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

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Summary & Conclusions and a Future Perspective

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Bone metastatic growth is characterized by tumor-induced bone resorption and subsequent tumor stimulation by factors released from the resorbed bone matrix. This feedback mechanism is known as the vicious cycle of bone metastases.

The complex interaction between bone metastases and the bone micro-environment requires a treatment interfering with multiple pathways. Bone resorption is an attractive treatment target to halt the vicious cycle of bone metastases. This, can be targeted alongside other processes such as tumor cell proliferation and survival, angiogenesis and immune modulation. Angiogenesis can be slowed down by inhibiting VEGF signaling and preventing the recruitment of circulating endothelial precursor cells (CEPs). The immune system could potentially be activated by targeting regulatory T-cells (T_{reg}). Transforming growth factor-β (TGF-β) is released and activated during bone resorption. Thus, TGF-β stimulation of the tumor can be stopped by inhibiting osteolysis. The inhibition of osteolysis can either be achieved by direct targeting of osteoclasts activity or by blocking osteoclast differentiation. Decreased levels of TGF-β will lead to a decrease in secretion of pro-osteolytic factors, such as parathyroid hormone-related protein (PTHrP) and macrophage colony-stimulating factor (M-CSF), by the tumor cells. In addition, the tumor will be left more susceptible to apoptosis. Evaluating the treatment result of such a combined intervention in a multi-factorial disease process requires simultaneous measurement and follow up of multiple processes.

Due to their non-invasive nature, whole body molecular imaging techniques are especially suitable for longitudinal studies. Imaging technique are unique tools. They enable the quantification of structural changes and disease related processes such as angiogenesis and tumor growth in a non-invasive manner and at multiple time points. Altogether, molecular imaging provides the researcher the possibility of evaluating the result of a therapeutic intervention in over time in the same animal. [1–3]

Radiography and optical imaging techniques are primarily two dimensional (2D). However, recent advances have resulted in fluorescence molecular tomography (FMT) and other three dimensional (3D) fluorescence and bioluminescence data capturing methods as well as specialized small animal micro-CT (μCT) scanners. [1,3–5] The transition from 2D to 3D results in more realistic data and possibly betters quantification. However, the navigation through 3D datasets is less intuitive than 2D datasets. Moreover, the determination of a volume of interest (VOI) is more complicated than the selection of a two dimensional region of interest (ROI). [6]

Chapter 2 of this thesis describes a method to generate normalized cross-sections and select VOIs in complex data. The method is based on the definition of a centerline through a long-bone of interest. The naturally curved centerline can then be "straightened" to generate a new, normalized volume. This new volume can then be used to extract normalized cross-sections or to define a normalized VOI. The actual measurement of the selected volume is performed in the original volume to prevent measurement artifacts due to the straightening procedure. Following this method,

VOIs are defined relative to the anatomy of the bone.[6]

An automated method has been developed in-house to follow longitudinal studies both qualitatively and quantitatively. This method, described in chapter 3, is based in part on a previously published method to fit an animal atlas over μCT scan data.[7,8] Subsequently, a predefined VOI can be segmented and measured. These volume measurements are performed fully automated. The acquired data does not differ significantly from manual segmentation and measurements.[9]

Using a center-line-based approach, sub-volume datasets of long-bones can be transformed into a stack of slices cutting through the bone under a 90° angle (Chapter 2).[6] A similar approach has been implemented in the automated method, but instead of defining a center-line per scan, the center-line of the segmented atlas bone is used. The thickness of the bone cortex can be determined in these orthogonal slices. All the cortical thickness data of the bone of interest can then be projected as color code on a volume rendering of the bone. This method of generating cortical thickness maps, described in chapter 3 is a valuable tool to study changes in the bone anatomy over time. The user of this method can identify the location of osteolytic and osteosclerostic regions.[9] This helps in defining regions of interest for further analysis of the acquired imaging datasets or for subsequent histological examination.

μCT gives a detailed insight in structural changes during the course of a disease and/or treatment. More insight into the direct molecular and mechanistic response can be acquired by combining structural imaging modalities with functional imaging. It is not only possible to acquire 3D optical data, but also to project these 3D optical data sets back onto scans of various other modalities (e.g. μCT, PET, SPECT and MRI). Combining multiple imaging modalities within one study offers unique research opportunities, but it also lies at the core of the major challenges in data analysis. Datasets are acquired using various highly specialized cameras and machines. Each one of these machines often have specific requirements in terms of anesthetics and animal positioning and produce data in various formats. Animal handling and moving animals from one machine to another introduce variations in animal posture between datasets. Most of these posture variations can be prevented by performing these actions with care. However, the posture variations are even more abundant in longitudinal studies where animals are imaged at multiple time points, often with intervals of several weeks. In these cases, specialized animal holders have proven to be insufficient. Also, during longer studies, for example five or six weeks, growth of the animal between scans becomes a problem.

Chapters 2 and 3 show how posture variation can be normalized in μCT scans. In chapter 4 an approach to integrated data handling has been described which uses the skeleton as a reference frame in order to compensate for posture variations in datasets acquired with other modalities. This approach is based on the registration and segmentation of μCT data. In this case 3D optical or nuclear imaging data (positron emission tomography (PET) and single photon emission computed tomography (SPECT)) is coupled to the μCT data and segmented alongside. Also the handling of MRI data is currently dependent on μCT, but this could be improved in

future since magnetic resonance imaging (MRI) provides skeletal contrast in addition to soft tissue contrast.

A great number of *in vitro* assays are available which are used to evaluate the effect of possible new treatments in a laboratory setting. There are many different ways to assess the anti-angiogenic capabilities of a compound. These assays include proliferation assays of human umbilical vein endothelial cells (HUVECs) and tube forming assays in three dimensional culture systems. Most of these *in vitro* models for angiogenesis include only one cell type, namely endothelial cells. However, angiogenesis is a complex process in which multiple cell types interact. Moreover, existing assays focus on quantifying the effect of compounds on vascular outgrowth and the formation of new vessels. The vascular disruption capability of a new compound is often not quantified.

A new vascular disruption assay is described in chapter 5. This assay is a variation to an angiogenesis assay developed within the LUMC by Deckers *et al.*[10] The angiogenesis and vascular disruption assay are unique in the sense that they consist of *ex vivo* bone explants containing all cell types involved in vasculogenesis. The newly formed vascular bed in these assays is a model for tumor vasculature which, much like the vessels in these assays, is poorly matured and often lacks support by pericytes and smooth muscle tissue.[10] The vascular disruption assay described in this thesis is the first multicellular assay which can be used to quantify treatment responses on newly established vasculature.[11]

The *in vitro* angiogenesis and vascular disruption assays and the methods of analyzing μCT scans have been used in chapter 6 for evaluation of the treatment effect of a combination treatment for bone metastases. The rationale behind the treatment design was to simultaneously target all the important processes during metastatic growth in bone, namely tumor growth, angiogenesis and bone resorption. The treatment consisted of ENMD-1198 (a 2-Methoxyestradiol (2ME2) derivate), low dose "metronomic" cycplophosphamide and the bisphosphonate risedronate.

2ME2 is a a microtubule targeting agent (MTA). As such 2ME2 has anti-proliferative effects against fast dividing cells such as cancerous cells and developing endothelial cells during angiogenesis.[12–15] Moreover, 2ME2 causes the phosphorylation of Bcl-2 and Bcl-xL, leading to an upregulation of the intrinsic and extrinsic apoptotic pathways, a mechanism shared with paclitaxel.[16]

The anti-angiogenic effects of 2ME2 are not solely caused by its cytotoxicity towards endothelial cells. It also inhibits hypoxia-inducible factor-1α (HIF-1α) expression.[17,18] HIF-1α is a pro-angiogenic factor regulating over 70 genes involved in many cancer related processes like angiogenesis, glycolysis, metastasis and cell growth, amongst which VEGF.[19,20]

Furthermore 2ME2 inhibited intra-osseous growth of 4T1 and MDA-MB-231 breast cancer cells and protects the bone from subsequent cancer-induced osteolysis.[21,22] This protective effect of 2ME2 on bone is not only a result of a reduced tumor burden. 2ME2 is also capable of suppressesing osteoclast differentiation and induces apoptosis of mature osteoclasts.[23] Moreover, 2ME2 effectively represses bone

loss in an animal model of post-menopausal osteoporosis[24] and it preserved bone and reduced the frequency and severity of arthritis in a model of postmenopausal rheumatoid arthritis.[25]

Chapter 6 describes a study where the 2ME2 derived compound ENMD-1198 was used to treat bone metastatic growth following intra-osseous inoculation with MDA-BO2 cells, a bone specific subclone of the MDA-MB-231 breast cancer cell line.[26] ENMD-1198 is a slightly modified form of 2ME2 which has better characteristics in terms of metabolic stability, anti-angiogenic activity and cytotoxicity.[27,28] We showed that ENMD-1198 has direct effects on tumor growth, angiogenesis and bone turnover.[29]

ENMD-1198 treatment was combined with low dose "metronomic" Cyclophosphamide (CTX) in order to improve the anti-angiogenic and anti-vasculogenic effects of the treatment. CTX is a cytostatic drug which is often in combination with other anti-neoplastic drugs to treat malignant lymphomas at the maximum tolerated dose. It is also used as adjuvant therapy in metastasized ovarian, breast and prostate cancers. CTX is not cytotoxic in its original form but requires activation by P450 metabolism in the liver.[30,31] Metabolism results in the active metabolite 4-Hydroxyperoxycyclophosphamide (4HC).[32] The low dose used in this study has been proposed as anti-angiogenic therapy rather than inducing direct tumor cell death.

It has already been shown that cytotoxic agents can have anti-angiogenic properties at low dose. In 1986, Polverini and Novak were the first to show this with low dosing of mitoxantrone and bisantrene, two cytotoxic agents which were in clinical trial at the time.[33] A dose dependent inhibition of angiogenesis was found using a vascularization model of the rat cornea. Interestingly, the authors explicitly note that there was no untoward toxicity to the tissue at the drug concentrations used in the experiment. There are three suggested mechanisms underlying the antiangiogenic effect of metronomic chemotherapy using CTX. First, by directly inducing apoptosis of proliferating tumor endothelial cells.[34] Secondly, by blocking or reducing the viability of circulating endothelial progenitor cells (EPCs).[35] Thirdly, by elevating the levels of cellular and circulating thrombospondin-1 (TSP-1).[36] The anti-angiogenic effect of metronomic chemotherapy seems to be much stronger in combination with anti-angiogenic compounds such as VEGF inhibitors.[37] In addition to the anti-angiogenic effects, metronomic cyclophosphamide causes a selective depletion of T_{reg}cells resulting in an enhanced tumor immune response.[38,39] Low dose CTX has no, or very low, effect on tissues that are otherwise highly sensitive to maximum tolerated dose CTX treatment.[40]

Both ENMD-1198 and metronomic CTX target angiogenesis and tumor vasculature. The main mechanism of ENMD-1198 is to render the tumor cells irresponsive to hypoxia by interfering with HIF-1α signalling; thus down-regulating VEGF expression.[27] On the other hand, the antiangiogenic effect of metronomic CTX is largely VEGF independent. It targets endothelial cells of tumor vasculature and vascular repair by circulating endothelial cells. The fact that these mechanisms of action are independent suggest a possible synergy between these compounds in combination

treatment. The date presented in chapter 6 however, showed only a mild benefit of combined treatment compared to ENMD-1198 treatment alone. The ENMD-1198 treatment alone was very effective leaving only little room for improvement. Moreover, the experiments were performed in an immune deficient model while part or the low dose CTX treatment effect works through alterations of the local immune system. This results in suboptimal treatment effects in the chosen model.[29]

In order to halt the vicious cycle of bone destruction, the treatment was combined with bisphosphonates (BPs). BPs are widely used in the clinic for the management of osteoporosis, metastatic bone disease and Paget's disease ever since patient with myositis ossificans was treated with BPs, called diphosphonates at the time, for the first time in 1969.[41–44]

BP treatment halts the cycle of bone metastatic growth by blocking osteoclast function.[45] BP treatment reduces skeletal complications and morbidity in patients with bone metastases, but it is not curative.[46] *In vivo* evidence suggests that BPs can prevent the development of new metastases. However, established metastases remain largely unaffected by BP treatment.[47] A possible explanation for this lies within the vicious cycle which promotes osteolytic bone metastatic growth. The positive feedback within this cycle is so strong that the cancer induced osteolysis becomes practically irresponsive to treatment.

Recent studies suggest that zoledronate treatment stimulates an anti-tumor immune response.[48–50] The immune activation of zolendronate is currently studied in phase I and II clinical trials.[48,51,52] Also, several studies indicate direct cytotoxic and anti-proliferative effects of BPs towards tumor cells.[53–55] The actual clinical relevance of these studies remains unclear as these studies are often performed with very high BP concentrations.[56]

Risedronate was added to the ENMD-1198 based combination therapy. It was postulated that, in a combination, BP and ENMD-1198 may be able to stop local osteolysis altogether. The results presented in chapter 6 were less optimistic. Adding a bisphosphonate to the mixture did not result in improved treatment efficacy. In addition, potentially adverse effects were observed in the animals receiving a combination of ENMD-1198, CTX and BPs.[29] Future work should be aimed at optimizing the dose of the individual treatments. Moreover, the role of a possible immune component should be investigated using tumor models suitable for immunological research. Chapter 6 is, apart from its biological significance, an example of how tools like those described in chapter 2, can facilitate research.

Taken together, this thesis describes methods to measure structural changes in the skeleton using μCT resulting in normalized qualitative and quantitative assessment of bone volume and thickness. In addition, normalized cross sections can be generated to allow side-by-side comparison of scan data. It has been shown that these methods can be used to identify biologically relevant changes. In addition, ENMD-1198 was identified as a promising compound for the treatment of bone metastases.

Future Perspective

Molecular imaging and image analysis are fast developing fields. Constant technological advances result in many new exciting tools and possibilities, but also new challenges to overcome. Much of the published work in the field of image analysis has been generated with custom made source codes and scripts. The translation into more user friendly interfaces often lacks behind. This is unfortunate because many good solutions to excisting problems stay unnoticed for people using imaging techniques as research tool as these people themselves are often not experts in image analysis. It is therefore important that more user friendly interfaces are developed.

One of the exciting aspects of the field of molecular imaging is the fact that these techniques are at the crossroads of pre-clinical and clinical practice and of diagnosis and treatment. Many of the image analysis techniques, both described in this thesis and in other publications, can potentially be used for clinical purposes as well. The reverse, where clinical applications can be used to solve pre-clinical problems, is often true as well. The use of a kinetic atlas to segment datasets and visualize changes over time is not yet used in the clinic. However, radiologists are positive to the idea of developing this approach for clinical applications.

Optical imaging modalities are becoming more important in diagnosis and treatment. Fluorescence imaging is already being used in operation theaters for sentinel lymph-node procedures. Also, peri-operative use of topically applied tumor specific probes on excised tissue is under clinical evaluation. Optical mammography is under clinical evaluation as a method of monitoring early treatment response to neoadjuvant therapy. Further development of clinically approved cancer specific probes could have a great impact on this field, in the operation theatre, for diagnosis and during patient follow up.

Currently, methods of aligning partial and whole body clinical CT and MRI data are being developed within the LUMC department of radiology. The basic principles of these techniques have their origin in the atlas based approaches developed for pre-clinical image processing.

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