1 antibodies (155). However, patients without PD-L1 staining still showed a response rate of 13-17%, compared to 39%-44% of patients with PD-L1 expression (155, 156).

**CTLA-4 – mechanism of action and preclinical data**

Cytotoxic T lymphocyte-associated antigen (CTLA)-4 is also present on T-cells and binds to CD80/CD86 on antigen presenting cells, thereby transmitting an inhibitory to the T-cell (Figure 1C.1 and 1C.2). Blocking of CTLA-4 by an inhibitory antibody led to tumor regression in vivo (157). Also in preclinical breast cancer models CTLA-4 inhibition was studied. Treatment with a CTLA-4 antibody reduced tumor growth and pulmonary metastases in a syngeneic mouse model (Figure 1C.3). CD8+ T-cell influx, stimulated by radiotherapy, was necessary to obtain an anti-tumor effect of CTLA-4 blocking.

**CTLA-4 – clinical data**

CTLA-4 levels, measured by immunohistochemistry and on mRNA level in 90 samples, were higher in breast cancer tissue compared to normal breast tissue (158). Tremelimumab and ipilimumab block the activity of T-cell suppressor CTLA-4 (Figure 1C.3). In a phase I study in which 26 MBC patients received tremelimumab and exemestane, 11 experienced stable disease as best response (159). In another phase I study in early phase breast cancer patients neoadjuvant ipilimumab was administered after cryoablation to induce antigen presentation (160). Analysis of the characteristics of TILs in the mastectomy specimens showed a higher ratio of CD8+/Ki67+ compared to regulatory T cells in the combined treatment group compared to either alone. Ipilimumab and tremelimumab studies in MBC patients are ongoing (Table 1).

No data regarding prediction of treatment response is currently available.

**TAMs – mechanism of action and preclinical data**

Another group of immune cells are TAMs. Originally thought to be derived solely from bone marrow progenitors, TAMs are currently subject of debate as recent syngeneic lung cancer and glioma mouse experiments have found TAMs originating from spleen, circulating monocytes and yolk sack progenitors (161-163). TAMs can increase the survival and proliferative capacity
of cancer cells by secreting growth factors such as epidermal growth factor, TGFβ and ILs (164) (Figure 1C.1 and 1C.2). In mouse models, TAMs are recruited to the primary tumor or metastases by chemokine ligand 2, colony stimulating factor (CSF)-1 and granulocyte macrophage-CSF (123, 165, 166). Genetic or pharmacological deletion of CSF-1(R) in MMTV-PyMT mice resulted in reduced tumor infiltrated TAMs (167, 168). Further, transgenic expression of CSF-1 in the mammary epithelium of PyMT mice resulted in an increased number of TAMs and more pulmonary metastases (169). As TAMs are derived from the same cell lineage as osteoclasts, the effect of bisphosphonates on TAMs was studied. In mice transgenic for HER2, the number of TAMs was lower in the tumor microenvironment in parallel with the decrease in tumor vascularization after bisphosphonate administration (reviewed in (170)) (Figure 1C.3).

**TAMs – clinical data**

In breast cancer, the presence of TAMs is associated with a worse prognosis (171). No markers to predict response to TAM targeted therapy are currently known.

In conclusion, key players in the immunological microenvironment of breast cancer are T-cells, IL-12, immune checkpoint receptors and TAMs. Modulation of adequate T cell response to breast cancer is effected by CTLA-4 and PD-1. Targeting this process, by compounds such as IL-12, tremelimumab, ipilimumab and PD-1/PD-L1 inhibitors, is a promising strategy in breast cancer treatment.

**STIMULATION OF TUMOR CELL MIGRATION**

After tumor cells have invaded into their surroundings, the next step of tumor progression is migration to and through the circulation. The breast cancer microenvironment contains several factors that stimulate tumor cell migration, including hepatocyte growth factor (HGF) and stromal derived growth factor (SDF-1) (Figure 1D). Also involved in the tumor cell migration is the process of EMT, which is discussed in section 3.

**HGF – mechanism of action and preclinical data**

HGF is a soluble factor that is being secreted by CAFs and adipocytes and binds to the c-mesenchymal-epithelial transition factor (cMET) tyrosine kinase receptor on cancer cells (172) (Figure 1D.1 (circle tumor cell) and 1D.2 (circle microenvironment)). Binding of the cMET receptor triggers several downstream pathways in tumor cells, including MAPK and PI3K, inducing proliferation and migration (173). Transcription of both HGF and cMET is induced by several stromal cytokines such as IL-1, IL-6, TNF-α and TGF-β (174). HGF added to breast cancer cells induced migration and invasion in vitro (175, 176). In vivo, lung metastasis formation was enhanced
when tumor cells were incubated with HGF before inoculation (177). Transgenic mice, in which HGF expression was elevated by HGF cDNA in their mammary epithelium, developed invasive mammary tumors and pulmonary metastases (178). Moreover, HGF can play a role in sensitivity to certain drugs. HGF lowered sensitivity to the HER2 and epidermal growth factor receptor 1 TKI lapatinib in HER2 positive breast cancer cells (179). Inhibiting HGF excretion by fibroblasts studied with hammerhead ribozymes reduced invasiveness of breast cancer cells \textit{in vitro} (180, 181). Using the same technique, cMET inhibition reduced migration and invasion of breast cancer cells \textit{in vitro} in response to HGF (180). In addition, growth of human breast cancer xenografts co-injected with cells from a human fetal fibroblast cell line in mouse models was decreased when HGF excretion by these fibroblasts was inhibited (180, 181). Tivantinib, a selective cMET inhibitor, reduced bone metastasis formation in mice after injection of MDA-MB-231 tumor cells into the systemic circulation (182) ((Figure 1D.3 (circle targeting)).

**HGF – clinical data**

High cMET expression determined immunohistochemically in 930 and 330 primary breast tumors, respectively, was more frequently present in deceased or metastasized patients (183) and correlated with worse disease related survival (184). Also, high cMET expression, based on protein arrays from lysates of 257 fine needle aspirates of primary breast cancers, is associated with worse disease free and overall survival (185). Presurgical serum HGF levels were 1.5 fold higher in 124 mainly stage II and III breast cancer patients compared to 35 women with benign breast tumors (186). Preclinical results have prompted clinical trials with compounds targeting the HGF/cMET axis (Figure 1D.3). In a phase 1 trial with the TKI tivantinib, 14 out of 51 advanced solid tumor patients had stable disease for over 4 months (187). Two other TKIs, cabozantinib and foretinib (both against vascular endothelial growth factor receptor (VEGFR)2 and cMET), are currently being tested in breast cancer patients with ER positive and HER2 overexpressing tumors (Table 1). Phase 2 trials are ongoing in TNBC patients with tivantinib, cabozantinib, foretinib and the cMET monovalent antibody onartuzumab (Table 1).

**HGF – prediction of treatment response**

With regard to the prediction of response to anti-cMET therapy, in a human glioblastoma xenograft in mice, the level of autocrine HGF excretion was predictive for the response to anti-cMET therapy by a TKI (188).

For molecular imaging, both cMET and HGF can be visualized pre-clinically. Mouse agonistic human cMET antibody DN30 was radiolabeled with $^{89}$Zr. In nude mice bearing a human gastric- or head-and-neck cancer cell line xenograft, $^{89}$Zr-DN30 injection resulted in a maximum tumor to blood ratio of 5 (189). Anti-HGF nanobodies (1E2-Alb8 and 6E10-Alb8) were labeled with $^{89}$Zr and used as a PET tracer in nude mice bearing human glioblastoma xenografts. Tumor uptake of the tracer remained stable, while blood levels of the tracer gradually decreased over time, suggesting specific tumor uptake. The nanobodies inhibited tumor growth (190). With regard to biomarkers for evaluating the effect of cMET or HGF targeting, no clinical molecular imaging data are available.
SDF-1 – mechanism of action and preclinical data
SDF-1 (also known as CXCL12) is produced by CAFs and acts as chemo-attractant for tumor cells expressing chemokine C-X-C motif receptor 4 (CXCR4) (191, 192) (Figure 1D.1 and 1D.2). Organs expressing SDF-1, such as lung, bone marrow and liver, can thus lure CXCR4 expressing tumor cells to migrate towards them (193). Targeting SDF-1 or CXCR4 with antibodies or peptide inhibitors decreased breast cancer cell motility in vitro and reduced tumor growth and metastasis formation in vivo (Figure 1D.3) (191, 194). To compare tumor levels of CXCR4 with the physiological expression, several CXCR4 targeting imaging agents have been developed in the preclinical setting (reviewed in (195, 196)).

SDF-1 – clinical data
High SDF-1 expression determined immunohistochemically in breast cancer tissue of stage I-III patients, was prognostic for worse disease free- and overall survival in three retrospective studies involving a total of 628 patients (197-199). The correlation between CXCR4 expression in breast cancer tissue and patient outcome has been studied frequently, with contradictory results (200-202). In the light of preclinical data, clinical data with the CXCR4 antagonist plerixafor also of interest for breast cancer though no clinical data for breast cancer exists to date. A phase I/II clinical trial using plerixafor, administered in addition to chemotherapy showed the safety of this combination in 46 relapsed acute myeloid leukemia patients (203).

SDF-1 – prediction of treatment response
Small molecules, antibodies and peptides directed against CXCR4 have been radiolabeled and show CXCR4 expression level dependent tumor uptake in several xenograft mouse models, including breast tumors (204-208). Copper-64 (64Cu) labeled plerixafor is currently being investigated as clinical tracer in cancer patients (Table 1).

In conclusion, stimulation of tumor cell migration by the microenvironment involves HGF and SDF-1 signaling. Intervention strategies including cMET, HGF and CXCR4 inhibitory agents are moving into the clinical arena in an investigational setting.

ANGIOGENESIS

The tumor microenvironment instigates new vessel formation in response to pro-angiogenic factors secreted by cancer cells (209) (Figure 1E). These tumor vessels show leakiness and a chaotic structure. As a result, angiogenic stimuli are increased which leads to an even more defective vascular system. In the earliest breast cancer stages, angiogenesis is already implicated.
**VEGF-A – mechanism of action and preclinical data**

VEGF-A is secreted by cancer cells and TAMs and binds to the VEGFRs on endothelial cells (209, 210) (Figure 1E.1 (circle tumor cell) and 1E.2 (circle microenvironment)). VEGF-A expression in normal glandular epithelial structures of the human breast is lower than in (pre)malignant lesions. Expression increased with tumor dedifferentiation (mean number of VEGF-A positive cells 2.5% ± 0.4% in normal lobules versus 10.4% ± 6.6% malignant lesions, P < 0.001) (211). In vivo experiments using different non-breast cancer human xenografts in mice showed anti-tumor effect of a monoclonal murine anti-human VEGF-A antibody (212) (Figure 1E.3 (circle targeting)). Anti-angiogenic agent sunitinib is a TKI against platelet derived growth factor receptors and VEGFRs (Figure 1E.3). In mouse models with human xenografts, sunitinib treatment resulted in tumor regression or growth inhibition (213). A broad panel of other TKIs targeting VEGFRs, with different specificity, have shown anti-tumor activity in preclinical breast cancer models (214-220). However, by mimicking the clinical treatment scheme in mouse models, anti-angiogenic therapy efficacy was diminished. Administration of sunitinib or pazopanib to mice that had metastatic disease and undergone surgery for primary tumor resection, did not result in increased survival, while treatment of mice with only a primary tumor showed an anti-tumor effect (221). This difference in treatment efficacy could explain the disappointing results of anti-angiogenic therapy in clinical trials.

**VEGF-A – clinical data**

Bevacizumab, the most widely used anti-angiogenic drug, is an anti-VEGF-A humanized monoclonal antibody (222) (Figure 1E.3). A meta-analysis of 7 trials involving 4,032 MBC patients, studied the effect of combining bevacizumab with first line chemotherapeutic drugs and showed a progression free survival prolongation of 1.4 to 5.8 months (HR 0.67; 95% CI 0.61 to 0.73) while overall survival was not increased (223). In the RIBBON-2 trial bevacizumab was combined with standard chemotherapy compared to chemotherapy alone as second line treatment in patients with HER2 negative MBC (224). Progression free survival was prolonged from 5.1 to 7.2 months, but again overall survival was not affected. A subgroup analysis of the RIBBON-2 trial in TNBC patients showed a trend towards increased overall survival (225). Whether there is truly a benefit with bevacizumab for this subtype in the metastatic setting is currently being investigated (Table 1). Also, no additional value was found when bevacizumab was combined with either anti-HER2 or endocrine therapy in a subset of HER2 positive and ER positive MBC patients (226, 227). The negative BEATRICE, BETH and E5103 trials do not support a role for bevacizumab in the adjuvant treatment for TNBC, HER2 positive and HER2 negative patients (228-230). Multiple adjuvant phase III trials with bevacizumab in breast cancer patients are still ongoing (Table 1). In addition to lack of treatment efficacy, bevacizumab treatment is accompanied by toxicity such as hypertension, bleeding and proteinuria (223).

In MBC patients, sunitinib did not prolong progression free survival in phase III trials, and monotherapy sunitinib had an inferior progression free survival compared to capecitabine (231, 232). Clinical trials with sunitinib in breast cancer patients are ongoing. Also other TKIs targeting
VEGFR have been studied in clinical trials. Phase II trials in different settings with motesanib, pazopanib, axitinib, cediranib and vandetanib in breast cancer patients showed no clinical efficacy while toxicity was present in most of the studies (233-237). Two other phase I trials and one phase II trial with nintedanib, tivozanib and apatinib respectively showed partial response or pathological response up to 50% of the patients (238-240). Further studies with TKIs targeting VEGFR are ongoing (Table 1).

Based on the phase III clinical trials, so far none of these anti-angiogenic drugs seems to play a clinically relevant role in any of the studied breast cancer subgroups. There are several hypotheses postulated to explain this phenomenon. The biology of angiogenesis is still incompletely understood. The vascularization mechanisms might differ between tumor types (241). It could be that VEGF is no driver for angiogenesis in breast cancer progression (242). This is supported by the low uptake of $^{89}$Zr-bevacizumab in breast cancer lesions, as mentioned below. Even if VEGF is a driver of angiogenesis, other angiogenic factors could take over its function when VEGF is solely blocked (243), although this is not specific for breast cancer (244). The discrepancy between preclinical and clinical effects might be caused by the difference in treatment regimen between preclinical models and clinical setting as described above.

**VEGF-A – prediction of treatment response**

While the clinical relevance of targeting VEGF in breast cancer is limited, measuring angiogenic factors may be used for tumor identification of a susceptible subtype. Plasma VEGF-A levels showed no clear relationship with clinical effect of angiogenesis inhibitors (245-247). However, circulating levels of VEGF-A, may not reflect what is happening at the level of the tumor since VEGF-A binds locally to the ECM (248). By radiolabeling the anti-VEGF antibody bevacizumab with $^{89}$Zr, VEGF can be visualized with PET. Preclinically, there was tumor specific uptake in human breast and ovarian xenografts in mice (249, 250). In patients, $^{89}$Zr-bevacizumab uptake on PET was shown in 25 of 26 primary breast tumors (251). Interestingly, $^{89}$Zr-bevacizumab uptake in the primary breast tumors was relatively low, compared to tumor uptake in a series of 22 metastatic renal cell cancers (mean SUVmax 1.85 vs 10.1) (251, 252). This difference in VEGF-A tumor levels between renal cell and breast cancer lesions, may possibly be related to the difference in efficacy of targeting angiogenesis in these tumor types.

In conclusion, angiogenesis in the microenvironment to support breast cancer growth, is effected by VEGF-A and its receptors. Targeting strategies, including bevacizumab and sunitinib, have been studied in breast cancer; however, so far limited effects have been seen for this therapeutic strategy in breast cancer treatment.
EXTRACELLULAR MATRIX REMODELING

The ECM prevents tumor cells from invading the surrounding tissues, and remodeling of the ECM is therefore an obvious process by which the microenvironment might support tumor cells. Numerous factors are involved in this process, including integrins, lysyl oxidase (LOX) and MMPs (Figure 1F).

**Integrins and MMPs – mechanism of action and preclinical data**
Integrins at the tumor cell membrane are a family of heterodimeric, transmembrane glycoproteins consisting of one α and one β subunit. Each family member binds multiple ECM ligands which activates intracellular signaling pathways (253) (Figure 1F.1 (circle tumor cell) and 2F.2 (circle microenvironment)). MMPs are a family of (secreted or membrane bound) proteolytic enzymes which are expressed by a variety of cells including cancer cells and CAFs and have the ability to degrade ECM components (162, 254, 255) (Figure 1F.1 and 1F.2). MMTV-neu mouse tumors as well as adjacent stromal tissue showed increased tissue stiffness compared to normal tissue which was characterized by an increase in elastic modulus, collagen and collagen cross-links (256). Increasing ECM stiffness combined with oncogenic stimuli resulted in increased invasion of breast cancer cells in 3D in vitro models as well as in MCF10A human xenografts in mice. This increase in invasion was blocked by treatment with integrin blocking antibody AIIB2 integrin (Figure 1F.3 (circle targeting)). Additionally, overexpression of integrin β1 in the MMTV-neu and MCF10A human xenograft mouse model resulted in an increased number of invasive colonies.

**Integrins and MMPs – clinical data**
Mammographic density, correlated to high collagen content and suggested to be correlated with ECM stiffening (72), is related to worse breast cancer survival (257). Moreover, a prospective study amongst 439 breast cancer patients, the presence of fibrotic foci evaluated on paraffin embedded tumor tissue resulted in decreased disease free and overall survival (258).

Despite a strong rationale for targeting factors involved in ECM remodeling, clinical trials with anti-integrin and anti-MMP strategies have so far failed to show meaningful results (259-264) (Figure 1F.3). Several phase I studies with a MMP inhibitor and integrin inhibitors are ongoing in metastatic solid cancer patients (Table 1).

No data regarding prediction of treatment response is currently available.

**LOX – mechanism of action and preclinical data**
A potential alternative might be to target LOX. Members of the LOX family are secreted by cancer and stromal cells in response to hypoxia and modify the ECM (Figure 1F.1 and 1F.2). LOX crosslinks collagen IV in the basement membrane of the ECM, recruits CD11b+ cells to distant metastatic sites and induces expression of MMPs. Evidence suggests that the extracellular activity of these proteins in remodeling the ECM facilitates tumor cell invasion and metastasis. Preclinical models showed a decrease in metastases formation after inhibition of LOX and LOX
ligand (LOXL)2, without affecting tumor growth (136) (Figure 1F.3). Moreover, in the ECM stiffness models described above, LOX elevation resulted in increased collagen expression, a stiffer ECM and increased tumor growth and invasion (256).

**LOX – clinical data**
Both up-and down regulation of LOX family members have been associated with cancer progression. This paradoxical role of the LOX family is possibly due to their multiple temporal and spatial expression patterns, which may confer differential functions (265). The LOXL2 antibody AB0024 has been tested in phase 1 clinical trial, results are awaited (Figure 1F.3).

No data regarding prediction of treatment response is currently available.

In conclusion, extracellular matrix remodeling by the microenvironment is effected by integrins, LOX and MMPs. Targeting strategies including anti-integrin, anti-MMP and up-and down regulation of LOX have so far not been successful in supporting the relevance of this process in breast cancer.

**DISCUSSION AND FUTURE PERSPECTIVES**

The focus of this review was to describe the current knowledge of processes involved in the breast cancer microenvironment, and how these processes affect breast cancer progression and metastasis. We described the formation of the metastatic niche, metabolic stimulation, stimulation of tumor cell migration, immune modulation, angiogenesis and matrix remodeling. Increasing evidence is supporting the significance of targeting the breast cancer microenvironment. However, different levels of evidence for targeting the described processes are apparent. Targeting the process of formation of the metastatic niche and metabolic stimulation by the breast cancer microenvironment, is already showing clinical efficacy. Intervening with stimulation of tumor cell migration and immune modulation by the microenvironment, is an upcoming field of great interest and research. In contrast, targeting of microenvironmental angiogenesis or matrix remodeling appears to be of limited clinical relevance in breast cancer treatment so far.

For clarity reasons, in this review we have described the role of microenvironment in modulating breast cancer behavior based on six separate processes. However, we are aware that the complexity of the microenvironment exceeds beyond this compartmentalization. The different processes are related to each other and there are factors that have functions outside the process they are described in. Modulation of the ECM, for example, modulates migration of cancer cells and IGF-1 can also induce migration. However, due to the complex nature of the microenvironment, creating overview by arranging the data is essential for using these concepts in optimizing breast cancer therapy.

To optimize targeting the microenvironment for maximal anti-cancer effect, more detailed knowledge of the interaction between environment and cancer is needed. Preclinical models
that allow investigation of this interaction in a species specific manner are currently in development. Furthermore, as many of the described processes take place in parallel, combining agents directed at multiple microenvironmental factors, administered in combination with standard anti-cancer directed treatments such as chemotherapy, may ensure the best clinical result. Combining microenvironment targeting agents, like immunotherapy, with local damage inducing agents might even work synergistically (168). In addition, differences between breast cancer subtypes are becoming more and more apparent (266) and selecting the appropriate study population could maximize treatment results.

In view of the fact that microenvironmental factors usually do not consistently have a “good” or “bad” impact during cancer progression, it is vital to study the optimal timing of administering microenvironment targeting agents in future studies. With regard to predictive markers for treatment response, tissue and blood assessments may be suitable for this purpose. However, these are static measurements that may not suit the dynamics of targeting microenvironment-cancer interactions. Molecular imaging, although not generally available, can provide local real time information about the in vivo interaction of the tumor and its microenvironment. As PET imaging results in whole body images, it may also offer information regarding intra- and inter lesion heterogeneity (170, 267).

In summary, targeting the breast cancer microenvironment is an upcoming field of research. For some of the processes involved in the tumor-stroma interaction, clinical evidence for useful intervention is already present, supporting the concept of targeting these processes. Further optimization of this approach is warranted, with regard to combinations of agents, timing, improved knowledge of breast cancer subtype specific- aspects, and predictive markers, to improve this approach for comprehensive implementation in breast cancer care.

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**Conflicts of interest**
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