Imaging in pre-clinical cancer research : applied to bone metastases

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General Introduction

Breast Cancer; a Perspective

Over the last decades, the prognosis of breast cancer has been much improved (Figure 1.1). For instance, the five year survival rate based on all cases registered by the United States Surveillance Epidemiology and End Results (SEER) program in 2001–2007 was 89% compared to 60% in the 1950s.[1] Based on the same SEER data, approximately 12% of the women born in the U.S. today will eventually develop breast cancer during their lifetime.

The chance of survival depends strongly on the stage of the disease at the moment of diagnosis. The 5 year survival rate of breast cancer patients with localized disease is 98% compared to only 23% for patients with distant metastases. This shows that a large number of patients carrying distant metastases cannot be cured. Consequently, treatment of these patients is mainly palliative, aimed at prolonging life and improving the quality of life.[1,2]

Autopsy revealed bone metastases in approximately 70% off all patients who died of breast cancer.[3,4] This preference of breast cancer to metastasize to bone, a characteristic shared with prostate cancer, has already been noted by Stephen Paget in 1889. As a metaphor describing this characteristic he wrote that "When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil".[5] This so called seed and soil hypotheses still holds true today, be it slightly rephrased to fit present day scientific knowledge.

Bone metastases are especially difficult to treat due to a strong positive feedback loop between the tumor and the bone micro-environment.[6] Tools to follow treatment response in a pre-clinical setting of both tumor and bone related processes such as tumor growth, angiogenesis, expression of enzymes and signaling molecules, osteolysis and bone formation are needed in research towards better treatment of bone metas-

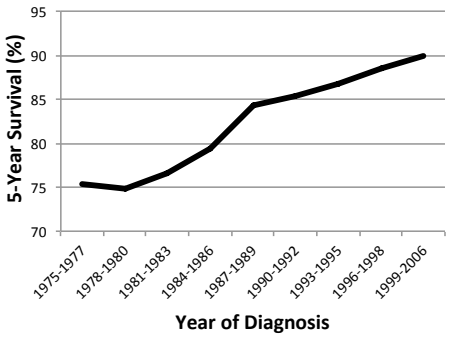

Figure 1.1: Five-year survival of breast cancer patients by year of diagnosis. The five-year survival of all female breast cancer patients has been steadily increasing over the last decades. This can be explained by better treatments on one hand and earlier diagnosis due to extensive mammography screening on the other hand.[1]

tases. Only approaches that are capable of following all of these processes will enable a researcher to get a complete understanding in disease progression and treatment efficacy.

Molecular imaging has become one of the main tools in cancer research. The possibility to perform both structural and functional imaging make molecular imaging modalities an attractive research tool. The integrated data handling of different imaging modalities and their possible role in cancer research are discussed within this thesis. The described approaches have been applied in the evaluation of a new compound, ENMD-1198, as possible beneficial compound in the treatment of bone metastases in a pre-clinical mouse model.

Metastatic Bone Disease

Before evaluating the use of various imaging approaches in the field of bone metastases research, it is important to have a general understanding of the biology and pathophysiology of this specific type of metastases. Both breast and prostate cancer have a strong preference to metastasize to bone. In the bone micro-environment, breast cancer is more likely to result in osteolytic lesions while prostate cancer results mainly in osteoblastic lesions, but also mixed lesions exist in some cases.[7,8] The complications caused by bone metastasis are vast; osteolytic lesions may result in severe bone pain, fracture, life-threatening hypercalcaemia and nerve compression, whereas, osteoblastic lesions can result in severe bone pain or fracture due to the reduced quality of the bone.

There is a multitude of crucial processes during bone metastatic growth. These include tumor growth and tumor–stroma interactions by direct contact and through signaling molecules (reviewed by Lorusso *et al.*[9] and Mundy[10]). The interactions and signaling between the tumor and its direct surroundings result in local pro-angiogenic signaling (reviewed by Voorzanger-Rousselot *et al.*[11] and Guise *et al.*[12]), local activation and infiltration of the innate immune system and local suppression of the adaptive immune system (reviewed by Lin *et al.*[13]). All of these processes have a positive feedback on tumor growth. Moreover, the skeletal metastatic sites are often characterized by a distortion of the delicate balance in bone turnover leading to osteolytic and/or osteoblastic lesions at the metastatic tumor site.

A Vicious Cycle

The bone matrix holds an abundant store of growth factors, which are released during bone resorption. Many different cell types are involved in the process of bone metastatic growth: tumor cells, endothelial cells and stromal cells, plus the bone specific osteoblasts and osteoclasts and their precursors. Each of these cell types fulfill their own key role in the context of bone metastasis.

Once settled in the bone micro-environment, breast cancer cels are capable of releasing various signaling molecules such as, bone morphogenic proteins (BMPs),

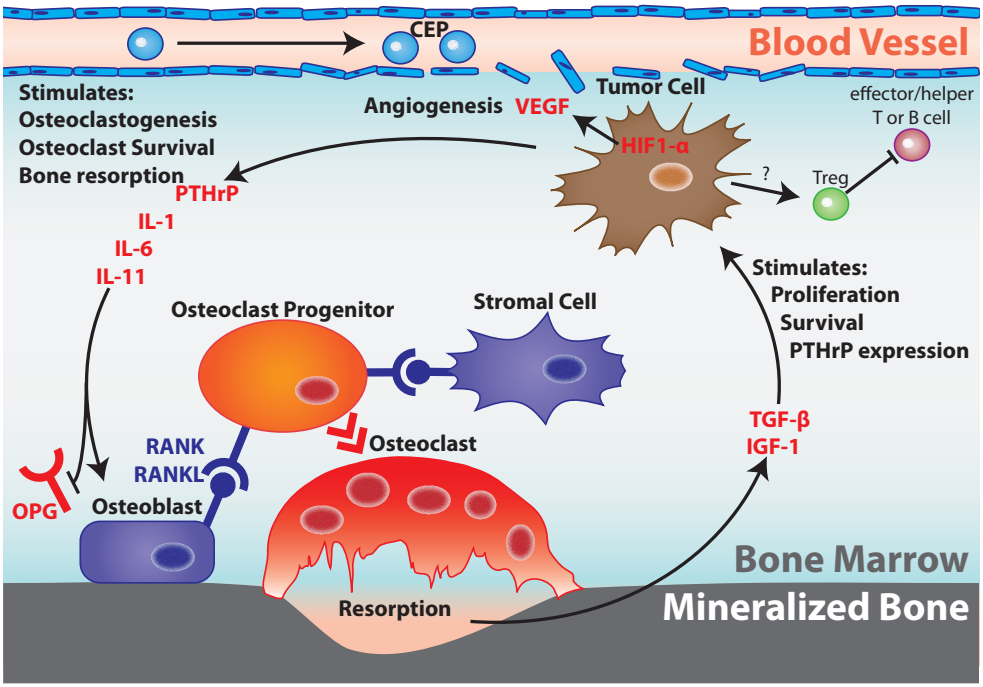

Figure 1.2: Schematic representation of the vicious cycle of bone metastasis. Tumor cells stimulate Osteoblasts and stromal cells to express RANKL by producing osteolytic factors as PTHrP, IL-1, IL-6 and IL-11. These factors lead to a downregulation in OPG expression, an inhibitor of RANKL. In turn, RANKL results in increased osteoclastogenesis and osteoclast survival. Mineralized bone matrix is rich in cytokines and growth factors including TGF-β. These factors are released in the bone marrow space upon bone resorption by osteoclasts. In turn, these factors stimulate tumor cell survival, growth, and production of PTHrP and other osteolytic factors which further stimulate osteoclastic resorption. In addition, bone metastases are generally hypoxic leading to an upregulation of HIF-1α and secretion of VEGF, a strong pro-angiogenic factor. CEPs are attracted by leaky tumor vasculature and further stimulates angiogenesis. Tumors recruit T_{reg} cells via a mechanism which is largely unknown. T_{reg} cells inhibit possible immune reactions against the tumor through the down regulation of T & B effector and helper cells.[6,10,14]

insulin-like growth factors (IGFs), transforming growth factor-β (TGF-β) and parathyroid hormone-related protein (PTHrP), which in themselves have an effect on bone.[7,15] PTHrP is a signaling molecule involved in mammary gland development and lactation, hence, its strong presence both in the healthy breast as well as in breast cancer. In addition, PTHrP is involved in many other processes as signaling molecule, amongst which the maintenance of a calcium homeostasis.[16–18] These multiple functions of PTHrP are at the core of the pathogenesis of osteolytic bone metastases of breast cancer.

PTHrP, released locally in the bone by metastases, stimulates the expression of receptor activator for nuclear factor-κB ligand (RANKL) on neighboring bone marrow stromal cells and osteoblasts.[10] RANKL signaling stimulates the maturation of osteoclasts from RANK positive precursor cells. Moreover, RANKL prolongs the survival of mature, active, osteoclasts.[19] Osteoclasts resorb the mineralized bone matrix, which in turn causes the release and activation of growth factors and cytokines present in the bone. TGF-β is such a factor which is highly present in bone.[20,21]

The released TGF-β stimulates tumor cells to produce more osteolytic factors (PTHrP, IL-6, IL-11) that can, in turn, further stimulate osteoclastic resorption and increase the TGF-β release from bone.[6,20,22] This feed-forward stimulation of osteoclastic bone resorption is referred to as the "vicious cycle" of bone metastasis (Figure 1.2).[15,22–24] The strong positive feedback between bone destruction and metastatic growth makes these lesions nearly impossible to treat.[10,14] The local bone destruction is the main cause or morbidity in metastatic bone disease.

Tumor Angiogenesis

Tumors cannot grow without sufficient blood supply making angiogenesis a critical process in tumor growth. In the adult, angiogenesis is a tightly regulated process occurring almost exclusively during wound healing and in ischemic areas. However, at a certain point during tumor growth there is a shift of balance towards angiogenesis. This shift has been called the "angiogenic switch", a result of crosstalk between the tumor and surrounding healthy tissue.[25]

In the case of bone metastases, angiogenesis is strongly driven by hypoxia. Hypoxia and the stabilization of hypoxia inducible factor-1α (HIF-1α) as a key initiators of (tumor-)angiogenesis has been studied extensively and reviewed by Liao and Johnson.[26] VEGF is one of the downstream targets of hypoxia signaling and the main factor involved in pro-angiogenic signaling.[27] Most of the vasculogenic and angiogenic effects of VEGF are mediated through the VEGF receptor 1 (VEGFR1, FLT-1) and VEGF receptor 2 (VEGFR2, Flk-1) expressed on endothelial cells.[28] During angiogenesis, including tumor angiogenesis, both VEGF and VEGFR2 expression are locally upregulated.[29,30]

The one sided pro-angiogenic, mainly VEGF mediated, signaling leads to the formation of an abnormal vascular network. The newly formed vessels are leaky, tortuous and often lack pericytes and a basement membrane.[31,32] Leaky, poorly formed,

vasculature and high levels of VEGF attract circulating endothelial progenitor cells (CEPs). These cells are able to differentiate into endothelial cells and are normally involved in vessel repair, angiogenesis and neo-vascularization, adding up to the already existing pro-angiogenic environment (Figure 1.2).[33,34]

Molecular Imaging

Molecular imaging is the term used to describe a wide range of imaging tools and techniques that enable the visualization of molecular processes and interactions (functional imaging) or structures and micro-architecture (structural imaging). Molecular imaging modalities can be based on light (e.g. optical imaging), on the use of radioactive tracers (e.g. PET and SPECT), on the use of ultrasound or on differences in magnetic resonance (e.g. MRI). These functional imaging modalities can be combined with structural imaging modalities which provide more anatomical detail such as radiography or computed tomography (CT).

When performing research on bone metastases, it is important to follow both structural and functional developments in and around the tumor. Structural imaging modalities are used to follow diseased induced changes to the skeleton whereas functional imaging is to follow processes such as matrix degeneration, tumor angiogenesis and tumor growth. The non-invasive character of optical imaging, imaging modalities based on detection of light, makes it possible to follow animals over time throughout the experimental period.

Whole Body Optical Imaging

Optical imaging of cancer presents a challenge because tumor cells usually do not have a specific optical quality that clearly distinguishes them from normal tissue. However, the field of whole body optical imaging has been transformed over the last decades by improvements in camera detection systems as well as better tools for making clonal cell lines or transgenic animal models with light-generating capabilities or specific fluorescent properties.

The term optical imaging includes all of the imaging techniques based on the detection of photons with wavelengths in the ultraviolet, visible, near-infrared and infrared parts of the spectrum. These photons are emitted from living cells, tissues or animals through either bioluminescence or fluorescence. As a result, optical imaging can be divided in: bioluminescence imaging (BLI) and fluorescence imaging (FLI). Despite the similarities in their applications, BLI and FLI both have their own characteristics, strengths and weaknesses such as differences in availability, sensitivity, signal to noise ratio (SNR) and interference by background emission from tissues.[35–38]

The choice of tools, such as whether to use FLI or BLI, is determined by the questions needing to be addressed, e.g. FLI allows total cells *in vivo* to be measured as well as *in vitro* and *ex vivo* analysis to be performed whereas BLI often gives an

indication of metabolizing cell activity. Therefore, BLI has evolved into a standard modality in pre-clinical research to follow tumor growth non-invasively over time.

X-rays and μCT

X-rays dominated the field of skeletal imaging ever since Rontgen's publication of a photo of his wife's hand and various other shadow images in Science back in 1896.[39–41] The subsequent work of people like Alessandro Vallebona and William Watson formed the basis of X-ray tomography. It is during the 1970s that X-ray-based imaging underwent revolutionary changes after advances in digital computing enabled the development of CT by Godfrey Hounsfield.[42,43]

Radiographs of small animals are made in the same way as their human counterpart. The technique is not much different from the method described by Röntgen. The subject is placed between a concealed photographic film or digital X-ray camera and an X-ray point source. The recorded image is a two dimensional (2D) shadow projection of the subject.

Relatively new are specialized small animal μCT scanners. These machines can produce high resolution three dimensional (3D) datasets of *in vivo* and *ex vivo* specimens. In general, 3D methodologies are preferable over their 2D counterparts as they give a better approximation of the real life situation. Moreover, μCT can potentially be used to quantify osteoblastic lesions as well as osteolytic lesions, something that is not possible with radiography. However, data analysis of 3D datasets can be tedious and only few standardized protocols for data analysis are in place since the imaging techniques are relatively new.[44–47]

Contribution and Outline of this Thesis

The aim of this work was to develop methods to measure structural changes in the skeleton using μCT. In addition, these new methods should be able to quantify biologically relevant changes. In order to do this, normalized methods to analyze μCT scans and perform quantitive measurements within these datasets are described in this thesis. These techniques were combined with a biological angiogenesis assay and used as research tools in a study comparing various different combination treatments of bone metastases.

Chapter 2 describes a manual μCT based method to asses specific changes in bone volume. The method allows the user to select normalized volumes of interest based on manual input. In addition, the user can generate normalized cross sections and longitudinal sections for side-by-side presentations, comparison of cortical thickness and validation of histological findings.

Chapter 3 describes an automated μCT based method to assess disease induce changes in bone volume and thickness. The segmentation and volume measurements are fully automated in order to minimize observer bias. The segmentation algorithm is able to find the region of interest in whole-body rodent μCT scans, regardless of the animal posture during the scan. The exact location of volumetric changes can be asses using automatically generated color coded cortical thickness maps.

Chapter 4 gives an overview of the advances made by the LUMC departments of Endocrinology and Radiology - Image Processing (LKEB) in multi-modality molecular imaging with an emphasis on μCT. This puts Chapters 2 and 3 in a broader perspective by linking μCT to other imaging modalities.

Chapter 5 describes an angiogenesis assay. The essay enables the differentiation between anti-angiogenic and vascular disrupting properties of compounds. In addition, the assay will indicate the main mechanism underlying the anti-angiogenic properties

Chapter 6 discusses the efficacy of the suggested combination treatment consisting of ENMD-1198, “metronomic” cyclophosphamide and bisphosphonates. In addition, this chapter is exemplary on how the described angiogenesis assay, μCT quantification techniques, radiographs and optical imaging can be combined in a set of experiments to answer biological questions and assess treatment efficacy.

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