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“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”

— J.R.R. Tolkien, The Hobbit

ABSTRACT

Mari Suominen

Dual inhibition of the vicious cycle and tumor growth in breast and prostate cancer bone metastasis – evaluation of various therapeutic approaches in experimental mouse models

University of Turku, Faculty of Medicine, Department of Cell Biology and Anatomy, Drug Research Doctoral Program

Annales Universitatis Turkuensis, Medica-Odontologica, 2018

Bone metastases affect the vast majority of advanced breast and prostate cancer patients with metastatic disease. Since the bone microenvironment can help cancer cells to resist treatment, it is important to find treatment strategies that could disturb this intimate relationship.

We studied different treatment strategies in breast and prostate cancer mouse models to test the efficacy and clarify the mode-of-action of the tested agents. It was found that the anti-neoplastic agent sagopilone effectively inhibits bone resorption, and that alpha-radiation emitting radium-223 is able to affect both tumor growth and tumor-induced bone reaction. Radium-223 was shown to be actively incorporated into the newly formed bone by osteoblasts, providing rationale for the efficacy in osteoblastic bone metastases. However, in a striking discovery, we observed radium-223 to be efficacious in both types of bone metastases: in osteoblastic prostate cancer as well as in osteolytic breast cancer, reducing both tumor burden and the bone reaction. These effects combined contributed to increased survival, whereas zoledronic acid, inhibiting only bone resorption, and doxorubicin, affecting only tumor growth, did not increase the survival in our breast cancer bone metastasis model. The effects of a sequential combination of doxorubicin followed by zoledronic acid were investigated in an osteolytic breast cancer model, but synergistic effects were not observed.

Taken together, it was shown that effectively disrupting multiple steps of the vicious cycle can result in markedly improved treatment results. However, efficacy varies between study settings, models and drug combinations, suggesting the need of careful optimization for maximal therapeutic effects.

Keywords: breast cancer, prostate cancer, bone metastasis, vicious cycle, sagopilone, radium-223, combination treatment

TIIVISTELMÄ

Mari Suominen

Luumuutosten ja syövän kasvun noidankehän katkaiseminen rinta- ja eturauhassyövän luuetäpesäkkeissä – erilaisten hoidollisten lähestymistapojen arviointi kokeellisissa hiirimalleissa

Turun Yliopisto, Lääketieteellinen tiedekunta, Solubiologia ja anatomia,
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Levinnyttä rintasyöpää tai eturauhassyöpää sairastavista potilaista suurin osa kärsii luuetäpesäkkeistä. Luussa rinta- ja eturauhassyöpäsolut saavat aikaan luuta hajottavien ja sitä muodostavien solujen aktivoitumisen, mistä taas seuraa syöpäsolujen kasvua edistävien kasvutekijöiden vapautuminen ja etäpesäkkeitten kasvun kiihtyminen, noidankehän muodostuminen. Koska mikroympäristö luussa voi auttaa syöpäsoluja myös vastustamaan hoitoa, on tärkeää löytää hoitostrategioita, jotka voisivat hillitä luusolujen ja syöpäsolujen välisen noidankehän aiheuttamaa etäpesäkkeitten kasvua ja vastustuskykyä syöpälääkkeille.

Tässä tutkimuksessa testattiin erilaisia lääkkeitä rintasyövän ja eturauhassyövän hiirimalleissa niiden tehon ja vaikutusmekanismin selvittämiseksi. Solumyrkky sagopilonin huomattiin estävän tehokkaasti syövän kasvun lisäksi luun hajoamista. Myös alfa-säteilijä radium-223 vähensi sekä syövän kasvua että syövän aiheuttamia luumuutoksia. Tutkimuksessa osoitettiin, että osteoblastit kerryttivät aktiivisesti radium-223:a muodostamaansa luuhun. Tämä vaikutusmekanismi selittää radium-223:n tehokkuutta uutta luuta muodostavissa eturauhassyövän etäpesäkkeissä. Lisäksi tehtiin yllättävä havainto, että radium-223 on tehokas myös rintasyövän luuta hajottavissa etäpesäkkeissä vähentäen sekä syöpäsolujen määrää että luun hajoamista. Nämä vaikutukset yhdessä pidensivät hiirten elinaikaa. Tsoledronihappo, joka estää luun hajoamista tai doksorubisiini, joka vähentää syöpäsolujen kasvua, eivät pidentäneet elinaikaa tässä rintasyövän osteolyyttisten luuetäpesäkkeiden hiirimallissa. Doksorubisiinin ja tsoledronihapon peräkkäistä annostelua tutkittiin samassa hiirimallissa, mutta merkittävää yhteisvaikutusta ei havaittu.

Tutkimuksessa osoitettiin, että parempien hoitotuloksien saavuttaminen on mahdollista katkaisemalla luu- ja syöpäsolujen muodostama noidankehä samanaikaisesti useasta kohtaa. Kuitenkin erilaisten tutkimusasetelmien, mallien ja lääkeyhdistelmien käyttö vaikuttaa tutkittavan hoidon tehokkuuteen. Siksi luuetäpesäkkeiden optimaalisen hoidon kehittäminen edellyttää uusien lääketutkimusten huolellista suunnittelua.

Avainsanat: rintasyöpä, eturauhassyöpä, luuetäpesäke, noidankehä, sagopiloni, radium-223, yhdistelmähoito

CONTENTS

ABBREVIATIONS

ADC	Antibody-drug conjugate
ADT	Androgen deprivation therapy
AI	Aromatase inhibitor
ALP	Alkaline phosphatase
BALP	Bone alkaline phosphatase
BMD	Bone mineral density
BMP	Bone morphogenic protein
BMU	Basic multicellular unit, also bone multicellular unit
CAF	Cancer-associated fibroblast
CCR7	C-C chemokine receptor type 7
CDK	Cyclin-dependent kinase
CRPC	Castration-resistant prostate cancer
CSF-1	Colony stimulating factor 1
CTIBL	Cancer treatment-induced bone loss
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
CXCR4	Chemokine receptor 4
CXCL12	Chemokine ligand 12
DCIS	Ductal carcinoma in situ
DSB	Double-strand break
DTC	Disseminated tumor cell
ECM	Extracellular matrix
EMA	European Medicines Agency
EMT	Epithelial-to-mesenchymal transition
EPHA3	Ephrin type-A receptor 3
ER	Estrogen receptor
ERK	Extracellular signal–regulated kinase
FGF	Fibroblast growth factor
FSCN1	Fascin 1
GAS6	Growth arrest-specific 6
GnRH	Gonadotropin-releasing hormone
HER2	Human epidermal growth factor receptor 2,
HIF	Hypoxia-inducible factor
HPG	Hypothalamic-pituitary-gonadal
HSC	Hematopoietic stem cell
i.v.	Intravenous
LET	Linear energy transfer
mCRPC	Metastatic castration-resistant prostate cancer
MDSC	Myeloid-derived suppressor cell
MEK	Mitogen-activated protein kinase kinase (also known as MAP2K and MAPKK)

MGT	Masson-Goldner trichrome
miRNA	MicroRNA
MSC	Mesenchymal stem cell
mTOR	Mechanistic target of rapamycin (formerly mammalian target of rapamycin)
ONJ	Osteonecrosis of the jaw
OPG	Osteoprotegerin
OS	Overall survival
OVX	Ovariectomy
PAP	Prostatic acid phosphatase
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PET	Positron emission tomography
PFS	Progression-free survival
PINP	Procollagen type I N-terminal propeptide
p.o.	Per os
PR	Progesterone receptor
PSA	Prostate-specific antigen
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
PTN	Pleiotrophin
Ra-223	Radium-223
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
s.c.	Subcutaneous
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
SDF-1	Stromal cell-derived factor 1, also known as CXCL12
SRE	Skeletal-related event
TAM	Tumor-associated macrophage
TBK1	TANK binding kinase 1
TGF-β	Transforming growth factor β
TNBC	Triple-negative breast cancer
TNFα	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
TRACP 5b	Tartrate-resistant acid phosphatase isoform 5b
uNTx	Urinary N-telopeptide
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VLA-4	Very late antigen-4
Wnt	Wingless and Int1 proteins

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the roman numerals I-IV. The original publications have been reproduced with the permission of the copyright holders.

I Mari I. Suominen, Jukka P. Rissanen, Rami Käkönen, Katja M. Fagerlund, Esa Alhoniemi, Dominik Mumberg, Karl Ziegelbauer, Jussi M. Halleen, Sanna-Maria Käkönen, Arne Scholz. Survival benefit with radium-223 dichloride in a mouse model of breast cancer bone metastasis. Journal of the National Cancer Institute (2013) 105:908-916.

II Mari I. Suominen, Katja M. Fagerlund, Jukka P. Rissanen, Yvonne M. Konkol, Jukka P. Morko, ZhiQi Peng, Esa J. Alhoniemi, Salla K. Laine, Eva Corey, Dominik Mumberg, Karl Ziegelbauer, Sanna-Maria Käkönen, Jussi M. Halleen, Robert L. Vessella, Arne Scholz. Radium-223 inhibits osseous prostate cancer growth by dual targeting of cancer cells and bone microenvironment in mouse models. Clinical Cancer Research (2017) 23:4335-4346.

III Mari I. Suominen, Rami Käkönen, Jukka P. Rissanen, Jussi M. Halleen, Pirkko Härkönen, Sanna-Maria Käkönen. Sequential treatment with doxorubicin and zoledronic acid has no additive effects in a mouse model of established bone metastases. Submitted.

IV Anne Strube*, Mari I. Suominen*, Jukka P. Rissanen, Dominik Mumberg, Ulrich Klar, Jussi M. Halleen, Sanna-Maria Käkönen. The anti-tumor agent sagopilone shows antiresorptive effects both in vitro and in vivo. Osteoporosis International (2011) 22:2887-2893.

**Equal contribution*

1 INTRODUCTION

Despite recent advances in cancer therapy, bone metastases remain incurable and very common in a variety of cancers, especially breast and prostate cancer. Bone metastases may also serve as a source of secondary metastases. The importance of the microenvironment is a re-emerging topic, beginning with the well-known "seed and soil" theory of S. Paget published in 1889 in Lancet (Paget, 1989). The first detailed parts of interaction of cancer cells and osteoclasts were described over a hundred years later, when cancer cells in bone were found to induce resorption, and increased resorption was found to aid cancer cell growth through the release of growth factors (Guise et al., 1996). In addition, it is now known that increased bone resorption, caused for example by hormone deprivation therapies of breast and prostate cancer, can fertilize the soil before the arrival of cancer cells and increase the number of bone metastases (Ottewell et al., 2015, Wang et al., 2015b). Furthermore, the soil has been shown to be able to confer both growth advantage and drug resistance (Roodhart et al., 2011) to the residing tumor cells. Drug resistance is a major problem in advanced cancer. While many resistance mechanisms are based on properties of cancer cells themselves and the clonal evolution of resistant clones, increasing evidence of the contribution of a protective microenvironment, like bone marrow, is emerging (Hemann, 2016). Bone marrow also harbors the hematopoietic stem-cell (HSC) niches that offer a safe haven for dormant tumor cells (Shiozawa et al., 2011). Especially in estrogen receptor positive (ER+) breast cancer, relapses can occur even 10 years after the diagnosis, and these relapses most often occur in bone. Events awakening the dormant cells seem to include activation of a bone remodeling unit and sprouting of microvessels, which is inherently linked to bone remodeling (Ghajar et al., 2013, Quayle et al., 2015). Thus, it is crucial to realize the importance of evaluating the bone effects of new drug candidates, and that many cancer treatments affect the bone marrow microenvironment in a way that may promote tumor growth. Furthermore, affecting both tumor growth and the bone microenvironment would provide the best strategy for eliminating the tumor cells or preventing the outgrowth of dormant cells.

2 REVIEW OF THE LITERATURE

2.1 Breast and prostate cancer

2.1.1 Incidence of breast and prostate cancer

Breast cancer is the most common cancer in women and prostate cancer is the second most common cancer after lung cancer in men. According to the GLOBOCAN report, an estimated 1.67 million new breast cancer cases, and 1.1 million new prostate cancer cases were diagnosed in the world in 2012. Together, their incidence accounts for 19.7% of all cancers, and because the 5-year survival rates are much shorter in many other cancers, including lung cancer, the 5-year prevalence rises much higher (Figure 1). (Ferlay et al., 2012, Bray et al., 2013).

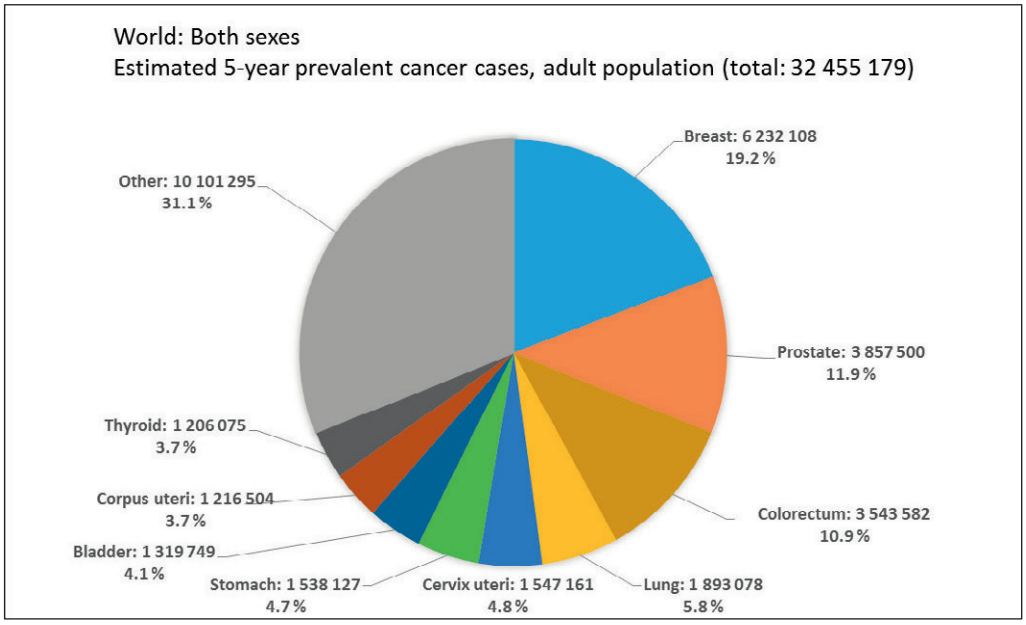

Figure 1. The 5-year prevalence on different cancers in the world. Breast and prostate cancer together account for 31% of all cancers. Adapted from GLOBOCAN 2012 database, accessed 23.2.2018, http://globocan.iarc.fr/old/pie_pop_prev.asp?selection=224900&title=World&sex=0&type=4&window=1&join=1&submit=%C2%A0Execute (Ferlay et al., 2012)

Survival rates in the Western world are high mostly due to early detection by mammography and measurements of prostate-specific antigen (PSA). Furthermore, the treatments are very effective, and the prognosis for 5-year survival is 90.6% in breast cancer and 93.5% in prostate cancer in Finland, according to statistics from 2013 to 2015 (Suomen_Syöpärekisteri, Accessed 25.2.2018).

2.1.2 Breast cancer subtypes and treatment

Treatment of breast cancer depends on the subtype of the tumor (Table 1). Breast cancer is divided into four subtypes characterized by the expression of hormone receptors as follows: 1) Luminal A is estrogen receptor (ER) and/or progesterone receptor (PR) positive (ER+/PR+), human epidermal growth factor receptor 2 negative (HER2-), 2) Luminal B is ER+/PR+, HER2+, 3) HER2 type is ER-, PR-, HER2+, 4) Triple negative is ER-, PR-, HER2-. After surgery and possible radiation treatment, the backbone of luminal A subtype treatment is an antihormonal approach, like antiestrogens, aromatase inhibitors (AIs) and gonadotropin-releasing hormone (GnRH) agonists. In addition, four targeted therapies are available: mechanistic target of rapamycin (mTOR) inhibitor everolimus (Lee et al., 2015a) and three inhibitors of cyclin-dependent kinases (CDK) 4 and 6: palbociclib, ademaciclib and ribociclib (Walker et al., 2016, Drugs.com, 2017, Syed, 2017). Ribociclib is accepted only in combination with an AI. Luminal B subtype has the same treatment options as luminal A, and in addition, it can be treated with anti-HER2 antibodies trastuzumab or lapatinib. Furthermore, trastuzumab linked to cytotoxic emtansine is currently available, and a tyrosine kinase inhibitor neratinib was recently approved for early stage HER2+ disease (Park et al., 2016). For the hormone-receptor positive cancers, a considerable number of different combination treatments are possible (Brufsky, 2017). For HER2 type, the anti-HER2 antibodies are used. For triple-negative breast cancer (TNBC), no targeted treatments have been approved yet, leaving conventional chemotherapy as the only option (cytotoxic agents in Table 1). There is a wide variety of chemotherapeutic agents available that are often used in different combinations. Furthermore, for TNBC (and luminal A) metastatic breast cancer, the anti-angiogenic drug bevacizumab can be used in combination with the chemotherapeutic agent paclitaxel.

Table 1. Approved drugs for breast cancer treatment. Source: https://www.cancer.gov/about-cancer/treatment/drugs/breast#2 accessed 23.2.2018. Bevacizumab is approved by European Medicines Agency (EMA).

Mode-of-action	**Drug**	**Subtype**
Antiestrogen (SERM)	tamoxifen, toremifene	Luminal A & B
Antiestrogen (SERD[1])	fulvestrant	Luminal A & B
GnRH agonist	goserelin acetate	Luminal A & B
PR agonist: HPG[2] axis downregulator	megestrol acetate	Luminal A & B
Aromatase inhibitor	anastrozole, exemestane, letrozole	Luminal A & B
HER2 inhibitor	trastuzumab, lapatinib ditosylate	Luminal B, HER2+
HER2-ADC[3] (mAb+ cytotoxic drug)	trastuzumab emtansine, pertuzumab	Luminal B
mTORC1 inhibitor	everolimus	Luminal A
CDK 4/6 inhibitor	palbociclib, ribociclib, abemaciclib	Luminal A
Tyrosine kinase inhibitor	neratinib	Luminal B, early stage
VEGF inhibitor	bevacizumab (approved by EMA only)	HER2 negative
Cytotoxic	capecitabine, cyclophosphamide, docetaxel, doxorubicin hydrochloridde, epirubicin hydrochloride, eribulin mesylate, 5-FU, gemcitabine, ixabepilone, methotrexate, paclitaxel, nab-paclitaxel, thiotepa, vinblastine sulfate	All

[1] selective estrogen receptor degrader
[2] hypothalamic-pituitary-gonadal
[3]antibody-drug conjugate

2.1.3 Treatment of prostate cancer

The first treatment for prostate cancer is radical prostatectomy or radiotherapy. Androgen deprivation therapy (ADT) is started either when biochemical recurrence (serum PSA) or metastasis is observed. The time from biochemical recurrence to metastasis reported in a retrospective review was 8 years in patients not receiving ADT, and only 34% of patients had metastasis in the timeframe of the study (Pound et al., 1999). In

metastatic patients, the duration of response to ADT is typically 13-20 months (Sharifi et al., 2005). In metastatic patients, prostate cancer almost always progresses despite the ADT and is then referred to as "castration-resistant". However, the disease is usually still dependent on AR signaling. Resistance can develop through intratumoral androgen synthesis, mutation of the AR or increase of certain splice variants of AR. Because of the dependency, further suppression of androgen levels by inhibiting androgen production in the tumor and in the host can be used. This is accomplished with the CYP17A1 inhibitor abiraterone acetate. The view of continuing hormonal dependence has really changed the way patients are treated, according to a recent study that reported a major shift from conventional chemotherapy (docetaxel) as first-line therapy in metastatic castration-resistant prostate cancer (mCRPC) in 2010 to hormonal therapy (abiraterone or enzalutamide) in 2013 (Flaig et al., 2016). Other current treatment options are radium-223 dichloride, studied in this thesis, immunotherapy and chemotherapy. Unlike in breast cancer, for prostate cancer, the list of approved drugs is very short. Only thirteen drugs have been approved so far, and six of them during the last ten years (Table 2).

Table 2. FDA-approved drugs for prostate cancer. (Source: https://www.cancer.gov/about-cancer/treatment/drugs/prostate, accessed 23.2.2018)

Mode-of-action	**Drug**
Antiandrogen	enzalutamide, bicalutamide, flutamide, nilutamide
GnRH agonist	leuprolide acetate, goserelin acetate
GnRH antagonist	degarelix
CYP17A1 inhibitor	abiraterone acetate
Immunotherapy	sipuleucel-T
Radionuclide therapy	radium-223 dichloride
Cytotoxic	cabazitaxel, docetaxel, mitoxantrone hydrochloride

2.1.4 Cancer treatment-induced bone loss

Cancer treatments have many adverse effects. The well-known side-effects of chemotherapy include leukopenia, nausea and vomiting, hair loss, diarrhea or constipation and fatigue. Depending on the type of chemotherapy used, it may also damage for example the heart, kidneys or the liver. Hormonal treatments in women cause menopausal symptoms, and in men hot flashes, loss of sexual desire, erectile dysfunction and increased

risk of diabetes and heart attack. All targeted treatments have their specific sets of side-effects. However, the most relevant side effect of cancer treatments regarding this thesis is cancer treatment-induced bone loss (CTIBL). CTIBL increases the risk of fractures, but the effects of changes in bone microenvironment on tumor dormancy and growth in bone and, thus, the success of treatment, have so far been largely unappreciated. The effects of different drugs and their combinations on bone microenvironment, unless they are bone-targeted, is practically uncharted territory. Furthermore, there is preclinical evidence that bone loss may actually increase the risk of bone metastases (Ottewell et al., 2014b, Wang et al., 2015b). New research emphasizes that we truly need to appreciate and understand the bone microenvironment, as the initiating step in the whole metastatic cascade is the expansion of hematopoietic stem and progenitor cells in the bone marrow, followed by their mobilization and formation of premetastatic niches (Giles et al., 2016). The treatments and CTIBL are discussed in more detail in chapter 2.4 "Bone effects of current breast and prostate cancer treatments".

2.1.5 Recurrence

Despite the very good 5-year prognoses, approximately 30% of breast cancers and 25% of prostate cancers are estimated to eventually metastasize (Cooperberg et al., 2004, O'Shaughnessy, 2005). A surprisingly large portion, 30%, of patients with newly diagnosed early stage breast cancer, namely the localized stage of ductal carcinoma in situ (DCIS), have disseminated tumor cells (DTCs) in their bone marrow (Sänger et al., 2011). Usually, metastasis occurs within 5 years of the diagnosis, but ER+ breast cancer has a peculiar tendency for late relapse, even decade(s) after diagnosis. DTCs and factors affecting their quiescence and outgrowth are discussed in chapter 2.1.6 "Dormancy". With current treatment options, patients live with their metastatic disease for 2 years on average. However, some patients live considerably longer, 10 years and more. For metastatic prostate cancer, the median overall survival (OS) is around 20 months (Fizazi et al., 2015). In a prospective study of metastatic breast cancer, the median OS in patients recurring between 1999 and 2001 was 22 months (Chia et al., 2007).

2.1.6 Dormancy

DTCs are found in the bone marrow of breast and prostate cancer patients at a very early phase: in 72% of prostate cancer patients prior to prostatectomy and in 57% after prostatectomy in patients with no evidence of disease (Morgan et al., 2009). About 30-40% of breast cancer patients, including patients with DCIS, have DTCs at diagnosis, and of those, approximately half will relapse (Braun et al., 2005, Sänger et al., 2011). DTCs are an independent factor of poor prognosis (Morgan et al., 2009, Braun et al., 2005). Thus, understanding what makes tumor cells dormant, what wakes them up and induces growth into metastases and how cancer treatments affect this process are vital.

The cancer stem cell theory states that some tumor cells hold stem cell-like properties, such as low proliferative activity, resistance to apoptosis, unlimited ability for self-renewal and differentiation (Bao et al., 2013). DTCs are known to harbor stem cell-like properties and to occupy stem cell niches supporting quiescence and survival. There is evidence of two stem cell niches in the bone microenvironment: the endosteal, i.e. osteoblastic, niche and the perivascular niche. Both hematopoietic and mesenchymal stem cells have been identified in both of these niches, and they most likely share the same progenitor. In fact, given the interdependence and close co-occurrence of angiogenesis and osteogenesis (Kusumbe et al., 2014), the endosteal and perivascular niches may even be different parts of the same system. This idea was further supported by two studies (Nombela-Arrieta et al., 2013, Wang et al., 2013). Hypoxia has been linked to maintaining quiescence in HSCs, but in a very convincing set of studies Nombela-Arrieta et al. found that the hypoxic state of HSCs is cell-specific and maintained despite the oxygen tension (Nombela-Arrieta et al., 2013).

Cancer cells were assumed to occupy the same niches as HSCs due to the existing knowledge of prostate cancer cells using the same homing to bone mechanisms as hematopoietic cells (Taichman et al., 2002). Shiozawa et al. were the first to report that prostate cancer cells compete with HSCs for the stem cell niche, and that they can dislodge the HSCs from their niche. They also showed binding of prostate cancer cells to osteoblasts through annexin II receptor, supporting the idea of an endosteal niche, and that this binding confers dormancy and resistance to chemotherapeutics to prostate cancer cells (Shiozawa et al., 2010), (Shiozawa et al., 2011). Inhibition of the mTOR pathway through TANK binding kinase 1 (TBK1) is another factor reported

to induce dormancy and drug resistance to prostate cancer cells in the HSC niche (Kim et al., 2013).

Ottewell et al. have shown that increased bone remodeling, and thus an increased number of BMUs, increases the number of bone metastases in both breast and prostate cancer mouse models. This increase was not due to an increased number of cancer cells homing to bone, but increased activation of the DTCs present (Ottewell et al., 2014b, Ottewell et al., 2014a). Very intriguing is the fact that the longest tumor recurrence times, of a decade and more, coincide with the cycle of renewal of all bone in the skeleton, taking 10-20 years (Hensel and Thalmann, 2016).

One of the early events in a new bone remodeling cycle is the formation of vessels in the remodeling site. Angiogenesis is necessary for normal bone remodeling as well as for growth and fracture repair, and Ghajar et al. showed that stable endothelium supports tumor dormancy, whereas activated, sprouting endothelium induces tumor growth. Binding to thrombospondin 1 on the stable endothelium was found to be crucial for dormancy, whereas transforming growth factor β1 (TGF-β1) and periostin expression were inducing tumor growth near the sprouting vessels (Ghajar et al., 2013, Ghajar, 2016). Accelerated bone turnover by parathyroid hormone (PTH) is also known to induce expansion of the HSC niche (Calvi, 2006). Thus, suppressing the level of bone turnover should help in keeping the DTCs in a dormant state, resulting in the prevention of bone metastases.

2.2 Bone metastasis

Almost all solid tumors can metastasize to bone, but bone metastases are most common, in the following order, in cancers of prostate, breast, thyroid, bladder, lung, kidney and melanoma (Macedo et al., 2017). Bone metastases co-localize with red bone marrow and cancellous bone, i.e. the ends of long bones, pelvis, ribs, the sternum, vertebrae and the skull. Early detection of bone metastases would be valuable for optimal treatment, but unfortunately bone metastases are typically found when they are already symptomatic. The most common and very sensitive method for detection of bone metastases is bone scintigraphy, showing sites of active bone metabolism. However, active bone metabolism may also be caused by other reasons, such as a fracture or inflammation, lowering the specificity of this technique somewhat (Łukaszewski et al., 2017). Positron emission tomography (PET)

with fludeoxyglucose F 18 (^{18}F-FDG) combined with computed tomography detects cells with high glucose uptake, and is as sensitive as whole-body magnetic resonance imaging in detecting bone metastases (Łukaszewski et al., 2017). Bone metastases can also be detected by classic X-ray imaging, although this method is much less sensitive. In X-ray images, the lesions are classified as osteolytic or osteosclerotic (also called osteoblastic). In osteolytic metastases bone is destroyed and in osteosclerotic metastases new, pathological bone is formed. Both types are observed in breast and prostate cancer, but breast cancer typically forms mostly osteolytic lesions and prostate cancer osteosclerotic. Differentiating between a new pathological sclerotic lesion and the healing of an osteolytic lesion is difficult.

2.2.1 Clinical significance in breast and prostate cancer

Bone is the most common site of metastasis in breast and prostate cancer. Breast cancer subtypes have different propensities of metastasizing to bone: in patients with metastatic disease, 55% of TNBC, 70% of HER overexpressing and 85% of ER+ tumors developed bone metastases (Savci-Heijink et al., 2015). In prostate cancer, 80% of patients with metastases have bone involvement (Tombal and Lecouvet, 2012). Patients with bone metastases have severe pain, pathologic fractures and nerve compression syndromes, even though bone-only metastasis carries a better prognosis than visceral metastasis in breast cancer (Lee et al., 2011). Pathologic fractures, nerve compression syndromes, bone pain requiring palliative radiotherapy and orthopedic surgeries to bone are called skeletal-related events (SREs)(Clemons et al., 2012), and SREs are linked to poor prognosis (Jensen et al., 2011). Also, less obvious parameters like bone formation marker serum alkaline phosphatase (ALP) or bone specific ALP (BALP) and bone resorption marker urinary N-telopeptide (uNTx), reflecting the extent of bone involvement, have been shown to independently predict OS (Fizazi et al., 2015). Recently, it was found that prostate cancer cells express tissue nonspecific alkaline phosphatase which induces epithelial-to-mesenchymal transition (EMT), regulates tumor growth and is associated to poor disease-free survival (Rao et al., 2017). In addition to the morbidity and mortality caused by bone metastases, tumor cells residing in bone can be the source of secondary metastases. In clinical trials, bone metastases have presented a challenge, because the treatment response cannot be assessed with the same, standardized RECIST criteria as visceral tumors (Scher et al., 2008). Furthermore, interpreting the technetium scans is a challenge, because

increased activity may infer to disease progression or bone healing. This has led to ambiguous results in clinical trials and most likely also hindered research focusing on bone metastases and even research on metastatic prostate cancer overall, because a limited number of patients have nodal or visceral metastases (Morris et al., 2015). Hence, modified criteria for assessing clinical activity in bone metastases have been in use from 2008 (Scher et al., 2008).

2.2.2 Bone metastatic process

The bone microenvironment is involved in the formation of all metastases, not just bone. It becomes involved before the tumor cells even leave the primary tumor, because facilitators of metastasis: monocytes (macrophages), mesenchymal stem cells (MSCs) and immature myeloid cells are expanded and mobilized from the bone marrow and guided to the primary tumor by cytokines secreted by the tumor (Ren et al., 2015, Giles et al., 2016). Through education by tumor cells, these cells become tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs) and myeloid-derived suppressor cells (MDSCs), which assist in tumor growth and metastatic process by inducing immune-evasion, angiogenesis, EMT and invasion (Giannoni et al., 2011, Barcellos-de-Souza et al., 2016). After entering the vasculature, the cells need to survive the shear stresses in the circulation and be able to attach to vessel walls and exit. Cell clusters are more likely to survive in circulation than single cells, because they are protected from anoikis. Even though cell clusters are rare when compared to single circulating cells, cell aggregates are much more likely to result in metastasis (Aceto et al., 2014). Cell aggregates can also consist of platelets and CAFs that can promote the formation of metastasis (Menter et al., 2014, Ao et al., 2015).

In addition to its obvious role as a part of the musculoskeletal system and a deposit for calcium and other minerals, bone is a hematopoietic organ. Hematopoiesis requires stem cell niches, a vascular network that is designed for cells entering and exiting the blood stream (the sinusoidal system) and the presence of several growth factors. Cancer cells can utilize all of these factors. The homing and colonization steps are discussed in more detail in chapter 2.2.4 "Characteristics of tumor cells metastasizing to bone".

Like in the primary tumor, tumor cells in bone induce changes in the surrounding cells, driving the formation of stroma. In bone, the affected cells

include osteoclasts and osteoblasts and their precursors. Depending on the activated cell types, tumor growth in bone results in either osteolytic (bone resorbing), osteoblastic (bone forming) or a mixed bone lesion (Figure 1). Breast cancer mainly forms osteolytic lesions in ~75% of the cases, while a majority of prostate cancer bone metastases are osteoblastic (Mundy, 2002). The signaling pathways leading to osteolytic or osteoblastic lesions are discussed in more detail in chapter 2.2.5 "The vicious cycle". The classification to osteolytic and osteoblastic lesions is based on the appearance of X-ray images (Figure 1). A majority of patients have both lesion types, and at the cellular level, bone resorption and formation are both ongoing (Guise et al., 2006). Activation of osteoclasts also in osteoblastic metastases has very practical consequences; antiresorptive treatments, i.e. treatments inhibiting osteoclast activity like bisphosphonates and receptor activator of nuclear factor κB ligand (RANKL) inhibitor, are effective in also osteoblastic metastases (Mundy, 2002, Fizazi et al., 2011). These treatments are discussed further in chapter 2.4 "Therapies targeting bone".

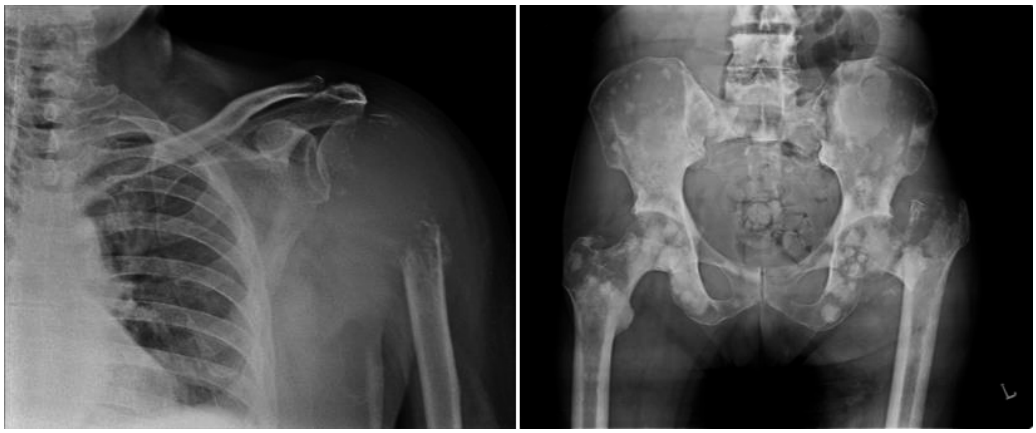

Figure 2. A) Osteolytic lesion in the humerus. Case courtesy of Dr Maulik S Patel, Radiopaedia.org, rID: 19359. B) Osteoblastic (osteosclerotic) abnormal bone growth in the pelvis. Case courtesy of Dr Nafisa Shakir Batta, Radiopaedia.org, rID: 38894. Both images: Creative Commons license CC BY-SA 3.0.

2.2.3 Microenvironment of bone metastases

Several components in the microenvironment of bone metastases contribute to the homing and colonization of tumor cells as well as to the

drug sensitivity and dormancy. The cellular compartment includes a high number of different mature and immature cell types, including immune cells. The extracellular matrix in the bone microenvironment has many unique properties not encountered elsewhere in the body. Finally, marked differences in oxygen tension between different compartments of bone create special circumstances in this microenvironment.

The multicultural cellular compartment

The cellular microenvironment in bone consists of bone cells (osteocytes, osteoblasts, bone lining cells, osteoclasts and their precursors), mesenchymal and myeloid stem cells, endothelial cells, nerve fibers, immune and blood cells and their precursors, macrophages, fibroblasts and adipocytes. Osteoblasts are cells of mesenchymal origin, which lay down the type I collagen and mineralize the new matrix. They secrete several proteins, e.g. osteocalcin and osteopontin, assisting in the mineralization process. Some osteoblasts differentiate further to osteocytes or quiescent bone lining cells. Osteoclasts are cells of hematopoietic origin that form through multiple cell fusions of their mononuclear precursors. These multinucleated cells attach to the bone surface and form a resorption lacuna, where low pH and proteases digest the inorganic and organic matrices.

Normal bone remodeling is dependent on angiogenesis (Parfitt, 2000), and normal hematopoiesis is in turn dependent on osteoblasts (Taichman, 2005). This intimate relationship is depicted in basic physiology, as red bone marrow in adults resides in midst of cancellous bone. Bone metastases occur almost exclusively in these areas of red bone marrow and high bone turnover. Bone remodeling occurs in the basic multicellular units (BMUs), where the bone lining cells retract and form a canopy over the BMU. Osteoblast progenitors are formed from MSCs or bone lining cells (Matic et al., 2016). Osteoblast progenitors then induce maturation and activation of osteoclast progenitors, which have arrived through a newly formed vessel, through the expression of RANKL in osteoblasts and its receptor receptor activator of nuclear factor κB (RANK) in osteoclasts and their progenitors. Osteoclasts attach to the bone surface and start resorbing. After a reversal phase, the osteoblast progenitors mature and fill up the resorption pit with new bone. Some of the osteoblasts get buried in the osteoid and differentiate to osteocytes, some differentiate to bone lining cells, and others die by apoptosis. The bone remodeling process is reviewed by Sims and Martin (2014). A basic principle in bone remodeling is the coupling effect, which

states that bone resorption induces the proper amount of osteoblasts to fill in the resorption pits (Parfitt, 1982). The ratio of RANKL and the secreted decoy receptor of RANK, osteoprotegerin (OPG) is a mechanism by which osteoblasts in turn regulate resorption. An imbalance leads to either osteoporosis, which is very common with ageing, or a much rarer problem, osteopetrosis. A schematic view of the remodeling cycle is depicted in Figure 3. Approximately 10% of the skeleton is renewed every year (Sims and Gooi, 2008). Breast and prostate cancer cells in bone have long been known to interact with osteoclasts and osteoblasts in a way that promotes tumor growth. These interactions are presented in chapter 2.3.3 "The vicious cycle". The contribution of bone marrow adipocytes on tumor growth and survival is unveiling as adipokines, fatty acid-binding proteins and other adipocyte-derived factors are found to play important roles (Morris and Edwards, 2016). Recent data also suggests the involvement of megakaryocytes; the number of platelets and megakaryocytes producing them is increased with the arrival of tumor cells (Jackson et al., 2017). However, this may be an effort to inhibit tumor growth because cancer progressed faster in animals with fewer megakaryocytes and platelets (Jackson et al., 2017). In contrast, reports of platelets promoting metastasis have also been published (Yu et al., 2014). The role of osteoblasts and endothelial cells in forming HSC niches is important in regulating the dormancy of cancer cells (Esposito and Kang, 2014).

Immune cells – corrupted or good?

The contribution of immune cells in cancer is under very active investigation, and some studies assessing the role of immune cells, especially in bone metastases, have been published. Osteoclasts and immune cells are all derived from the myeloid lineage. T-cells express RANKL and are known to activate osteoclasts in estrogen deficiency and autoimmune disorders, and B-cells have been found to protect bone homeostasis by secreting osteoprotegerin [OPG, reviewed in (Fournier et al., 2006)]. A preclinical experiment has shown that CD4+ T-cells (Th17 cells) of tumor-bearing mice induce bone resorption before tumor cells arrived to the bone, and that they actually prepare a pre-metastatic niche and enabled bone metastasis (Monteiro et al., 2013). Wu et al. (2016) have found that tumor-associated macrophages and osteal macrophages in regions of pathological bone were present in high density in all studied clinical samples of prostate cancer bone metastasis. T-cells were present only in 3/7 of the samples and B-cells in 1/7 samples. The distribution of the T- and B-cells was inconsistent, ranging from aggregates to scattered single cells. The

group also depleted macrophages in an immunocompetent intratibial prostate cancer mouse model and found out that the tumor-induced bone formation was inhibited, even though the tumor itself grew well (Wu et al., 2016). Conversely, Soki et al. (2015) have found that macrophage depletion results in decreased tumor size and increased trabecular bone. However, they utilized a wide depletion of macrophages, also depleting osteoclasts and an osteolytic prostate cancer cell line (Soki et al., 2015). These details prevented them from observing the role of macrophages on tumor-induced bone formation. The importance of macrophages in bone metastases may be based on the role of osteal macrophages in normal bone turnover (Sinder et al., 2015). One reason behind the low prevalence of T- and B-cells observed by Wu et al. (2016) might be the abundant TGF-β released from bone. In addition to a host of other effects, TGF-β drives immune tolerance by inducing regulatory T-cells and MDSCs (Johnston et al., 2016). These findings in osteo- and oncoimmunology have opened new venues for drug development, such as direct counteraction of TGF-β or blockade of immune check points, including cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) on regulatory T-cells.

Extracellular matrix composition like no other

Extracellular matrix (ECM) of bone consists of mostly of mineralized type 1 collagen. Minerals are stored in the form of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, which is an insoluble salt of calcium and phosphorus. Hydroxyapatite forms 50% of bone volume and 70% of bone weight. Bone contains 99% of calcium and 85% of phosphorus present in the body. Collagen is secreted by osteoblasts and forms triple helixes which bond together to fibrils by pyridinium crosslinks. Fibrils form a lamellar network, and hydroxyapatite is deposited between the fibrils. Osteoblasts also secrete other proteins that are deposited to the bone matrix such as osteocalcin, osteonectin, fibronectin, vitronectin, thrombospondin, matrix-gla-protein, osteopontin and bone sialoprotein. Osteocalcin and osteonectin are involved in mineralization, whereas matrix-gla-protein inhibits it. Osteopontin, fibronectin and bone sialoprotein bind to integrins and have a role in bone homeostasis, angiogenesis and hematopoiesis (Young, 2003, Granito et al., 2015, Lazar-Karsten et al., 2011, Nilsson et al., 2005).

Osteoid, i.e. unmineralized bone, has a rigidity (elastic modulus, E) of 25-40 kPa, and mineralized bone – 100-10^6 kPa (Engler et al., 2006). For comparison, smooth muscle E is ~10 kPa. Higher ECM rigidity (on the soft tissue scale) induces many basic processes in cells such as evasion of

apoptosis, migration and invasion. Engler et al. (2006) have shown that on the rigidity scale of soft tissue to osteoid, ECM rigidity can regulate stem cell renewal and determine the direction of differentiation. In softer ECM, MSCs differentiated to adipocytes, and in stiffer ECM, representing osteoid rigidity, they took on osteoblastic differentiation path, even when the cell culture medium supported the other path (Engler et al., 2006). The very high rigidity of mineralized bone has been shown to upregulate proteins in breast cancer cells, that are important in inducing osteolysis (Ruppender et al., 2010).

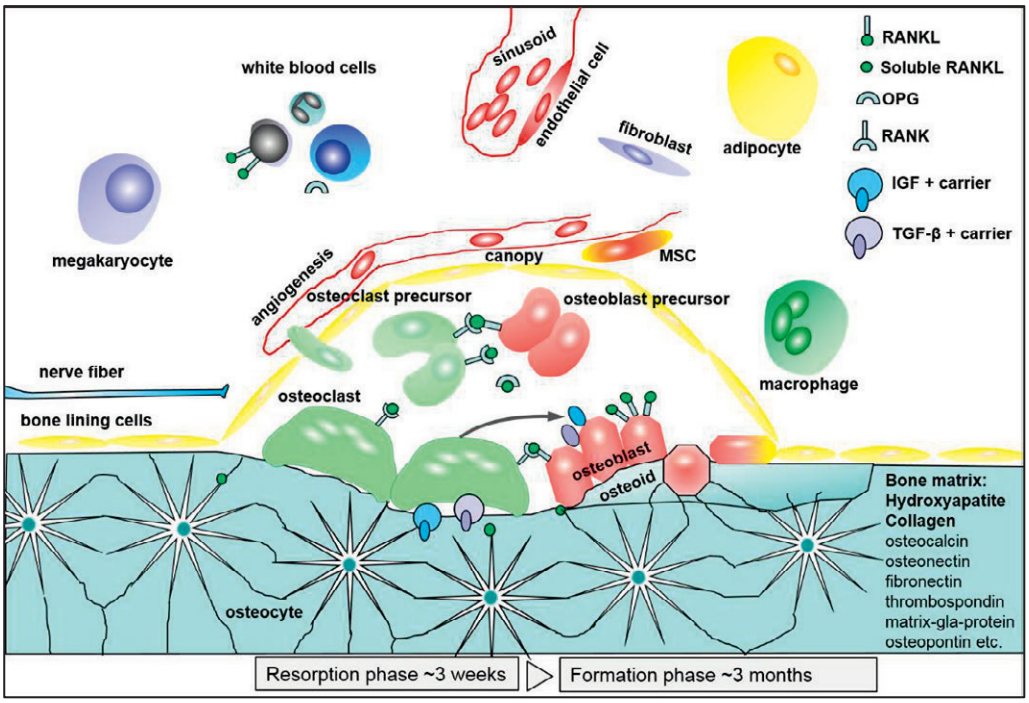

Figure 3. Schematic view of the remodeling cycle and the components of bone microenvironment. Abbreviations: MSC: mesenchymal stem cell, RANK and RANKL: receptor activator of necrosis factor kappa B and its ligand, OPG: osteoprotegerin, IGF: insulin-like growth factor, TGF-β: transforming growth factor β.

Oxygen tension is setting the scene

Despite ample vascularization, bone marrow has been shown to be a hypoxic environment by measuring hypoxia-inducible factors (HIFs) and hypoxia-sensitive staining (Parmar et al., 2007). The suggested contributing factors

in the development and maintenance of hypoxia are the high cellularity and slow blood flow in the sinusoids (Parmar et al., 2007). More recent measurements of local oxygen tension in living animals have supported these results and increased our understanding of the blood flow and oxygen gradient (Spencer et al., 2014). Instead of decreasing from the sinusoidal region to the endosteum, the extravascular oxygen tension is higher in the endosteal region due to perfusion by endosteal arterioles, which decreases towards the sinusoidal region (Spencer et al., 2014).

Since solid tumors usually have hypoxic areas, the arriving tumor cells may already have upregulated the machinery necessary for survival in hypoxic conditions, including induction of angiogenesis. HIF-signaling in tumor cells is known to facilitate metastasis in many ways, e.g. inducing migration and invasion (Rankin and Giaccia, 2016). Hypoxia in the primary tumor may have dramatic distant consequences; it has been reported that hypoxia-induced lysyl oxidase (LOX) secretion in the primary tumor induces bone resorption and formation of osteolytic lesions, even before the arrival of the tumor cells to bone. Thus, the hypoxic conditions in the primary tumor pave the way for tumor cell homing to bone. Importantly, they also showed that LOX was able to induce bone resorption without the presence of RANKL (Cox et al., 2015). Hypoxia has been known to induce parathyroid hormone-related peptide (PTHrP) expression (Manisterski et al., 2010) and thus induce resorption through the RANK pathway (discussed more closely in chapter 2.2.4 "Characteristics of tumor cells metastasizing to bone"). Therefore, the new LOX-induced RANK-independent pathway further strengthens the importance of hypoxic conditions in the primary tumor and bone microenvironment in bone metastases. Hypoxia also induces the secretion of VEGF and ET-1, which are inducers of angiogenesis, but also osteogenesis (Hall et al., 2005). HIFs are major players in the cell's response to hypoxia. HIF-2α is responsible for the PTHrP induction, whereas HIF-1α can induce chemokine receptor 4 (CXCR4) expression (Johnson et al., 2015), important in the homing and colonization processes discussed in the next chapter (2.2.4 Characteristics of tumor cells metastasizing to bone). Further evidence for the role of HIF-induced CXCR4 signaling in bone colonization and remarkably also in the growth and metastasis of a distant tumor was recently found, when Devignes et al. increased HIF signaling in osteoblast-lineage cells in mice. They found increased expression and plasma levels of the stromal cell-derived factor 1 (SDF-1, also known as CXCL12) and were able to link this finding to increased orthotopic mammary tumor growth, lung metastasis and colonization of the bone (Devignes et al., 2018). HIF-1α

has also been shown to increase tumor growth and the number of blood vessels in a preclinical mouse model of breast cancer bone metastases using MDA-MB-231 cells expressing non-degradable HIF-1α (Hiraga et al., 2007). A growth factor abundant in bone microenvironment, TGF-β, is known to decrease HIF-1α degradation (Kingsley et al., 2007). Osteolytic metastases may be more hypoxic than osteoblastic metastases, because oxygen consumption in osteoblastic metastases is lower due to low cellularity and the large proportion of bone (Cook et al., 1998).

2.2.4 Characteristics of tumor cells metastasizing to bone

Chemokines and adhesion molecules are important in homing and colonization

Homing denotes the ability of a cell to exit circulation when reaching the target organ, to stay and survive there. The target organs may be selected before the tumor cells even leave the primary tumor. This phenomenon is called the "formation of the pre-metastatic niche" (Kaplan et al., 2005). It has been shown that tumor cells secrete soluble factors, such as proteins and exosomes containing mRNA, that are able to condition the target organ to be more susceptible to tumor growth. These soluble factors also recruit MDSCs and CAFs that, in addition to preparing the premetastatic niche, also increase the mobility and invasive properties of cells in the primary tumor and their ability to survive in certain microenvironments (Peinado et al., 2012, Kaplan et al., 2006). For instance, bone-marrow-derived CAFs in the primary tumor have been shown to induce increased Src activity in tumor cells, which in turn increases their survival in the bone microenvironment (Zhang et al., 2013). In extravasation and adherance to stromal cells in bone marrow, breast and prostate cancer cells partly utilize the same chemotactic cues and cell adhesion molecules to hematopoietic progenitor cells and leukocytes. Several of these molecules have been identified, such as CXCR4 and C-C chemokine receptor type 7 (CCR7), annexin II receptor, RANK, CD44, very late antigen-4 (VLA-4) and a mixture of integrins (Esposito and Kang, 2014) among others. CXCR4 and CCR7 bind to SDF-1 expressed in bone marrow stromal cells and osteoblasts (Müller et al., 2001, Taichman et al., 2002). Annexin II is expressed in osteoblasts and bone marrow endothelial cells (Jung et al., 2007, Shiozawa et al., 2008) and several integrin-binding proteins, such as osteopontin and thrombospondin, are expressed by many stromal cells, osteoblasts and deposited in the bone ECM (Nilsson et al., 2005, Young, 2003).

Colonization denotes the ability of the resident tumor cells to proliferate, finally forming overt metastasis. The initial binding of cancer cells to stromal cells upon arrival to bone helps the tumor cells to survive and often times also supports proliferation. Sometimes, however, it can induce quiescence of the tumor cells. Some of the intrinsic and environmental factors helping the tumor cells in colonization have been identified. SDF-1/CXCR4 signaling has also been proposed to be essential in the colonization step (Shiozawa et al., 2011). Comparing cell lines with sublines possessing more osteotropic properties, differences on the level of gene expression, microRNA (miRNA) expression and protein secretion have been identified. Increased secretion of IL-11 in response to TGF-β (Pollari et al., 2012a), increased serine production (Pollari et al., 2011) and decreased expression of miRNAs 204, 211 and 379 (Pollari et al., 2012b) have been shown to separate the osteotropic subline MDA-MB-231(SA) from the parental MDA-MB-231 breast cancer cell line.

RANK is a cornerstone in osteoclastogenesis

RANK belongs to the family of tumor necrosis factor receptors (TNFR). It was first recognized in cells of the myeloid lineage: myeloid-derived dendritic cells, activated T-cells and osteoclast progenitors (Anderson et al., 1997). It has two ligands, RANKL (also called TRANCE, OPGL and ODF) and a soluble decoy receptor called OPG. RANKL is membrane-bound, but can be cleaved by matrix metalloproteinases (MMPs). It can activate RANK in both membrane-bound and soluble forms, although the membrane-bound form is more efficient in inducing osteoclastogenesis (Nakashima et al., 2000). RANKL and OPG are expressed by osteoblasts and regulate the differentiation, activation and survival of osteoclasts (Khosla, 2001). NFκB activation, as per the name of the receptor, is involved in all three processes. PI3K activation through Src is involved in survival and resorption, and the MEK-ERK pathway is involved in differentiation and survival. Later, RANK/RANKL pathway has also been found to be important in mammary gland development and during carcinogenesis. It has been recognized as a link between the breast cancer cells and bone environment, promoting breast cancer cell metastasis to bone (Blake et al., 2014). Furthermore, RANK expression has also been found in primary prostate cancer samples and prostate cancer cell lines (Li et al., 2014). Even though prostate cancer mainly induces osteoblastic lesions, both processes, bone resorption and formation, are present in prostate cancer bone metastases (Roato et al.,

2008). Osteoclasts are important because osteoblasts need a resorbed bone surface to initiate bone formation.

PTHrP is possibly anti-metastatic in the primary tumor, but guilty of bone damage

PTHrP was first discovered as a factor in serum, causing humoral hypercalcemia of malignancy (HHM) through increased bone resorption in the absence of bone metastases (Suva et al., 1987). Since then, PTHrP has been also recognized as important in many physiological processes, including the development of mammary glands and lactation (reviewed in (Guise and Mundy, 1996, McCauley and Martin, 2012)). Thus, it is no surprise that around 60% of primary breast cancers express PTHrP (Southby et al., 1990). However, HHM without bone metastases is very rare in breast cancer (Martin and Moseley, 2000). One reason for this discrepancy might be the differential expression of PTHrP; it can either be secreted or transcribed without the signaling region for secretion, thus leaving the protein within the cell. The effects of nuclear PTHrP and PTHrP binding to its cell surface receptor are often opposing (review by (Wysolmerski, 2012). Whether intracellular PTHrP expression in the primary tumor is a good or bad prognostic factor remains a controversial issue, as there are many studies with opposing results (Henderson et al., 2001, Henderson et al., 2006, Takagaki et al., 2012). However, when the breast cancer cells are in the bone microenvironment, PTHrP expression and secretion is upregulated, leading to increased local bone resorption, i.e. osteolytic lesions. The upregulation of PTHrP in breast cancer bone metastases almost seems a rule, as 92 % of bone metastases express PTHrP, including patients with PTHrP-negative primary tumors (Powell et al., 1991). One key factor in this upregulation is the rigidity of the mineralized bone matrix (Page et al., 2015, Ruppender et al., 2010).

PTHrP is also expressed by prostatic epithelial cells and prostate adenocarcinoma (Cramer et al., 1996b, Iwamura et al., 1993).

2.2.5 The vicious cycle

The term “vicious cycle” in the context of bone metastases refers to the self-amplifying sequence, where factors secreted by the tumor induce the breakdown of bone or the formation of new bone, leading to a release of growth factors, further supporting tumor growth. For clarity, the osteolytic and osteoblastic forms of the vicious cycle are presented separately, even

though both processes often co-exist in the same patient and even in the same metastatic site.

Osteolytic metastases

The first molecular players identified in cancer-induced osteolysis were PTHrP and TGF-β. PTHrP production and secretion by breast cancer cells is upregulated in the bone through TGF-β signaling, and PTHrP increases resorption indirectly by binding to the PTH receptor in osteoblasts (Guise et al., 1996). Osteoblasts in turn secrete less OPG and more RANKL, which binds to the RANK on osteoclasts and induces differentiation and bone resorption. In addition, factors secreted by cancer cells, such as LOX, colony stimulating factor 1 (CSF-1), tumor necrosis factor α (TNFα), VCAM-1, MMP1, Jagged 1 and interleukins 8 and 11, are capable of inducing the activation of osteoclasts, some of them directly, independently of RANK (Morgan et al., 2004, Sotiriou et al., 2001, Cox et al., 2015, Hensel and Thalmann, 2016, Ren et al., 2015). In osteolytic metastases, bone resorption and formation can become uncoupled, because some of these cancer cell secreted factors are able to induce osteoclast formation directly. In addition, cancer cells secrete factors inhibiting osteoblasts and physically disrupt the canopy over BMU (Roodman and Silbermann, 2015). As osteoclasts resorb bone, growth factors such as TGF-β, IGF-1 and bone morphogenetic proteins (BMPs) that are deposited to bone, are released. They enhance the growth of cancer cells and the secretion of osteoclast activation factors. In addition, osteoclast-derived miRNAs have been identified as messengers between osteoclasts and cancer cells (Esposito and Kang, 2014). TGF-β is deposited as part of an inactive complex and can be activated by proteases such as plasmin or cathepsins, glycoprotein thrombospondin-1 or the acidic environment present in the resorption lacuna (Oreffo et al., 1989, Khalil, 1999). In addition to its effects in bone, TGF-β is important in a plethora of cellular functions, such as EMT, invasion, angiogenesis and immune tolerance, but on the other hand, it suppresses the proliferation of normal cells (Juárez and Guise, 2011). Moreover, calcium itself can act as a growth factor, at least in prostate cancer cells that have a calcium-sensing receptor (Liao et al., 2006). Furthermore, immune cells are known to play an important part in the vicious cycle. Activated T-cells express RANKL and are able to induce the differentiation of osteoclasts *in vitro* without any supporting chemokines. TGF-β in turn inhibits the proliferation of T-cells, helping tumor cells to evade their effects (D'Amico and Roato, 2015).

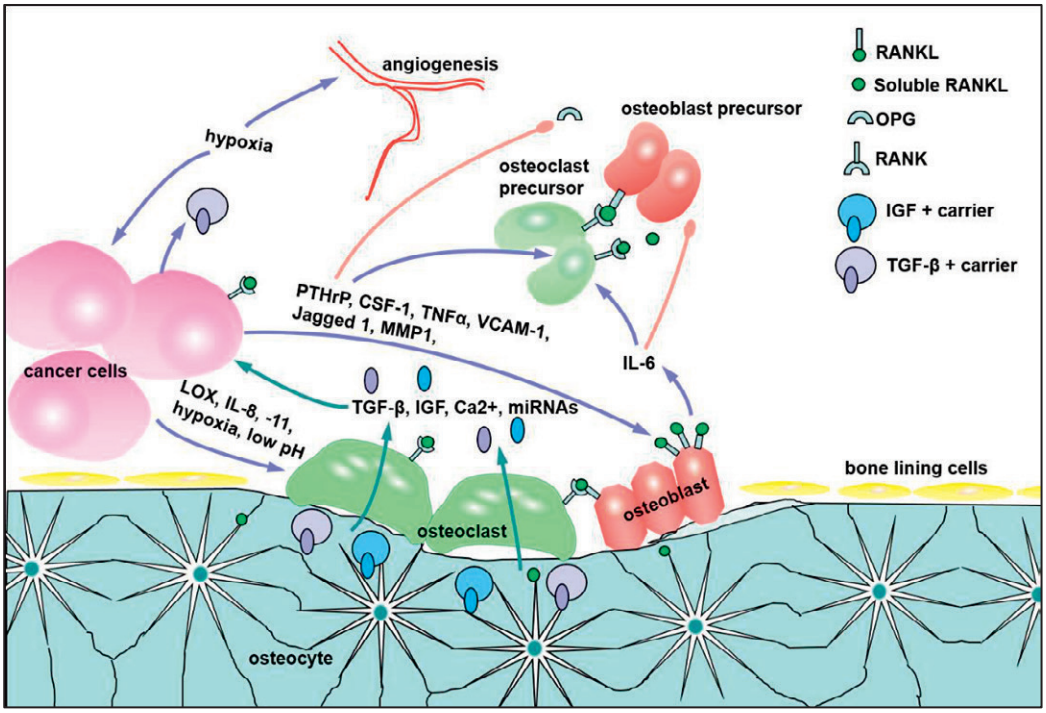

Figure 4. Vicious cycle of osteolytic metastasis. The breast cancer cells secrete factors that induce pre-osteoblasts and osteoblasts to express more RANKL and IL-6 but less OPG. This increases osteoclast differentiation and activity. Other factors secreted by breast cancer cells or osteoblasts enhance osteoclast differentiation directly. TGF-β, IGF and calcium released by resorbing osteoclasts induce tumor growth and further secretion of osteoclastogenic factors. Additionally, osteoclasts secrete miRNAs, which may regulate tumor cells. Hypoxia induces the secretion of TGF-β from breast cancer cells and angiogenesis. Abbreviations: PTHrP: parathyroid hormone-related protein, CSF-1: colony stimulating factor 1, TNFα: tumor necrosis factor α, VCAM-1: vascular cell adhesion molecule 1, MMP1: matrix metalloproteinase 1, TGF-β: transforming growth factor β, IGF: insulin-like growth factor, IL-6, -8 and -11: interleukins 6, 8 and 11, LOX: lysyl oxidase.

The concept of the vicious cycle has received some criticism based on the fact that a disruption of the cycle by preventing bone resorption, for example by bisphosphonates, is not sufficient to inhibit tumor growth, and thus, other factors must be important (Hensel and Thalmann, 2016). However, even though the tumor is not dependent on the vicious cycle at a stage of overt growth, it may be crucial in shifting from dormancy or the micrometastastic stage to growth. Preclinical studies dosing bisphosphonate prior to inoculation of the cancer cells into circulation have

revealed the inhibition of formation of bone metastases (van der Pluijm et al., 2005, Ottewell et al., 2014b, Ottewell et al., 2014a). Furthermore, emerging clinical evidence supports the notion of the importance of resorption inhibition in the prevention of bone metastases (Coleman et al., 2014b). In addition, the vicious cycle may be important in situations where cancer treatment has weakened the tumor, and it needs the support of the microenvironment to survive. This thesis examines the importance of the vicious cycle in treatment response.

Osteoblastic metastases

Prostate cancer in bone behaves differently from breast cancer. Most breast cancers are primarily osteolytic, whereas prostate cancers tend to be osteoblastic, forming new, unorganized and weak bone, or a clear mixture of the two types. One factor behind the osteoblastic reaction seems to be PSA. Inducing the expression of PSA through a transgene in a PSA-negative prostate cancer cell line has been shown to shift the bone reaction from osteolytic to osteoblastic (Cumming et al., 2011). PSA is a serine protease and cleaves many proteins important in bone metabolism, releasing, e.g., insulin-like growth factor (IGF) and TGF-β2 from their binding proteins to stimulate osteoblasts (Réhault et al., 2001), as well as inactivating PTHrP, a strong inducer of bone resorption (Cramer et al., 1996a). However, in osteoblastic metastasis of prostate cancer, strong osteoclastic resorption is also typical, and bone resorption marker levels in prostate cancer patients can be even higher than in osteolytic breast cancer patients (Mountzios et al., 2010, Roato et al., 2008). Osteoblasts need a resorbed bone surface onto which new bone can be formed. Thus, an increase in the number of BMUs and dysregulation of their spatiotemporal locations is required, in addition to the increased osteoblast number and activity within one BMU, in order to achieve the vast amount of new, pathological bone observed in osteoblastic metastases. An increase in the BMU number results in increased resorption, leading to high resorption marker levels. In addition, the amount of bone available for resorption is high due to constant formation of new bone. Furthermore, the cancer-induced new bone might also be more prone to resorption because of its disorganized and pathological nature. Thus, the efficacy of anti-resorptive treatments in osteoblastic metastases is explained by the prevention of new BMU formation, also resulting in decreased bone formation. It also suggests that the resorption and formation are still at least partly coupled in osteoblastic metastases, since there is still a certain sequence of events within each BMU.

Prostate cancer cells increase the number of osteoblasts by pushing MSCs toward osteoblastic differentiation by secreting endothelin 1 (ET-1), wingless and Int1 proteins (Wnts), BMPs, transforming growth factors, fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs). In addition, angiogenesis is increased by these factors, expanding the HSC niche that prostate cancer cells can inhabit (Hensel and Thalmann, 2016). ET-1 has also been found to be secreted from breast cancer cells producing osteoblastic lesions, underlining its overall significance in the osteoblastic lesion type (Yin et al., 2003). Prostate cancer cells themselves can act like osteoblasts, which is called osteomimicry, even to the degree of forming mineralized bone nodules (Koeneman et al., 1999, Lin et al., 2001). Özdemir et al. studied the stromal response by utilizing a species-specific microarray in two osteoblastic prostate cancer xenograft models and found a gene signature representing the osteoblastic stromal response. They found, as one could expect, that the most prominent processes were angiogenesis and osteogenesis, with TGF-β as the most significant pathway mediator. In the osteoblastic stromal signature, among the most expressed genes were pleiotrophin (PTN), ephrin type-A receptor 3 (EPHA3) and fascin (FSCN1), all identified as mediators of osteoblast differentiation and activation (Özdemir et al., 2014a). However, it is unclear what factors the osteoblasts provide that support the growth of cancer cells (Hensel and Thalmann, 2016). Along the tightly linked processes of angio- and osteogenesis, the periosteal/vascular HSC niche is expanded and new stem cell niches become available for the cancer cells (Özdemir et al., 2014a). These newly formed niches do not promote quiescence, but proliferation, since niches adjacent to sprouting vasculature, awaken cancer cells and induce proliferation (Ghajar, 2016). Thus, maybe the osteoblast-derived factors driving cancer cell proliferation have been so elusive, because there are either none or only a few. Maybe the osteoblastic response is not a driver in the vicious cycle, but a passenger due to its inherent coupling to angiogenesis. From another angle, the osteoblastic response could be observed as desmoplastic stroma, able to exert both tumor-promoting and tumor-suppressive effects (Özdemir et al., 2014a, Özdemir et al., 2014b).

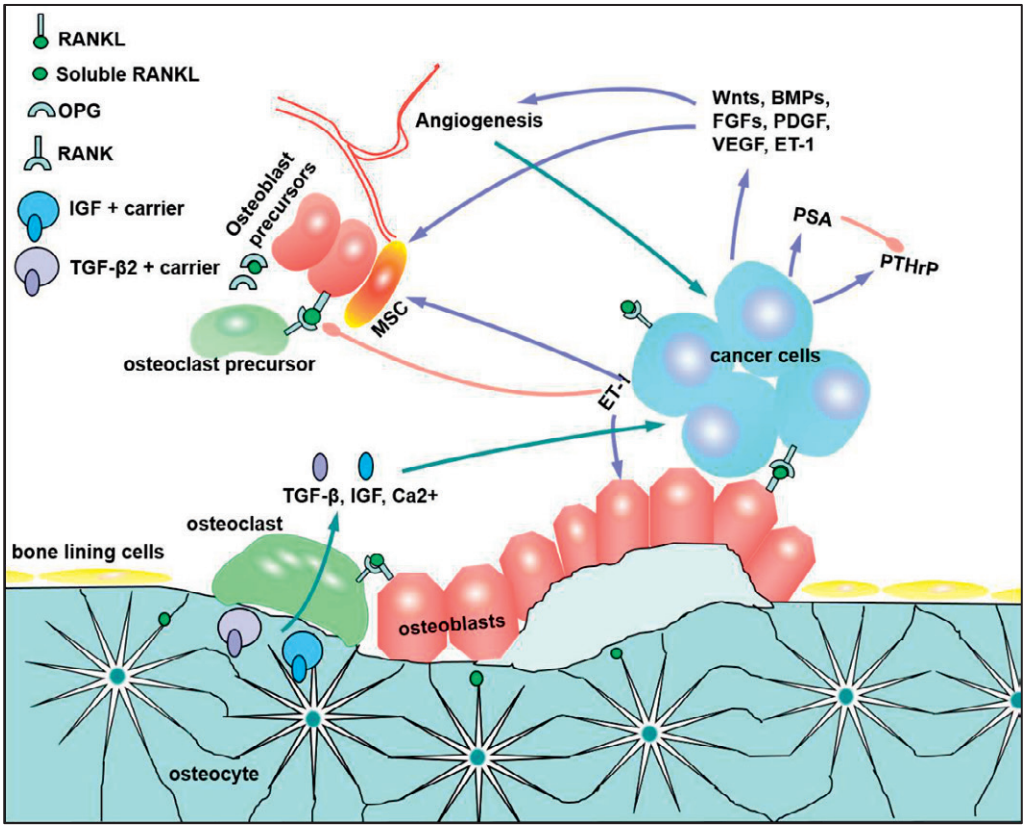

Figure 5. Vicious cycle of osteoblastic metastasis. Prostate cancer cells secrete factors that induce angiogenesis and differentiation of MSCs to osteoblasts. Prostate cancer cells utilize the perivascular niches offered by the sprouting endothelium. Osteoblasts induce differentiation and activation of osteoclasts, and released growth factors support the growth of tumor cells. PSA inactivates the PTHrP, downregulating the osteoclast activation by osteoblasts. ET-1 decreases RANKL expression and increases OPG expression. Abbreviations: BMPs: bone morphogenetic proteins, FGFs: fibroblast growth factors, MSC: mesenchymal stem cell, PDGF: platelet-derived growth factor, VEGF: vascular endothelial growth factor, ET-1: endothelin 1, PSA: prostate-specific antigen, PTHrP: parathyroid hormone-related protein, TGF-β: transforming growth factor β, IGF: insulin-like growth factor.

2.2.6 The role of the bone microenvironment in resistance to cancer therapies

Drug resistance presents a major challenge in clinical oncology (Holohan et al., 2013). The bone microenvironment can help tumor cells survive cancer treatments in several ways: *1. By inducing dormancy*. Dormant cells

are resistant to chemotherapy, because chemotherapy affects rapidly dividing cells. Dormant cells are also metabolically less active and may use different metabolic routes than actively dividing cancer cells. Dormant cells inhabit a niche that inherently provides many survival cues such as prostaglandin (Hemann, 2016). Ghajar reported that with lapatinib treatment, HER2-expressing breast cancer cells decreased in mouse bone marrow stroma, but the dormant tumor cells in perivascular niches in bone marrow were not affected. This resistance was shown to be contact-dependent, and sensitivity to therapy was regained with integrin-targeting therapy (Ghajar, 2016). *2. Through interaction with stromal cells.* Stromal cells provide anti-apoptotic signals, chemokines and growth factors. For example, myeloma cells become resistant to dexamethasone through stromal IL-6 secretion (Grigorieva et al., 1998). This can occur within the vicious cycle; Lee et al. recently reported that Growth Arrest-Specific 6 (GAS6) secreted by osteoblasts prevents docetaxel-induced apoptosis in prostate cancer cells (Lee et al., 2016). The survival cues can also be evoked by chemo- or radiotherapy itself, as shown by endothelial cells secreting IL-6 and Timp1 in response to chemotherapy, thereby supporting tumor cell survival (Gilbert and Hemann, 2011). These factors can help the cancer cells circumvent their "addiction" of, for example, estrogen or a kinase depleted by a targeted treatment. Using a genetically modified mouse model of acute myeloid leukemia, Hemann (2016) created chemotherapy-resistant AML cells, which very interestingly were resistant only *in vivo*, and the resistance was not due to any new mutation. The dissemination of resistant cells in mice was different from the parent cells, as they no longer colonized the spleen but homed to bone marrow. These findings are in line with the clinical findings that recurrent AML does not show the appearance of new mutations (Hemann, 2016). *3. Through hypoxic environment.* Many treatments rely on generating oxygen radicals and with low oxygen levels, this process is inefficient. Hypoxia also induces the secretion of many survival factors from the bone marrow stromal cells.

2.3 Effects of cancer treatments on bone

2.3.1 Hormonal treatments

The hormone-responsive form of breast cancer is treated with ablation of sex hormones by GnRH agonists, AIs or antiestrogens. Despite the longstanding knowledge of the adverse effects of hormonal cancer

treatment on bone health, the problem did not receive much attention until the last decade (Aapro, 2006, Reid et al., 2008). A deficiency of sex steroids, especially estrogen, causes osteoporosis by increasing bone resorption. CTIBL is more rapid than in normal menopause, because AIs also prevent local estrogen production (Hadji, 2015). In addition to exposing the patients to an increased risk of fracture, increased bone resorption has quite recently been shown to increase the frequency of breast and prostate cancer bone metastases in animal models (Ottewell et al., 2015, Ottewell et al., 2014b). Increased bone resorption due to antihormonal treatments may also partly explain the discrepancy of ER-positive breast cancers being more likely to develop bone metastases than ER-negative breast cancers in humans (Lee et al., 2011). Animal models of bone metastasis are usually run in young animals with naturally high bone turnover, but most of them use ER-negative cells. Antiestrogens tamoxifen and toremifene, which are selective estrogen receptor modulators (SERMs), and fulvestrant, which is selective estrogen receptor downregulator (SERD) are not so detrimental to bone (Love et al., 1988, Turner et al., 1988, Agrawal et al., 2009, Goss et al., 2007b), and tamoxifen can even protect from adverse bone effects of the simultaneous GnRH agonist (Sverrisdóttir et al., 2004). Differences have also been found between non-steroidal and steroidal AIs (letrozole and anastrozole vs. exemestane). The steroidal exemestane is thought to support bone formation through the androgen receptor (Goss et al., 2007a). However, because androgens are not as important in preserving bone integrity, exemestane treatment still causes bone loss (Hadji, 2015).

Like breast cancer, recurrent prostate cancer is treated with sex hormone ablation, either by chemical castration through GnRH agonist, antagonist or antiandrogens such as bicalutamide. With GnRH agonists/antagonists, serum testosterone and estradiol levels decrease, as opposed to increased serum testosterone and estradiol levels with bicalutamide treatment. GnRH agonist/antagonist treatment increases bone metabolism, but the effects of inhibiting androgen receptor activity on bone metabolism are not as grave as withdrawal of estrogen. Unchanged bone metabolism markers during bicalutamide treatment (Smith et al., 2003) and stable bone mineral density (BMD) during enzalutamide treatment (Merseburger et al., 2015) are evidence of the less severe effects. When a tumor becomes resistant to standard ADT, CYP17A1 inhibitor abiraterone acetate and chemotherapy can be used. Because abiraterone acetate prevents the synthesis of both androgens and estrogens, it is reasonable to expect severe bone effects. However, clinical studies have not reported BMD results, whereas studies of

the direct effects on bone cells by abiraterone acetate are emerging. CYP17A1 inhibitor abiraterone was shown to inhibit osteoclasts and stimulate osteoblasts *in vitro*, with corresponding effects on bone markers CTX and ALP in prostate cancer patients (Iuliani et al., 2015). Whether these effects are enough to prevent the deleterious effects of sex hormone deficiency remains to be seen. A part of abiraterone's efficacy in bone metastatic patients might thus come from effects on the bone microenvironment. These results also emphasize that even though prostate cancer produces mostly osteoblastic lesions, a compound decreasing resorption, even though simultaneously increasing formation, can still disrupt the vicious cycle and help in gaining homeostasis.

2.3.2 Chemotherapy

Common chemotherapeutic agents in breast cancer are taxanes, doxorubicin, 5-fluorouracil, cyclophosphamide, methotrexate and cisplatin. The newest class of chemotherapeutics is epothilones, of which ixabepilone is approved for metastatic breast cancer. Chemotherapy has been shown to have deleterious effects on bone and induce CTIBL either through direct effects on bone cells or chemotherapy-induced menopause (Ratcliffe et al., 1992, Fogelman et al., 2003). Direct effects may be marked, as up to 60% loss of cancellous bone upon cytotoxic therapy have been reported in preclinical studies (Hadji, 2015). The proportion of premenopausal breast cancer patients experiencing long-term amenorrhea after chemotherapy or chemotherapy + tamoxifen was 35% (Valentini et al., 2013). Failure of ovarian function can be considered a desirable effect in hormone-receptor positive cancers. However, in bone, the consequences of estrogen deprivation are destructive, and probably fueling the homing and/or colonization of the cancer cells to bone as described in the previous chapter. Furthermore, because chemotherapy is directed towards rapidly dividing cells, it always carries some degree of myelotoxicity, and thus, it also severely disturbs the bone microenvironment. Importantly, microtubule stabilizing agents, such as taxanes and epothilones might also inhibit the osteoclasts relying on the dynamic cytoskeleton to enable resorption. However, the effects or chemotherapeutic agents on bone health are rarely investigated. In addition, glucocorticoids, which are often used in hematological malignancies and as an adjuvant treatment to control chemotherapy-induced nausea in solid tumors, are known to increase bone resorption, impair bone formation and induce apoptosis of osteocytes (O'Brien et al., 2004). Differences in bone effects are likely to exist among

common chemotherapies and these differences should be investigated to gain more optimized treatments for patients.

2.3.3 Immunotherapy

Sipuleucel-T (Provenge®) is the first immunotherapy approved for (bone) metastatic prostate cancer. A patient's dendritic cells are trained against the fusion antigen, consisting of prostatic acid phosphatase (PAP) and granulocyte-macrophage-colony-stimulating factor (G-MCSF) *ex vivo*, and injected back (Kantoff et al., 2010). The efficacy in prostate cancer patients with bone metastasis suggests that the trained dendritic cells were able to induce considerable immune response even in the presence of immune-suppressive TGF-β. TGF-β has also been shown to be important in a mouse model of osteoblastic prostate cancer bone metastases (Mishra et al., 2011). The bone effects observed in clinical trials of sipuleucel-T have been related to the prostate cancer-induced bone changes, but with the rise of osteoimmunological research, other underlying mechanisms might also be revealed in the future. For example, activated T-cells might serve as an additional cue for increased resorption. However, it is worth mentioning that the results of the sipuleucel-T phase III trial (Immunotherapy for Prostate Adenocarcinoma Treatment, IMPACT) have been criticized for a possible negative impact of the placebo treatment, leading to wrong conclusions of the study results (Huber et al., 2012). Since then, however, several additional studies have proven the efficacy of sipuleucel-T in prostate cancer (Hu et al., 2016).

2.3.4 Targeted treatments and new candidates

Hardly any reports of bone effects from targeting HER2 with a monoclonal antibody, such as trastuzumab, are available. However, one small clinical study reported by Pilanci et al. (2015) has suggested that when administered simultaneously with zoledronic acid, trastuzumab may increase the risk of developing osteonecrosis of the jaw (ONJ). Trastuzumab also acts as an angiogenetic inhibitor, and because bisphosphonates affect angiogenesis in bone as well, the cumulative effect may be behind the increased risk (Pilanci et al., 2015).

HER2 is also targeted by dual tyrosine kinase inhibitors, such as lapatinib, afatinib and neratinib that simultaneously target EGFR. Inhibition of tyrosine kinases is considered to have multiple effects on bone, and the net

result for each tyrosine kinase inhibitor will likely depend on the inhibition profile of different tyrosine kinases. A class effect of inhibiting bone resorption has been suggested (Alemán et al., 2014). Thus, several tyrosine kinase inhibitors are presented under section 2.4. Bone targeted therapeutics in chapter 2.4.4. New candidates.

Inhibition of mTOR is especially efficient in hormone responsive breast cancers, because the hyperactivity of the mTOR-pathway is essential in endocrine resistance. Inhibition of the mTOR pathway also has direct bone effects, as it decreases osteoclast survival and affects the cathepsin K production of osteoclasts. The mTOR inhibitor everolimus has indeed shown bone protective effects in clinical trials (Hadji et al., 2013).

Inhibition of CDK4/6 is likely to have direct bone effects in breast cancer patients, because CDK6 is a key element in regulation of osteoblast and osteoclast differentiation (Grossel and Hinds, 2006). Indeed, inhibition of osteolytic lesion progression in a multiple myeloma mouse model has been reported (Feng et al., 2009). However, no results of the bone effects of the CDK4/6 inhibitor palbociclib, used in combination with AIs or anti-angiogenic drug bevacizumab, have yet been reported.

Antibody-drug conjugates (ADCs), linking a targeting antibody and usually a chemotherapeutic agent, are likely to have fewer side effects on bone than conventional chemotherapy due to more targeted exposure.

Several immunotherapeutic agents are in clinical trials for breast cancer. On ClinicalTrials.gov site (https://clinicaltrials.gov), a search with terms "breast cancer" AND "immunotherapy" returned 158 interventional studies as of February 23rd, 2018. Because of the lack of targeted treatments and the presence of tumor-infiltrating lymphocytes, TNBC has been the most popular subtype in immunotherapeutic trials (Marmé, 2016). The most advanced in clinical development for TNBC and already approved for other cancers are compounds that try to activate a patient's T-cells by using antibodies against inhibitory signaling molecule PD-1 or its ligand (PD-L1), namely pembrolizumab and atezolizumab, respectively (ClinicalTrials.gov identifiers: NCT02555657 and NCT02425891). The immunological checkpoints are also important for normal bone metabolism. PD-1-deficient mice present mild osteopetrosis, whereas CTLA4 deficiency results in osteopenia (Nagahama et al., 2004). However, these observations were made in non-tumor-bearing mice, and the mice were deficient throughout

their lives. Even though it is likely that clinical use of checkpoint inhibitors will not result in any dramatic changes in the bone mass of cancer patients, it is an intriguing thought that CTLA4 inhibitors increasing bone resorption might be more suitable in osteoblastic tumors and PD-1 inhibitors – in osteolytic tumors.

AR degradators and receptor antagonists targeted at mutated AR are in development. Because AR signaling inhibits apoptosis in osteoblasts and osteoclasts (Vignani et al., 2016), AR degraders may have unwanted bone effects, whereas receptor antagonists of mutated AR would not affect normal receptors in bone cells.

No anti-angiogenic drugs are currently approved for prostate cancer. Sunitinib and bevacizumab increased progression-free survival (PFS) and cabozantinib – radiographic PFS but not OS in prostate cancer (Michaelson et al., 2014, Smith et al., 2016, Kelly et al., 2012). In breast cancer, sunitinib increased overall response rate but did not affect PFS or OS (Bergh et al., 2012). Bevacizumab received accelerated approval in breast cancer, which was later revoked by FDA, but it is still approved by EMA for use in metastatic breast cancer, in combination with paclitaxel or capecitabine. Tasquinimod, which targets MDSCs and reduces angiogenesis, but not through VEGFR inhibition, also increased PFS but not OS in metastatic prostate cancer (Sternberg et al., 2016). Despite these drawbacks, a number of clinical studies with angiogenesis inhibitors are ongoing. Because of the tight connection of osteogenesis and angiogenesis, angiogenesis inhibitors are likely to have bone effects. Indeed, increased risk of ONJ has been linked to the use of angiogenetic inhibitors (Pilanci et al., 2015). Otherwise, because the patients are adults, inhibition of the remodeling cycle is likely to cause slow and subtle changes in normal bone, possibly resolving with the cessation of anti-angiogenic treatment. In accordance with the modest results in metastatic breast cancer patients, bevacizumab had no effects on tumor growth in bone or osteolysis in a mouse model of bone metastasis in a study by Bachelier et al. (2014). Interestingly, good inhibition of tumor growth and osteolysis was achieved when combined with vatalanib, a tyrosine kinase inhibitor of VEGFRs 1-3 (Bachelier et al., 2014).

2.4 Therapies targeting bone

2.4.1 Bisphosphonates

Bisphosphonates are pyrophosphate analogs that bind to hydroxyapatite. They inhibit bone resorption but also calcification in high concentrations. They are divided into two classes according to the presence or absence of nitrogen. Nitrogen-containing bisphosphonates are more effective in inhibiting resorption. Their mode-of-action is inhibition of the mevalonate pathway in osteoclasts (Amin et al., 1992). Bisphosphonates are currently used as the main treatment of osteoporosis, and as adjuvant treatment in patients with bone metastases, because they have very few side-effects and are efficient in decreasing pain and protecting bone, thus preventing skeletal-related events (Body et al., 2016, Kanis et al., 2013).

The direct anti-tumor effects of nitrogen containing bisphosphonates have been studied widely, and many *in vitro* studies have shown clear anti-tumor effects, mostly affecting through the mevalonate pathway as in osteoclasts (Clézardin, 2005). The effects on viability, angiogenesis and invasion have been observed. However, since bisphosphonates *in vivo* bind to bone rapidly and tightly, the concentrations necessary to observe these effects are only high enough in the resorption lacuna, and thus, these anti-tumor effects should not be observed *in vivo*. Interestingly though, there is evidence that bisphosphonates get recycled, as small amounts of alendronate have been found in the plasma of patients many weeks after dosing (Porras et al., 1999). They may enter the blood after detachment from bone or after being resorbed and released via the osteoclasts and may again bind to bone at another site (Nancollas et al., 2006). This "recycling" seems to depend on the osteoaffinity of the compound, as does the original distribution between active and quiescent bone surfaces and osteoid (Roelofs et al., 2012). For a long time, the anti-tumor effects of bisphosphonates on primary tumors were under much debate due to conflicting results (Ottewell et al., 2012, Clézardin, 2005). Macrophages were suspected as mediators of the anti-tumor effects, and this was finally confirmed when bisphosphonates were tagged with fluorescent labels and followed in Balb/c mice carrying syngeneic 4T1 tumors in the mammary fat pad. They showed that bisphosphonates attached to microcalcifications in the tumor and were taken up by tumor-associated macrophages in quantities high enough to kill them, and this depletion of macrophages slowed down the primary tumor growth (Rogers, 2012, Junankar et al., 2015). The role of macrophages was soon confirmed by another group (Rogers et al., 2013).

Two large clinical trials, AZURE and ABCSG-12, have been performed to study the efficacy of a nitrogen containing bisphosphonate zoledronic acid (ZA) in preventing breast cancer bone metastasis. AZURE concluded that zoledronic acid increases disease-free survival (DFS) in postmenopausal but not in premenopausal women, and ER status had no effect (Coleman et al., 2014b). ABCSG-12 results indicate that women over 40 benefit from ZA; premenopausal women with ER-positive tumors also benefited (Gnant et al., 2011). It is interesting to note that the ER-positive cases were treated with GnRH agonist goserelin, while in AZURE, ER-positive patients did not receive goserelin. Both postmenopausal and premenopausal patients receiving goserelin have increased bone resorption (Fogelman et al., 2003), which is blocked by ZA. Thus, inhibiting increased resorption can prevent cancer cells either from finding refuge in the bone metastatic niche or awakening from dormancy. Indeed, a recent study reported increased distant disease-free survival in postmenopausal breast cancer patients on AI therapy with oral osteoporosis treatment (Lipton et al., 2017)

2.4.2 RANKL inhibitor

The notion of critical importance of the RANK – RANKL / OPG pathway in bone resorption led to the development of a monoclonal antibody inhibiting RANKL. The fully human RANKL antibody denosumab is approved for treatment of osteoporosis and bone metastases of breast and prostate cancer. It has proven to be very effective in inhibiting bone resorption and, thus, also skeletal-related events in breast and prostate cancer patients with bone metastases (Fizazi et al., 2011, Lipton et al., 2012). There is now also data of RANKL inhibition preventing the occurrence of bone metastases in prostate cancer patients (Smith et al., 2013). When used as an adjuvant treatment in breast cancer patients receiving AI therapy, the RANKL inhibitor increases the time to first fracture, but the intermediate report of the ABSG-18 trial did not differentiate fractures due to bone metastases or other causes (Gnant, 2015). When the ABSG-18 trial matures, it will produce data on the effects of a RANKL inhibitor on OS.

Since RANK is expressed in normal breast epithelium and in breast cancer cells, inhibiting RANKL might also decrease the risk of developing breast cancer, and/or effect the tumor cells directly (Gonzalez-Suarez et al., 2007). Furthermore, the RANK/RANKL system is involved in the regulation of inflammation and immunity and has been suggested to be a key regulator of

epithelial stemness (González-Suárez and Sanz-Moreno, 2016). Recently, simultaneous high serum RANKL/OPG ratio and high progesterone levels were linked to a high risk of developing breast cancer in postmenopausal women (Kiechl et al., 2017). One clinical study investigating the effects of the RANKL inhibitor on Ki-67 index of primary breast cancer was started, but it was unfortunately terminated in 2017 due to poor recruitment (ClinicalTrials.gov Identifier: NCT01864798). A clinical study investigating the effects of RANKL inhibition on the breast cancer occurrence of BRCA1 or BRCA2 mutation carriers is ongoing (Sousa and Clézardin, 2018). A large-scale study, retrospective or follow-up, of breast cancer risk in women taking a RANKL inhibitor for osteoporosis would be interesting, but to my knowledge, such study has not yet been reported or initiated.

2.4.3 Radioisotopes

The development of bone-targeted radioisotopes has utilized the inherent bone affinity of alkaline earth elements calcium (Ca), strontium (Sr), barium (Ba) and radium (Ra).

Beta-emitters samarium-153 (Sm-153), strontium-89 (Sr-89) and rhenium-186 (Re-186) are in clinical use for pain palliation of bone metastases (Koutsikos and Leondi, 2008, Bączyk, 2011). They have not shown effects on OS in clinical studies. Sr-89 is a calcium analog with a half-life of 50.5 days, and it is deposited to the bone during new bone formation. Sm-153 is linked to tetraphosphate for targeting the bone metastasis by hydroxyapatite binding and has a half-life of only 1.9 days. Myelotoxicity is a severe side-effect of both isotopes (Bączyk, 2011).

Radium-223 (Ra-223) is an alpha-emitter with a 11.4-day half-life and calcium mimetic properties. Alpha radiation has significantly shorter range and higher linear energy transfer (LET) than beta radiation. Shorter range results in milder myelotoxicity (Henriksen et al., 2003, Bączyk, 2011) and higher LET in stronger induction of DNA double-strand breaks (DSBs) in tumor cells (Kassis and Adelstein, 2005). Ra-223 dichloride was approved by FDA and European Medicines Agency (EMA) for treatment of patients with CRPC with symptomatic bone metastases and no known visceral metastatic disease in 2013. In addition to pain management and a decrease in skeletal-related events, it increases OS ('1.5-Year post-treatment follow-up of radium-223 dichloride (Ra-223) in patients with castration-resistant prostate cancer (CRPC) and bone metastases from the phase 3 ALSYMPCA

Study 9,' 2014, Wissing et al., 2013). Phase II clinical studies in bone metastatic breast cancer are ongoing and promising first results, as well as a case report, have been published (Coleman et al., 2014a, Takalkar et al., 2014).

Also, the physical site of DTCs in bone microenvironments may play an important role in the efficacy of radiation therapy: Wang et al. (2014) reported that prostate cancer cells in mouse bone were situated in osteoblast-rich areas and always less than 40 µm from an endosteal surface. This is important information when radiotherapeutic pharmaceuticals are considered for use in patients at risk of bone metastases, as they are known to concentrate in osteoblast-rich areas, and the maximum range of, for example, Ra-223 alpha particles is 100 µm. The idea of preventive radiotherapy has become reasonable just recently with the development of radiotherapies with only mild side-effects such as radium-223 dichloride. However, there might be an increased risk of secondary cancers based on an increase of malignancies following the administration of radium isotope 224 for ankylosing spondylitis (Nekolla et al., 2010). Therefore, risks versus benefits need to be carefully considered. The low risk of side effects also increases the possibilities for combination treatment with chemotherapy and immunotherapy.

The possibility of utilizing targeted alpha-particles as an anti-angiogenic treatment, both destroying the existing tumor neo-vasculature and inhibiting the outgrowth of new vessels, has been suggested (Akabani et al., 2002) and tested *in vitro* (Zhu et al., 2010).

2.4.4 New candidates

A few new drug candidates targeting bone metastases in breast or prostate cancer are currently in clinical development after the disappointing results of Src family kinase inhibitor dasatinib in a phase III prostate cancer study (Araujo et al., 2013) and endothelin receptor antagonists atrasentan and zibotentan also in Phase III prostate cancer studies (Miller et al., 2013, Quinn et al., 2013). However, Yu et al. showed a positive effect of dasatinib on bone metastases using PET imaging (Yu et al., 2015). Src kinase inhibitors KX2-391 and saracatinib failed due to poor clinical efficacy in phase II (Lara et al., 2009, Antonarakis et al., 2013). However, one study investigating saracatinib effects of on bone pain is ongoing (Clinical Trials.gov Identifier: NCT02085603). Src family kinases are important both in tumorigenesis and

in osteoclast function (Boyce and Xing, 2011). Endothelin-1 has been shown to be essential in bone formation, and elevated levels are found in prostate cancer patients (Yin et al., 2003).

Much of recent activity has been in the class of integrin-targeting agents. α_V integrins are expressed in tumor cells and osteoclasts, and antagonizing their effects has shown preclinical efficacy on cancer cell homing and colonization, cancer-induced osteolysis and neoangiogenesis (Schneider et al., 2011). The first to report in the clinical phase II, abituzumab, unfortunately did not show effects on the primary endpoint of progression-free survival, but subgroups of patients may have benefited (Élez et al., 2015). Etaracizumab phase II results are pending publication (Clinical Trials.gov identifier: NCT00072930). GLPG0187 and MK-0429 were tested in phase I, but no further development has been reported (Raab-Westphal et al., 2017).

Cathepsin K is a cysteine protease that is important for the bone resorptive function of osteoclasts. Cathepsin K inhibitor odanacatib showed a decrease in bone turnover marker in a phase II study (Jensen et al., 2010), but was withdrawn from further studies in oncological indications. Later, it was also withdrawn from development for osteoporosis due to an increase in risk of cardiovascular events (Mullard, 2016).

Although cabozantinib, a multiple tyrosine kinase inhibitor, including VEGFR-2 and MEK, may not usually be considered as a bone-targeting agent, it has clear bone effects (Haider et al., 2015). Since angiogenesis, bone growth and remodeling are tightly linked, this finding is not surprising. Cabozantinib showed progression-free survival in phase III trial in mCRPC patients, but did not meet the primary endpoint of increase in OS (Smith et al., 2016). In breast cancer, cabozantinib increased PFS in ER+ disease and increased markers of immune activation in TNBC disease in phase II trials (Tolaney et al., 2016, Tolaney et al., 2017). Even though bone scans were implemented for ER+ patients, the effects on bone metastases have not been reported so far. Further studies are ongoing.

Bortezomib is a proteasome inhibitor originally approved for treatment of multiple myeloma. It has been shown to induce osteoblast differentiation in preclinical studies (Oyajobi et al., 2007). Bortezomib did not show efficacy in phase II trial for mCRPC (Morris et al., 2007).

Guanabenz is an anti-hypertensive drug that inhibits reactive oxygen species. It has shown to induce osteoblastogenesis and suppress osteoclastogenesis and to protect osteoblasts and osteocytes from glucocorticoid-induced apoptosis *in vitro* and *in vivo* (Hamamura et al., 2015), (Sato et al., 2015). Unfortunately, the clinical trial investigating its effects on bone markers in patients with bone metastases was terminated in 2018 due to low accrual (Clinical Trials.gov identifier: NCT02443103).

A bi-functional macromolecular polybisphosphonate called OsteoDex was in development for treatment of bone metastastatic CRPC. It had shown preclinical efficacy on mammary fat pad tumor xenografts (Alaiya et al., 2014), but the phase IIb clinical trial was terminated due to low accrual (Clinical Trials.gov identifier: NCT02378870).

TGF-β pathway inhibitors are considered mainly microenvironment-targeting due to the pleiotropic effects of TGF-β on cancer cells (Neuzillet et al., 2015). Clinical studies with galunisertib in combination with enzalutamide in prostate cancer (NCT02452008) and fresolimumab in combination with radiotherapy in breast cancer (NCT01401062) are ongoing.

RON tyrosine kinase inhibitor has shown inhibition of cancer-induced osteolysis in preclinical models and a decrease of bone turnover markers in a phase I clinical study (Andrade et al., 2017).

Selected molecules involved in the formation and growth of bone metastases and thus possible targets for drug development are presented in Table 3.

Table 3. Potential new targets for developing therapies to prevent and treat bone metastases. These molecules are important in the bone metastatic process, but some of the targets are very challenging for drug development. Modified from Krzeszinski and Wan (2015) and Esposito and Kang (2014).

Molecular target	Role in bone metastatic process	Reference
Escape from primary tumor		
Snail*	Stimulate EMT and tumor-bone vicious cycle	(Burton et al., 2015)
TWIST1*	Promote breast cancer bone metastases through mir-10b	(Croset et al., 2014)
Notch1	Signature gene for the acquisition of EMT in prostate cancer bone metastases	(Sethi et al., 2010)
Homing to bone		
Tumor cell secreted factors		
CXCR4/SDF-1	Attract cancer cells to HSC niche	(Hirbe et al., 2010, Richert et al., 2009, Smith et al., 2004)
CXCL10/CXCR3	Enhances tumor growth and osteoclast differentiation	(Lee et al., 2012)
Adrenomedulin	Increase osteoclast activity	(Siclari et al., 2014)
TGF-β2, GDF15, FGF3, FGF19, CXCL1, galectins, β2-microglobulin	Highly expressed in the conditioned medium from isolated osteoblastic bone lesion cancer cells	(Lee et al., 2015b)
DDR1	Associate with cancer cell survival and homing on bone	(Valencia et al., 2012)
HDAC4, PITX1, ROBO1	Promote lung adenocarcinoma bone metastases	(Luis-Ravelo et al., 2014)
Canonical WNT/TCF	Enhance both bone and brain metastases	(Nguyen et al., 2009)
HSC niche		
PTH**	Expand HSC niche	(Calvi et al., 2003)
TBK1	Inhibits mTOR function and enhances cancer cell survival	(Kim et al., 2013)
Gas6	Induces cancer cell dormancy	(Shiozawa et al., 2010)
BMP2, 6	Promote osteoblast differentiation, maintain HSC niche and regulate cancer cell dormancy	(Jung et al., 2008, Joseph et al., 2012, Grassinger et al., 2007, Kobayashi et al., 2011)
Bone cell secreted factors		
IGFs	Modulate both bone microenvironment and cancer cells	(Yakar et al., 2010, Hiraga et al., 2012)

Molecular target	Role in bone metastatic process	Reference
PDGFR	Associate with cancer bone homing and osseous colonization	(Catena et al., 2011)
CCL5	Promote cancer cell migration and invasion	(Bai et al., 2014)
Adhesion of tumor cells to bone cells		
cadherin-11	Mediate binding of cancer cells to osteoblasts	(Kawaguchi et al., 2001, Huang et al., 2010, Chu et al., 2008, Tamura et al., 2008)
hAJs	Mediate the interaction between cancer cells and osteogenic cells	(Wang et al., 2015a)
P-selectin	Induce strong adherence to cells expressing CXCR4	(Hedges et al., 2014)
CD44	Bind to ECM and primarily to HA	(Hiraga et al., 2013)
Bone microenvironment and macrometastases		
miR-16, miR-378	Potential circulating biomarkers for osteolytic bone metastases	(Ell et al., 2013)
miR-34a	Inhibit osteoclast differentiation by suppressing Tgif2 and impede cancer progression at both pre- and post-metastatic sites as a p53 target and a CD44 inhibitor	(Hermeking, 2007, Liu et al., 2011, Krzeszinski et al., 2014)
miR-192	Inhibit angiogenesis and decrease osteolytic lesions	(Ell and Kang, 2014)
cathepsin B	Inhibition reduces lung and bone metastasis	(Withana et al., 2012)
c-fms	Osteoclast differentiation	(Ohno et al., 2006)

** transcription factors are difficult to target*

*** contraindication in patients with history of breast or prostate cancer*

3 AIMS OF THE STUDY

This project aimed to clarify the effects of existing drugs and new drug candidates on the bone microenvironment and the tumor growth of breast and prostate cancer bone metastases. The contribution of the microenvironment to tumor growth in various treatment modalities at different disease stages was studied to provide information for drug development aimed at taking advantage of the synergistic effects on tumor cells and bone microenvironment.

The specific aims were:

1. To clarify whether the anti-neoplastic microtubulin stabilizer sagopilone has direct effects on bone cells and whether it can inhibit bone destruction *in vivo*.
2. To study the effects and efficiency of Ra-223 dichloride in osteolytic breast cancer.
3. To clarify the mode-of-action of Ra-223 dichloride in prostate cancer bone metastases using two mouse models.
4. To study whether combining zoledronic acid and doxorubicin for dual inhibition of the vicious cycle in an osteolytic breast cancer bone metastasis model provides synergistic advantages over respective monotherapies.

4 MATERIALS AND METHODS

4.1 *In vitro* assays

4.1.1 Osteoclast assays (I, IV)

In vitro effects of drug candidates on osteoclast differentiation and activity were studied by culturing primary human osteoclast precursor cells (Lonza, Verviers, Belgium) on bovine bone slices. In the differentiation assay, tartrate-resistant acid phosphatase 5b (TRACP 5b) was measured using the BoneTRAP® kit (IDS Ltd, Boldon, UK) after culturing the osteoclast precursors for seven days in differentiating conditions. TRACP 5b in culture media can be used as a marker of osteoclast number (Rissanen et al., 2008). Ra-223 dichloride (50-1600 Bq/ml, Algeta ASA, Oslo, Norway) was added at the beginning of culture. Sagopilone (0.1 – 10 nM) or paclitaxel (0.5 – 20 nM) were also added at the beginning of the culture but were washed off after 2 hours of exposure. OPG (5 nM, PeproTech EC Ltd, London, UK) was used as a reference compound in all differentiation assays.

Osteoclast activity was studied by letting the differentiated osteoclasts resorb bone for three days and measuring CTX-I (Serum Crosslaps ELISA, IDS Ltd. Boldon, UK) from the culture medium. The ratio of CTX on day 10 to TRACP 5b on day 7 represents the activity of each individual osteoclast. A known inhibitor of osteoclast activity, cysteine protease inhibitor trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64, 1 µM, Sigma-Aldrich, St. Louis, MO), was used as a reference compound in osteoclast activity assays. Sagopilone (2.5 – 50 nM), paclitaxel (2.5-50 nM) or Ra-223 dichloride (50-1600 Bq/ml) were added on day 7. To investigate the short-term effects of sagopilone and paclitaxel, the compounds were washed off after 2 hours of exposure on culture day 7. E64 was again used as a reference compound. Cytotoxicity was measured at day 10 using the Toxilight® BioAssay kit (Lonza).

4.1.2 Osteoblast assays (I)

Mouse mesenchymal KS483 cells (Percuros B.V., Enschede, The Netherlands) were used to study the effects of Ra-223 dichloride on osteoblast maturation and bone formation and to clarify the mechanism of

Ra-223 corporation into bone matrix. Assays were performed on collagen-coated 96-well plates (BD, Franklin Lakes, NJ, USA).

Differentiation was studied by culturing the immature osteoblastic cells in the presence of ascorbic acid (50 µg/ml, Fluka, Sigma-Aldrich) and β-glycerophosphate (5 mM Sigma-Aldrich) for eight days. The culture medium was collected and cell lysates were prepared for measuring ALP activity as previously described (Lowry et al., 1954), and total protein (Protein Assay, Bio-Rad Laboratories Inc. Hercules, CA, USA),

Bone-forming activity was studied by continuing the culture for 13 days. On day 11, procollagen type I N-terminal propeptide (PINP, Rat/Mouse PINP EIA kit, IDS Ltd) was measured as a marker of formed organic bone matrix from the culture medium, and on day 13, the amount of calcium deposited was measured from the bone matrix dissolved in HCl (Calcium assay, Roche Diagnostics, Basel, Switzerland). In the differentiation and bone formation assays, Ra-223 dichloride (50-1600 Bq/ml, Algeta ASA) or reference compound 17β-estradiol (E2, 10 nM, Sigma-Aldrich) were added in the beginning of the culture period and always in conjunction with medium change (every 3-4 days).

The incorporation of Ra-223 to the bone matrix was investigated in the bone formation assay performed, as described above, but in the presence of 10 nM E2 to potentiate bone formation. Ra-223 dichloride (100 Bq/ml) was added to the medium on days 0, 4, 8 and 11. On day 13, the culture medium was removed, and the cells were frozen in water at -20°C overnight. After the addition of HCl, the amount of Ra-223 was quantified using the Hidex 300 SL Automatic TDCR Liquid Scintillation Counter (Hidex Ltd, Turku, Finland).

4.1.3 Cancer cell proliferation assay (I)

A proliferation assay using MDA-MB-231(SA) human breast cancer cells (kindly provided by Dr. Theresa Guise) was used to study the effects of Ra-223 dichloride on breast cancer cell growth. Cells were plated on 96-well plates (100 µl/well). After 24 hours, Ra-223 dichloride (50-1600 Bq/ml, Algeta ASA) or the reference compound doxorubicin (1 nM, Sigma-Aldrich) were added. Proliferation was measured in 24-hour intervals using the WST-1 proliferation assay (Roche Diagnostics).

4.2 Animal models

All animal experiments were performed under valid animal experimentation licenses. In subproject I, the experiment was conducted in accordance with the German Animal Welfare Act of 1998, and in subprojects II, III and IV, the licenses were granted by the Animal Experiment Board of Finland and performed according to guidelines of the European Union directive 2010/63/EU. Group sizes were determined based on prior experience of the tumor take rate (where applicable) and the mean and variation of the main parameters in the animal models used.

4.2.1 Mouse ovariectomy (OVX) model (IV)

The effect of sagopilone on the loss of BMD in mice was investigated using the mouse ovariectomy (OVX) model. The 3-month-old C3H/HeN mice were stratified into four groups with a similar body weight (SHAM, OVX, OVX+E2, OVX+sagopilone, n=10). Body weights were determined three times a week. Treatment was started four days after surgical operations with either sagopilone [8 mg/kg, intravenously (i.v.), injection every 14 days] or estrogen [subcutaneously (s.c.) implanted pellet 0.01 mg/60 days; Innovative Research of America, Sarasota, FL, USA]. Six weeks after OVX, the mice were sacrificed, uteri weighed and hind limbs collected.

4.2.2 Osteolytic breast cancer bone metastasis model (I, III)

The efficacy of several therapeutic agents on breast cancer bone metastases was investigated using a model of intracardiac inoculation of bone-seeking breast cancer cells. Triple negative breast cancer MDA-MB-231(SA) cell line is a bone-tropic subline of MDA-MB-231 cells that has been transfected with green fluorescent protein (GFP) (pTurboGFP-N vector, Evrogen JSC, Moscow, Russia). The cells were harvested at 70% confluence, and 10^5 cells in 100 µl of phosphate buffered saline (PBS) were inoculated to the left cardiac ventricle of 4-6-week-old female nude mice (Harlan Laboratories B.V., Horst, The Netherlands). The cells primarily home to bone and induce lytic bone lesions, paraplegia and cachexia within four weeks. The mice were randomly categorized into groups either before inoculation or on day 1 based on body weight or on day 14 based on radiographic bone lesions and body weight. The mice were sacrificed on day 25, or in survival studies, individually, when sacrifice criteria were fulfilled, but on day 50 the latest.

Sacrifice criteria were weight loss over 20%, paraplegia, difficulty breathing or other severe morbidity. Mice were sacrificed with cervical dislocation under anesthesia. They were given analgesics once before the inoculation and for the last 5 days of the study or when needed (0.1 mg/kg buprenorphine s.c. twice a day or 0.02 mg/ml in drinking water). Blood samples for serum marker measurements were collected from the saphenous vein before randomization and on day 24. Terminal blood samples for measuring ionized calcium were drawn by cardiac puncture into heparinized syringes.

Table 4. Experimental set-up of studies performed with breast cancer bone metastasis model.

Study	Random. day	Sacrifice day	Groups	n	Orig. publ.
1	14	25	Vehicle, Ra-223 dichloride[1] doses 300, 600 and 1200 kBq/ml	7	I
2	14	25	Vehicle, Ra-223 dichloride 300 kBq/kg	7	I
3	-1	50 survival	Vehicle, Ra-223 dichloride 300 kBq/kg on days -1, 2 or 15	12	I
		2	Satellite group (inoculated, no treatment)	3	
4	14	50 survival	Vehicle, Ra-223 dichloride 300 kBq/kg, DOX[2] 5 mg/kg, their combination, ZOL[3] 0.1 mg/kg and ZOL combined with Ra-223 dichloride.	9-10	I
5	14	25	Vehicle, DOX 2.5 mg/kg, ZOL 0.1 mg/kg and their sequential combination	8	III

[1] A single i.v. dose of Ra-223 dichloride (Algeta ASA) was administered in all studies.
[2] Doxorubicin (Ebewe Pharma GmbH, Unterach, Austria), dosed intraperitoneally (i.p.) once a week.
[3] Zoledronic acid (Novartis Pharma GmbH, Nürnberg, Germany), a single s.c. dose.

4.2.3 Osteoblastic prostate cancer bone growth model (II)

The efficacy of Ra-223 dichloride on the growth of prostate cancer cells in bone and the formation of osteoblastic lesions were studied in models of intratibial inoculation of LuCaP 58 (University of Washington, Seattle, USA) and LNCaP (ATCC, Manassas, VA, USA) prostate cancer cells into male SCID (Charles River, Germany and Harlan, Italy) and NOD SCID mice (NOD.CB17-Prkdcscid/NcrCrl, Charles River, Germany), respectively. LuCaP 58 is a serially transplantable tumor, originating from the rapid autopsy series of the University of Washington. LNCaP is a widely used and well-

characterized cell line originating from a lymph node metastasis. For LuCaP 58 intratibial inoculations, subcutaneous tumors were harvested and processed into single cell suspension as described above (Corey et al., 2002). In both models, $2x10^6$ cells in 20µl of PBS were inoculated into the tibia of 6-8-week-old immunocompromised male mice. Six to nine weeks after the intratibial inoculation, the mice were stratified into groups with a similar serum PSA and mean radiographic lesion area and treated for 6 weeks. Blood samples and X-ray images were obtained before randomization and every second week thereafter. Mice were sacrificed with cervical dislocation after CO_2 asphyxiation. They were given analgesics for 2 days in conjunction with the intratibial inoculation and for the last 5 days of the study or when needed (0.1 mg/kg buprenorphine s.c. twice a day or 0.02 mg/ml in drinking water).

Table 5. Experimental set-up of studies performed with prostate cancer bone growth models.

Study	Cells	Random. week	Time from dose to sacr.	Groups	n	Orig. publ.
1	LNCaP	6	6 weeks	Vehicle, Ra-223 dichloride[1]	13-14	II
2	LuCaP 58	7 or 9	24, 48, 72h	Vehicle, Ra-223 dichloride	12	II
3	LuCaP 58	7 or 9	6 weeks	Vehicle, Ra-223 dichloride	15-17	II
4	LuCaP 58	7 or 9	6 weeks	Vehicle, Ra-223 dichloride, abiraterone[2]	17	II

[1] Ra-223 dichloride (Institute for Energy and Technology, Oslo, Norway) dose in all studies was 300 kBq/kg i.v. every 4 weeks.
[2] Abiraterone (Janssen Biotech, Horsham, PA, USA) dose was 200 mg/kg by p.o. daily.

4.2.4 Serum marker and blood Ca^{2+} measurements (I, II, III)

Blood samples were processed into serum and stored at -70°C, except for the terminal whole blood samples. Resorption marker TRACP 5b was measured in breast cancer bone metastasis studies using the MouseTRAP® ELISA kit (IDS Ltd). Hypercalcemia was detected by measuring ionized calcium (Ca^{2+}, corrected to pH 7.4 by the internal algorithm of the instrument) in whole blood using the ABL835 Flex blood gas analyzer (Radiometer Medical ApS, Bronshoj, Denmark) immediately after sacrifice. In the prostate cancer studies, bone formation marker PINP was measured using the Rat/Mouse PINP EIA kit (IDS Ltd), and tumor growth followed by

measuring PSA using Quantikine Human Kallikrein 3/PSA ELISA kit (R&D Systems).

4.2.5 X-ray and GFP imaging methods (I, II, III)

Osteolytic and osteoblastic lesions were detected using X-ray imaging. The mice were X-rayed in the prone position with Faxitron Specimen Radiographic system MX-20 D12 (Faxitron Bioptics LLC, Tucson, AZ, USA), and the images were analyzed for the number and area of lesions in both hind limbs using MetaMorph™ image analysis software (Molecular Devices, Sunnyvale, CA, USA).

The tumor burden by GFP-expressing cells was quantitated using GFP imaging. The fluorescence emitted by the MDA-MB-231(SA)-GFP cells was detected using the LT 9 GFP-imaging system LT-MACIMSYSPLUSC (Lightools Research, Encinitas, CA; excitation wavelength 470/40 nm band-pass, emission 515 nm cut-off). The exposure time was 2.34 s, gain 6.7 and offset 238. From the images gained, the fluorescent area was determined by using MetaMorph™ image analysis software (Molecular Devices).

4.2.6 *Ex vivo* pQCT and microCT measurements (II, IV)

The BMD in the tibia of ovariectomized mice was measured using peripheral quantitated tomography (pQCT, X-CT Research SA+, Stratec Medizintechnik, Pforzheim, Germany). Metaphyseal BMD was measured 1.6 mm distally from the articular surface, and diaphyseal (cortical) BMD 10 mm from the articular surface of excised tibiae. Total BMD, trabecular BMD from metaphysis and cortical BMD from diaphysis were determined.

Bone volume and architecture of the tumor-bearing tibiae were determined using micro-computed tomography (μCT, Skyscan 1072 scanner, Bruker, Kontich, Belgium) and CTAn analysis software (Bruker). The measurement started 0.2 mm below the growth plate and extended 6 mm distally. The used voxel size was 7 μm. Trabecular and cortical bone volumes could not be assessed separately due to the severe destruction of normal bone architecture. Thus, only total bone was analyzed using the lower intensity threshold of 80.

4.2.7 Histological methods (I, II, III)

Formalin-fixed bone samples were decalcified in 10% EDTA for two weeks and processed into paraffin blocks. Midsagittal 4 µm sections were cut and stained with hematoxylin-eosin (HE), Masson-Goldner Trichrome (MGT) and for TRACP activity. Tumor, necrotic tumor and bone areas were measured, and the number of osteoblasts was counted from MGT-stained midsagittal sections. Osteoclasts were counted from the tumor-bone interface as TRACP-positive multinucleated cells at bone surface using the DM4000 B Leica Research Microscope (Leica Microsystems, Wetzlar, Germany) with 20x objective, normalized for tumor-bone interface length.

The amount of DSBs was analyzed by counting positive tumor cells in immunohistochemical staining for γ-H2AX molecules (JBW301, Millipore). For analysis of DSBs in osteoclasts, a double staining for TRACP activity and γ-H2AX molecules was performed. DSB positive osteoblasts were analyzed from the same sections. Apoptotic cells were counted as TUNEL-positive cells with apoptotic morphology. Analyses were performed using the Leica DM4000 B Research Microscope with 40x objective. Proliferation of tumor cells was analyzed by counting Ki-67 positive cells using the ImageJ open source programme (https://imagej.net). The staining and analyses were performed at BioSiteHisto (Tampere, Finland).

The presence of breast cancer cells in bone marrow 2 days after intracardiac inoculation was examined in 18 bone sections by immunohistochemical stainings of pancytokeratin (clones AE1/AE3, Dako, Glostrup, Denmark) and GFP (rabbit polyclonal primary antibody against denatured TurboGFP, Evrogen).

Autoradiography of plastic-embedded histological sections was performed in order to observe the location and range of alpha-particles. Briefly after plastic removal, the slides were dipped into an Ilford K5 emulsion (Polysciences Inc., Warrington, PA, USA) and the exposure was carried out for three days in a light-protected box. After stopping and fixing the autoradiography reaction, the slides were counterstained with MGT.

4.3 Statistical methods

The data were analyzed using SPSS (IBM) and R (www.r-project.org). All statistical analyses were performed as two-sided tests, and p-values smaller than 0.05 were considered significant.

Datasets with only one data point per sample were first evaluated for normality and homogeneity of variance. When necessary, either log, square root or reciprocal transformation was applied. In case of a comparison of two groups, Student's t-test was used, and in case of multiple groups, analysis of variance (ANOVA) was utilized. When the ANOVA result was statistically significant, a suitable post hoc test, such as Tukey's HSD or Dunnett test, was performed. If the data was unfit for parametric tests, rank-transformation was applied, and the non-parametric Kruskal-Wallis test followed by a Mann-Whitney *U*-test for pairwise comparisons were utilized.

The frequency data were analyzed using the Fischer exact test, and the time to euthanasia was analyzed by Kaplan-Meier survival analysis (log-rank test).

Datasets with multiple datapoints per sample, such as body weight and serum markers, were analyzed using either fixed or mixed models (estimated using R package nlme). Comparisons between groups at specific time points were examined using model contrasts, adjusting the p-values for multiple comparisons when necessary.

5 RESULTS AND DISCUSSION

5.1 Efficacy and mode-of-action of Ra-223 in osteolytic breast cancer (I)

Radium is a calcium-mimetic and naturally bone-seeking as it binds to hydroxyapatite. As a refinement of the known bone-seeking properties, active incorporation by osteoblasts and accumulation in the growth plate regions had been suggested (Henriksen et al., 2002, Salmon et al., 1999). At the time of these studies, the alpha-emitting Ra-223 had shown promising results and a favourable safety profile in phase II clinical studies in prostate cancer patients. However, the effects and mode-of-action in osteolytic metastases were not clear. Furthermore, because of the favourable safety profile, there was a growing interest in possible combination therapies. Another aspect resulting from the favourable safety profile is the prospect of a preventive treatment in patients at high risk for bone metastases. Because bone metastases are still incurable, being able to prevent their occurrence is highly desirable. The disseminated tumor cells have been shown to localize in the HSC niches close to endosteal bone surface (Wang et al., 2014). Dosimetric calculations of Ra-223 show high and localized radiation dose within approximately 70 μm range from bone surface (Henriksen et al., 2003). This range covers the 40 μm distance of most disseminated tumor cells from the endosteal surface (Wang et al., 2014). Thus, we aimed to study the mode-of-action of Ra-223 dichloride as well as it's efficacy in preventive and treatment settings in an osteolytic breast cancer bone metastasis model.

5.1.1 Ra-223 dichloride inhibits osteoclast and osteoblast differentiation and cancer cell proliferation

The hypothesis of active incorporation of Ra-223 in newly formed bone was tested in a 13-day culture of mineralizing osteoblasts. Furthermore, the effects of Ra-223 dichloride on bone cells were studied by differentiating human osteoclast precursors and mouse osteoblast precursors in the presence of Ra-223 dichloride. Also, the activity of mature osteoclasts and osteoblasts was studied. Finally, the efficacy of Ra-223 dichloride on inhibiting proliferation of MDA-MB-231(SA) cells was tested in a simple, 5-day proliferation study. In the cancer cell proliferation assay it was found that Ra-223 dichloride inhibited the proliferation of a MDA-MB-231(SA)

cells dose dependently on 400, 800 and 1.600 kBq/ml concentrations (I: Figure 1). In the osteoblast activity assay the Ra-223 incorporation to bone by osteoblasts was confirmed by measuring the radioactivity of the newly formed bone matrix. Furthermore, Ra-223 dichloride was found to stimulate osteoblast activity on low concentrations (100 and 200 kBq/ml, I: Figure 2 B and C). However, on concentrations 800 and 1600 kBq/ml, Ra-223 dichloride decreased osteoblast activity (I: Figure 2 B and C) and it also inhibited the differentiation of osteoblasts on the same doses than cancer cell proliferation was inhibited: 400 kBq/ml and higher (I: Figure 2 A). Ra-223 dichloride dose dependently inhibited the differentiation of osteoclasts on all tested doses (I: Figure 2 D), but it did not affect the resorption activity of mature osteoclasts (data not shown).

The increase in osteoblast activity at low doses is likely comparable to adding calcium, which is known to stimulate osteoblasts (Yamaguchi et al., 1998, Ahlstrom et al., 2008). The inhibitory activity observed in both osteoblast and osteoclast differentiation as well as in cancer cell proliferation suggested that Ra-223 could be efficacious in both osteoblastic and osteolytic lesions. Having activity in both lesion types is clinically meaningful, because patients very often have both types of lesions present. These results warranted further preclinical testing.

5.1.2 Ra-223 dichloride inhibits the progression of established tumors and osteolytic lesions

In a dose-finding study in tumor-bearing animals, Ra-223 dichloride doses 300, 600 and 1200 kBq/kg were tested; the 300 kBq/kg dose was found as effective as the higher doses (data not shown). To test the efficacy on established bone metastases, when the osteolytic lesions were visible, we stratified the mice into two groups with a similar body weight and osteolytic lesion score. The occurrence of tumor-associated cachexia, determined by weight loss ≥20%, curved spine and/or dehydration, was totally prevented and the onset of weight loss was delayed in the Ra-223 dichloride treated group. At sacrifice, Ra-223 dichloride was found to effectively reduce bone destruction: the osteolytic lesion area in X-ray images was smaller, and the trabecular bone area in histological sections was higher (I: Figure 3 C, E, D and F). Also, whole-body tumor burden analysed by fluorescence imaging was lower (I: Figure 3 B). The bone protection was at least partly due to the decreased number of osteoclasts (I: Figure 4 A and B). The decreased tumor burden could be a result of the disruption of the vicious cycle, but direct anti-

tumor effects were observed as increased number of double-strand break positive tumor cells (I: Figure 4 D, E).

Ra-223 dichloride was very effective in decreasing bone destruction and fully prevented cachexia. Cachexia is closely linked to tumor growth in bone in this model and it does not occur when the MDA-MB-231 tumor grows orthotopically in the mammary fat pad (Regan et al., 2017). Furthermore, also tumor burden was decreased. One important consideration is the age of the animals; because the mice used in this model were growing fast, the active bone formation might result in a higher Ra-223 content in bone compared to a clinical situation with osteolytic lesions. However, the binding of Ra-223 to the revealed hydroxyapatite on eroded surfaces may also occur. Clinical evidence for the latter was recently reported when the co-localization of Ra-223 and osteolytic lesions in a breast cancer patient was observed (Costa et al., 2018). These results confirmed the dual effect on both bone resorption and tumor growth *in vivo*, and the efficacy of Ra-223 dichloride in an osteolytic setting.

5.1.3 A survival study with combination treatments

A survival study was run to confirm that the observed effects on bone lysis and tumor burden actually convert to increased survival. In addition, the possibility of combination treatments was explored. To test different types of combination treatments preclinically, two additional compounds, doxorubicin and zoledronic acid, were included in the study as monotherapies and in combination with Ra-223 dichloride. Based on our previous studies, doxorubicin decreases tumor burden but not osteolytic area in this model when measured at day 25, whereas zoledronic acid decreases osteolytic area but not tumor burden (unpublished data). The median survival time in the control group was 25 days, and Ra-223 dichloride as monotherapy increased the median survival to 29 days ($p<0.05$). Doxorubicin or zoledronic acid did not increase survival as monotherapies, but in combination with Ra-223 dichloride they increased the median survival to 32 and 31 days, respectively (both $p<0.001$ vs control) (I: Figure 7). However, the increase of survival in the combination groups was not significant when compared to Ra-223 dichloride monotherapy.

The results were in line with the only previously published preclinical data of increased survival in Ra-223 treated nude rats with bone metastases

(Henriksen et al., 2002). Bisphosphonates and denosumab are currently used in prevention of SREs in breast cancer patients, but they do not increase overall survival. In this study, Ra-223 dichloride increased the survival of mice with bone metastases, while zoledronic acid did not have an effect on survival. These results suggest that Ra-223 dichloride could be efficacious in the treatment of breast cancer patients with osteolytic lesions, and that combining Ra-223 dichloride treatment with chemotherapy or a resorption inhibitor is well tolerated, feasible and might be beneficial.

5.1.4 Early administration of Ra-223 dichloride prevents the occurrence of bone metastases

The effect of Ra-223 dichloride treatment before inoculation of the cancer cells or at the micrometastatic stage was examined in the intracardiac MDA-MB-231(SA) breast cancer model. The presence of breast cancer cells in bone marrow two days after intracardiac inoculation was confirmed with IHC stainings against pancytokeratin and GFP of 18 sections altogether out of three control animals. Tumor cells were found in all sections (I: Supplementary Figure 3). When mice were sacrificed 25 days after the inoculation of the cells, we did not find any tumor growth, only single cells in the histological sections of femur and tibia of animals dosed with Ra-223 dichloride one day before or two days after cell inoculation (I: Figure 5 C). In a survival study with the same dosing schedule, we found that the preventive dosing increased median survival from 24 days in the control group to 39 days, and dosing in the micrometastastic stage to 36 days (both $p<0.001$) (I: Figure 6). Three animals in the preventive group survived the maximum length of the study, 50 days. Two of these mice had only minimal residual disease based on the fluorescence imaging, and no tumors were found in any of the three animals in histological sections of the hind limbs.

These results provide evidence that early treatment with Ra-223 dichloride is able to clear micro deposits of cancer cells from the vicinity of bone, as proposed by Henriksen et al. (2003). However, an increased risk for secondary malignancies is the major factor preventing the administration of Ra-223 dichloride to patients at a high risk of developing bone metastases. The follow-up of almost 900 patients treated with radium-224 in 1945-1955 for tuberculosis or ankylosing spondylitis revealed an increase of several secondary malignancies (Nekolla et al., 2010, Spiess, 2010). The received doses were high, with a maximum of 5 MBq/kg (Chmelevsky et al., 1988). In another study including ankylosing spondylitis patients from 1948 to 1975

treated with a lower dose (170 kBq/kg, weekly for 10 weeks), an increase in solid tumors was not observed, but a significant increase in leukemia and myeloid leukemia was noted (Wick et al., 1999, Wick et al., 2008). Ankylosing spondylitis typically manifests in the early twenties, also reflected in the division of the age of exposure to two classes, under and over 20 years of age, in the reports. However, since the target population of cancer patients is markedly older, and the observed latency times were quite long, the increase in risk of developing leukemia might be smaller than reported by Wick et al. for radium-224. In addition, the Ra-223 dichloride dose used for cancer patients is 55 kBq/kg in four-week intervals (maximum of 6 cycles), resulting in a much lower total exposure. Furthermore, due to the longer half-life and higher retention of decay products in bone, Ra-223 is likely to deliver a smaller fraction of the total radiation dose to soft tissues (Henriksen et al., 2003). Nevertheless, better prognostic factors on who will develop overt bone metastasis are needed to enable further consideration of preventive Ra-223 dichloride treatment.

5.2 Efficacy and mode-of-action of Ra-223 dichloride in osteoblastic prostate cancer (II)

Bone metastases with and without SREs are linked to poor survival in prostate cancer (Nørgaard et al., 2010). Bone targeted treatments zoledronic acid and denosumab decrease SREs, but even though denosumab also increases the bone metastasis free survival, it does not affect the overall survival (Smith et al., 2012). Thus, more drugs especially designed to target the bone metastases are needed. In study II, we showed that Ra-223 is actively incorporated into mineralizing bone by osteoblasts *in vitro*. Because prostate cancer forms predominantly osteoblastic bone metastases, Ra-223 was expected to effectively target those lesions. To support the then ongoing clinical studies, the effects and mode-of-action of Ra-223 dichloride were studied in two models of prostate cancer growth in bone.

5.2.1 Mode-of-action in prostate cancer

When studying the time course of changes induced by the Ra-223 dichloride treatment in the LuCap 58 model, we found that double-strand breaks in tumor cells were increased already 24 hours after the administration of Ra-223 dichloride to tumor-bearing animals, and the amount of DSBs was on the same level in 48- and 72-hour time points (II: Figure 5 E). These effects

were also reflected in the serum PSA level, which was decreased 72 hours after dosing (II: Figure 5 C). In osteoblasts, DSBs also increased 24 hours after administration and were somewhat decreased, although still significantly elevated, at 72 hours, whereas in osteoclasts, the increase of DSBs was observed later – 72 hours after administration (II: Figure 5 F-G). Alpha-particles were found to localize mainly in bone and osteoblasts, but also some tumor cells (II: Figure 5 A).

A recent study investigated the distribution of Ra-223 in humans and prostate cancer mouse models and reported that Ra-223 localizes in areas of active bone metabolism and not the tumor itself (Abou et al., 2016). Our findings support this result in showing that most alpha-particles reside in or in close contact to bone. However, since the technique we utilized enabled the localization of alpha-particles and their tracks on the cell level, we were also able to observe alpha-particles in prostate cancer cells. Thus, the dual mode-of-action of Ra-223 dichloride in osteoblastic prostate cancer bone metastases was confirmed.

Alpha-particles can also have an anti-angiogenic mode-of-action (Akabani et al., 2002, Zhu et al., 2010). Even though Ra-223 dichloride is not targeted to vasculature, it is possible that the delivery to sites of bone formation will also result in anti-angiogenetic effects, especially when taking into account the intimate relationship of osteo- and angiogenesis.

5.2.2 Efficacy in osteoblastic prostate cancer models

Two intratibial prostate cancer models with osteoblastic lesions were successfully set up. Approximately 45% of animals inoculated with LuCaP 58 cells reached the inclusion limit (serum PSA at least 1 ng/ml) 7-9 weeks after inoculation, whereas approx. 65% of animals inoculated with LNCaP cells were included in study based on bone lesion score and serum PSA.

The effects of Ra-223 dichloride on tumor-induced osteoblastic bone formation were investigated measuring bone formation marker PINP from serum every second week. After sacrifice, the bone volume and architecture were studied by microCT. We found that in both models, Ra-223 dichloride at dose 300 kBq/kg given twice with a 4-week interval reduced serum PINP and bone volume of the tumor-bearing tibia (II: Figure 2). For further investigation, mid-sagittal histological sections were prepared, and the number of osteoblasts and osteoclasts was counted. Ra-223 dichloride was

found to decrease the number of osteoblasts (II: Figure 3 F and G). Ra-223 dichloride also decreased the number of osteoclasts at tumor-bone interface in the LNCaP model but not in the LuCaP 58 model (II: Figure 3 H and I). However, the length of tumor-bone interface was decreased in both models (II: Figure 1 G and H), and thus, the total number of osteoclasts was decreased also in the LuCaP 58 model (data not shown), reflecting the overall reduction in tumor and bone volume.

To follow the Ra-223 dichloride effects on tumor burden, serum PSA was measured every second week, and finally, the tumor area was measured from histological sections. In addition to changes in bone, Ra-223 dichloride also decreased tumor growth in both models as measured by serum PSA (II: Figure 1 A and B). The tumor area in histological sections was decreased in the LNCaP model, but the decrease in the LuCaP 58 model did not reach statistical significance ($p=0.098$) (II: Figure 1 C and D). Very interestingly, macroscopically visible metastases were observed in the LuCaP 58 inoculated mice at sacrifice, mostly in the kidneys and liver. These visceral tumors were observed in 9/17 (53%) and 6/18 (33%) of vehicle and Ra-223 dichloride-treated mice, respectively. Since the tumor growth in bone was already established at the time of treatment, we wanted to see whether animals with high or low tumor burden at the time of treatment had a different rate of visceral metastases. Thus, we stratified animals according to serum PSA being under or over 5 ng/ml at the time of treatment start. In the vehicle group, 5/10 animals with PSA under 5 ng/ml had visceral tumors, while in the Ra-223 dichloride treated group, only 2/10 animals had visceral tumors. However, the difference was not statistically significant (II: Figure 4).

The proportion of mice with established LuCaP 58 tumors in bone (45%) was not optimal for preclinical efficacy studies, but it did resemble the clinical situation, where according a study of Morgan et al, 48% of prostate cancer patients positive for DTCs after radical prostatectomy develop biochemical recurrence (Morgan et al., 2009).

In the analyses of the bone effects, the decrease of osteoblasts suggests that the effects of Ra-223 dichloride on PINP and bone volume occurred through inhibition of osteoblast differentiation. This is in line with *in vitro* results in study II, showing decreased osteoblast differentiation with higher doses of Ra-223 dichloride. Due to accumulation in bone, the radiation dose received by precursor cells in close proximity to bone can be high (Henriksen et al.,

2003). Similar to its effects in the breast cancer model, Ra-223 dichloride also decreased the number of osteoclasts at tumor-bone interface. However, this effect was observed only in the LNCaP model and surprisingly not in the LuCaP 58 model. This difference might be due to the overall severity and stronger bone changes observed in the LuCaP 58 model. Still, overall reduction in the pathological bone changes by Ra-223 dichloride was very clear in both models.

In the analyses of the effects on tumor growth, Ra-223 dichloride decreased the serum PSA and the total tissue area in both models and the tumor area in the tibia in the LNCaP model. Because only one section of each sample was analysed, the tumor area measurement may not represent the tumor burden as well as the serum PSA does. Furthermore, any extraosseous and visceral tumor growth would have also contributed to the serum PSA. When the presence of visceral tumors was stratified according to high or low PSA at randomization, the mice treated with Ra-223 dichloride when PSA was still low had the smallest incidence of visceral tumors. However, the possibility that Ra-223 dichloride could inhibit secondary metastases from bone when administered early enough needs further investigation.

Part of the efficacy of cytotoxic therapies, such as chemo- and radiotherapy, is due to the formation of neo-antigens, which can enhance the host's immune reaction against the tumor. Ra-223 dichloride was also recently shown to induce T-cell mediated killing of several cancer cell types (Malamas et al., 2016). However, because immunocompromised mice were used in these models, any T- or B-cell mediated responses could not be investigated. Further investigation in either syngeneic or humanized prostate cancer models with tumor growth in bone would be warranted. An ongoing phase 2 clinical study with sipuleucel-T and Ra-223 dichloride will provide more information on the usefulness of the combination (ClinicalTrials.gov identifier NCT02463799).

5.3 Combination of doxorubicin and zoledronic acid in established bone metastatic breast cancer (III)

Zoledronic acid is a potent inhibitor of bone resorption and very useful in treatment of CTIBL as well as bone metastases. In the latter situation, zoledronic acid decreases bone pain and time to first skeletal-related event, although it does not increase OS (Coleman et al., 2014a). There is some

preclinical evidence that the sequence of zoledronic acid and chemotherapy is important, and with the right timing, zoledronic acid can potentiate the effects of the chemotherapy regimen, both in the bone environment and in the primary tumor (Ottewell et al., 2008a, Ottewell et al., 2009, Ottewell et al., 2010, Ottewell et al., 2008b). Furthermore, a retrospective clinical study in a neoadjuvant setting also had reported benefits of sequential combination in a subgroup of patients (Coleman et al., 2010). No additive effects on the tumor could be observed in clinical trials testing a combination treatment in a neoadjuvant setting (but not the sequential combination) in stage II/III breast cancer (Charehbili et al., 2014, Hasegawa et al., 2015, Horiguchi et al., 2013).

This study aimed to test the possible additive or synergistic effects of a sequential combination treatment with doxorubicin and zoledronic acid on established MDA-MB-231(SA) bone metastases. We found that doxorubicin increased the number of apoptotic cells in the tumor compared to control (III: Figure 2 E), but was not sufficient to reduce the tumor burden, as analysed by fluorescence imaging and histology (III: Figures 1 C and 2 D). Zoledronic acid, on the other hand, decreased osteolytic lesion area analysed from X-ray images and increased trabecular bone area compared to control (III: Figures 3 B and C). In addition, ionized calcium levels in the blood and serum TRACP 5b activity were lower than in the control group (III: Figures 4 C and D). However, zoledronic acid did not affect tumor burden or the number of apoptotic tumor cells (III: Figure 2 E).

These results suggest that the sequential combination treatment may not be effective in very aggressive tumor types. Our model is markedly faster than the MDA-MB-231/BO2 and MDA-MB-436 models, lasting 6-9 weeks compared to the 25 days in the MDA-MB-231(SA) model (Ottewell et al., 2008a, Ottewell et al., 2009). In established MDA-MB-231/BO2 tumors, doxorubicin reduced the tumor area, although no increase in caspase-3 positive cells was observed (Ottewell et al., 2008a). We have observed that 2.5 mg/kg doxorubicin is able to decrease tumor burden quantitated by fluorescence imaging in the MDA-MB-231(SA) model, when the dosing was started before the tumors had been established (Käkönen et al., 2008). However, this dose was considered low in the established setting, but chosen in order to best observe the possible additive and synergistic effects. The notion that a single clinically relevant dose of zoledronic acid protects bone but does not affect tumor burden or survival is in line with several studies (Daubiné et al., 2007, Ottewell et al., 2008a, Suominen et al., 2013).

In the combination group, the effects of both doxorubicin and zoledronic acid were observable, but additive or synergistic effects were not observed in any of the parameters. The striking difference between the BO2 and (SA) sublines of MDA-MB-231 cells is intriguing, though not unexpected. Marked differences in speed and frequency of metastases as well as metastatic sites have been observed between MDA-MB-231 subpopulations (Kang et al., 2003). In this study, sequentially combining zoledronic acid and doxorubicin did not produce any synergistic effects; whole-body tumor burden stayed at the level of doxorubicin monotherapy and osteolytic lesion area at the level of zoledronic acid monotherapy. The results show that once established, the MDA-MB-231(SA) cells are capable of growing in the bone microenvironment even when the vicious cycle has been disrupted by inhibiting resorption. The results do not provide any evidence of direct anti-tumor effects of bisphosphonates in this model, but simultaneously highlight the divergence of the two sublines (SA) and BO2 originating from the same parental cell line MDA-MB-231.

5.4 Effect of sagopilone on bone (IV)

CTIBL is a significant clinical problem, especially in cancers treated with hormone deprivation therapy (Saad et al., 2008). Furthermore, chemotherapy-induced ovarian failure may result in adverse bone effects (Hadji, 2015). CTIBL may also have consequences beyond the increased fracture risk: In preclinical models increased bone resorption has been shown to increase the incidence of bone metastases and tumor growth in bone. Earlier studies had shown that anti-neoplastic, microtubule-stabilizing new epothilone called sagopilone was very effective in several tumor models, including a breast cancer bone metastasis mouse model (Strube et al., 2009, Hammer et al., 2010, Hoffmann et al., 2008, Klar et al., 2006). In the bone metastasis model, the cues of direct bone effects were observed, but in the animal model, it was not possible to definitively distinguish whether the beneficial effects were solely due to decreased tumor burden, or whether direct effects also played a role. However, preliminary *in vitro* studies indicated direct effects on osteoclast activity (Strube et al., 2009). These interesting findings led to further investigation of sagopilone bone effects in an osteoclast culture *in vitro,* and an *in vivo* model of estrogen deficiency –induced bone loss.

In the *in vitro* studies, sagopilone was compared to paclitaxel, a standard chemotherapy drug with the same mode-of-action. The *in vitro* results

confirmed direct effects on bone cells. Osteoclast activity was inhibited in physiologically relevant concentrations (IV: Figure 1 A-B). Furthermore, since the clearance of sagopilone from blood is significantly more rapid in mice than in humans, the effects of a short-term treatment of only two hours were also studied. The short-term treatment resulted in an inhibitory effect on osteoclast differentiation but not on activity (IV: Figure 1 E-F). Sagopilone showed less cytotoxicity and more inhibition of activity in osteoclast cultures than paclitaxel (IV: Figure 1 C, D, G and H). The biological relevance of this inhibition was studied *in vivo* by treating ovariectomized mice with sagopilone for six weeks. Ovariectomy caused a massive decrease of trabecular BMD, which was totally prevented and even over-compensated by estradiol dosing, when compared to the SHAM group (IV: Figure 2). Sagopilone was able to significantly inhibit the trabecular bone loss induced by OVX, even though the BMD of sagopilone-treated mice was not very close to the level of the SHAM group (IV: Figure 2).

The inhibition of osteoclast differentiation and activity might be due to inhibition of non-mitotic functions of microtubules, such as exocytosis, polarization and adhesion. Indeed, adhesion of endothelial cells is impaired by paclitaxel (Davis et al., 2005). Tight adhesion to the bone matrix is essential for the formation of the resorption lacuna, and exocytosis is necessary in delivering the lysosomal proteases to the lacuna (Zhao, 2012). These results confirmed that sagopilone has direct inhibitory effects on osteoclast resorption and thus also a dual effect in bone metastases. The bone protective effects observed in the OVX model were statistically significant, but did not fully prevent the bone loss. However, given the fact that many cancer treatments are detrimental to bone, these results strengthen the status of sagopilone and other compounds with similar mode-of-action as a drug candidate and support its further development.

6 CONCLUSIONS

In this thesis work, several treatment options for bone metastases of breast and prostate cancer were studied in preclinical models. The efficacy and mode-of-action of new drug candidates, as well as synergistic effects of existing treatments were investigated. Based on these experiments, the following conclusions can be drawn.

1. The efficacy of Ra-223 dichloride in osteolytic breast cancer model further supports the development of Ra-223 dichloride in the treatment of breast cancer bone metastases.
2. Ra-223 dichloride was shown to have a dual mode of action, affecting bone cells both in osteolytic and osteoblastic settings and inducing DSBs in tumor cells. These dual effects that interrupt the vicious cycle in multiple pathways were able to reduce tumor burden in bone in breast and prostate cancer metastases and increase the survival of mice with breast cancer bone metastases.
3. The efficacy of Ra-223 dichloride in preventive and micrometastatic settings warrants further research into the possible use of Ra-223 dichloride for breast and prostate cancer patients at a high risk of bone metastases.
4. The combination of doxorubicin and zoledronic acid in an aggressive bone metastasis model did not produce any synergistic effects. It suggests that a disruption of the vicious cycle may not be enough to restrain the growth, and that MDA-MB-231(SA) tumor cells were not sensitive to the cytotoxic agent used.
5. The anti-resorptive effects of sagopilone emphasize the possibility and importance of developing anti-cancer treatments with simultaneous bone-protective effects.

Taken together, these studies highlight the importance of studying the effects of cancer treatments on tumor growth in the bone microenvironment and also on the bone itself in order to develop the most effective therapies to treat and also possibly to prevent bone metastases. The clinical significance of these results lies in the observation that even when both aspects, tumor and bone, were covered, established bone metastases could not be cured. The only way to eradicate bone metastases would seem to be the prevention of the homing or colonization processes.

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