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Speaker Abstracts

Numbers beginning with 'S' refer to Speaker Abstracts
Numbers beginning with 'P' refer to Poster Presentations

PRE-METASTATIC NICHES / CANCER STEM CELLS

S1
Metastasis Is… When Cancer Stem Cells Find Their Niches

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Metastatic growth in distant organs is the major cause of cancer mortality. We have now identified two key mechanisms essential for the initiation of metastasis: first, a small population of cancer stem cells (CSCs) that is critical for metastatic colonization. These CSCs represent a stable population of tumour cells which selectively survive and proliferate upon metastatic seeding and thereby drive the initial expansion of cancer cells at the secondary site. In contrast, nonCSCs fail to grow, are rapidly lost and do not dedifferentiate into CSCs *in vivo*.
Second, we find stromal niche signals to be crucial for this process. We identify the extracellular matrix (ECM) component POSTN to be expressed by fibroblasts in the normal tissue and in the stroma of the primary tumour. Importantly, infiltrating tumour cells need to induce stromal POSTN expression in the secondary target organ to initiate colonization. This ECM protein is required to allow cancer stem cell maintenance and blocking POSTN function prevents metastasis. POSTN recruits Wnt ligands and thereby increases Wnt signalling in cancer stem cells. We suggest that the education of stromal cells by infiltrating tumour cells is an important step in metastatic colonization and that preventing denovo niche formation may represent a novel treatment strategy against metastatic disease.

S2
Suppression of Metastasis by MicroRNAs

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Metastasis and metastatic progression are complex and fascinating processes that are driven by the concerted activities of many genes expressed by cancer cells. These genes can have both cell-intrinsic effects on cancer cells and cell-extrinsic effects on the tumor microenvironment. Metastatic cells employ various mechanisms in order to over-express these genes. One such mechanism is the silencing of small non-coding RNAs (microRNAs). We previously discovered a set of such non-coding elements that target metastasis promoting genes in breast cancer. These metastasis suppressor miRNAs are silenced in breast tumors of patients that develop metastasis to bone and lung, while their re-expression in highly metastatic cells robustly suppresses metastasis. We have found that one of these miRNAs, miR-335, suppresses a set of genes that promote invasion and migration of cancer cells while the other miRNA, miR-126, suppresses a set of genes that are secreted by cancer cells and act to recruit endothelial cells to the metastatic niche—thus activating metastatic colonization. These miRNAs thus represent key regulators of metastatic progression, serve to delineate the molecular and cellular mechanisms underlying cancer metastasis, and reveal novel genes as candidates for therapeutic targeting in cancer.

S3
Targeting Breast Cancer Stem Cells

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Studies describing the tumor as a hierarchically organized cell population have changed the classical oncogenesis view and propose new therapeutic strategies. Cancer stem cells (CSCs) are thought to sustain tumor initiation/maintenance, therapy resistance, and systemic metastases. Targeting this tumor cell population is crucial to achieve a true cancer cure. A large research effort is now aiming to develop drugs targeting CSCs.
In order to identify cancer stem cell regulatory pathways and potential therapeutic targets we realized a gene expression profiling of the breast CSC population. We obtained distinct 'cancer stem cell' signatures leading to the identification of several therapeutic targets. Using human primary tumor xenograft models we have functionally prove that we can efficiently target the CSC population and inhibit tumor regrowth and metastasis formation.
These studies have important implications for elucidating the role of cancer stem cells in tumorigenesis, metastasis and treatment resistance.

S4 **P1**

Fibronectin Originating from Osteoblasts Affects Immune Cell Signatures and Tumor Growth

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Fibronectin is a matrix protein involved in cell differentiation and migration. Osteoblasts produce an isoform containing the EDA domain. This isoform has been shown to attach to immune cells. We therefore hypothesized that EDA originating from the osteoblasts might affect immune cell differentiation and hence tumor growth.
To test this, we conditionally deleted fibronectin in osteoblasts using the collagen-α1(I) promoter. This resulted in a significant decrease in EDA fibronectin in the bone marrow by 50%, associated with a decrease in CD11b+ cells both in the bone marrow (27%, $p<0.05$) and in the spleen (15%, $p<0.05$). Furthermore, ly6C+ cells were significantly increased (63%, $p<0.005$). Cells expressing this marker have been viewed as either being precursors of dendritic cells or expressed on the proinflammatory macrophages (M1). We therefore hypothesized that tumor growth would be suppressed in these mice. Injecting the bone seeking MDA-MD-231B/luc+ cells intratibially resulted in a significant decrease in tumor growth (61%, $p<0.05$). This was associated with a decrease in CD11b+ cells in the tumor from cKO mice (43%, $p<0.05$), an increase in ly6C+ cells (35%, $p<0.05$), and an increase in osteoclasts on the bone surface adjacent to the tumor (15%, $p<0.05$). In line with the increase in osteoclasts circulating osteoprotegerin levels were decreased in cKO mice (12%, $p<0.05$). Furthermore, TGF-β was significantly decreased in tumor tissue (24%, $p<0.05$) and this was associated with a decrease in proliferation in the tumor (17%, $p<0.05$). Blood vessel formation was not affected.
In contrast, knockdown of fibronectin (kd) in tumor cells resulted in a significant decrease in growth (by 83%, $p<0.005$), without a change in CD11b+ cells or the cytokines examined. Instead, there was a significant decrease in blood vessel formation by 40% ($p<0.05$). We therefore hypothesized that the combination of an immune environment not conducive to tumor growth together with an inability to stimulate angiogenesis might result in failure of tumor growth. Indeed, injecting knockdown tumors in cKO mice resulted in the development of only two out of 11 tumors ($p<0.05$ in a Fisher's exact test).In summary, fibronectin originating from the osteoblasts affects immune response to tumors. Furthermore, the combination of fibronectin loss in the osteoblasts and in the tumor itself results in failure of tumor development. Thus fibronectin seems to fulfill multiple functions in tumor formation.

S5 **P61**

MicroRNA-34c Inversely Couples the Biological Functions of the Bone-specific Transcription Factor RUNX2 and the Tumor Suppressor P53 in Osteosarcoma

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Osteosarcoma (OS) is the most common malignant bone tumor. RUNX2, a key regulator of osteoblast differentiation and suppressor of cell growth, is aberrantly expressed in OS. The latter suggests a context dependent function of RUNX2 as tumor suppressor or oncoprotein. We examined pathological roles of RUNX2 in the etiology of OS and the mechanisms by which RUNX2 expression is deregulated to account for its tumor-related elevation. RUNX2 is expressed in OS tissue micro-arrays and positively correlates with increased staining of the proliferation marker Ki67, suggesting that RUNX2 may support osteosarcoma cell growth. Indeed, knockdown of RUNX2 inhibits cell growth in U2OS osteosarcoma cells. The frequency of RUNX2 elevation in OS (~50%) is comparable to the predisposing inactivation of tumor suppressor p53 in ~50-70% of osteosarcomas. Consistently, RUNX2 levels are elevated in the absence of p53 in distinct OS cell lines and diverse proliferating mesenchymal cells. Conversely, activation of p53 by DNA damage and stabilization of p53 with the MDM2 inhibitor Nutlin-3 drastically decrease RUNX2 protein levels. Thus, RUNX2 expression is inversely linked to loss of p53. The strong reduction of RUNX2 protein expression can only partially be explained by transcriptional control demonstrated by the absence of p53 and RUNX2 binding sites in the respective promoters for RUNX2 and p53, the two-fold decrease in Runx2 mRNA and less than 30% declined RUNX2 transcription activity after Nutlin-3 exposure, suggesting a regulation by post-transcriptional mechanisms. To address the possible contribution of microRNAs (miRNAs) to modulations in RUNX2 levels we measured expression of validated miRNAs that directly target RUNX2 in human OS cells and mesenchymal progenitor cells. Expression analysis revealed that elevated RUNX2 protein expression is directly linked to diminished expression of several RUNX2 targeting miRNAs. The p53 dependent miR-34c is the most significantly down-regulated RUNX2 targeting miRNA in OS. Ectopic expression of miR-34c markedly decreases RUNX2 protein levels, and 3'UTR reporter assays confirmed RUNX2 as a direct target of miR-34c in OS cells. Importantly, stabilization of p53 with Nutlin-3 increases miR-34c expression while decreasing RUNX2 protein levels. We propose a novel RUNX2-p53-miRNA regulatory network that controls proliferation of osseous cells and is compromised in osteosarcoma.

BONE MARROW MICROMETASTASIS

S6

CTC and DTC-Markers of Response and Prognosis or New Therapeutic Targets?

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Clinically evident cancer consists of constantly evolving cancer cells, recruited stroma, blood vessels and extracellular matrix. Cancer cells are constantly shed into the circulation; however, the majority of these cells do not form metastases. Indeed, most patients with operable cancers have detectable circulating tumor cells (CTC) at diagnosis, but most do not develop metastases. Systemic adjuvant chemotherapy is administered to most patients with locally advanced epithelial cancers such as breast, lung, prostate, colon, stomach, pancreas, and ovary after definitive surgical and radiation treatments. Theoretically, adjuvant therapy targets microscopic tumor cells that have

escaped from the primary tumor. Currently, the presence of microscopic disseminated tumor cells (DTC) is difficult to detect except in lymph nodes and bone marrow and as CTC in blood and is not routinely performed. Present detection of these cells occurs through immunohistochemical evaluation of hematopoietic and lymphoid tissues where epithelial cells are rare.
The persistence of CTC after cancer therapy (but not at diagnosis) is associated with decreased survival. Whereas, the presence of DTC in the bone marrow at diagnosis is associated with a poor prognosis. Interestingly, DTC are rarely affected by cytotoxic chemotherapy, despite significant clinical responses in the primary tumor. Genomic analyses of these relatively rare CTC and DTC are difficult, but demonstrate heterogeneity between the primary tumor and CTC/DTC. DTC often express stem cell markers and have low proliferation rates. Enthusiasm exists for using CTC and DTC as a 'real time' biopsy to target therapy for individual patients. However, it is unclear whether CTC/DTC are the cause for poor prognosis or rather a marker of overall disease burden and biologic aggressiveness. Numerous questions remain about the nature of CTC and DTC. Are persistant DTC the source for late relapses? Where do DTC reside in the bone marrow? Bone-homing tumor cells can compete for and reside in the hematopoietic stem cell niche. Can this be exploited to mobilize DTC out of the protective niche and into the circulation as CTC? CTC numbers correlate with treatment responses but DTC do not. Does this mean that CTC are more susceptible to therapy? Are DTC really cancer stem cells that need different therapies? The current clinical utility of CTC and DTC and future role in cancer therapy will be discussed.

S7

Circulating Tumor Cells Predict Survival in Early Breast Cancer Patients

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Background: Circulating tumor cells (CTCs) have been shown to predict shortened progression-free and overall survival in metastatic breast cancer. We evaluated whether the presence of CTCs increases the likelihood of subsequent relapse and death in early breast cancer.
Methods: CTCs were analyzed in 2,026 patients with early breast cancer using the FDA-approved CellSearch System (Veridex, USA) before the patients began adjuvant chemotherapy. The patients represented a patient subset randomized to in the SUCCESS clinical trial. The patients were followed for a median of 35 months (range: 0 to 54 months). Cox regression models were used to assess the prognostic significance of CTCs for disease-free and overall survival. (EUDRA-CT number 2005-000490-21).
Results: CTCs were detected in 21.5% of the patients (n=435; median: 1.3, range: 1-827). Lymph node involvement was more prevalent in patients with CTCs ($p<0.001$); however, no association was found with tumor size, grading or hormone receptor status. There were 114 instances of recurrence, and 66 patients died during follow-up. The presence of CTCs was an independent predictor of poor disease-free survival (DFS; $p<0.0001$), distant disease-free survival (DDFS; $p<0.001$), breast cancer-specific survival (BCSS; $p=0.0079$) and overall survival (OS; $p=0.0002$). The prognosis was worst in patients with at least 5 CTCs (hazard ratio (HR): 4.0 [95% CI: 2.21-7.07] for DFS and HR: 3.1 [95% CI: 1.51-8.28] for OS).
Conclusions: This is the first study to demonstrate the independent prognostic relevance of CTCs in a large prospective trial of patients with primary breast cancer.

S8 **P16**

Macrophages and Prostate Cancer Skeletal Metastasis

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Tumor associated macrophages are implicated in the pre-metastatic niche, tumor establishment, and growth; yet mechanisms for their contribution to the pathogenesis of cancer metastasis are entirely unclear. Macrophages play a multitude of functions in normal development and homeostasis, including mediating efferocytosis. Efferocytosis is the phagocytosis of apoptotic cells, an integral process by which potentially harmful by-products of dead and dying cells are removed to create a pro-resolving environment. Rapidly growing tumors have an extensive amount of apoptosis yet the mechanisms for clearance of dying tumor cells have not been explored. The purpose of this project was to determine the impact of macrophages and their phagocytotic capacity in prostate cancer metastasis. The general role of macrophages was determined using the genetic Mafia mouse model which upon pharmacologic administration of AP20187 results in fas-induced apoptosis of c-fms (CSF-1 receptor) positive macrophage lineage cells. The Mafia model was optimized for AP20187 administration and reduction in mature bone marrow macrophages validated by FACs (GR1loF4/80+CD115+SSCint/lo CD11bhi cells ~2.5% in control vs 0.2% in AP-treated). In mice with subcutaneous RM-1 tumors there was an increase in CD11b+Gr1+ (immature myeloid) cells in the bone marrow and a higher increase in macrophage ablated mice. When tumors were co-implanted with neonatal murine vertebrae (vossicles) in AP-treated mafia mice there was a significant increase in TUNEL positive apoptotic cells (likely due to inefficient clearance). A significant reduction in tumor growth was noted in tibiae when macrophages were ablated after intratibial tumor inoculation. To evaluate macrophage efferocytosis *in vitro* immunofluorescence was performed to validate and quantify the ability of RAW 264.7 (macrophage cell line) or bone marrow CD11b+

cells to phagocytose apoptotic RM-1 cells and a significant increase in phagocytosis found 5hrs after co-culture. Macrophage polarization using CD206 and CD86 markers revealed a shift from the M1 to M2 phenotype when macrophage lineage cells encountered apoptotic tumor cells. In addition, treatment of macrophage lineage cells with zoledronic acid reduced their efferocytotic capacity *in vitro*. These studies identify a new mechanism by which tumor associated macrophages support prostate cancer growth and offer a more targeted potential for therapeutic intervention in skeletal metastasis.

S9 **P17**

Thrombospondin-1 Contributes to the Vicious Cycle of Bone Metastasis in Both the Tumor and Host Microenvironment Compartments

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Thrombospondin-1 (TSP1), known primarily as an anti-angiogenic, is a secreted ligand for 12 receptors including CD47. Under healthy remodeling, osteoclasts (OCs) and osteoblasts (OBs) are tightly regulated. In skeletal metastasis, however, tumor cells secrete factors that promote OC and OB activity, resulting in the release of growth factors that stimulate local tumor growth, leading to a vicious cycle of aberrant bone cell activation and tumor cell proliferation. *We demonstrate that TSP1/CD47 ligation is essential for healthy OC/OB coupling and contributes to the vicious cycle of bone metastasis, both in the tumor and in the host microenvironment.* TSP1-/- mice have 25% higher bone volume than wild type (WT) at 6 months. TSP1-/- mice also show decreased OC activity by serum CTX. TSP1-/- OC formation is disrupted in culture, and OC formation is increased upon addition of CD47-binding peptide. Nitric oxide (NO) exerts a biphasic effect, with toxic effects at high and low concentrations. TSP1 antagonism in OC cultures causes 30-fold increased expression of iNOS. Pharmacological inhibition of NOS restores WT levels of serum CTX in TSP1-/- mice. TSP1-/- mice also exhibit OB dysfunction *in vitro*, consistent with decreased bone formation *in vivo*. TSP1 is a component of the mineralized bone matrix. We show that OCs do not resorb TSP1-/- bone normally compared to WT bone. This OC dysfunction leads to an imbalance in remodeling, leading to the TSP1-/- osteopetrotic phenotype. TSP1 signaling in OC and OB coupling also plays a critical role in bone metastasis. Contrary to their typical anti-tumor roles, TSP1 and CD47 are induced in bone metastatic cell line C42b compared to the parental non-metastatic LnCaP. We employed tumor models to uncover a paradoxical role for TSP1 in cancer progression in primary versus metastatic disease. Tumoral-derived TSP1 promotes OC activity and bone cell derived TSP1 promotes tumor cell proliferation. These data indicate a role for both tumoral- and host-derived TSP1 in bone metastatic tumor progression and osteolysis. These findings are especially important in light of research to develop TSP1 mimetics for cancer treatment. Our data indicate that these mimetics may have detrimental effects on metastatic disease. *We show that TSP1 is a critical coupling factor, both to promote homeostatic remodeling and to promote tumor growth and osteolysis in skeletal metastasis.*

S10 **P18**

miR-192 Impairs Metastatic Angiogenesis by an Exosomal Transfer Mechanism

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Recent findings suggest that miRNAs (miR) can modulate a complex gene network in cell-intrinsic and extrinsic manner through their secretion into exosomes and cargo transfer to target tissues. We previously identified miR192 as heavily downregulated in different highly metastatic subpopulations (HMS) isolated from bone metastases, but its mechanistic contribution remains unknown.
Overexpression of miR192 in HMS led to stunted decrease invasiveness and metalloproteolytic activity as compared to mock transduced cells whereas cell growth kinetics was unaltered. To delineate the pleiotropic functions of miR192 in metastatic activity, after intracardiac inoculation (i.c.) in nude mice, bioluminescence imaging (BLI) showed a dramatic decrease in skeletal tumor burden in mice injected with miR192 and a marked reduction in osteolytic lesions assessed by X-rays and µCT scans. To explore its role in bone colonization, we intratibially injected (i.t.) miR192 cells. Significant decrease in BLI was associated with a decrease in osseous tumor burden in mice injected with miR192 cells. Growth kinetics and apoptosis of tumor cells were unaffected *in vivo*. Interestingly, the number TRAP+ cells were impaired in mice injected with miR192 cells. Transcriptomic analysis identified MCP-1 as a prosteoclastogenic factor severely repressed in miR192 derived tumors. Consistently, incubation with conditioned medium derived from miR192 tumor cells, but not with exosomes, showed a decrease TRAP+ cells *in vitro*. Since miR frequently modulate angiogenesis, we explore the possibility that miR192 could act via exosomal transfer. Interestingly, metastasis-induced angiogenesis was also severely deranged in mice i.t. injected with miR192 cells. To assess the contribution of miR192 released in exosomes, transfer to endothelial cells (HUVEC) was demonstrated after exosome-fluorescent labeled incubation. Cargo transfer to HUVEC was demonstrated by incubation with exosomes derived from cells overexpressing a non-human miR. Incubation of HUVEC with miR192-derived exosomes led to decreased endothelial proliferation, migration and tubulogenesis. Moreover, miR192 in HUVEC repressed key proangiogenic IL8, CXCL1 and ICAM1 factors leading to impaired angiogenesis.Thus, bone metastatic colonization is achieved by novel multimodal mechanisms governing tumor cell dependent functions, and non-cell autonomously regulated tumor-induced osteolysis, and angiogenesis by an exosomal transfer mechanism.

CANCER AND BONE TARGETED THERAPIES (I)

S11

Future Strategies of Molecular Targeted Therapies for the Treatment of Bone Metastases from Solid Tumors

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Breast, prostate and lung cancer are prone to metastasize to bone. Once metastatic cells are in the bone marrow, they do not, on their own, destroy bone. Instead, they alter the functions of bone-resorbing (osteoclasts) and bone-forming (osteoblasts) cells and hijack signals coming from the bone matrix, resulting in skeletal complications that cause pathological fractures and bone pain. The realization that metastatic cancer cells in the bone marrow stimulate bone destruction has led to the use of therapies that inhibit the activity of osteoclasts [bisphosphonates, monoclonal antibody directed against NF-κB ligand (RANKL), cathepsin K inhibitors, integrin alphav beta3 antagonists, tyrosine kinase inhibitors. However, the activity of osteoblasts may be also stronly impaired during bone metastasis formation. Indeed, metastatic cancer cells produce negative regulators of osteoblast differentiation (dickkopf-1, activin A, sclerostin), suggesting that therapies targeting these negative regulators might offer additional opportunities for skeletal restoration. Furthermore, the bone marrow is a reservoir of factors (vascular endothelial growth factors, transforming growth factor-beta, chemokine CXCL-12 and its receptor CXCR4) that provides a permissive niche to metastatic cancer cells, and targeting these bone marrow-derived factors is therefore also a promising therapeutic strategy. The clinical exploitation of these drugs targeting not only the bone compartment but also the tumor and stroma compartments will undoubtedly offer promise for the treatment of skeletal lesions from solid tumors.

S12

Targeting the Tumor Ecosystem: Inhibiting CCL2 as an Example of Targeting Chemokines to Inhibit Cancer-host Interactions

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There has been an increasing recognition that the tumor microenvironment contains host non-cancer cells in addition to cancer cells, interacting in a dynamic fashion over time. The cancer cells compete and/or cooperate with non-tumor cells, and the cancer cells may compete and/or cooperate with each other. It has been demonstrated that these interactions can alter the genotype and phenotype of the host cells as well as the cancer cells. The interaction of these cancer and host cells to remodel the normal host organ microenvironment may best be conceptualized as an evolving ecosystem. Describing tumors as ecological systems defines new opportunities for novel cancer therapies ('ecological therapy'). Chemokines are a family of small and secreted proteins that play pleiotropic roles in inflammation-related diseases, including cancer. Chemokine (C-C motif) ligand 2 (CCL2) is of particular importance in cancer development since it serves as one of the key mediators of interactions between tumor and host cells. CCL2 induces the recruitment of monocytes to the tumor and their subsequent differentiation into tumor associated macrophages (TAMS). TAMs subsequently are major mediators of angiogenesis and matrix remodeling. Multiple studies have revealed that inhibition of CCL2 substantially decreases macrophage infiltration, decreases osteoclast function, and inhibits prostate cancer growth in bone in preclinical animal models. These preclinical data have been successfully translated to the clinic. SWOG study S0916, for example, was a phase II, window trial of an anti-CCR2 antibody in patients with bone metastases. Preliminary results suggest that administration of anti-CCR2 antibody resulted in decreases in urinary NTX in a significant number of patients. These data suggest that altering the tumor microenvironment through inhibition of CCL2 may be a viable strategy for interfering with cancer cell activity and growth. This example of translation from bench to bedside demonstrating how an understanding chemokine effects on the tumor ecosystem can serve as a paradigm for other cytokines, chemokines, and cell interactions within the tumor microenvironment.

S13

Not Available

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S14 **P19**

Cabozantinib Reduces Breast Cancer Bone Metastases and Improves Survival in a Mouse Model

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Breast cancer commonly metastasizes to bone, causing pain and fracture. During tumor development, cells within the expanding mass of the tumor are frequently deprived of oxygen as their distance from existing blood vessels increases. As a consequence, tumor hypoxia develops, which results in induction of key mediators of angiogenesis including VEGF, MET and VEGFR2. MET is expressed by most carcinomas and its elevated expression relative to normal tissue has been detected in a number of cancers including prostate, breast and colorectal tumors. Both osteoblasts and osteoclasts express MET and VEGFRs, and respond to HGF and VEGF. Cabozantinib (cabo, XL184) is a balanced inhibitor of MET and VEGFR2, with activity against other receptor tyrosine kinases including RET, AXL, KIT, and FLT3. Cabo treatment in preclinical models results in tumor regression and blockade of tumor invasiveness and metastasis. Cabo has shown clinical activity in patients with tumors that metastasize to bone, such as

castration-resistant prostate cancer and metastatic breast cancer, where a complete or partial resolution of lesions on bone scan, reduced bone pain, and soft tissue tumor regressions were observed. To elucidate the mechanisms underlying some these clinical observations, the effects of cabo were studied in a human breast cancer bone xenograft model. Female nude mice were inoculated with MDA-MB-231 cells into the left cardiac ventricle and treated with cabozantinib (10 and 60 mg/kg/day via oral gavage). Treatment was initiated 13 days after tumor inoculation when osteolytic lesions were detectable on x-ray and continued to a maximum of 11 days. Mice treated with cabo (60 mg/kg/d) did not exhibit as much weight loss as vehicle-treated mice ($P<0.05$) and showed a reduction in osteolytic lesion area as measured by x-ray ($p<0.05$). Cabo treatment at both doses reduced the intensity of photon emission from tumors as measured by optical imaging using a Cathepsin K-linked fluorescent probe ($p<0.01$). Mice treated with cabo at both doses showed significantly improved survival compared with vehicle treated mice ($p<0.01$). In conclusion, cabo reduced osteolytic lesions and improved survival in mice with established breast cancer bone metastases. Studies to further characterize the molecular mechanisms underlying these effects are ongoing.

S15 **P20**

Evaluating the Safety and Efficacy of Denosumab Treatment for Giant Cell Tumor of Bone

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Giant cell tumor of bone (GCTB) is a rare, aggressive tumor. Currently, no definitive therapy exists for patients (pts) with unresectable or metastatic GCTB. Surgery for resectable GCTB may be effective, but aggressive and morbid procedures are often required to reduce the risk of recurrence. Denosumab is a fully human monoclonal antibody against RANKL. In a previous phase 2 study, denosumab reduced the number of giant cells by ≥ 90% in the majority of GCTB pts. We report safety and efficacy results from a prespecified interim analysis of a second open-label phase 2 study of denosumab in GCTB pts. Pts with surgically unsalvageable GCTB (Cohort 1), resectable GCTB with planned surgery (Cohort 2), or who transferred from a previous phase 2 study (Cohort 3) received SC denosumab (120 mg, Q4W). Investigator-determined disease status was assessed at each visit. Investigator-determined assessments were based on clinical status, radiologic assessments, or pathological response. For Cohort 2 pts, the timing and type of surgical intervention were also recorded and compared with the planned surgery at baseline.
Enrolled pts (N=282) were 58% female; median age 33 (range, 13-83) yrs; 47% had recurrent unresectable disease; median time on study 10 (range, 0-29) months. Adverse events were reported in 236 pts (84%); most frequently arthralgia 20%; headache 18%; nausea 17%; fatigue 16%; back pain 15%, and extremity pain 15%. Osteonecrosis of the jaw was reported in 3 pts (1%); 2 of 3 cases resolved by the analysis cut-off date with conservative therapy. One pt died of respiratory failure, not denosumab-related. Non-serious hypocalcemia was reported in 15 pts (5%). Based on investigator's assessment of disease status, 163 of 169 evaluable pts (96%) in Cohort 1 had no disease progression at any time on study. Based on best response, 158 of 159 (99%) had achieved stable disease or better. Among 100 Cohort 2 pts who had planned surgery at baseline, 64 of 71 eligible pts (90%) had not undergone their planned surgery by month 6. At the analysis cut-off date, 74 (74%) had no surgery and 16 (16%) underwent less morbid surgery than initially planned.
Conclusion: In the largest study of GCTB therapy to date, denosumab appeared to be well tolerated and delayed disease progression in the majority of pts. Denosumab also prolonged the time to surgery and reduced the need for morbid surgery in many pts. Denosumab continues to be studied as a potential treatment for GCTB.

S16 **P21**

Involvement of the Robo 1 and 4 Proteins in Breast Cancer Bone Metastasis

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The Roundabout (ROBO) receptors and their ligands, SLIT glycoproteins, were originally identified as important axon guidance molecules.
A comparative transcriptomic study between the MDA-MB-231 breast cancer cell line and its osteotropic subpopulation, B02 cells, has shown an overexpression of the Robo1/4/Slit2 pathway.In order to characterize the implication of Robo1 or 4 receptors on bone metastasis formation, Robo1 or 4 expression was silenced in B02 cells using ShRNA strategy.
An intraveinous injection of ShRobo1 cells into nude mice induces a 50% increase of osteolytic lesions, compared to the control cells, whereas no difference is observed with the ShRobo4 cells. Moreover, a fatpad injection of ShRobo1 leads to a 3-fold increase of the tumoral volume compared to the control cells. Inversely, with ShRobo4 cells, tumor weight displays a 50% decrease, along with a tardive tumor appearance. Surprisingly, both depleted cell lines induce identical osteolytic lesions after an intra-tibial injection.
In order to decipher molecular events underlaying these *in vivo* observations, *in vitro* experiments were also performed. The silencing of Robo1 and 4 in B02 cells show opposite effects: Robo1 depletion induces a gain in migratory and

invasive capabilities, whereas Robo4 inhibition leads to a strong loss of the invasion property. In addition, a bone marrow flush at D7 after IV injection shows a tremendous decrease of the bone marrow colonization capacity of the depleted Robo4 cells, whereas Robo1 silencing has no effect.
The analysis of ShRobo4 cells conditioned media, by cytokine array, shows an overproduction of the pro-osteoclastic cytokines MCP1, IL8 and PlGF. This cytokines upregulation is associated with an increase of the *in vitro* osteoclastogenesis performed with these conditioned media. These suggest that the ShRobo4 cells are able to stimulate the osteoclastogenesis *in vivo* explaining the identical extend of osteolysis observed, despite the bone marrow delayed colonization.
These results prompt us to propose that Robo1 and 4 are involved in the bone metastasis formation and development. We hypothesis that Robo1 may have an anti-invasive role, while Robo4 could act as a pro-invasive factor in B02 cells.

IMMUNOLOGY AND CANCER

S17
Immune Regulation of the Tumor/Bone Vicious Cycle

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In the bone metastasis field it is established that there is a mutual interaction between the osteoclasts (OC) and the cancer cells, known as the tumor/bone vicious cycle. This vicious cycle model is based on pre-clinical studies performed in immune compromised animals showing amelioration of bone metastases by targeting the OC. However, clinical studies demonstrate only a 28% reduction in skeletal related events in patients with bone metastases treated with OC inhibitors and currently there is no evidence supporting anti-resorptive therapies in reducing the incidence of bone metastases. In this study, we show that modulation of antitumor T-cell responses alters tumor growth in bone, regardless of the OC status. PLCγ2-/- mice, with dysfunctional OC and impaired T-cell activation, have increased bone tumor burden despite protection from bone loss. Importantly, injection of WT CD8 T cells in PLCγ2-/- mice reduces tumor growth to levels of untreated, tumor-bearing WT mice. Consistent with a role of T cells in tumor growth in bone, T-cell activation diminishes skeletal metastasis whereas T-cell depletion enhances it, even in the presence of zoledronic acid.
Since PLCγ2 is primarily expressed in myeloid cells, but not T cells, we hypothesized that cells of myeloid origin could restrain T-cell activation in PLCγ2-/- mice, thus promoting tumor growth in bone even with poorly functional OC. Myeloid Derived Suppressor Cells (MDSC) are enriched in patients with advanced malignancies and potently suppress the T-cell responses favoring tumor growth. Thus, MDSC are likely candidates to restrain T cell activation in PLCγ2-/- mice. We find that tumor-bearing PLCγ2-/- mice have an increased MDSC with greater tumor-promoting abilities than WT MDSC. PLCγ2-/- MDSC suppress T cell activation more potently than WT MDSC, due to increased ROS and NO production. PLCγ2-/- MDSC display reduced β-catenin levels *in vivo* and *in vitro* and consistent with a functional role for this protein, deletion of β-catenin in myeloid cells leads to greater MDSC expansion and enhanced tumor burden.
In conclusion, our findings indicate that the current tumor/bone vicious cycle model should be expanded to include T cells and MDSC as important positive regulators of tumor growth in bone. Considering that bone metastases are often refractory to current cancer treatments, a better understanding of the role of the immune system in skeletal metastases could lead to better therapeutic strategies.

S18
The Bone Marrow in Multiple Myeloma: A Paradigm Example of Concurrent Cancer-induced Bone Disease and Immune Dysfunction

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Multiple Myeloma (MM) is a prototypic disease in which disease progression is dependent on genetic and epigenetic features intrinsic to myeloma cells, but also on the interactions that tumor cells establish with non-tumor cells in the bone marrow (BM), such as stromal cells, endothelial cells, osteoblasts, osteoclasts and others. These cells are functionally driven not only to generate very disruptive symptoms such as bone lesions, but also to establish a protumoral local microenvironment which promotes myeloma cell growth and resistance to chemotherapy. Several data indicate that cells of innate and adaptive immunity are also involved in this interplay via direct or indirect mechanisms. As a consequence of this competitive interplay, host immunity progressively looses its capacity to counteract myeloma cells and is turned into a key factor contributing to myeloma cell growth and survival. This is a late event coming after a variable time period during which the immune system recognizes tumor cells and mounts effective antitumor immune responses. Epidemiological, clinical, and experimental evidences strongly support this postulate. Some of the immuno editing mechanisms operated by myeloma cells in the BM will be reviewed in this presentation with special regard to a specific subset of innate effector cells (V9gammaVdelta2 T cells) which has concurrently been studied in the peripheral blood and the BM of MM patients. These cells have a natural inclination to recognize and kill tumor cells of B-cell origin, but this capacity is selectively lost by V9gammaVdelta2 T cells isolated from the BM. These data highlight the peculiarity of the BM as a privileged site where tumor cells operate to evade immune recognition, defuse naturally reactive effector cells like Vgamma9Vdelta2 T cells, and eventually convert immune cells from antitumoral into protumoral players.

S19 P22
The PPARγ Ligand Badge Reduces Tumour Burden and Increases Bone Marrow Adiposity in Multiple Myeloma *In Vivo*

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Interactions between myeloma (MM) cells and cells of the bone marrow microenvironment promote tumour growth,

survival and osteolytic bone disease. A better understanding of these interactions is essential to identify new therapeutic approaches for this fatal malignancy. The role of osteoclasts and osteoblasts are well studied, however the contributions of other cell types, including bone marrow adipocytes, are poorly understood. We hypothesized that a change in adipogeneis within the bone marrow microenvironment may play a role in MM development. Peroxisome proliferator-activated receptorγ (PPARγ) promotes adipocyte differentiation. C57Bl/KaLwRij mice were treated with Bisphenol-A-DiGlycidyl-Ether (BADGE, 30 mg/kg daily i.p.), a compound reported to regulate PPARγ activity and adipogenesis, or vehicle, and inoculated with 5TGM1 myeloma cells. BADGE treatment significantly decrease tumour growth rate (as measured by serum IgG2bK concentrations, $p<0.01$), reduced tumour burden within the bone marrow by 55% ($p<0.001$), and increased MM cell apoptosis of MM-bearing mice, compared to vehicle. No significant difference in trabecular bone volume was found, however a significant increase in the number of bone marrow adipocytes was observed ($p<0.05$). No significant difference was found in non-tumour mice. A trend towards an increase in total body fat percentage was observed with BADGE treatment both in non-tumour and MM-bearing mice, as compared to vehicle. In support of an increase in adipogenesis, BADGE treatment increased PPARγ mRNA expression ($p<0.01$), downstream targets adiponectin ($p<0.001$) and CCAAT/enhancer binding protein α (C/EBPα), $p<0.01$ in the bone marrow of MM-bearing mice, as compared to vehicle treated mice. *In vitro* studies demonstrated a trend towards an increase in PPARγ mRNA expression in mouse bone marrow stromal cells, $p<0.08$, but with no significant difference in adipocyte differentiation in response to BADGE. BADGE dose-dependently decreased MM cell viability, with no effect on viability of osteoblasts or bone marrow stromal cells. This direct anti-tumour effect of BADGE in MM cells was independent of PPARγ expression, which was not detected in 5TGM1 MM cells. Taken together, our studies demonstrated a striking reduction in tumour burden in response to BADGE, associated with an increase in bone marrow adiposity, raising the possibility that BADGE may have a potential therapeutic benefit in the treatment of myeloma.

S20

Late-Breaking Abstract Presentation

Please see the late breaking abstract supplement for full abstract information.

S21 **P3**

Bone Marrow Mesenchymal Stromal Cell-derived Exosomes Mediate Oncogenesis in Multiple Meyloma

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Bone marrow (BM)-derived mesenchymal stromal cells (MSCs) support multiple myeloma (MM) cell growth, but little is known about the putative mechanisms that may regulate the interaction between MM cells and the surrounding BM milieu. Exosomes are known to mediate cell-to-cell interaction. We therefore studied the role BM-MSCs-derived exosomes in supporting MM biology *in vivo* and *in vitro*. We found that primary normal and MM BM-MSCs release CD63+/CD81+ exosomes, as confirmed by electron microscopy and immunogold labeling. BM-MSCs DiD-labeled exosomes were transferred into MM cells, as shown by time-lapse confocal microscopy. This transfer was further validated in human MM cell lines incubated with murine (C57BL/6 miRNA-15a/16-1-/- and wild type) BM-MSCs-derived exosomes: qRT-PCR showed presence of murine miRNAs in human MM cells. The impact of BM-MSCs-derived exosomes on MM cell behavior *in vivo* was next evaluated. Normal and MM BM-MSCs-derived exosomes were loaded into tissue-engineered bones (TEB) with MM.1S-GFP+/Luc+ cells, and implanted s.c. into mice: MM cell homing and tumor growth were tested by using *in vivo* confocal microscopy and bioluminescence imaging. MM cells co-cultured with MM BM-MSC-derived exosomes induced rapid tumor growth at the site of TEB implant, and rapid dissemination to distant BM niches. In contrast, MM cells co-cultured with normal BM-MSC-derived exosomes led to minimal tumor growth and cell dissemination. We next explored the mechanisms by which exosomes may modulate MM biology: miRNA expression profiling was performed on exosomes isolated from normal and MM BM-MSCs. We found that miRNA15a is significantly lower in exosomes derived from BM-MSCs of MM patients ($P<.05$). We previously showed that miRNA15a shows lower expression in primary MM cells. We therefore sought to examine whether lack of transfer of tumor suppressor miRNA15a can modulate tumor growth and dissemination in MM. We over-expressed miRNA15a in BM-MSCs, and found inhibition of MM cell proliferation and adhesion to fibronectin. Exosomes isolated from BM-MSC-pre-miRNA15a-transfected cells both inhibited MM cell proliferation and adhesion. These findings demonstrate the existence of exosome-driven interactions between the BM milieu and MM cells, suggesting that exosomes constitute a novel mechanism for intercellular transfer of genetic information in the form of miRNAs in MM.

BONE MICROENVIRONMENT AND CANCER

S22

Could the Osteocyte be Playing a Role in Cancer Metastasis?

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Breast and prostate cancer frequently metastasize to bone, and once within bone are difficult to treat. Bone appears to support cancer cell growth resulting in a vicious cycle of cancer cell growth and bone destruction. It has been established that cross-talk occurs between cancer cells such as multiple myeloma and bone cells such as osteoclasts and osteoblasts, but it is not clear if such interactions occur between cancer cells and osteocytes. The osteocyte has been shown to be a multifunctional cell acting as an orchestrator of bone remodeling, a regulator of mineral homeostasis, and a mechanosensor. This raises the question of whether cancer cells can modify the function of osteocytes. There is some evidence to support this concept as it has been shown

that bone metastasizing tumors induce different genetic profiles in osteocytes compared to multiple myeloma (Eisenberger *et al.*, 2008), however, it is not known if cancer cells can modify osteocyte function. And conversely, do osteocytes send signals that modulate cancer cell viability, proliferation, and invasiveness? Do cancer cells modify or enhance those signals? Cancer cells are known to acquire antigens expressed by certain bone cell types such as bone sialoprotein, an osteoblast marker, and it also appears that cancer cells can acquire antigens expressed by osteocytes. For example, sclerostin, a marker for the late osteocyte, is overexpressed by plasma cells from multiple myeloma patients (Brunetti *et al.*, 2011). Another antigen expressed in early osteocytes known as E11/gp38/podoplanin is highly expressed in tumors such as osteosarcoma and this membrane molecule may play a role in tumor cell migration (Kunita *et al.*, 2011; Ariizumi *et al.*, 2010; Kashima *et al.*, 2011). Finally, can an osteocyte embedded in bone become transformed? Can they form osteosarcomas or other bone tumors? There are many unanswered questions regarding the potential roles that osteocytes may play in cancer biology. As osteocytes make up over 90-95% of all bone cells in the adult skeleton, it is highly likely that they are also take part in the 'vicious cycle'.

S23

Vascular Endothelial Cells as a Source of Stem Cells for Osteoblast Differentiation

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It is well known that formation of heart valves during cardiac development is based on the process of endothelial-mesenchymal transition (EndMT). In this process, requiring the BMP/TGFβ receptors ALK2 and ALK5, endothelial cells in the heart tube are converted to mesenchymal stem cells, which in turn differentiate into the cells of the heart valves and their support structures. That this capacity of endothelial cells to transition into mesenchymal stem cells are not limited to the developing heart, is illustrated by recent studies of heterotopic bone formation in humans and mice and the development of white and brown fat in mice.

In patients with the autosomal disorder Fibrodysplasia Ossificans Progressiva, carrying heterozygous activating mutations in ALK2, EndMT contributes to the formation of the heterotopic bone formed in muscles and soft tissues of these patients. Studies of the molecular mechanisms involved suggest that vascular endothelial cells carrying such ALK2 mutations activate multiple signaling pathways, including Smad pathways downstream of both ALK2 and ALK5 activation. This results in induced expression of the transcription factor Snail and several other 'mesenchymal' transcription factors. These factors stimulate conversion of the cells to mesenchymal stem-like cells, which in turn differentiate to chondrocytes and osteoblasts. The same process can be induced in wild-type endothelial cells when they are stimulated with cytokines, such as TGFβ2 or BMP4, that have the ability to activate both ALK2 and ALK5. However, the process is inhibited when cells are treated with BMP7 (activating only ALK2) or VEGF or when they are exposed to inhibitors of any one of many major signaling pathways.

These studies raise the possibility that EndMT of vascular endothelial cells may be used clinically to increase the pool of mesenchymal stem cells for cartilage and bone formation during skeletal repair and regeneration. Further studies of the molecular mechanisms of EndMT and the factors responsible for differentiation of endothelial-derived stem cells to chondrocytes and osteoblast should help define strategies that will make this possible.

S24

The Niche and Bone Metastasis – What Are We Missing

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Metastasis of cancer to bone is commonly described as inscribed in the 'seed and soil' paradigm. Mimicking events normally occurring within the interaction of blood borne hematopoietic progenitors and the bone marrow stroma, metastati sseeding of tte bone marrow is thought to arise from the interaction of blood-borne cancer cells competent to establish secondary growth and the extracellular environment in bone. Seminal work in the last 10 years has been centered on endosteal surfaces and vascular walls as sites, and on osteoblasts and endothelial cells as defining cells, of the niche dwelled by hematopoietic stem cells. More recent data have shifted the focus on skeletal stem cells (also known as mesenchymal stem cells). Both in humans and mice, MSCs have been shown to be critical regulators of hematopoietic function; furthermore, their nature as osteoprogenitors and their position at the ablumenal surface of sinusoids, togehter define a singel cell apt to reconcile the osteogenic nature and vascular position of the 'niche'. The line of experimental work that led to formulate the niche hypothesis has ever since beem dominated by transplantation of HSC as the pivotal experimental approach. The experimental approach best suited to investigate the 'niche' function, however, both in hematopoietic physiology and in mechanisms of cancer metastasis, rests with transplantation of cells able to establish a 'niche' *in vivo* in the mouse. Refined models of transplantation of human MSCs now allow to prove conclusively a role of human MSCs in regulating hematopoietic physiology and in modeling bone metastasis. These studies can be complemented by *in situ* analysis of early events in natural bone metastasis in selected series of patients. Taken together, these two approaches offer a glimpse of a more dynamic scene than previously hypothesized.

S25 **P4**

Antagonism of Inhibitor of Apoptosis Proteins Increases Bone Metastasis via Unexpected Osteoclast Activation

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Inhibitor of Apoptosis (IAP) proteins play a central role in many types of cancer, and IAP antagonists are in development

as anti-cancer agents. IAP antagonists cause apoptosis in many cells, but they also activate alternative NF-κB signaling through NIK, which regulates osteoclasts and bone turnover. We therefore hypothesized that the bone microenvironment might impact the anti-tumor efficacy of IAP antagonists. Bi-weekly treatment with bivalent IAP antagonist BV6 prevented growth of subcutaneously (SQ) implanted MDA-MD231 tumors in nude mice, while intratibial (IT) tumors continued to expand, although at a slower rate than controls. In order to focus on the effect of these drugs on bone, we turned to the 4T1 mouse breast cancer line, which is resistant to IAP antagonists. BALB/c mice were treated with BV6 or vehicle weekly for 4 weeks, and 4T1 cells were injected IT or into the left cardiac ventricle (LV) between the 3rd and 4th doses. A control set of mice received tumor cells SQ. Remarkably, BV6 increased tumor growth in bone by 5.5-fold (IT) and 3.2-fold (LV) on day 10 after tumor inoculation. In contrast, there was no significant difference in the growth of SQ implanted tumor cells or in visceral tumor growth following LV injection, indicating that tumor-enhancing effects of BV6 are specific to bone. To further elucidate the effects of BV6 on bone, we treated tumor-naïve mice with BV6 for 4 weeks, and found that bone mass was decreased by 25-35% in both C57/bl6 and BALB/c mice. Indices of both osteoclast and osteoblast activity were significantly increased, indicating high turnover osteoporosis. A monovalent IAP antagonist, 52S, also decreased bone mass by 25% after 2 weeks of daily injection, and similar to BV6 increased osteoclast differentiation *in vitro*. To determine whether osteoclasts were responsible for increased bone metastasis, we gave zoledronic acid (ZA) and BV6 prior to LV injection of 4T1 cells. In BV6-treated mice, ZA increased bone mass and decreased tumor burden in bone to the levels in mice receiving only vehicle.

Conclusion: The bone microenvironment is a frequent target of metastasis and is an important reservoir from which disseminated tumor cells seed other organs. Our findings that IAP antagonists promote the growth of tumor cells in bone, via the osteoclast, suggest that care should be taken to address the tissue-specific tumor promoting actions of these potentially useful anti-cancer agents.

S26 **P5**

Loss of Expression of PMEPA1, a Negative Regulator of Transforming Growth Factor-beta Increases Bone Metastases from Prostate Cancer

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In contrast to its growth-inhibitory properties early in tumor development, TGFβ increases bone metastases as shown by mouse models of breast cancer and melanoma bone metastases. We previously found that a small molecule inhibitor of the TGFβ type I receptor, SD208, inhibits bone metastases caused by PC3 prostate cancer cells. To identify TGFβ-regulated genes in prostate cancer, we analyzed gene expression in PC3 cells ± TGFβ (5 ng/mL, 48 h) by Affymetrix gene array and qPCR. Up-regulated genes included PTHrP, IL11, CTGF and ADAM19, which increase bone resorption and MMP13 and TSP1, two TGFβ activators. The most increased gene was PMEPA1 (23x, $P<0.03$). Alternate splicing gives rise to membrane-bound and cytosolic isoforms of PMEPA1, both increased by TGFβ in prostate (PC3, DU145), breast (MDA-MB-231) and lung (A549) cancer cells. Overexpression of membrane-bound PMEPA1 decreased TGFβ signaling assayed by $(CAGA)_9$ promoter-luciferase. Inhibition of TGFβ signaling was prevented when mutating PPxY domains and Smad interaction motif (SIM) in PMEPA1 that interact with Smurf ubiquitin ligases (regulators of Smad degradation) and Smad2/3, respectively. In coimmunoprecipitation experiments, Smad2-Smurf2 interaction was increased by PMEPA1, suggesting a role as scaffold protein. Despite having SIM, cytosolic PMEPA1 neither interacted with Smad2/3 nor inhibited TGFβ signaling. PMEPA1 knockdown with siRNA increased Smad2 phosphorylation and expression of IL11, MMP13 and ADAM19 mRNA induced by TGFβ in PC3 cells. PMEPA1 expression was consistently increased in primary tumors of patients with prostate cancer, as well as in breast and lung cancers, compared to normal tissue, using Oncomine. In the Yu dataset (GSE6919), PMEPA1 mRNA was significantly increased in primary prostate cancers (2.0x, $P<0.001$) but decreased in distant metastases (-1.8x, $P<0.001$). To test whether PMEPA1 is functionally important for bone metastasis, we stably silenced its expression in PC3 cells with shRNA. PMEPA1 knockdown significantly increased osteolytic lesion area on x-ray compared to mice receiving shRNA control cells via the left cardiac ventricle (8.8±2.8 and 3.9±1.6 mm^2 for 2 shPMEPA1 clones vs 0.3±0.1 and 0.4±0.2 mm^2 for 2 shCtrl clones, $P<0.001$). Our results suggest that high PMEPA1 decreases TGFβ signaling in the primary tumor, while loss of PMEPA1 augments TGFβ signaling in bone metastases. PMEPA1 may regulate Smad-mediated TGFβ signaling in both early tumorigenesis and late metastases.

S27 **P6**

Discovery of the Genetic Basis of a Hereditary Form of Osteosarcoma and Demonstration of the Gene's Dysregulation in Sporadic Osteosarcoma

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Hereditary cancer syndromes represent a powerful biologic system for identifying cancer-causing genes. Though the syndromes themselves may be rare, their study can provide insights into the basis of the more common sporadic forms of the cancer. Diaphyseal medullary stenosis with bone sarcoma is an autosomal dominant bone dysplasia/bone cancer syndrome characterized by bone infarctions, cortical growth

abnormalities, and pathologic fractures. Most notably, 35% of affected individuals develop either malignant fibrous histiocytoma (MFH) of bone or osteosarcoma (Figure 1). Using a linkage based approach, we previously mapped the tumor suppressor gene locus to chromosome 9p21-22 (1). We now demonstrate that this osteosarcoma syndrome results from mutations in the most proximal of three previously unrecognized terminal exons of the methylthioadenosine phosphorylase (MTAP) gene and leads to defects in alternative splicing of the gene (2).

MTAP is a ubiquitously expressed homotrimeric-subunit enzyme critical to polyamine metabolism and adenine/methionine salvage pathways. Our studies demonstrate that two of the novel exons arose from early and independent retroviral integration events in the primate genome at least 40 MYR ago. Since their integration they have gained a functional role. The six distinct retroviral-sequence containing MTAP isoforms, each of which can physically interact with the wild type form of MTAP are expressed in all tissues, including bone. The disease-causing mutations result in exon skipping and dysregulated alternative splicing of all MTAP isoforms.

Based on these findings in a hereditary form of bone sarcoma, we then analyzed the expression of these MTAP isoforms in a sample set (n=16) of sporadic osteosarcoma samples. All tumor samples expressed similar levels of the wild type MTAP RNA sequence but the expression pattern of the splice variants varied markedly between nearly all the samples. The majority of samples did not express MTAP splice variant 1 (SV1; n=11/16) and nearly half did not express SV6 (n=9/16). Taken together, these results identify the first gene involved in the development of both hereditary and sporadic forms of bone MFH/osteosarcoma and have potential implications for the treatment of this human cancer.

References:
1. *Am J Hum Genet* 1999;64:801-7.
2. *Am J Hum Genet* 2012;90:614-27.

IMAGING TECHNOLOGIES

S28
PET-scan in Monitoring Bone Metastases

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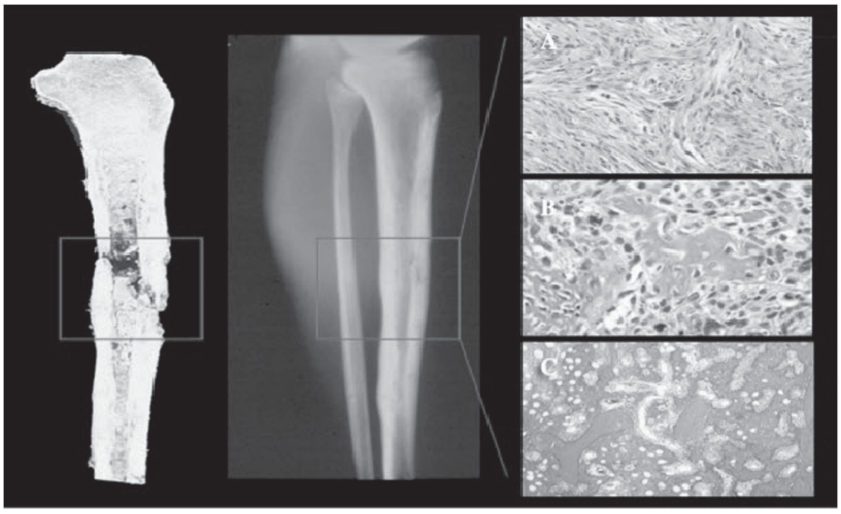
The talk will cover the basics of metabolic imaging by means of radio-isotopes, and will highlight the clinical applications of monophotonic and positron imaging for the workup of bone metastases, as well as the potential preclinical applications, including small animal imaging.

Bone scintigraphy using 99mTc-labelled diphosphonates is being used as the first-line imaging modality in the workup of bone metastases of many osteophilic cancers, such as breast and prostate. Bone scan is reflecting the osteoformation, the radiopharmaceutical being complexed within hydroxyapatite crystals, and is thus dependent on the osteoblastic activity. Conventional 'planar' bone scintigraphy allows for whole-body screening of bone metastases, but it remains a 'qualitative' imaging modality, even if its diagnostic accuracy has been improved with the use of tomography (SPECT) and the more recent addition of fusion SPECT-CT imaging. The quantification of bone metabolism is still not possible with this technique.

PET-scan (positron emission tomography) is another scintigraphic technique that relies on the detection of photons emitted by 'positron-electron' annihilation reaction. PET, and more recently combined PET-CT, allows for dynamic and quantitative imaging of functional of pathologic processes. Multiple tracers labelled with positron emitters are available. For bone imaging, we can used NaF, which will act as a 'enhanced bone scintigraphy'. PET-NaF has been used to quantify bone metabolism in benign and malignant disorders, and it can be used to closely monitor the bone response to anti-cancer or bone-targeted therapies. Other PET tracers allow for the quantitative imaging of



[S27] **Figure 1** Histopathological Analysis of an Hereditary Osteosarcoma. (**A**) Histologic analysis revealed that 95% of the studied tumor specimen displayed the typical pattern of malignant spindle (fibroblastic) cells of bone MFH. (**B**) Malignant cells forming neoplastic bone. (**C**) Focal sheets of neoplastic bone within the tumor are shown to be entrapping pre-existing bone trabeculae, and the overtly malignant cells are shown to produce bone. All sections stained with H&E.

tumoral biology. The most widely used is 18F-fluorodeoxyglucose, a glucose analogue. PET-CT with FDG is now a standard of care in many oncological conditions, e.g. initial staging of lung and head and neck cancers, detection of breast or colorectal cancer recurrence, therapy monitoring in lymphoma. Combination of NaF and FDG imaging has been used by some teams to enhance the accuracy of whole-body staging of cancers. Other tracers are targeted at specific metabolic process, such as DNA synthesis (FLT), cell membrane formation (choline), or receptor expression (somatostatin, ...).
The use of multiple tracers allow for separately monitoring the effect of therapy on bone and on the tumoral component of the bone metastasis.

S29 **P7**

Very Late Antigen-4 (VLA-4) Targeted PET Imaging of Multiple Myeloma

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Purpose: Clinical imaging plays an important role in diagnosing, staging and monitoring multiple myeloma (MM). Very late antigen-4 (VLA-4) promotes MM cell trafficking and drug resistance. Development of VLA-4 targeted PET probes for novel imaging of MM is crucial for monitoring a key molecular signature reflective of disease progression and treatment response.
Experimental Design: For the proof-of-principle imaging experiments with a VLA-4 targeted radiopharmaceutical, ^{64}Cu-CB-TE1A1P-LLP2A, cell binding/uptake assays were performed with 5TGM1 murine myeloma cells. Small animal PET/CT imaging and biodistribution studies were achieved in KaLwRij mice inoculated with 5TGM1 cells subcutaneously or intraperitoneally.
Results: Cell uptake studies with ^{64}Cu-CB-TE1A1P-LLP2A (40 MBq/μmol) demonstrated receptor specific uptake ($P<0.0001$) in VLA-4 positive 5TGM1 cells. Saturation binding assays gave a K_d of 2.2 nM (±0.1) and B_{max} of 136 pmol/mg (±19). Biodistribution of ^{64}Cu-CB-TE1A1P-LLP2A in 5TGM1 tumor bearing KaLwRij mice at 2 h post injection demonstrated high radiotracer uptake in the tumor (12 ± 4.5%ID/g) and in the VLA-4 rich organs spleen (8.8 ± 1.0 %ID/gram) and marrow (11.6 ± 2.0 %ID/g). Small animal PET/CT imaging with ^{64}Cu-CB-TE1A1P-LLP2A at 2 h post injection (~27 pmol) demonstrated high uptake in the 5TGM1 tumors (SUV 6.6 ± 1.1) and spleen was the key clearance organ. There was a 3-fold reduction in the tumor uptake in the presence of blocking agent (6.6 ± 1.1 vs 2.3 ± 0.4).
Conclusions: The results demonstrate novel VLA-4 targeted imaging of MM tumors in mouse models that emulate human MM using a high affinity PET imaging probe.
Translational Relevance: There are currently no specific MM imaging agents clinically or in development. The present study demonstrates the ability of a VLA-4 targeted PET imaging radiotracer to detect MM tumors with high sensitivity in mouse models that closely emulate human myeloma bone disease and progression. VLA-4 expression in MM tumors is a marker of viability, and novel VLA-4 targeted molecular imaging of MM would shift the paradigm toward better diagnosis and treatment management.

CANCER AND BONE TARGETED THERAPIES (II)

S30

The Paradigm of Bone-seeking Anti-resorptive Drugs: Evidence That Myeloid Cells Other Than Osteoclasts are also Cellular Targets of Bisphosphonates *In Vivo*

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Bisphosphonate drugs rapidly target the skeleton and are generally considered to specifically affect only bone-resorbing osteoclasts. The molecular target of nitrogen-containing BPs in osteoclasts is FPP synthase. Inhibition of this enzyme prevents the synthesis of isoprenoid lipids necessary for the prenylation of small GTPases, thereby causing the accumulation of the unprenylated form of these proteins and fundamentally affecting cell function and survival. BPs have become the standard treatment for metastatic bone disease, but N-BPs such as zoledronic acid (ZOL) also have anti-tumour effects in some preclinical models of skeletal and non-skeletal tumours. More recently, ZOL had effects on survival in some clinical trials of patients with breast cancer. However, the exact mechanisms underlying these anti-tumour effects *in vivo* are still unknown, and the extent to which ZOL is internalised by cells other than osteoclasts (especially other cells of the myeloid lineage) is unclear.
We have recently used a novel, fluorescently-labeled BP (AF647-RIS) to determine the cell types capable of internalising BP in the 4T1 murine breast cancer model. 24 hours after a subcutaneous injection of AF647-RIS, we observed uptake by Gr1+CD11b+ myeloid derived suppressor cells (MDSCs) and CD11b+F4/80+ monocytes/macrophages in blood, spleen and bone marrow. Uptake of AF647-RIS was detectable in MDSCs in peripheral blood as quickly as 30 min after drug injection. The presence of AF647-RIS in tumours was evident (particularly in the tumour capsule, which was rich in F4/80+ macrophages by immunostaining) by imaging the whole tumours *ex vivo* 24 hours after injection. Flow cytometric analysis of cell suspensions from tumours showed no evidence of cellular uptake by tumour cells, but some uptake by tumour-associated macrophages (TAMs). Furthermore, 7 days after a single injection of a clinically relevant dose of ZOL into tumour-bearing mice, we detected a significant reduction in the number of MDSCs in the spleen.
In summary, BPs can no longer be considered to affect only osteoclasts *in vivo* and we provide conclusive evidence that BP can be internalized *in vivo* by myeloid cells (MDSCs and macrophages). Given the important role of these cells in the progression of tumour growth and metastasis, our studies suggest that these may be the major cellular targets accounting for the anti-tumour effects of bone-targeted BPs such as ZOL.

S31

Antiresportive Agents in Skeletal Metastasis and Osteoperosis

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With an ageing population and improving cancer therapies, the two most common benign and malignant bone diseases, osteoporosis and bone metastases, will continue to affect an increasing number of patients. Our expanding knowledge of the molecular processes underlying these conditions has resulted in novel bone targets that are currently being explored in clinical trials. Clearly, the approval of denosumab, a monoclonal antibody directed against RANKL, has just marked the beginning of a new era for bone therapy with several additional new therapies lining up for clinical approval in the coming years. Potential agents targeting the osteoclast include cathepsin K, currently in phase 3 trials, and src inhibitors. This presentation will provide a comprehensive overview of the most promising agents currently explored for the treatment of skeletal metastasis and osteoporosis.

S32

Bone-targeted Agents Impact on Relapse and Mortality in Early Breast Cancer

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Bone-targeted treatments with bisphosphonates and denosumab reduce bone resorption and are used in clinical breast cancer care because they reduce the risk of skeletal complications and prevent treatment-induced bone loss. In addition, they can positively impact clinical outcomes in breast cancer patients, as has recently been demonstrated in large clinical trials. These effects are most pronounced in breast cancer patients with established menopause at diagnosis and in premenopausal women with endocrine-responsive disease who received treatment with goserelin, which suppresses ovarian function by inhibiting the production of ovarian hormones. While the routine adjuvant use of adjuvant anti-resorptives remains controversial, these drugs can not only in the fight against bone metastases modify the course of bone destruction via inhibitory effects on the 'vicious cycle' of growth factor and cytokine signaling between tumor and bone cells within the bone marrow microenvironment, but obviously also affect the fate of dormant tumor micro metastases in the stem cell niche in early breast cancer. Zoledronic acid was found to improve disease-free survival and overall survival in some adjuvant breast cancer settings. In the prostate cancer setting, antiresorptive therapy was reported to delay the development of overt bone metastases. Ongoing studies will provide further insight regarding the anticancer potential of antiresorptive agents.

S33 **P8**

Prevention of Tumor Growth and Bone Destruction in Myeloma by Pim Kinase Inhibition

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Devastating bone destruction in multiple myeloma (MM) still remains a significant clinical problem for which there is, as yet, no effective treatment to restore bone. In pursuing factors responsible for MM expansion and bone destruction, we have found that the serine/threonine kinase Pim-2 is overexpressed in MM cells as an anti-apoptotic mediator, and further up-regulated when MM cells are cocultured with bone marrow stromal cells and osteoclasts (Leukemia, 2011). In the present study, we explored the impact of Pim-2 inhibition on tumor growth and bone destruction in MM. Pim-2 protein expression was up-regulated in MC3T3-E1 preosteoblastic cells by addition of cytokines known as inhibitors of osteoblastogenesis in MM, including IL-3, IL-7, TNF-alpha, TGF-beta and activin A as well as MM cell conditioned media, suggesting Pim-2 as a common downstream mediator of these inhibitory factors. Treatment with Pim-2 siRNA or the Pim inhibitor SMI-16a facilitated mineralized nodule formation by BMP-2 in MC3T3-E1 cells; enforced expression of Pim-2 abrogated the mineralized nodule formation, suggesting antagonism of bone formation by Pim-2. The Pim-2 knockdown up-regulated Smad1/5 and p38MAPK phosphorylation as well as Osterix expression by BMP-2. Of note, the Pim inhibition restored mineralized nodule formation suppressed by MM cell conditioned media. Furthermore, treatment with SMI-16a at 20 mg/kg mouse every other day for 30 days markedly decreased MM tumor size without apparent loss of bone both in MM mouse models with intratibial injection of murine 5TGM1 MM cells and in SCID-rab MM models with human INA6 MM cells. These results demonstrate that Pim-2 up-regulated in bone marrow stromal cells acts a negative regulator for bone formation in MM, and suggest that Pim inhibition is able to prevent bone destruction and restore bone formation besides the suppression of tumor burden in MM.

S34 **P9**

Chondroadherin Is a Novel Regulator of Bone Metabolism that Antagonizes Cancer-related Disease through its Integrin Alpha2beta1 Binding Domain

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cCHAD is a cyclic peptide representing the alpha2beta1 integrin binding sequence of the matrix protein chondroadherin (CHAD). We found that this peptide inhibited osteoclast formation and bone resorption by impairing preosteoclast motility (-80% vs control), without affecting the intracellular signals to osteoclastogenesis or the molecular mechanism of bone

dissolution. The effect of cCHAD was mediated by transcriptional downregulation of genes involved in cell motility, including Nitric Oxide Synthase (NOS)2, migfilin and vasp. Consistently, treatment with the NO donor S-nitroso-N-acetyl-D,L-penicillamine blocked the effect of cCHAD rescuing normal preosteoclast motility, while the NOS2 inhibitor N5-(1-iminoethyl)-l-ornithine mimicked the inhibitory effect of the peptide. *In vivo*, cCHAD reduced osteoclast number and activity, counteracting bone loss in ovariectomized mice treated by both preventive and curative protocols. Balb/c nu/nu mice, intracardially injected with the human breast cancer cell line MDA-MB-231 and treated with 10 mg/kg cCHAD 5 days/week by an adjuvant protocol, showed a significant decrease of cachexia (vehicle 87%; cCHAD 31%; P=0.004) and incidence of bone metastases (vehicle 54%; cCHAD 19%; P=0.004), with a trend of reduction of visceral metastasis volume (mm^3: vehicle 23±8; cCHAD 9±8). When administered together with a suboptimal dose of doxorubicin (DXR, 0.1 mg/kg), cCHAD significantly improved survival (71% P=0.01) and reduced visceral metastases volume (3.5±1.4 mm^3) compared to 0.1 mg/kg DXR alone (survival 15%; volume 29±14 mm^3), reaching the effect of the optimal dose of DXR (0.2 mg/kg) and suggesting the ability of cCHAD to potentiate the outcome of chemotherapy. In mice orthotopically injected with MDA-MB-231 cells, cCHAD was also able to reduce tumor volume by both adjuvant and curative protocols of treatment. cCHAD impaired motility of tumor cells as well, was well tolerated even when administered for prolonged time and, while showing an effect on bone loss and development of bone metastases similar to that of alendronate, it induced a better improvement of cachexia and inhibition of tumor growth in visceral organs. Taken together, these pre-clinical data suggest that cCHAD could be employed in the management of bone loss and breast cancer-related diseases, underscoring an important translational impact of our study.

S35 **P10**

Regulation of Breast Cancer Induced Osteolysis by IKKβ

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IκB Kinase β (IKKβ), a key component of NFκB signaling, plays an important role in bone remodelling and cancer. IKKβ deficiency increases bone mass in mice whereas hyperactivation causes the formation of sclerotic bone lesions. Here, we studied the role of IKKβ in breast cancer-induced osteolysis *in vitro*, *ex vivo* and *in vivo*. Targeted inhibition of IKKβ kinase activity (87% reduction in IκB phosphorylation, p=0.001) in MDA-MB-231 cells by small interfering RNA significantly reduced conditioned medium-induced osteolytic bone loss in the mouse calvarial organ culture system (35% gain in bone volume, p<0.05, Figure 1c) and in adult mice (21% gain in bone volume, p<0.05). *In vitro*, silencing of IKKβ activity in MDA-MB-231 inhibited cell migration (64% reduction, p<0.01, Figure 1a) and significantly reduced the ability of these cells to stimulate osteoclast formation (87% reduction, p<0.01, Figure 1b) and to inhibit osteoblast differentiation (22% increase, p<0.05). Interestingly, inhibition of IKKβ activity in MDA-MB-231 also reduced TGFβ release (49% reduction, p<0.01), suggesting that IKKβ-driven TGFβ production by cancer cells may contributes to breast cancer cell behaviour in bone metastatic environment. Functional studies in osteoclasts showed that the selective IKKβ inhibitors BMS-345541 (BMS) and IMD-0354 (IMD) (10 μM) totally prevented osteoclast formation induced by a variety of mouse and human breast cancer cells or their derived factors. In osteoblasts, inhibition of IKKβ activity markedly reduced (p<0.01) RANKL/OPG ratio induced by conditioned medium, and significantly increased alkaline phosphatase levels (BMS: 90% increase and IMD: 67% increase, p<0.01) and bone nodule formation (BMS: 27% increase and IMD: 15% increase, p<0.05). Mechanistic studies in breast cancer cells, osteoclasts and osteoblasts showed that inhibition of IKKβ activity prevented cytoplasmic sequestering of the pro-apoptotic transcription factor FoxO3a,

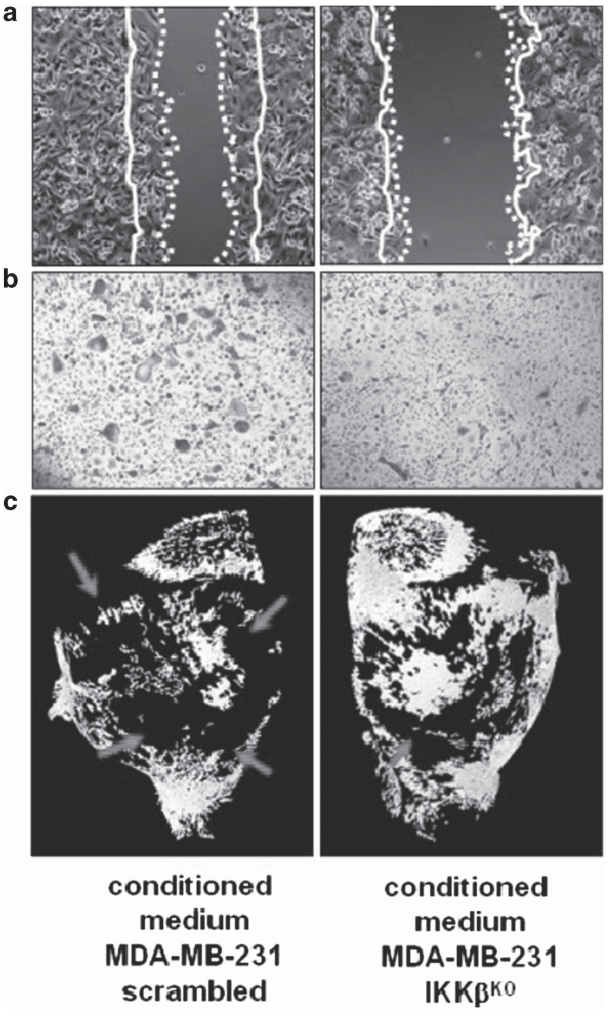

Figure 1 Targeted inhibition of IKKβ kinase activity inhibited MDA-MB-231 migration (panel **a**) and significantly reduced the ability of MAD-MB-231 cells to stimulate osteoclast formation (panel **b**) and to cause osteolytic bone damage in the mouse calvarial organ culture system (panel **c**).

suggesting a novel role of IKKβ/FoxO3a axis in breast cancer - bone cell interaction. Collectively, these findings reveal a novel role of IKKβ in the bidirectional crosstalk between breast cancer and bone cells, and suggest that IKKβ inhibitors, as anti-resorptive, anti-migratory and osteoanabolic agents might be effective in the treatment of metastatic bone disease.

GREGORY R. MUNDY MEMORIAL LECTURE

S36

Bone Targeted Treatments in Early Cancer – Has the Seed and Soil Hypothesis been Fulfilled?

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The processes involved in metastasis are complex and only partially understood. At the cellular level, metastasis is a rare event that appears to be dependent not only on the biological characteristics of the cancer cell(s) escaping from the primary tumour site, but also on the contributions from host stem cells and cancer cell-host cell interactions within target organs. The bone microenvironment would appear to be central to metastasis to the skeleton and also, potentially, to both other distant and loco-regional sites.

The use of bone targeted agents in early cancer has become increasingly important to prevent adverse effects of cancer treatments on bone health. In addition, and perhaps more importantly, they may also modify the course of the disease and disrupt the metastatic process via indirect inhibitory effects on the 'vicious cycle' of growth factor and cytokine expression within the bone marrow microenvironment. In the adjuvant, early cancer setting, there is increasing evidence that bone targeted treatments can modify the course of the disease, especially in the context of low levels of reproductive hormones. Improvements in both disease free and overall survival have been reported in several large clinical trials of adjuvant bisphosphonates in breast cancer, and beneficial effects on the development of bone metastasis seen with the use of denosumab in men with castrate resistant prostate cancer. In the breast cancer studies, the effects of bone targeted therapies appear to be at least as great on disease recurrence outside the skeleton as they are on development of bone metastasis. Intriguingly, it is the extra-skeletal effects that appear to be particularly influenced by levels of reproductive hormones, with marked heterogenity of effect according to menopausal status. Ongoing work, both within the clinic and in preclinical models is trying to explain these somewhat unexpected observations. The seed and soil hypothesis, first described by Stephen Paget in the 19th century and refined by Greg Mundy and others 20-30 years ago, appears to be still of great relevance in understanding the spread of cancer. However, only in the last few years has the evidence accumulated to support the inclusion of bone targeted agents that modify the soil into adjuvant treatment strategies that hitherto focussed purely on the seed.

CANCER RELATED BONE COMPLICATIONS (I)

S37

Cancer-associated Muscle Dysfunction: Role of Ryanodine Receptor 1 Remodeling

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Muscle weakness is common in advanced cancers and is a cause of significant morbidity yet the mechanisms of cancer-associated muscle dysfunction are unknown. Ryanodine receptor (RyR1) is the skeletal muscle sarcoplasmic reticulum calcium release channel required for excitation-contraction coupling. RyR1 undergoes remodeling in disease states resulting in leaky channels characterized by oxidation and nitrosylation and loss of the stabilizing subunit, calstabin1. We hypothesized that impaired muscle function in cancer could be due to remodeling of RyR1.

We used a mouse model of breast cancer bone metastases bone during which mice develop significant weight loss. Female nude mice were inoculated with MDA-MB-231 cells via intra-cardiac inoculation and compared to non-tumor bearing controls. Mice developed osteolytic lesions 12 days after inoculation. Tumor bearing mice lost significant weight by 4 weeks compared to controls (20.5±0.6 vs 23.2±0.4; $p<0.0002$). This was associated with a significant reduction in total body tissue, lean mass and fat in tumor bearing mice, as assessed by DXA ($P<0.01$). Muscle specific force production of the extensor digitorum longus (EDL) muscle was significantly decreased in tumor bearing mice compared to controls ($p<0.001$) and the reduction in muscle force correlated with larger osteolytic lesions ($p<0.05$). In addition, transmission electron microscopy showed dysmorphic mitochondria in tumor bearing mice. RyR1 from EDL of tumor bearing mice was oxidized, nitrosylated and depleted of calstabin1, consistent with leaky channels. Muscle function was also significantly impaired in animals harboring small bone osteolytic lesions visible by X-ray ($p<0.001$) during the early stages of weight loss and disease progression. Importantly, mice with primary MDA-MB-231 tumors in the mammary fat pad (without bone metastases) did not lose weight or exhibit muscle weakness. Our data show that MDA-MB-231 bone metastases are accompanied by severe muscle dysfunction and remodeling of RyR1 channel complex resulting in leaky channels. Similar remodeling of RyR1 has been shown to cause muscle weakness in muscular dystrophies and sarcopenia. Targeted therapy against leaky RyR1 channels improves muscle function and exercise capacity in murine models of muscular dystrophy and sarcopenia and may be an effective therapy for cancer-associated muscle weakness.

S38 **P11**

Radium-223 Completely Blocks Osteolytic Disease Progression and Increases Survival in a Mouse Model of Breast Cancer Bone Metastases

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Radium-223 chloride (ra-223; Alpharadin; Bayer HealthCare/Algeta ASA, Oslo, Norway) is an alpha-emitting radiopharmaceutical that has been shown to improve overall survival in the phase III clinical study (ALSYMPCA) in the treatment of castration resistant prostate cancer with bone metastases. Ra-223 has also shown efficacy in markers of bone metabolism in breast cancer bone metastasis patients. As calcium mimetic, ra-223 localizes to bone, where the emission of alpha-particles provide an efficient and localized radiation treatment to metastatic skeletal tumor lesions. In this study, we investigated the effects of ra-223 on survival and development of breast cancer bone metastases *in vivo* when administered in preventive and treatment settings and on differentiation and activity of human osteoclasts *in vitro*.
The *in vivo* effects were studied using a model where human MDA-MB-231(SA)-green fluorescent protein (GFP) cells were inoculated into nude mice via left cardiac ventricle at day 0. In the bone metastasis study, the animals were dosed with either vehicle or a single dose of ra-223 (300 kBq/kg) at day -1 (preventive setting) day 2 (micro-metastatic setting), or day 15 (treatment setting). Radiography, fluorescence imaging and histomorphometric analysis were performed at sacrifice (day 25). In the survival study, the animals were dosed with either vehicle or a single dose of ra-223 (300 kBq/kg) at day -1 or 2. In the *in vitro* study, human osteoclasts were cultured on bovine bone slices. The differentiation and resorption activity of osteoclasts were quantitated by measuring the amount of secreted tartrate-resistant acid phosphatase isoform 5b (TRACP 5b) and secreted C-terminal cross-linked telopeptides of type I collagen (CTX). Ra-223 decreased osteolysis by 98, 99.6 and 82%, when administered at days -1, 2 or 15. Median survival was 24.5 days in the control group, 39.5 days ($p < 0.001$ vs ctrl) in the group administered at day -1, and 35.5 days ($p<0.001$ vs ctrl) in the group administered at day 2. Ra-223 inhibited dose dependently differentiation of osteoclasts *in vitro*, but had no effect on resorption activity of mature osteoclasts. In summary, ra-223 administered in a preventive or micro-metastatic setting completely prevented osteolytic disease progression and increased survival in this mouse model of breast cancer bone metastases. These findings support the clinical development of ra-223 for patients at risk of developing bone metastases.

S39 **P13**

Mechanical Loading Decreases Osteolysis and Tumor Formation via Direct Effects on Bone Remodeling

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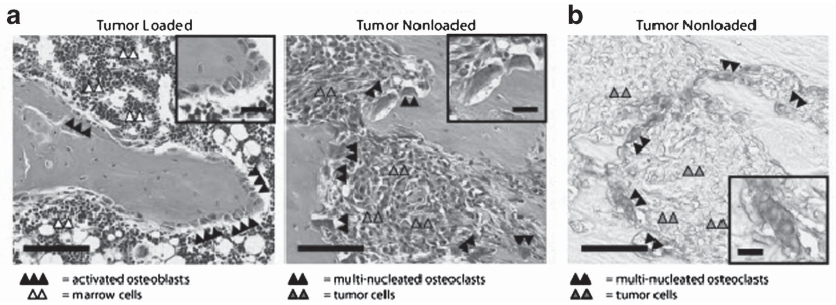
The skeleton is subject to mechanical stimulation; however, the effect of loading on the 'vicious cycle' of breast cancer bone metastasis remains unclear. We established a mouse model of human intratibial breast cancer to study metastatic tumor growth and bone degradation as a function of mechanical stimulation. Additionally, we developed a 3D culture model of bone metastasis to probe tumor-bone cell interactions in response to loading under well-defined conditions *in vitro*.
To assess the ability of loading to modulate metastasis dynamic tibial compression (1200 cycles, 4Hz, 5d/wk) was applied to 6wk old SCID mice following intratibial injection of human breast cancer cells (MDA-MB231, 0.5e6). Intratibial localization and lack of metastasis to other sites was determined via bioluminescence imaging (BLI). After 6 weeks, microCT and histological analysis were performed. To determine the preventative capability of loading, tibiae were subjected to preloading for 2 weeks prior to tumor cell injections. For *in vitro* studies, cyclic hydrostatic pressure (6MPa, 1Hz, 2h/d for 3d) was applied to hydroxyapatite (HA)-containing 3D polymeric scaffolds seeded with 1.5e6 MDAs, pre-osteoblasts (pre-Obs; human mesenchymal stem cells), or pre-osteoclasts (pre-Ocs; RAW 246.7). Tumor cell growth and secretion of pro-osteoclastogenic interleukin-8 (IL-8) was determined via DNA analysis and ELISA, respectively. Oc and Ob differentiation were assessed via analysis of TRAP secretion and intracellular ALP and osteocalcin mRNA, respectively. Additionally, histological and microCT analysis were conducted.
BLI suggests that injected MDAs localize to the tibiae without inducing secondary metastases. Loading dramatically



[S39] (**a**) Representative H&E cross-sections from loaded and nonloaded tumors after 6 weeks. Activated osteoblasts (inset bar = 25 μm) covered skeletal surfaces in loaded tibiae, whereas presence of multi-nucleated osteoclasts (inset bar = 25 μm) indicated bone degradation in nonloaded animals. Bar = 100 μm. (**b**) The presence of osteoclasts was further confirmed via subsequent TRAP staining of adjacent sections (inset bar = 25 μm). Bar = 100 μm.

inhibited metastatic tumor growth and bone degradation accompanied by greater Ob activation and reduced formation of multinucleated Ocs. Changes in tumor growth were likely related to altered bone rather than tumor cell behavior as loading did not affect tumor cell growth and IL-8 secretion in HA scaffolds *in vitro*. However, loading elevated Ob differentiation as indicated by increased tissue formation, and ALP. Correspondingly, loading negatively impacted Oc differentiation and decreased the number of large, multi-nucleated, TRAP-releasing cells. In conclusion, loading decreases tumor-mediated bone degradation via direct effects on bone rather than tumor cells, and may present a novel preventative measure against metastasis.

CANCER RELATED BONE COMPLICATIONS (II)

S40

Cancer-induced Bone Pain

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Cancer-induced bone pain (CIBP) is the most frequent type of cancer pain. CIBP adversely affects quality of life, performance status and mood and is associated with increased morbidity. CIBP rarely exists as a single entity, but instead exists as background (tonic) pain, spontaneous pain at rest and incident-related pain. While the former is thought to be responsive to traditional analgesics, the latter are problematic. Palliative radiotherapy (XRT) remains the gold standard and most effective treatment of CIBP. However, the analgesic effect may vary and is not immediate. As XRT is only effective in a proportion of patients predicting who will gain an analgesic benefit would be of great value. There is not currently a diagnostic test or marker that will predict those likely to benefit from XRT.

Following a pilot study, 60 patients receiving XRT for CIBP were recruited and assessed before and 6 weeks after XRT. In addition to pain and function, detailed somatosensory testing over the painful bone was carried out. On multivariate regression analysis, thermal changes were predictive of an analgesic response to palliative radiotherapy. These findings were congruent with our animal model results.The use of somatosensory testing has been used to evaluate novel analgesic approaches in our animal model of CIBP and in our translational clinical studies. An example using the recently completed translational pregabalin for CIBP randomised controlled trial will be presented.

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S41

Quality of Life Issues Related to Bone Metastasis and Antiresoptive Compounds

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Approximately 75% of patients with advanced cancer, especially those with common solid tumors of the breast and prostate will develop metastatic bone disease. Without adequate bone targeted therapy around half of these patients will experience one or more skeletal related events (SREs) such as radiation or surgery to bone, pathologic fracture and spinal cord compression. Perversely as modern anti-cancer therapy now extends survival times, this also increases patients' exposure to the risk of developing SREs. Not only are SREs life-threatening but they also compromise health-related quality of life (HRQoL), impairing mobility, causing significant pain and increasing the need for strong opioid medication. The negative impact of bone metastases and complications is wide ranging creating substantial burdens on both families and healthcare systems as well as a diminution of patients' HRQoL. Newer bone-targeted agents including the fully human monoclonal antibody denosumab may offer worthwhile benefits to patients over other oral or intravenous bisphosphonates. For example 3 pivotal trials in common solid tumors have shown that compared with zolendronic acid, denosumab reduced the time to first and subsequent on-study SREs and conveyed many HRQoL advantages including delaying reports of severe pain, reducing interference from pain on activities of daily living and the need for strong opioids. In this presentation I will consider some of the outcomes beyond efficacy that are relevant to patients such as: the advantages and disadvantages associated with the different routes of administration of bone-targeted agents, costs including extra testing, travel to hospital and burden on care-givers, as well as factors influencing adherence and patient preference.

S42 **P14**

Increased Adiponectin Reduces Nerve Growth Factor Expression in Myeloma-bearing Mice; a Novel Therapy to Combat Bone Pain in Cancer-bone Disease

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Bone pain is a major clinical feature of multiple myeloma. Nerve growth factor (NGF) promotes the growth and responsiveness of nociceptive fibers and positively correlates with pain sensitization. NGF is secreted by bone marrow stromal cells (BMSCs), and it has been shown that blocking NGF can prevent bone pain without altering tumor burden in murine models of prostate cancer. Interestingly, the adipokine adiponectin (ADPN) negatively correlates with NGF and bone pain in osteoarthritis. We hypothesized that ADPN suppresses NGF within the bone microenvironment and upregulation of ADPN may be used therapeutically to treat myeloma-associated bone pain. We utilized the 2T3 pre-osteoblast cell line to test the effect of ADPN on NGF *in vitro*. 5TGM1 and

Rag2-/- murine models of myeloma were used to establish the relevance of targeting NGF for myeloma-associated bone pain. Mice were treated with apolipoprotein peptide mimetic L-4F to increase ADPN *in vivo*. Established myeloma therapies (Velcade, Melphalan) were used to determine the effect of tumor burden on serum NGF. We first showed that 2T3 cells express ADPN receptors. Treatment of 2T3 cells with LPS led to a significant increase in NGF expression and secretion. This was inhibited by ADPN (1 ug/mL). We have previously shown that ADPN is decreased in patients who progress to myeloma and in myeloma-permissive KalwRij mice. Consistent with a role for ADPN to decrease NGF expression, KalwRij mice had increased serum NGF in the presence and absence of tumor. NGF was undetectable in WT and Rag2-/- mice. Inoculation of 5TGM1 myeloma cells into the myeloma-permissive Rag2-/- mice increased serum NGF. 5TGM1 myeloma cells do not express NGF, confirming that tumor cells are not responsible for the increase in NGF following tumor inoculation. Treatment of myeloma-bearing mice with L-4F increases serum ADPN and decreases tumor burden. Importantly, L-4F treatment also resulted in a significant decrease in NGF; KalwRij and Rag2-/- mice treated with L-4F both exhibited a 45% decrease in serum NGF ($p<.01$ and .05, respectively). Velcade and Melphalan reduced tumor burden, but failed to significantly alter serum NGF suggesting that decreased NGF with L-4F treatment was not due to decreased tumor. These data show that increasing ADPN *in vitro* and *in vivo* results in reduced NGF levels. Our results suggest that agents that increase ADPN, such as L-4F, may have therapeutic potential for the treatment of myeloma induced-bone pain.

S43 **P15**

Effects of Everolimus on Disease Progression in Bone and Bone Marker Levels: Outcomes from the BOLERO-2 Trial

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Background: BOLERO-2, a multinational, double-blind, placebo-controlled, phase 3 study in postmenopausal women with estrogen-receptor-positive breast cancer (BC) progressing after nonsteroidal aromatase inhibitors (NSAIs), showed significant clinical benefits with the addition of everolimus (EVE) to exemestane (EXE) (Baselga J *et al*. *NEJM* 2012;366: 520-9). As bone resorption is an important factor in BC (due to bone metastases [mets] as well as endocrine therapies, such as aromatase inhibitors), it is interesting to study bone-related effects of EVE. In preclinical studies, mTOR inhibition was associated with decreased osteoclast survival and activity. Exploratory analyses in BOLERO-2 evaluated the effect of EVE vs placebo (PBO) on bone marker levels and BC progression in bone.

Methods: Eligible patients were treated with EXE (25 mg/day) and randomized (2:1) to EVE (10 mg/day) or PBO. Bone turnover markers were exploratory endpoints analyzed at 6 and 12 weeks after treatment initiation, and included bone-specific alkaline phosphatase, amino-terminal propeptide of type I collagen, and C-terminal cross-linking telopeptide of type I collagen. Progressive disease in bone was defined as worsening of a preexisting bone lesion or development of a new bone lesion.

Results: Baseline disease characteristics, including baseline bone mets (EVE: n=371, 76%; PBO: n=185, 77%), were well balanced between arms (N=724), although baseline bisphosphonate (BP) use slightly favored the control arm (EVE 44% vs PBO 55%). Zoledronic acid (29% EVE vs 34% PBO) and pamidronate (6% EVE vs 7% PBO) were the most commonly used BPs at baseline. At 18 months' median follow-up, progression-free survival (primary endpoint), overall response rate, and clinical benefit rate ($P<.0001$, all) were significantly higher with EVE (n=485) vs PBO (n=239). Bone marker levels at 6 and 12 weeks increased vs baseline with PBO, but decreased with EVE. The cumulative incidence rate of BC progression in bone was consistently lower for EVE vs PBO in the overall population and in patients with bone mets at baseline (n=556). Fracture incidence was numerically lower in the EVE arm (2.3%) versus PBO (3.8%).

Conclusions: Exploratory analyses from BOLERO-2 suggest that adding EVE has beneficial effects on bone turnover and BC progression in bone in patients receiving EXE therapy for BC progressing after NSAIs.

Poster Presentations

P1 **S4**

Fibronectin Originating from Osteoblasts Affects Immune Cell Signatures and Tumor Growth

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Abstract selected for oral presentation. See Speaker Abstracts.

P2

Host-derived MMP-7 Decreases Myeloma Progression *In Vivo*: An Unexpected Role for MMP-7 and Osteopontin in Myeloma Pathogenesis

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Defining interactions within the host bone marrow microenvironment that promote the development of multiple myeloma (MM) is essential to identify new therapeutic approaches. Matrix metalloproteinases (MMPs) are key regulators of tumour: host interactions due to their ability to alter the activity of multiple substrates. Increasing evidence supports a critical role for host-derived MMPs in cancer progression and MMP7 has been implicated in breast and prostate cancer-mediated osteolysis. We hypothesized that host-derived MMP7 may play a role in MM bone disease *in vivo*. We have previously shown that inoculation of 5TGM1 MM cells into C57Bl/RAG2-/- mice resulted in MM development with osteolytic bone disease identical to that in the syngeneic C57Bl/KaLwRij strain. We used Rag-2/MMP7 null (MMP7-/-) mice to investigate the role of host-derived MMP7 in MM *in vivo*. Inoculation of 5TGM1-GFP MM cells into MMP7-/-mice unexpectedly resulted in a significant increase in the rate of tumour growth, as measured by serum IgG2bk concentrations, when compared to MM-bearing Rag2-/- wild-type mice (WT). This surprising finding suggested that host MMP7 plays a protective rather than a contributory role in MM. FACS analysis of GFP-positive MM cells confirmed a 72% increase in tumour burden within bone in MM-bearing MMP7-/-mice, $p<0.01$, and microCT analysis demonstrated a 47% decrease in BV/TV and 67% increase in osteolytic lesions in MM-bearing MMP7-/- mice as compared with MM-bearing WT mice, $p<0.01$. No significant difference in osteoclast or osteoblast number was found. In support of an effect of host-derived MMP7 on tumour growth and/or survival, a 51% decrease in MM cell apoptosis was observed in MM-bearing MMP7-/-mice, $p<0.05$. Using a candidate list of MMP7 substrates, we identified that MMP7-/-mice had increased serum osteopontin (OPN), compared to WT, $p<0.001$. *In vitro* studies demonstrated that MMP-7 cleaved OPN increased MM cell apoptosis, but full length OPN had no effect. To determine the clinical relevance of our findings, we used western blotting to assess cleavage of OPN in serum of patients with MM, demonstrating a significant increase in the ratio of full-length:cleaved OPN as compared to control, $p<0.05$. Furthermore, we observed an 86% increase in the MMP inhibitor TIMP-1 in serum from MM patients, $p<0.0001$. Our studies suggest MMP-7 activity contributes to MM pathogenesis and that host-derived MMP-7 inhibits MM growth *in vivo*, in part via processing of OPN.

P3 **S21**

Bone Marrow Mesenchymal Stromal Cell-derived Exosomes Mediate Oncogenesis in Multiple Meyloma

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Abstract selected for oral presentation. See Speaker Abstracts.

P4 **S25**

Antagonism of Inhibitor of Apoptosis Proteins Increases Bone Metastasis via Unexpected Osteoclast Activation

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Abstract selected for oral presentation. See Speaker Abstracts.

P5 **S26**

Loss of Expression of PMEPA1, a Negative Regulator of Transforming Growth Factor-beta Increases Bone Metastases from Prostate Cancer

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Abstract selected for oral presentation. See Speaker Abstracts.

P6 **S27**

Discovery of the Genetic Basis of a Hereditary form of Osteosarcoma and Demonstration of the Gene's Dysregulation in Sporadic Osteosarcoma

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Abstract selected for oral presentation. See Speaker Abstracts.

P7 **S29**

Very Late Antigen-4 (VLA-4) Targeted PET Imaging of Multiple Myeloma

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Abstract selected for oral presentation. See Speaker Abstracts.

P8 **S33**

Prevention of Tumor Growth and Bone Destruction in Myeloma by Pim Kinase Inhibition

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Abstract selected for oral presentation. See Speaker Abstracts.

P9 **S34**

Chondroadherin Is a Novel Regulator of Bone Metabolism That Antagonizes Cancer-related Disease Through its Integrin Alpha2beta1 Binding Domain

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Abstract selected for oral presentation. See Speaker Abstracts.

P10 **S35**

Regulation of Breast Cancer Induced Osteolysis by IKKβ

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Abstract selected for oral presentation. See Speaker Abstracts.

P11 **S38**

Radium-223 Completely Blocks Osteolytic Disease Progression and Increases Survival in a Mouse Model of Breast Cancer Bone Metastases

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Abstract selected for oral presentation. See Speaker Abstracts.

P12

Circulating Fibronectin Controls Tumor Growth and Fibronectin Content in Tumors Correlates with Survival

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Fibronectin exerts multiple roles in biology affecting proliferation, apoptosis, and differentiation. The roles of locally produced and circulating fibronectin were examined to better understand their role in malignancy.
Fibronectin conditional knockout mice on a nude background (cKO) received intracardial or intratibial injections of breast (MDA-MB-231B/luc+) cancer cells. Deletion of fibronectin in the circulation and bone marrow using the Mx promoter (Mx-cKO) resulted in prolonged survival (increased 25%, $p<0.05$) and diminished growth (CT: 20±4 vs cKO: 5±2 $x10^7$

RLU, p<0.01) that was due to decreased blood vessel formation (by 50%, p<0.01). Proliferation was significantly diminished (BrdU+ cells were diminished by 23%, p<0.05) and apoptosis was increased due to an increase in proapoptotic Bax-2 (p<0.05 by Western). Despite the key role attributed to fibronectin originating from endothelial cells in blood vessel formation, deletion of fibronectin in the circulation only using the albumin promoter attached to cre (Alb-cKO) resulted in the same decrease in blood vessel formation and growth as seen in Mx-cKO mice. This suggests that circulating fibronectin plays a key role in blood vessel formation during tumor growth.
Circulating fibronectin was able to infiltrate tumor tissue and increase endogenous fibronectin production both by cancer and stromal cells, qPCR analysis, both p<0.05). Because fibronectin is required for storage of VEGF in the matrix, VEGF in the tumors was decreased by 32% (p<0.05) in the absence of circulating fibronectin. Furthermore, endothelial cell proliferation and activation of signaling molecules (e.g., AKT) was significantly higher when cells were exposed to both fibronectin and VEGF than when exposed to either fibronectin or VEGF alone. Fibronectin content in tumors correlated with both blood vessel formation and tumor growth (r=0.6 and 0.9 respectively, p<0.05). Examination of two arrays from patients with prostate cancer showed that the strongest predictor of tumor related death was the presence of strong fibronectin staining intensity. An array of breast cancer biospies revealed a similar relationship. This shows that an increase in the amount of fibronectin in tumors reflects poor prognosis.
In summary, circulating fibronectin modulates blood vessel formation and tumor growth by affecting the amount of VEGF protein. Determination of fibronectin content is useful as a prognostic biomarker in prostate and breast cancer.

P13 **S39**

Mechanical Loading Decreases Osteolysis and Tumor Formation via Direct Effects on Bone Remodeling

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Abstract selected for oral presentation. See Speaker Abstracts.

P14 **S42**

Increased Adiponectin Reduces Nerve Growth Factor Expression in Myeloma-bearing Mice: A Novel Therapy to Combat Bone Pain in Cancer-bone Disease

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Abstract selected for oral presentation. See Speaker Abstracts.

P15 **S43**

Effects of Everolimus on Disease Progression in Bone and Bone Marker Levels: Outcomes from the BOLERO-2 Trial

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Abstract selected for oral presentation. See Speaker Abstracts.

P16 **S8**

Macrophages and Prostate Cancer Skeletal Metastasis

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Abstract selected for oral presentation. See Speaker Abstracts.

P17 **S9**

Thrombospondin-1 Contributes to the Vicious Cycle of Bone Metastasis in Both the Tumor and Host Microenvironment Compartments

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Abstract selected for oral presentation. See Speaker Abstracts.

P18 **S10**

miR-192 Impairs Metastatic Angiogenesis by an Exosomal Transfer Mechanism

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Abstract selected for oral presentation. See Speaker Abstracts.

P19 **S14**

Cabozantinib Reduces Breast Cancer Bone Metastases and Improves Survival in a Mouse Model

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Abstract selected for oral presentation. See Speaker Abstracts.

P20 **S15**

Evaluating the Safety and Efficacy of Denosumab Treatment for Giant Cell Tumor of Bone

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Abstract selected for oral presentation. See Speaker Abstracts.

P21 **S16**

Involvement of the Robo 1 and 4 Proteins in Breast Cancer Bone Metastasis

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Abstract selected for oral presentation. See Speaker Abstracts.

P22 **S19**

The PPARγ Ligand Badge Reduces Tumour Burden And Increases Bone Marrow Adiposity in Multiple Myeloma *In Vivo*

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Abstract selected for oral presentation. See Speaker Abstracts.

P23

Chondrosarcoma, Mast Cells and Angiogenesis

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For all tumours the centre is history and the edge is the future – particularly so for chondrosarcomas in which nodules of avascular tumour are separated by fibroconnective tissue septa. In a systematic glycoprofiling study, sections of 36 conventional intraosseous chondrosarcomas were stained with a panel of lectins and for mast cell tryptase. Tumour cells, septal mast cells and endothelial cells invariably stained with IPHA (Phaseolus vulgaris leukagglutinin) lectin. Only endothelial cells stained with PTL-II (Psophocarpus tetragonolobus) lectin. Mast cell and blood vessel counts increased significantly with tumour grade. Increased mast cell numbers and angiogenesis have been related to tumour progression of breast, lung and gastric carcinoma but there are no previous corresponding data for sarcoma. The receptor for IPHA is a branched glycan structure with increased beta1,6GlcNAc linkages produced by the glycosyltransferase GnTaseV. Carcinoma cells express IPHA receptors which are thought to increase metastatic potential although causative mechanisms are unclear. IPHA receptor expression has not previously been reported in chondrosarcomas. GnTaseV also has an indirect angiogenic effect. It is cleaved by gamma-secretase in the Golgi apparatus producing a secreted form which releases fibroblast growth factor (FGF-2) from extracellular matrices (including cartilage) and which in turn stimulates endothelial cell proliferation. Thus a possible novel pathway for angiogenesis in relation to chondrosarcoma is suggested.
The enzyme responsible for the PTL-II receptor is beta1,3galactosyltransferase (T synthase). Gene-targeted mice lacking

T synthase have disordered incompetent blood vessels and the enzyme is a requirement for vascular tube formation. This also indicates an unexpected requirement for O-glycosylation in tumoural vasculogenesis.
Therefore, at the edge of chondrosarcomas, tumour cells, mast cells and blood vessels are linked to facilitate tumour progression.

P24

Evaluating Denosumab Treatment for Giant Cell Tumor of Bone with an Independent Imaging Assessment

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Giant cell tumor of bone (GCTB) is a rare osteolytic tumor that tends to be locally aggressive. Currently no standard therapy exists for patients (pts) with unresectable or metastatic GCTB and no well-established tumor response criteria are available for evaluating GCTB treatments. The aim of this analysis was to provide an independent imaging assessment of tumor response for patients (pts) in a phase 2, open-label study of denosumab 120 mg (Q4W) for the treatment of GCTB, based on pre-specified response criteria. Objective tumor response (OR; complete or partial response) was evaluated retrospectively by an independent imaging facility (CoreLab Partners, Inc.) for patients who had CT, MRI, PET, or PET/CT as part of their standard of care. OR was summarized based on the best response using one of the following criteria: Response Evaluation Criteria in Solid Tumors (RECIST) v1.1, to evaluate tumor burden based on the size of lesions on MRI or CT scans; modified European Organization for Research and Treatment of Cancer (EORTC) to evaluate metabolic response based on Standardized Uptake Value by Body Weight (SUVbw) on PET scans; and modified, inverse Choi criteria to evaluate lesion density and size using Hounsfield units based on CT or MRI. Duration of response was evaluated in pts who had an OR, and durable responses were evaluated in patients with ≥ 2 evaluable time point responses that were at least 12 weeks apart. A total of 190 patients had ≥ 1 evaluable time point response. Patients were 55% female, age 35 (SD 13) years. An OR was observed in 136 patients (72%) based on the best response with any criteria (25% [47 of 187] by RECIST; 96% [25 of 26] for EORTC; and 76% [134 of 176] for Density/Size). The median (95% CI) time to OR was 3.1 months (2.9, 3.7). A total of 76/111 (69%) patients had OR sustained for up to 24 weeks, and 139/144 (97%) patients had tumor control (complete or partial response or stable disease) sustained for at least 12 weeks, based on the best response using any tumor response criteria. A total of 179/190 patients (94%) had no disease progression based on imaging criteria alone. The median time to disease progression was not reached at a median follow-up of 13.4 months.
Conclusion: In the first independent imaging assessment of a GCTB therapy to date, the majority of patients who received denosumab had a sustained, objective tumor response and tumor control. Denosumab continues to be studied as a potential treatment for GCTB.

P25

Targeting the E3 Ubiquitin Ligase C-Cbl Decreases Osteosarcoma Cell Growth and Survival and Reduces Tumorigenesis

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Deregulations of Receptors Tyrosine Kinase (RTKs) signaling are often associated with cancer. Targeting RTK degradation may therefore be an interesting approach to reduce RTK cell signalling in bone cancer. Osteosarcoma is the most common primary malignant bone tumor of bone and is characterized by a highly malignant potential with lung metastases as a major clinical outcome. We investigated whether targeting the E3 ubiquitin ligase c-Cbl which is implicated in the negative regulation of RTKs, could reduce RTK signaling, cell proliferation and survival in osteosarcoma. We first showed that increasing c-Cbl expression using lentiviral infection decreased osteosarcoma cell replication and survival, and reduced cell migration and invasion in murine and human osteosarcoma cells. Conversely, c-Cbl inhibition using short hairpin RNA (shRNA) increased osteosarcoma cell growth and survival, as well as invasion and migration, indicating that c-Cbl plays a critical role as a bone tumor suppressor. Importantly, we found that the anticancer effect of increasing c-Cbl expression was related specifically to the downregulation of EGFR and PDGFR alpha levels. In a murine bone tumor model, increasing c-Cbl expression in osteosarcoma cells also reduced EGFR and PDGFR alpha expression, resulting in decreased tumor cell proliferation and survival and reduced tumor growth *in vivo*. Moreover, forced c-Cbl expression in invasive cells dramatically decreased the incidence of lung metastasis in mice. Tissue microarray analysis revealed that low c-Cbl protein expression in human osteosarcoma is associated with elevated EGFR and PDGFR alpha protein levels in patients with poor outcome, which strengthens the importance of c-Cbl in human bone cancer cell development. In summary, our results show that selective targeting of c-Cbl expression represses specific RTKs, resulting in reduced osteosarcoma cell growth and survival and decreased bone tumor growth. More importantly,

targeting c-Cbl reduces osteosarcoma cell invasiveness *in vitro* and *in vivo*. This study supports the therapeutic interest of targeting c-Cbl to reduce bone malignancy in cancers involving abnormal EGFR or PDGFR alpha expression. This finding may lead to the development of mechanism-based drugs targeting c-Cbl-RTK interactions in bone cancers, which may have important implications for future therapeutic applications in conjunction with other anti-cancer therapies.

P26

Breast Cancer Induced Bone Pain

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Breast cancers are the most common source of metastases to bone of which cancer-induced bone pain is a frequent pathological feature. Cancer-induced bone pain is a unique pain state with a multiplicity of determinants that remains to be well understood and managed. Current standard treatments are limited by dose-dependent side effects that can depress the quality of life of patients. Glutamate is a neurotransmitter and bone cell-signalling molecule that has been found to be released via the system xC- cystine/glutamate antiporter on cancer cells of types that frequently metastasize to bone, including breast cancers. This project examines the hypothesis that limiting glutamate release from cancer cells metastasized to bone will reduce bone tissue disruption and cancer-induced bone pain. A mouse model of cancer-induced bone pain was established with intrafemoral human breast cancer cells (MDA-MB-231), and behavioural measurements were taken for weight bearing and induced paw withdrawal thresholds. The system xC- inhibitors sulfasalazine and (S)-4-carboxyphenylglycine both attenuated glutamate release from cancer cells in a dose-dependent manner *in vitro*. Treatment with sulfasalazine induced a moderate delay in the onset of behavioural indicators of pain in mouse models, and treatment with (S)-4-carboxyphenylglycine had no apparent results. This data suggests that the limitation of extracellular glutamate released from cancers in bone may provide some alleviation of the often severe and intractable pain associated with bone metastases.
In cancer cells, glutamate release is understood to be a side-effect of the cellular response to oxidative stress to upregulate the expression and activity of system xc to allow the increased uptake of cystine. The clinical implications of the attenuation of both cystine uptake and glutamate release extend to multiple types of cancers and to other disease states.
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P27

Allograft Arthrodesis of the Knee for Giant Cell Tumors

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Giant cell tumors of bone are aggressive benign tumors. Wide resection is reserved for a small subset of patients with biologically more aggressive, recurrent, and extensive tumors. For patients affected with giant cell tumors who are young or middle-aged adults with normal life expectancies and high levels of activity, arthrodesis is an attractive option for reconstruction after resection. We retrospectively studied 40 patients (mean age, 33.1 years) with Campanacci grade III giant cell tumors around the knee (12 distal femoral and 28 proximal tibial) that were treated with wide resection and allograft arthrodesis using compression plating between January 1998 and January 2008. At an average follow-up of 4.3 years (range, 2-10 years), no patient had local recurrence, malignant transformation, or pulmonary or distant metastases. The grafts united proximally and distally in 35 (87.5%) patients. Average limb-length shortening was 2 cm (range, 1.5-5 cm). No patient needed a lengthening procedure. Functional outcomes according to the Musculoskeletal Tumor Society measure were successful, with an average score of 26.3 points (range, 22-30 points).
Wide resection with allograft arthrodesis of the knee is a treatment option in young, active patients with Campanacci grade III giant cell tumors around the knee. Wide resection and reconstruction with knee allograft arthrodesis for giant cell tumors can achieve excellent control of disease, high fusion rates, acceptable functional results, and low complication rates.

P28

Incidence of Hypocalcemia Among Denosumab-treated Patients Enrolled in Three Registrational Phase 3 Trials

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Denosumab is a potent inhibitor of osteoclast formation, function, and survival and significantly reduces bone turnover. Inhibition of bone turnover may decrease serum calcium (Ca) levels. We evaluated the incidence of hypocalcemia in denosumab-treated patients (pts) with cancer and bone metastases enrolled in 3 pivotal phase 3 trials. Pts with solid tumors (breast, prostate, or other) with ≥1 bone metastasis or multiple myeloma (MM) with ≥1 bone lesion were enrolled in 1 of 3 identically designed, double-blind, double-dummy phase 3 trials.

Pts were randomized to receive denosumab (120 mg) or zoledronic acid (ZA; 4 mg, dose adjusted for renal function) every 4 weeks. Daily supplements of Ca and vitamin D were strongly recommended. Intake of these supplements was collected by pt report. Albumin-corrected serum Ca levels were measured by central lab. This analysis includes both adverse events (AEs) of hypocalcemia per investigator report (including preferred search term hypocalcemia) and grade 3/4 decreases in serum Ca levels (<7 mg/dL; <1.75 mmol/L) per central lab. Overall, 2841 pts received ≥1 dose of denosumab and 2836 pts received ≥1 dose of ZA. Adverse events of hypocalcemia were reported for 273 (9.6%) denosumab pts and 141 (5.0%) ZA pts, of which 41 (15.0%) and 18 (12.8%) respectively were deemed serious. 88 (3.1%) denosumab and 38 (1.3%) ZA pts had a grade 3/4 decrease in serum Ca levels per central lab. No fatal cases of hypocalcemia were reported. Among denosumab pts who experienced hypocalcemia, the median (Q1, Q3) time to first on-study hypocalcemia was 2.8 (1.0, 7.1) months. IV Ca supplementation was given to 38.1% (104/273) of denosumab pts with hypocalcemia events (3.7% of all denosumab pts). Hypocalcemia was reported in 5.6% of pts with breast cancer, 8.6% of pts with lung cancer, 12.8% of pts with prostate cancer, 14.0% of pts with MM, and 12.4% of pts with other types of solid tumors. The percentage of denosumab pts with hypocalcemia events was lower among pts who reported taking Ca and vitamin D supplements than among the pts who did not. In summary, hypocalcemia is an expected adverse reaction with bone-targeted agents, including denosumab 120 mg. Incidence of hypocalcemia was lower in pts who reported taking Ca and vitamin D supplements. Ca levels should be corrected prior to denosumab initiation and monitored as necessary on treatment. Pts should be advised to adequately supplement with Ca and vitamin D while receiving denosumab.

P29

Evaluation of Hypofractionated Palliative Radiotherapy Regimen for Painful Bone Metastases

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Background: Bone metastases are the most common cause of cancer-related pain. Radiotherapy (XRT) plays a major role in the palliation of pain in patients with bone metastasis. Several schedules of short and long fractionation XRT are used in clinical practice, with hypofractionated treatments being even more attractive for practical reasons. A considerable body of evidence supports the clinical use of short schedule of radiotherapy
Aims and Objectives: To evaluate the efficacy of short course fractionated schedule of 625cGy/fraction for 3 fractions in relieving pain in patients with multiple uncomplicated bone metastasis.
Method: Patients coming to the department of radiotherapy and oncology SMS Medical college were included in the study From January to December 2011, 60 patients with painful bone metastasis were treated with palliative localized radiotherapy. There were 42 males and18 females with a median age of 58 years (range 28-84). The commonest sites of treatment were the spine (59.6%) and pelvis (14.4%). The primary endpoint was clinically significant pain relief in the first six months of follow-up evaluated with the IAEA (International Atomic Energy Agency) pain measurement score measuring pain severity and pain frequency. Analgesic use was also recorded before and after treatment as drug frequency and drug severity. Patients with painful bone metastasis from any primary tumor site were irradiated. Treatment schedule consisted of a short course of radiotherapy with 625cGy/fraction/week for 3 weeks (total dose: 18.75Gy).
Results: Complete pain relief was achieved in 20/60 lesions (33%). The overall response (complete + partial) was 37/60 lesions (61%). The minimum, maximum and median follow-up was 3, 23, and 9 months. The actuarial median duration of pain relief was: 4.5 months. No particular side effects were recorded.
Conclusions: Palliation of pain was obtained in approximately two thirds of patients with this schedule. With regard to pain response these data justify a recommendation for the use of a more simple and convenient 625cGy for 3 fractions for the palliation of uncomplicated bone metastasis.
Conflict of interests: Nil.

P30

ErbB3 Silencing Inhibits Osteosarcoma Cell Proliferation and Tumor Growth *In Vivo*

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Osteosarcoma is the most common primary bone tumor in children and adults. The identification of the molecular signals that contribute to the aberrant osteosarcoma cell growth may provide clues to develop new therapeutic strategies for chemoresistant osteosarcoma. The ErbB family of receptor tyrosine kinase plays an important role in the growth of various organs. ErbB3 lacks an intrinsic kinase activity and forms preferred heterodimerisation with ErbB2 to activate signaling. The implication of ErbB2/ErbB3 in osteosarcoma cell growth and tumorigenesis is unknown. Here we investigated the implication of ErbB3 in bone tumor cells using short hairpin RNA (shRNA)-mediated inhibition of ErbB3. We found that silencing ErbB3 using lentiviral infection decreased cell growth by 50% in murine K7M2 osteosarcoma cells. In contrast, ErbB3 silencing did not significantly increase cell apoptosis in these cells. Using standard *in vitro* assays, we showed that ErbB3 silencing reduced cell migration by 40% and decreased cell invasion by 30% in K7M2 cells which are highly metastatic. These results indicate that ErbB3 plays a role in bone tumor cell growth and invasiveness *in vitro*. We thus investigated whether ErbB3 silencing may reduce tumor progression in a mouse model. In this assay, parental and shErbB3-transduced murine K7M2 cells were injected in BALB/C mice and tumor growth was determined after 5 weeks. In this murine bone tumor model, ErbB3 silencing in K7M2 cells strikingly reduced tumor growth and number compared to control tumors. Histological analysis of the developing ectopic bone tumors showed that shRNA-targeted ErbB3 expression decreased cell replication by 40% as determined by Ki67 staining, whereas cell apoptosis

evaluated by Tunel staning was not increased. Taken together, our results indicate that ErbB3 plays an essential role in bone tumorigenesis. The finding that ErbB3 depletion greatly reduces bone tumor cell growth and invasiveness *in vitro* and *in vivo* raises the potential therapeutic interest of targeting ErbB3 to impact bone tumors in which ErbB3 is highly expressed. Since our data indicate that inhibition of ErbB3 expression exerts anti-cancer effects in osteosarcoma, the use of specific ErbB3 antagonists such as therapeutic antibodies in conjunction with other therapies may prove to be useful for reducing osteosarcoma cell growth and malignancy.

P31

Prevention of Osteonecrosis of the Jaw (ONJ) in Cancer Patients Receiving Bisphosphonates: Empty Promise or Effective Strategy?

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Background: Guidelines for the prevention of ONJ in cancer patients (pts) receiving bisphosphonates recommend a screening dental visit and avoidance of invasive dental procedures. However, these guidelines are based on retrospective cohort/case-control studies and prospective trials supporting prevention have been hampered by small patient numbers, precluding robust estimates of overall benefit.
Methods: A systematic review of published papers and conference proceedings was performed, and a meta-analysis of relevant prospective comparative studies was conducted. The risk ratios (RR) for ONJ were pooled using the Mantel-Haenszel method in a fixed-effects model.
Results: Out of 382 identified references, 31 were deemed relevant and reviewed in detail. After excluding non-prospective (n=3), non-comparative (n=11), and duplicate publications (n=10), 7 eligible studies were included for meta-analysis. All studies were single-center, non-randomized, with a prospective intervention cohort and a retrospective control group. The median study size was 186 pts (range 84-966), with a median follow-up in intervention and control groups of 11.2 and 11.4 months, respectively. Individually, only 2 out of 7 trials reported a statistically significant reduction in ONJ risk with prevention. Analysis of pooled data on 2,243 pts (934 prevention arm; 1,309 no prevention arm) showed a relative reduction of 68% of ONJ risk (RR 0.32; 95% CI 0.20-0.50; p<0.001) using a preventive strategy (table 1). The weighted mean ONJ risk without prevention was 11.8% (95% CI 3.7-20.0). Overall, between study heterogeneity was not statistically significant (Cochran's Q 7.23; I^2 17%; p=0.30). A test for interaction revealed no difference in prevention benefit between pts with multiple myeloma (n=212) or with bone metastases from solid tumors (n=2,031) (p=0.38). There was weak evidence for small study-effect bias (Harbord p=0.07).
Conclusion: Screening dental visits and avoidance of invasive dental procedures appear effective to reduce ONJ risk in pts with solid tumors and multiple myeloma receiving bisphosphonates. Further preclinical research efforts are required to advance our understanding of ONJ pathophysiology and improve prevention.

TABLE 1	Prevention		No prevention		RR ONJ (95% CI)	P
	ONJ	Total	ONJ	Total		
Solid tumors	17	803	56	1228	0.36 (0.21 - 0.61)	<0.001
Multiple myeloma	6	131	14	81	0.22 (0.09 - 0.55)	0.001
Overall	23	934	70	1309	0.32 (0.20 - 0.50)	<0.001

[P31]

P32

***In Vitro* Evaluation of the Anti-tumoral and Anti-osteoclastogenic Effect of Combined Therapy with Zoledronic Acid and Saracatinib in Breast Cancer**

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Background: Breast cancer (BC) often spreads into bone leading to increased morbidity and mortality. Bone metastases (BMs) derive from a vicious cycle established between tumor cells and the bone microenvironment, favoring osteolysis and tumor growth. At this stage of disease the use of anti-resorptive drugs, like bisphosphonates (BPs) or denosumab decreases osteolysis, the incidence of skeletal-related events, and associated morbidity. Both agents are effective in reducing levels of NTX (a collagen type I fragment derived from the activity osteoclasts' cathepsin). In a phase II trial, the Src kinase inhibitor saracatinib (AZD0530) was not superior to BPs in reducing NTX levels but has shown to decrease the bone resorption marker ICTP (MMP-1 cleavage of type I collagen), which raises the hypothesis of an osteoclast-independent effect. Here, we tested *in vitro* the combination of zoledronic acid (ZA) (the most potent BP) and saracatinib in BC cell lines and in osteoclasts.
Objectives: To determine the anti-tumoral and anti-osteoclastogenic effects of ZA and saracatinib, in mono or combined therapy, in a BC *in vitro* model.
Methods: Human BC cells MDA-MB-231 were treated with 25 μM ZA (Novartis), 1 μM saracatinib (AstraZeneca) or ZA+saracatinib, to assess the effect of these compounds in cell proliferation, apoptosis, and MMP-1 expression. For osteoclastogenesis assays, mouse monocytic cell line RAW 264.7 was stimulated with 100 ng/μL RANKL to induce differentiation. Cells were incubated with 25 μM ZA, 1 μM saracatinib, or ZA+saracatinib, and the effect in osteoclastogenesis was assessed by TRAP staining.
Results: BC cells treatment with ZA significantly decreased BC cell proliferation (p<0.01), but had no effect on MMP-1 expression. Saracatinib significantly decreased BC cell proliferation (p<0.01), and significantly decreased MMP-1

expression ($p<0.01$). Treatment with ZA+saracatinib significantly decreased BC cell proliferation and MMP-1 expression ($p<0.05$ and $p<0.01$, respectively) when compared with ZA or saracatinib alone. Both drugs impaired osteoclastogenesis, an effect increased by the use of the two drugs simultaneously. Conclusions: ZA and saracatinib may have increased anti-tumoral and anti-osteoclastogenic effects when used in combination, which could potentially improve the clinical outcome of patients with BMs. Saracatinib down-regulates MMP-1 expression in BC tumor cells, favoring an osteoclast-independent effect in the tumor compartment of BMs.

P33

The Involvement of Osteoprotegerin (OPG) in Prostate Cancer Cell Migration Induced by Receptor Activator of Nuclear Factor Kappa-b Ligand (RANKL)

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Majority of patients with advanced stage of prostate cancer develop skeletal metastasis that lead to pain and pathological bone fractures. Receptor Activator of Nuclear Factor κB Ligand (RANKL) is a key regulator of osteoclastic bone resorption. In addition, RANKL has been shown to induce directional migration of breast and prostate cancer cells to bone. Osteoprotegerin (OPG) is a decoy receptor for RANKL, which blocks RANKL from its osteoclastogenic action, and impedes the development of skeletal metastasis. However, serum OPG levels in prostate cancer patients exhibit significant positive correlation with severity of bone metastasis. We hypothesized that OPG produced by cancer cells positively regulates RANKL-induced directional cell migration by controlling RANKL availability and maintaining its gradient. We first assessed RANK, RANKL and OPG expressions in human prostate carcinoma PC3 cell line and found that all the components were expressed by this cell line. Moreover, OPG was found to be secreted by PC3 cells. We next examined if RANKL induces directional migration of PC3 cells using transwell migration assay. We observed that when increasing concentrations (0-50 ng/ml) of RANKL were added to the lower chamber of transwell system, migration of PC3 cells through the insert membrane increased in a dose dependent manner (reaching maximum at 50 ng/ml) without increasing effect on cell viability. Further increase in the RANKL concentration resulted in a gradual decrease in PC3 cells migration. When OPG was added to the lower chamber, migration of PC3 cells was not effective in the absence of RANKL. However, in the presence of RANKL, OPG blocked RANKL-induced PC3 cells migration. We next performed transfection assay to examine how changes in OPG expression in PC3 cells can regulate cell migration. After silencing OPG with small interfering RNA (siRNA), and confirming the decreased level of OPG expression by western blot; migration assay was performed in the presence of RANKL. We observed that migration was not induced by RANKL in the OPG siRNA transfected cells. These data suggest that cancer cells-produced OPG act to augment RANKL-induced directional migration, and may increase the range in which cancer cells are responsive to RANKL gradient.

P34

Osteoblast Behaviour During Prostate Cancer Bone Metastases: A Role for Protease Activated Receptor 2?

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Osteoblasts express the G protein-coupled receptor, protease-activated receptor 2 (PAR_2). Prostate cancer cells express three known PAR_2 agonists, the soluble serine protease kallikrein related peptidase 4 and the membrane anchored serine proteases matriptase and transmembrane protease serine 2. As we have previously demonstrated a role for PAR_2 in bone development and repair, we have hypothesised that PAR_2 is required for the osteoblastic bone formation seen in prostate cancer bone metastases. To test this hypothesis, we have treated primary mouse $PAR_2^{+/+}$ and $PAR_2^{-/-}$ osteoblasts with control media and media conditioned by the osteolytic human prostate cancer cell lines PC3, DU145 and the osteoblastic human prostate cancer cell line MDA-PCa-2b. Following treatment, osteoblasts were assessed for proliferation using BrdU incorporation assays. Effects on differentiation were investigated using alkaline phosphatase activity assays and quantitative PCR assessment of expression of osteoblast-associated genes. Treatment of $PAR_2^{+/+}$ and $PAR_2^{-/-}$ osteoblasts with media conditioned by the MDA-PCa-2b cell line, but not the PC3 and DU145 cell lines, promoted cell proliferation and osteocalcin expression. Interestingly, media conditioned by the MDA-PCa-2b cell line also significantly increased expression of the markers of osteoblast differentiation, alkaline phosphatase, RUNX2, collagen type I, and osteopontin by $PAR_2^{+/+}$ osteoblasts, but had no effect on the expression of these markers by $PAR_2^{-/-}$ osteoblasts.
The results presented here suggest that activation of osteoblastic PAR_2 by proteases secreted from prostate cancer cells may play a role in the formation of osteoblastic lesions seen in prostate cancer bone metastases.

P35

Evaluating the Efficacy of De-escalated Bisphosphonate Therapy in Metastatic Breast Cancer Patients at Low-risk of Skeletal Related Events. Triumph: A Pragmatic Multicentre Trial

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Background: Optimal bisphosphonate (BP) dosing intervals for breast cancer patients (pts) with bone metastases (BM) remain unknown. BP are usually prescribed q3-4 wk regardless of individual risk for skeletal related events (SREs). Amadori (*J Clin Onc* 2012 suppl; abstr 9005) showed q12 wk BP is as effective as q4 wk in pts previously treated with >9 cycles of q4 wk BP. Further evaluation of modified BP dosing strategies is warranted. The objective of the current study was

to show in pts with biochemically defined low-risk bone disease (serum CTx <600 ng/L) that IV BP use every q12 wk for 1 year is sufficient to maintain stable serum c-telopeptide (CTx).
Methods: Eligible pts with BM, who had received >3 months of q3-4 wk IV BP were enrolled. Biochemical failure was defined as CTx levels >600 ng/L at baseline, weeks 6, 12, 24, 36 or 48. Evaluation of palliative benefit of 12-wk IV BP therapy was measured by SREs, analgesic use, and self-reported pain.
Results: From Oct. 2010–Sept. 2011, 85 pts were screened, with 13 found ineligible. In the 71 accrued pts: mean age 60 (SD 13), median time from breast cancer diagnosis to development of BM 4 months (IQR 82), median duration of prior BP therapy 14 months (IQR 19), and mean number of SREs/yr prior to entering study 0.35 (SD 0.76). Baseline median CTx was 120 ng/L (IQR 240) and BSAP 9.2 IU/L (IQR 3). To date: 26/71 pts (36%) remain on study. Reasons for coming off study include; study completion (18), elevation of CTx >600 ng/L (10), or on study SRE (3). A rise in CTx between baseline and wk 6 was associated with coming off study early (P=0.008). For pts who had had an SRE before study entry the odds ratios for coming off study early due to on study SRE was 0.0245 (CI 0.061-0.094; p=0.046). Of 8/13 pts ineligible due to baseline CTx >600 ng/L, 6 had an SRE within 1 year of screening.
Conclusion: De-escalating BP therapy to 12 weekly in low risk pts has advantages for both the pt and health care system. Individual risk of SREs is highly variable, however baseline serum CTx levels <600 ng/L is associated with a low risk of subsequent SREs. Larger trials are required to assess whether increasing CTx with de-escalated therapy will lead to higher rates of SREs or not (Coleman, *J Clin Onc* 2012 suppl; abstr 511). However, the results of this study and Amadori *et al*. would suggest that de-escalated BP treatment will likely become a new standard of care after a limited period of q 4wk treatment.

P36

Multiple Myelomas Express the Membrane Receptor Klotho and Respond to its Bone-derived Ligand, FGF23

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Purpose: To determine the expression and function of klotho and FGF23 in multiple myeloma (MM).
Experimental Design: Bone marrow aspirates were stained by immunohistochemistry (IHC) for klotho. Soluble klotho (s-KL) and intact FGF23 were measured using ELISAs in sera from controls and MM patients. Klotho and FGF23 expression were determined in human MM cell lines and primary cells using real-time PCR. MM cells treated with recombinant FGF23 were evaluated for klotho:FGFR-activated signaling.
Results: Marrow samples from 42 newly diagnosed MM patients all stained positively for klotho, while plasma cells from 8 controls and 3 patients with monoclonal gammopathy of unknown significance (MGUS) were negative. S-KL is shed by proteolysis from membrane klotho and also secreted as a splice variant. Human MM cell lines RPMI-8226, U266, H929 and MM.1s expressed membrane klotho mRNA but did not make sufficient s-KL protein to detect. Serum s-KL levels were similar in MM to healthy controls. Klotho forms, with a canonical FGF receptor (FGFR), a complex with high binding affinity for FGF23, which is secreted by bone cells. Intact FGF23 in sera from MM patients was 3-fold higher than controls, but MM cells lacked significant FGF23 mRNA, suggesting the increased serum FGF23 comes from a non-tumor source, such as osteocytes. 100 ng/ml rhFGF23 rapidly and significantly induced the early gene response transcription factor EGR1 mRNA 2-10x in MM cell lines, showing that the FGF23/FGFR/klotho signaling pathway is functional in MM cell lines.
Discussion: Bone involvement is a major cause of morbidity and mortality for patients with multiple myeloma (MM), but the tropism of MM cells for bone remains poorly understood. FGF23 is an endocrine factor released from bone that binds to cells expressing an FGFR and the coreceptor klotho. This is the first report of klotho expression and a molecular response to FGF23 by myeloma cells. MM interaction with bone could increase FGF23 secretion and activate signaling in tumor cells, driving myeloma bone disease. The klotho/FGF23 axis may provide a novel target for therapeutic intervention against MM.
Conclusions: Multiple myeloma cells express klotho, the specificity co-receptor for FGF23, and respond to FGF23, which is significantly increased in myeloma patients.

P37

3D Polyurethane Scaffolds (3D-PURS) with Defined Architecture and Rigidity for Analysis of Tumor-induced Bone Disease

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Tumor-induced bone disease is regulated by a complex interaction between tumor cells and the microenvironment. Recently, we published that the rigidity of 2D substrates can influence tumor cell gene expression. We hypothesize that a combination of the rigid bone matrix and the cellular content of the bone marrow are required for tumor cells to induce bone destruction. Studying these interactions *in vivo* is complicated. Furthermore, the limitations of 2D experiments are well known. Therefore, we have developed a model that uses a 3D polyurethane scaffold (3D-PURS) mimicking the bone microenvironment. 3D-PURS are generated by reactive liquid molding of polyurethane precursors in a prefabricated polystyrene mold, Figure 1a-b. The resulting architecture is 100% interconnected, Figure 1c. Pore sizes of 300 or 500 μm were utilized in this study, which were taken from trabecular spacing data measured by μCT analyses in mice with healthy or tumor-damaged bone. The mechanical properties of the 3D-PURS are controlled by the precursors and can be tailored to mimic soft tissue (<30 MPa) or bone (>500 MPa). Our initial studies indicated that tumor cells were able to adhere to the 3D-PURS by fluorescence and electron microscopy, Figure 1d-e. PTHrP (parathyroid hormone related protein) gene expression in 3D was measured. *In vitro* testing with soft and rigid 3D-PURS seeded with MDA-MB-231 cells ($1x10^6$ cells/scaffold) verified a significant increase in PTHrP expression with rigidity after 24 hours in culture, Figure 1g. The differences are greater *in vivo*, where similarly seeded 3D-PURS were implanted subcutaneously in mice. Tumors were observed after 21 days by

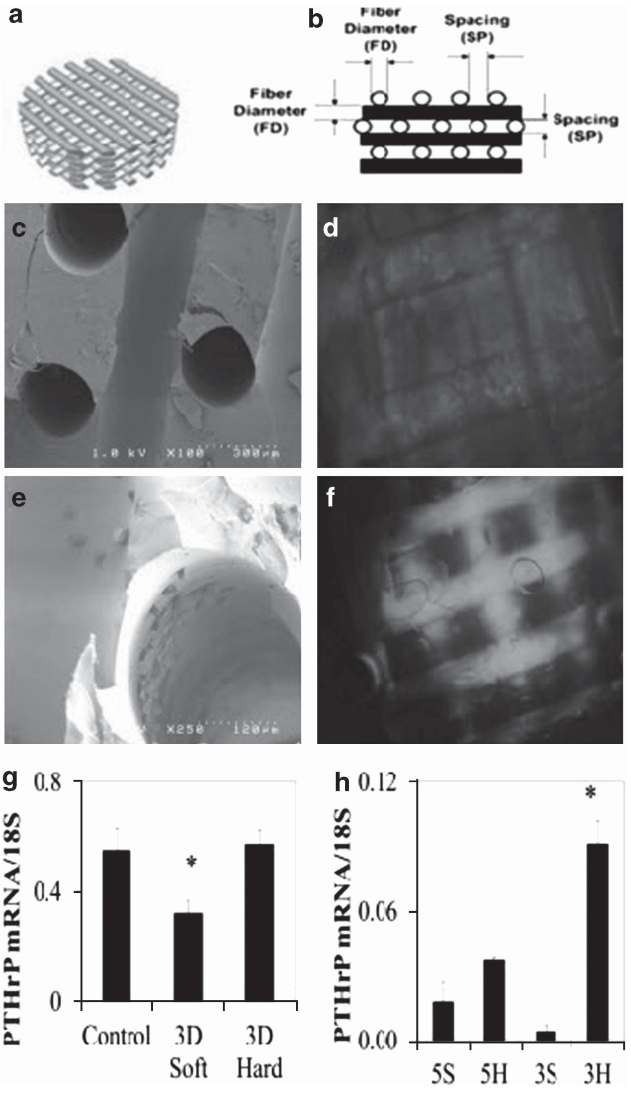

Figure 1 Analysis of metastatic cancer cells in a novel 3D polyurethane scaffold. (**a** and **b**) Diagram of prefabricated polystyrene scaffolds utilized as a mold for the reactive polyurethane; (**c**) SEM image of the 3D-polyurethane scaffold (3D-PURS) after the polystyrene mold is removed; (**d**) Fluorescent image of MDA-231 cells transfected with green fluorescent protein seeded into 3D-PURS; (**e**) SEM image of MDA-231 cells adhered to channel walls of 3D-PURS; (**f**) *Ex-vivo* fluorescent images of MDA-231 cells in 3D-PURS after 21 days *in vivo*. (**g**) *In vitro* PTHrP expression of MDA-231 cells culture on 3D-PURS (300 µm pores), the control is 2D tissue culture plastic; (**h**) *In vivo* PTHrP expression of MDA-231 cells culture on 3D-PURS after 21 days (5 = 500 µm pores, 3 = 300 µm pores, S = soft, H = hard). *p<0.05.

in vivo fluorescence imaging, at which point RNA was harvested from the excised scaffold, Figure 1f, using TRIzol. RT-PCR data showed a larger difference, Figure 1h, in PTHrP expression between soft and rigid scaffolds with 300 µm pores. Furthermore, both soft and rigid scaffolds with 500 µm pores had significantly lower expression of PTHrP than the rigid 300 µm scaffolds. The changes in PTHrP expression with differences in rigidity and pore size point to an active response to environmental cues by metastatic cancer cells. This model is ideal for studies of the microenvironmental effects on tumor cells. Specifically, this model will allow for molecular signaling studies and testing of potential inhibitors of the mechanotransduction pathway that can be clinically translated.

P38

Combination of Antiangiogenic Therapies Reduces Osteolysis and Tumor Burden in Experimental Breast Cancer Bone Metastasis

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Introduction: The clinical efficacy of anti-angiogenic monotherapies in metastatic breast cancer is less than originally anticipated and it is not clear what the response of bone metastases to anti-angiogenic therapies is. Moreover, the evaluation of the combined use of antiangiogenic agents is still in its infancy. Therefore, we examined here the impact of neutralizing tumor-derived VEGF and/or VEGF receptor activity in a mouse model of breast cancer bone metastasis.
Methods: VEGF expression in human MDA-MB-231/B02 breast cancer cells, which metastasize to bone, was invalidated using RNA interference strategy. The effects of VEGF silencing in B02 cells (Sh-VEGF) were compared with that of mock-transfected cells (Sc-VEGF) using *in vitro* cell migration and invasion assays and a model of tumor angiogenesis following subcutaneous implantation of Sh-VEGF and Sc-VEGF transfectants in nude mice. The effects of VEGF receptor tyrosine kinase inhibitor vatalanib were also investigated on bone metastasis formation caused by Sh-VEGF and Sc-VEGF transfectants. Additionally, the effects of vatalanib and VEGF-neutralizing antibody bevacizumab, used as single agents or in combination, were studied in the mouse model of bone metastasis caused by parental B02 breast cancer cells. Bone destruction was measured by radiography and histomorphometry. Skeletal tumor burden was measured by bioluminescence imaging and histomorphometry. All statistical tests were two-sided.
Results: VEGF silencing severely impaired the motility and invasiveness of B02 breast cancer cells *in vitro* and decreased tumor angiogenesis *in vivo*, leading to growth inhibition of subcutaneous tumor xenografts in animals. VEGF silencing in B02 cells did not however inhibit the formation and progression of experimental bone metastases. Similarly, vatalanib did not inhibit bone metastasis formation caused by Sc-VEGF transfectants. By contrast, it did inhibit bone metastasis formation in animals bearing Sh-VEGF tumors. Moreover, B02 tumor-bearing mice treated with vatalanib + bevacizumab showed a decreased bone destruction and a reduced tumor burden compared with vehicle, whereas a single agent therapy had no effect.
Conclusion: A combined therapy targeting both VEGF and its receptors efficiently reduces not only bone destruction but also skeletal tumor growth in a mouse model of breast cancer bone metastasis.

P39

Combination Treatment with Sunitinib and Docetaxel Provides Effective Therapy for Lytic Bone Metastasis of Human Prostate Cancer

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Bone metastases of prostate cancer cause bone deterioration with numerous clinical manifestations, including pain and fractures. We propose that appropriately selected combination therapy directed may have improved therapeutic outcomes over single agents or therapy directed at bone resorption. Combination therapy with the VEGF inhibitor Sunitinib and the chemotherapeutic Docetaxel was selected for these studies. Twelve week old male nude rats were injected in the tibiae with 22RV1 human prostate cancer cells tagged with luciferase. Rats were randomized to the following treatment groups at day 14: 1) Naïve Control, 2) Tumor Control, 3) Docetaxel 4) Sunitinib and 5) Docetaxel plus Sunitinib combination. IVIS and Faxitron images and blood samples were collected weekly throughout the study. Body weight was collected twice per week. Dynamic weight bearing data was collected at day 0, 14, and 42. At termination, hind limbs were collected for microCT, immunocytochemistry, and routine histology. While Sunitinib and Docetaxel both reduced tumor burden, Sunitinib was more effective than Docetaxel as a single agent. The combination of both drugs was more effective than either agent used alone. Tumor re-bound was observed in all groups by four weeks following cessation of treatment. However, the re-bound occurred more slowly in the combination group than single therapy groups. Bone volume in tumor bearing legs was reduced as measured by microCT analysis, thereby confirming the lytic nature of the tumors. Dynamic weight bearing assessment at Day 42 demonstrated less weight bearing on the affected limb amongst tumor bearing control animals when compared to naïve controls. This effect was not seen in any of the drug treatment groups indicating that therapeutic intervention preserved normal weight bearing. Standard H&E and TRAP staining demonstrated that tumor bearing rats had numerous osteoclasts in the tibial metaphysic as well as, decreased trabecular bone, when compared to naïve controls. Animals treated with Sunitinib, or the combination of Sunitinib and Docetaxel had fewer osteoclasts and preservation of trabeculae. In conclusion, the results suggested that the combination of Docetaxel and Sunitinib was more effective than either drug used as a single agent in the prostate tumor model.

P40

Bone Metastatic Prostate Cancer Cells Exhibit Adipomimetic Properties

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Bone metastases often occur in advanced prostate cancer. The invasion of prostate tumor cells in bone predominantly causes osteoblastic bone lesions, with increased bone mineral density at the lesion site. The precise mechanisms underlying the development and progression of prostate cancer bone metastases are poorly understood. More recently, obesity and adipokines have been associated with an increased risk of developing aggressive prostate cancer. Although adipokines such as adiponectin and leptin have been shown to increase the motility and the proliferation of prostate cancer cells, their role in prostate cancer bone disease is unknown. There is increasing evidence to suggest that, although originally identified as secreted from adipocytes, adipokines are expressed by a number of different cell types. Furthermore, prostate cancer cells have recently been shown to have the potential to differentiate into adipocyte-like cells. Therefore, we hypothesized that prostate cancer cells may exhibit an adipomimetic phenotype that may contribute to the development of bone metastases. To investigate this, we used cytokine arrays, PCR and western blotting to assess the expression profile of adipokines in human prostate cancer cells that have high (e.g. ARCaP M) or low (e.g. ARCaP E) osteoblastic bone-metastatic potential. Cytokine array analysis of conditioned media from ARCaP M cells and ARCaP E cells revealed differences in the expression level of several adipokines including adiponectin and leptin. The differential expression of adiponectin and leptin was further demonstrated at mRNA level by PCR analysis, with increased expression of both adipokines in ARCaP M cells as compared with ARCaP E cells. Western blots of total cell lysate demonstrated higher expression level of adiponectin protein in ARCaP M cells as compared with ARCaP E cells. In conclusion, our data for the first time demonstrate a differential expression pattern of adipokines between prostate cancer cells that have high osteoblastic bone-metastatic potential or low bone-metastatic potential, with the finding that adiponectin and leptin are expressed at a higher level in prostate cancer cells that are more metastatic to bone. The adipomimetic profile of bone metastatic prostate cancer cells suggests that adipokines may play an important role in the bone metastases of prostate cancer.

P41

N-cadherin Is an Important Mediator of Interactions between Myeloma Cells and Osteoblasts

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Introduction: Multiple myeloma is a plasma cell malignancy that causes extensive osteolytic bone disease. Present treatments target end stage disease but understanding how bone lesions are initiated may offer new approaches to prevent/suppress colonization. It is clear that myeloma cells form specific interactions with the bone microenvironment, where they can remain dormant and protected from current therapy to eventually proliferate and cause disease progression.
N-cadherin is an adhesion molecule that allows haematopoetic stem cells (HSCs) to localize to 'niches' containing osteoblasts on endosteal bone surfaces. In this study, we have tested the hypothesis that myeloma cells utilise N-cadherin to

adhere to osteoblasts *in vitro* and *in vivo* during colonization of bone and hijack the HSC niche.
Methodology: Primary cultures of calvarial mouse pre-osteoblasts were differentiated to mature osteoblasts in osteogenic medium. 5T33 and 5TGM1 myeloma cells were co-cultured with osteoblasts and adhesion evaluated using immunofluorescent microscopy. RT-PCR, flow cytometry and immunocytochemistry were used to assess N-cadherin expression in osteoblasts and myeloma cells. Immunohistochemistry and gene array were used to evaluate N-cadherin distribution/expression in myeloma cells growing in a mouse model of myeloma.
Findings: N-cadherin mRNA and protein were expressed by osteoblasts and myeloma cells. We showed focal expression of N-cadherin in less than 5% of myeloma cells, whereas expression was observed contiguously on the membranes of adjacent osteoblasts. N-cadherin expression significantly increased during osteoblastogenesis ($p<0.05$). Blocking N-cadherin mediated interactions, using specific antibodies raised against the ectodomain of N-cadherin, significantly reduced adherence of myeloma cells to osteoblasts *in vitro* ($p<0.05$). Gene array analyses showed significant increases in N-cadherin expression in the 5T33MM-bearing mice when compared to naïve mice not bearing tumour cells ($p<0.05$). Furthermore, immunohistochemistry demonstrated staining of N-cadherin when 5TGM1 cells were in contact with osteoblast *in vivo*.
Conclusion: These studies provide evidence that adherence of myeloma cells to osteoblasts is mediated by N-cadherin *in vitro* and *in vivo*, suggesting that myeloma cells may occupy a niche similar to that used by HSCs in bone.

P42

Metallothionein 2A Modulates Human Osteosarcoma Cell Resistance to Chemotherapy

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Osteosarcoma is the most common primary malignant tumor of bone characterized by a highly metastatic potential and a high propensity to develop strong resistance to chemotherapeutic agents. We previously showed that statins, which are HMG-CoA reductase inhibitors, exhibit *in vitro* anti-tumoral effects on osteosarcoma cells. Using microarray analyses, we identified metallothionein 2A (MT2A) as a target gene of statin. MT2A encodes a low molecular weight cystein-rich protein which plays a key role in the organism, especially in detoxification. Western blot and qPCR analyses confirmed that statins up-regulated MT2A gene and protein expression in several osteosarcoma cell lines. We therefore investigated the effects of MT2A over-expression on cell apoptosis, cell migration and invasion. U2OS human osteosarcoma cells were transduced with a lentiviral vector encoding the complete sequence of MT2A (LV-MT2A). No significant effects of MT2A forced expression were observed on cell migration or invasion. On an other hand, we found a decrease in cell replication, evaluated by BrdU incorporation assay, and an increase in apoptosis process, evaluated by caspases activity assay, leading to a global reduced cell viability, evaluated by the MTT test, in LV-MT2A cells compared to parental cells. Zinc is a well known regulator factor of many cellular functions. We found that forced expression of MT2A reduced the concentration of free intracellular zinc, evaluated by fluorimetric assay. The medium supplementation with increasing doses of ZnCl2 gradually counterbalanced the negative effect of MT2A over expression on cell viability, indicating that MT2A modulates cell vialibity through its chelating potential. We then evaluated the effect of MT2A forced expression on cell viability in the presence of different classical clinicaly used chemotherapeutic agents, namely cisplatin, doxorubicin and vincristin. For tested compounds, a significant lower effect on cell viability reduction was detected in LV-MT2A cells compared to parental U2OS cells. These findings show for the first time that MT2A is a target of statins and that MT2A plays a dual role as an anti-tumor protein by reducing proliferation and cell viability under favorable culture conditions, and as a pro-tumor protein by protecting osteosarcoma cells against apoptotic cell death induced by either serum depletion or chemotherapy agents. This provides a novel molecular target for therapeutic intervention in osteosarcoma.

P43

Characterisation of a Novel Bone Seeking Breast Cancer Cell Line Reveals Differential Expression of Metastasis-associated Proteins *In Vitro* and *In Vivo*

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Background: 70% of patients with late stage breast cancer develop bone metastasis and this condition is currently incurable. Little is understood about the molecular alterations acquired by tumour cells that promote homing and colonisation of bone. Identification of specific molecular determinants involved in these metastatic processes is essential for the development of relevant therapeutics. We have established a novel bone-seeking clone of the MDA-MB-231 cells that form bone tumours following intra-venous inoculation in nude mice. We have identified specific changes in gene and protein expression associated with bone homing and colonisation.
Methods: Mice were inoculated with either bone-seeking MDA-MB-231-IV cells by intra-venous or parental cells by intra-cardiac injection and culled 0, 7, 14, 21, 28 or 35 days later. Bone structure and tumour volume were measured by µCT and on histological sections. RNA from bone tumours and cell lines were analysed using human metastasis gene expression arrays; alterations >3 fold between the lines/sites were significant. Protein expression was analysed by immunohistochemistry. Changes between cells grown *in vitro* versus *in vivo* were associated with bone colonisation, whereas changes between IV and parental cells *in vivo* were associated with bone homing.
Results: MDA-MB-231-IV cells were identically tumorigenic to parental cells in mouse long bones, causing an equivalent decrease in bone volume over time with a concomitant increase in tumour volume compared to naïve mice. Differential

gene and protein alterations were observed between MDA-MB-231-IV and parental cells grown *in vivo* and *in vitro* implying that specific alterations are associated with bone homing and colonisation. Homing was associated with an increase in expression of the cell adhesion and migration molecule fibronectin, and the migration and calcium binding protein A4 (S100A4), whereas, colonisation was associated with a decrease in both fibronection and S100A4 and an increase IL1, a molecule known to effect cell proliferation, differentiation, and apoptosis. Upregulation of molecules influencing signal transduction pathways and breakdown of extracellular matrix including HRAS and MMP9 were associated with both tumour cell homing and colonisation of bone.
Conclusions: Specific molecular profiles are associated with different stages of metastasis. Molecules involved in bone homing are not the same as those involved in colonisation.

P44

Ovariectomy-induced Changes to the Microenvironment Triggers Growth of Established Breast Tumour Cells in Bone

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Background: Dormant tumour cells can be detected in the bone marrow of breast cancer patients several years after resection of the primary tumour. However, how these tumour cells are triggered to proliferate and form metastases remains to be established. One potential mechanism whereby tumour cells are stimulated to escape from dormancy is by changes to the bone microenvironment. We have shown that ovariectomy promotes osteoclastic bone resorption and bone loss in *in vivo* models. We hypothesise that increasing bone turnover by ovariectomy initiates bone metastasis.
Methodology: Intracardiac inoculation of MDA-MB-231 cells into 12-week-old mice results in tumour cell bone homing but no growth of bone metastases. 1 week after tumour cell implantation, animals underwent ovariectomy or sham operations. To assess effects of bone turnover on tumour take ovariectomised/sham operated animals were treated 1x per week with 100 ug/kg zoledronic acid. Tumour growth was measured by luciferase and multiphoton imaging; bone density was analysed by μCT; numbers and activity of osteoclasts/osteoblasts were analysed following TRAP staining and TRAP and P1NP ELISA, respectively.
Findings: Ovariectomy resulted in increased activity of osteoclasts, decreased activity of osteoblasts and decreased bone volume compared with sham operation. Ovariectomy also induced growth of established breast cancer cells in 78% of mice with an average of 2.6 tumours detected in the long bones. In contrast, tumour growth at this site was only detected in 18% of sham-operated mice and 10% of control mice. Stimulation of tumour growth following ovariectomy was reversed by zoledronic acid treatment. Multiphoton microscopy revealed individual cancer cells present in the long bones of all animals that did not develop tumours supporting that zoledronic acid inhibits ovariectomy induced proliferation of breast cancer cells. Interestingly, increased tumour burden following ovariectomy was specific to long bones, as numbers of tumours detected in other tissues were comparable between ovariectomised, sham and control mice.
Conclusions: 1) Ovariectomy-induced changes to the bone microenvironment triggers growth of breast cancer cells in bone, and this may represent a potential mechanism for initiation of breast cancer bone metastasis. 2) Inhibition of bone resorption following zoledronic acid treatment may prevent breast cancer bone metastases in postmenopausal breast cancer patients.

P45

Osteoblast and Osteoclast Numbers do not Reflect Changes in Bone Density and Turnover in Balb/c Nude Mice Following Ovariectomy or Castration

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Background: We aimed to characterise bone cell distribution and bone turnover following castration and ovariectomy in Balb-c/nude mice, to establish the correlations between changes in these parameters and the resulting effects on bone structure. Experiments designed to identify alterations in bone turnover commonly rely on counting numbers of TRAP positive osteoclasts and columnar shaped osteoblasts lining the bone surface. Osteoblast and osteoclast activity is assessed by the serum markers P1NP and TRAP, respectively. When comparing theses parameters, we observed discordance between the effects of ovariectomy and castration on numbers of osteoblasts and osteoclasts and the alterations in bone density.
Methods: 12-week old balb/c nude mice were castrated, ovariectomised or sham operated and culled 0, 1, 2, 3, 4, 5 or 8 weeks following operation (n=5 per group). Numbers of TRAP positive osteoclasts and columnar shaped osteoblasts were scored on histological sections from three separate regions of the tibia; medial and lateral endocortical surface and trabecular surface. Osteoblast and osteoclast activity was measured by serum P1NP and TRAP ELISA, respectively, and bone volume was analysed following μCT imaging of the tibiae.
Results: Ovariectomy and castration resulted in a significant and sustained reduction in trabecular bone volume from 2 weeks following operation. The reduction in bone volume following castration was accompanied by a significant decrease in serum P1NP and increase in serum TRAP, showing reduced activity of osteoblasts and increased activity of osteoclasts. In ovariectomised mice, serum P1NP was reduced 2 weeks following operation, however, serum TRAP levels were not significantly altered, showing that the ovariectomy-induced reduction in bone volume appears to primarily result from decreased osteoblast activity in this model. Interestingly, despite ovariectomy and castration having major impact on bone volume, no differences in osteoblast/osteoclast numbers were observed between operated and sham operated mice at any time point measured.
Conclusion: Assessment of numbers of osteoblasts and osteoclasts lining the bone surface is not indicative of their

activity, and is therefore not a representative measure of bone effects. Serum levels of P1NP and TRAP reflect the contribution of osteoblast and osteoclast activity and more accurately reflect the level of bone turnover.

P46

Histomorphometric and Microarhitectural Analyses Using the 2mm Bone Marrow Trephine in Metastatic Breast Cancer Patients on Long Term Bisphosphonate Therapy

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Background: Despite this widespread use of potent bone-targeted agents relatively little is known about their *in vivo* effects on bone homeostasis, bone quality, and bone architecture in humans. Traditionally bone quality has been assessed using a transiliac bone biopsy with a 7mm 'Bordier' core needle. The large needle size clearly limits use. We decided to evaluate whether using a 2 mm 'Jamshidi' core needle was more feasible.
Methods: A pilot study assessing bone quality and microarchitecture using a 2mm bone marrow trephine was conducted. Patients received tetracycline hydrochloride 500 mg BID on days 1 and 13 and the posterior iliac crest biopsy was performed between day 16 and 18. Samples were analyzed for bone microarchitecture, bone density, histomorphometry, and routine pathology assessment. All patients underwent dual energy X-ray absortiometry (DEXA).
Results: Twelve patients were enrolled, median age 56 (range 45-72), 11 pts were postmenopausal. Of the 11 patients with bone metastases, 10 were receiving bone-targeted agents for a median of 16.5 months (range 2 to 34 months). Bone mineral density scan results were in the osteopenic (4) and osteoporotic (1) range. Other treatments at the time of biopsy included aromatase inhibitors (7), tamoxifen (1), chemotherapy (3) and trastuzumab (1). Cancer cells were identified on bone biopsy in 5/12 patients. All samples provided sufficient data to detect excellent preservation of the trabecular structure using both microCT and histomorphometry. The sample quality was sufficient to perform other analyses including immunohistochemistry and confocal microscopy.
Discussion: Use of 2mm biopsies for assessment of bone quality is feasible and no major adverse events occurred. Assessment of reproducibility, effect of bisphosphonate on bone quality and novel information on bone/tumour interaction requires additional samples. Updated data will be presented.

P47

The Incidence and Consequences of Bone Metastases in Lung Cancer Patients Treated in the Non-trial Setting

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Background: Bone metastases (BM) in patients (pts) with advanced non-small cell lung cancer (NSCLC) can be associated with significant patient morbidity. However, despite randomised trial results showing significant reductions in skeletal related events (SREs), widespread uptake of bone-targeted agents has not been adopted in the NSCLC population. Given the paucity of literature around BM incidence, behaviour and outcomes in pts with advanced NSCLC we have reviewed our practice to gain additional insights.
Methods: Charts were reviewed for all NSCLC pts referred to our institution between January 1st 2007 and December 31st 2008. Collected data included date of NSCLC diagnosis, date of first relapse, sites of first distant metastases (including bone) and overall survival. In pts with BM we assessed; sites of disease, incidence and type of SREs, survival (in pts with and without SREs) and bone-targeted agent use.
Results: As part of a larger cohort, we report results from the first 80 eligible pts. 14 were excluded due to other concurrent malignancies. Median age was 67 years (45-88), and 42% were male. Of the remaining 66 pts, 41 presented with advanced disease, 13 relapsed after curative treatment for early stage disease and 12 remain disease free after curative treatment. Of the 54 pts with metastatic disease, 26 (48%) had BM during their clinical course; of these 21/26 had multiple BM.
Of pts with BM, 76% (20/26) developed an SRE. Median time from diagnosis of BM to SRE was 1 month (range 0-53). 50% of pts had at least 2 SREs (range 1-11). Types of SRE were radiotherapy (69%), pathological fracture (20%), spinal cord compression (6%) and surgery to bone (2%). 20/26 pts were documented as requiring opioids analgesia for BM. 13 pts required hospitalisation for pain control from BM, or treatment of SREs (1 to 3 admission per patient). No patients received bone-targeted therapy at any time, regardless of presence or absence of SRE.
Conclusions: Bone metastases are common in advanced NSCLC patients. In these patients, high rates of SRE were observed. In contrast to other malignancies, bone-targeted therapies were not used at all. Strategies are required to enhance the care of these patients. Data collection continues.

P48

Bone Metastastic Prostate Cancer Cells Impair Osteoblast Differentiation to Enhance Their Own Growth

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Metastases to the bone are often the most unfavorable and yet incurable final outcomes of tumor growth. The aim of our study is to unravel whether the interaction between prostate metastatic cells and osteoblasts depends on the differentiation stage of the osteoblast. We used an *in vitro* differentiating pre-osteoblast cell line (SV-HFO) and two prostate metastases cell lines, a bone (PC-3) and a lymph metastasis (LNCaP). Interaction was studied both after direct and indirect contact between tumor cells and osteoblasts during three differentiation stages of the osteoblasts (pre-mineralization, early and late mineralization). After 7 or 21 days of co-culture osteoblast growth and differentiation and tumor cell growth were studied. For assessing effects on cell growth in direct contact studies stably GFP expressing PC-3 and LNCaP cells were generated, to distinguish tumor cells from osteoblasts. 7 Days co-culture during the various osteoblast differentiation stages showed that pre-mineralization osteoblasts stimulated growth while early and late mineralization osteoblasts inhibited growth of both PC-3 and LNCaP cells. Growth stimulation was similar after direct or indirect contact whereas growth inhibition was only observed after direct contact. PC-3 cells significantly inhibited alkaline phosphatase activity after 7 days of co-culture, especially during the pre-mineralization stage and strongest after direct contact. No effect was found on osteoblast growth, either after direct or indirect contact co-culture. LNCaP cells had no effect. Interestingly, during 21 days of direct co-culture starting at the early osteoblast differentiation stage, PC-3 cells kept osteoblastic alkaline phosphatase levels very low and inhibited mineralization. LNCaP cells had no effect on osteoblast differentiation. Conclusion: Early osteoblasts stimulate and late osteoblasts inhibit prostate metastasis growth, independent of origin of metastasis. Only the bone metastasis cells impair osteoblast differentiation and keep them in an early differentiation and tumor cell growth stimulating stage. In the presence of the lymph metastasis cells the osteoblast differentiation progressed to the growth inhibitory mineralization stage. These findings form an important basis for identifying osteoblast derived factors and processes important in bone metastases. Currently gene and protein profiling of these specific osteoblast differentiation stages is performed aimed to identify these factors.

P49

Zoledronic Acid Combined with an Ultrasound Treatment Achieves Antitumoral Effect in Breast Cancer Bone Metastases

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As a treatment of breast cancer bone metastases, zoledronate (ZOL) acts as an inhibitor of osteoclast-mediated resorption. ZOL binds avidly to bone mineral. High doses of ZOL that have demonstrated antitumoral effects in animal studies are incompatible with clinical dosing regimens approved for the treatment of cancer patients with bone metastases. Here we report the feasibility of combining low intensity ultrasound treatments and ZOL to enhance the bioavaibility of this drug for tumor cells. Sixty-nine mice bearing breast cancer bone metastases were randomized into 6 groups. Eighteen days after tumor cell injection, a single dose of ZOL (calculated equivalent to the 4-mg clinical dose administered every 3-4 weeks) was combined with daily pulsed or continuous ultrasound (US). Pulsed US creates mechanical effects to stimulate bone formation. Thermal effects were produced by continuous US in order to increase the temperature of 5°C. Each treatment was applied 30 minutes per day and lasted fifteen days. Efficacy of treatments were measured by radiography (area of the osteolytic lesions in mm^2), histomorphometry (TB/STV and BV/TV) and TRACP5b in the serum. US alone (7.4±2.7 mm^2 for continuous US and 6.2±2.7 mm^2 for pulsed US) do not have any effect compared with the vehicle group (6.6±1.6 mm^2). ZOL combined with pulsed US do not significantly decrease the extent of osteolytic lesions (2.4±0.7 mm^2) or tumor burden (TB/STV=37%) when compared with ZOL alone (3.0±0.9 mm^2, TB/STV=46%). In sharp contrast, we found a statistically significant decrease of bone destruction and skeletal tumor burden in mice that received a daily treatment of continuous US with ZOL (1.3±0.4 mm^2, TB/STV=11%) compared with vehicle (6.6±1.6 mm^2, TB/STV=62%) and, most importantly, with ZOL alone (TB/STV=46%). TRACP5b levels were statistically significantly lower in animals treated with ZOL and daily continuous ultrasound (2.6 ±0.2 U/L) than in animals with ZOL alone (3.2±0.2 U/L). Daily application of low intensity continuous ultrasound in combination with a single clinically relevant dose of ZOL allows a synergistic effect, leading to a significant decrease of skeletal tumor burden. In addition, an increase of bone volume and a decrease in osteoclast activity both suggest that zoledronate is more bioavailable for tumor cells and osteoclasts *in vivo*.

P50

Trail Sensitivity in Ewing's Sarcoma Patients Is Modulated by the Expression of Death Receptor 4 and its New Short Isoform

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Ewing's sarcoma (EWS) is a high-grade neoplasm typically arising in the bones of children and adolescents. Survival rate decreases from greater than 50% to only 20% after 5 years for patients not responding to treatment or presenting metastases at diagnosis. Among new therapeutic approaches, TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily with a strong antitumoral activity and a minimal toxicity to most normal cells is a promising candidate. However, its use in clinics is currently limited due to resistance process developed in more than 30% of patients.
Cell viability experiments (XTT assay) showed that 4 out of 7 EWS cell lines were resistant to TRAIL induced cell death. We previously demonstrated that TRAIL administered by gene

transfer is able to limit EWS development in a xenograft model induced by sensitive cells in nude mice.
Western-blotting and flow cytometry analyses revealed that DR5 was uniformly and highly expressed by all EWS cell lines whether they are TRAIL-sensitive or not. In contrast, DR4 levels were higher in two sensitive cell lines than in the 5 others. In TRAIL-sensitive TC71 cells, knockdown of DR4 by shRNA was associated with a loss of sensitivity to TRAIL in spite of DR5 presence. During the cloning step of DR4 cDNA into lentivirus vector, a new transcript variant was identified, cloned and sequenced. This transcript designed bDR4 results from an alternative splicing and encodes a 310 aa protein which corresponds to the 468 aa aDR4 but truncated of aa 11-157 within the extracellular TRAIL-binding domain. According to modelling studies based on DR5-TRAIL crystal structure and DR5-DR4 similarities, the contact of bDR4 with TRAIL appeared largely preserved. The overexpression of this new DR4 isoform (named bDR4) in a TRAIL-resistant cell line re-sensitized these cells to TRAIL (as shown by XTT assay and Apo2.7 staining).
In conclusion, we demonstrated that TRAIL represents a promising therapeutic agent in Ewing's sarcoma as it significantly delays tumor development in mice. TRAIL sensitivity seems to be related to DR4 expression in EWS, as its down-regulation or overexpression is able to modulate respectively TRAIL-sensitivity and TRAIL-resistance, despite constant and high expression of DR5 in EWS cell lines. At last, we identified a short isoform of DR4 which is able to transduce TRAIL-apoptotic signal. Its function regarding sensitivity needs to be addressed.

P51

Biomarker Discovery for Breast Cancer Related Bone Metastasis Using Silac

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Background: There is a need for new biomarkers to identify breast cancer patients at most risk of developing bone metastases. We aim to discover such biomarkers using high-sensitivity proteomic approaches and now report on SILAC (stable isotope labelling by amino acids in cell culture) applied to an established cell model of human breast cancer bone metastasis, developed from MDA-MB-231 cells kindly supplied by J Massague.
Experimental: SILAC enables the identification and quantification of proteins from two or more cell lines in one experiment. Proteins from a control breast cancer cell line (MDA-MB-231) were metabolically labelled by growth in medium containing naturally abundant 12C- and 14N-labelled arginine and lysine ('light') while the bone metastatic cells were grown in isotopically 'heavy' 13C- and 15N-labelled arginine and lysine. The cell lines were reciprocally labelled to ensure confident quantification. Equal amounts (1:1) of light and heavy labelled proteins from the cell lysates were mixed together prior to peptide extraction and analysis of labelled peptides using a high-resolution Orbitrap mass spectrometer.
Results: Approximately 3,600 proteins were identified, of which, over 250 proteins were >1.5 fold up- or down-regulated in the bone metastatic cells compared to control cells. Gene Ontology of the differentially regulated proteins showed the majority of proteins up-regulated to be of cytoplasmic origin and involved with transcriptional and signal transduction processes, while the down-regulated proteins were associated with oxidation/reduction processes and were predominantly integral to membranes. A 'focus' list of five proteins of significant potential interest was generated following rigorous data and literature analyses.
Discussion: SILAC proteomics enables the discovery of proteins that may have potential as biomarkers of breast cancer bone metastases. Of the proteins chosen for further investigations, one plays a known significant role in breast cancer bone metastasis while the other four are potentially novel in the context of this disease and are known to have functional roles in angiogenesis, cell migration, cell-cell adherence, or cell cycle regulation. Candidate biomarkers are being investigated further using a range of techniques (such as TMAs, ELISAs) in experimental and clinical samples so as to assess the potential of these proteins to identify individuals at high risk of bone metastases.

P52

Role of Connexin43 in Tumor Development of Ewing's Sarcoma

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Ewing Sarcoma (ES) is a primary bone tumor characterized by a chromosomic translocation between the EWS gene and a member of the ETS gene family, most commonly FLI1, which leads to an aberrant transcription factor EWS-FLI1. Gap junctions are intercellular channels, composed of connexin (Cx), that allow direct intercellular communication between adjacent cells. Many studies have shown that tumorigenesis may be associated with a loss or reduction of gap junctional intercellular communication (GJIC). The aims of this work were to study the expression level of connexin43 (Cx43) in ES cells and to determine the role of this protein in tumor development of ES.
A loss of Cx43 expression in ES cell lines (TC32, A673, TC71, EW7, EW24, SKNMC, RDES and SKES1) was observed at protein and mRNA levels, in comparaison to those measured in human mesenchymal stem cells. In addition, A673 cells stably transfected with shRNA for EWS-FLI1 showed an increase of Cx43 level (at mRNA, protein and transcriptionnal levels) and GJIC. These results demonstrate that EWS-FLI1 alters Cx43 mRNA and protein expression and consequently GJIC in ES cells.
In an osteolytic murine model of ES, the over-expression of the Cx43 in A673 or TC71 ES cells dramatically reduce the tumor growth leading to a significant increase in animal survival 17 days after tumor induction. In addition, the microarchitectural parameters which were assesed by radiography and a microscanner analysis showed an increased trabecular bone volume when

Cx43 expression was enhanced. Histological analysis demonstrates that the increased expression of Cx43 in ES tumor cells inhibits the osteoclast activity and therefore the bone resorption. Together these results demonstrate the crucial role of: i) the transcription factor EWS-FLI1 in the down-regulation of Cx43 in Ewing tumor cells, and ii) Cx43 in tumor development of Ewing Sarcoma.

P53

TGF-β Promotes Tumor Growth in Osteosarcoma: Over-expression of Smad7 as a New Therapeutic Strategy

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Osteosarcoma, the main malignant primary bone tumor, affects a 'young' population composed of children and young adults (<40 years, median age 18 years). Current treatment consists of tumor resection associated with chemotherapy. In many cases, a lack of response to anti-tumor drugs is observed, leading to development of metastases and the patient's death. TGF-β plays a complex role in carcinogenesis, since it acts both as a tumor-suppressor and as a pro-oncogenic factor. It was particularly observed that TGF-β stimulates the metastatic process by promoting cell migration and invasion. By transient transfection of cells with the construct (CAGA)9-lux, and by measuring the expression of TGF-β target genes (PAI-1, CTGF, COL1A1) by Q-PCR, we demonstrated that the Smad pathway is functional in the osteosarcoma cell lines tested (HOS, SaOS2, U2OS, CAL72, SJSA1 and 143B). By cell counting and cell cycle analysis, we showed that TGF-β does not affect cell proliferation. The use of Boyden chambers (in the presence or absence of Matrigel®), shows that TGF-β stimulates cell migration and invasion, two key functions in tumor progression. In this context, by Q-PCR and by zymography, we observed that the TGF-β stimulates the expression and activity of MMP-2. By transfection of cells with (CAGA)9-lux, by measuring the level of Smad3 phosphorylation, and by measuring the PAI-1, CTGF and COL1A1 expressions, we demonstrated that the overexpression of Smad7 (inhibitory Smad) inhibits the transcriptional response mediated by Smad3 in osteosarcoma cell lines.
Furthermore, the use of Boyden chambers, showed that Smad7 over-expression inhibits the ability of TGF-β to stimulate cell migration and invasion, in part by inhibiting its ability to stimulate the expression and activity of MMP2. *In vivo*, using a murine model of osteosarcoma induced by intramuscular injection of HOS cells overexpressing Smad7 or not, we showed that inhibition of the TGF-β signaling pathway significantly slows the growth of the primary tumor and increases mice survival.
To resume, we demonstrated that, in contrats with the effects observed in tumors of epithelial origin (such as prostate, breast ...), TGF-β does not exert tumor-suppressor properties in osteosarcoma (mesenchymal origin), but mainly oncogenic properties (migration and invasion). Moreover, the results obtained by overexpression of Smad7 *in vitro* and *in vivo* suggest that TGF-β could be a therapeutic target in osteosarcoma tumor progression.

P54

Predictive Markers of Bone Metastasis in Primary Breast Tumors

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Background: Patients with breast cancer frequently develop bone metastases, which are responsible for high morbidity and reduced quality of life. It is known that numerous molecular markers and molecules with bone-like phenotype are involved in metastasization to bone and that only cancer cells with a specific molecular profile are capable of reaching and colonizing bone tissue. The timely identification of patients with a high probability of relapse to this site would enable them to receive tailored therapy with bone-specific drugs such as bisphosphonates or RANK-L inhibitors. The aim of the present study was to identify a pattern of tissue markers from primary breast cancer capable of predicting bone metastatization.
Materials and methods: The expression of different markers was retrospectively analyzed in frozen breast cancer tissue samples from 90 patients (30 cases with no evidence of disease (NEDP), 30 with bone metastases (BMP), and 30 with visceral metastases (VMP)). Eight transcripts were analyzed by Quantitative Real time PCR: trefoil factor 1 (TFF1), bone sialoprotein (IBSP), heparanase (HPSE), SPARC, connective tissue growth factor (CTGF), B2 microglobulin (B2M) and receptor activator of Nf-kB (RANK).
Results: The analysis of marker expression in the 3 different subgroups showed at least twofold higher median values of all markers in the BMP group respect to NEDP and VMP groups, reaching statistical significance for TFF1, B2M and CXCR4. In particular, median TFF1 values in the BMP arm was 430.64 compared to 115.83 and 32.79 in VMP and NEDP, respectively. Considering markers as dichotomous variables, TFF1 expression in BMP reached 60% with respect to 21% and 23% in NEDP and VMP, respectively. Combination analysis of TFF1 with B2M, CTGF or RANK showed that positivity to at least one of the 4 markers was observed in 79% of cases in the BMP group, but only in 21% and 30% of cases in NEDP and VMP subgroups, respectively.
Conclusions: Our preliminary study identified a gene expression pattern in primary breast cancer that could potentially predict patients destined to bone relapse. Such patients could consequently benefit from adjuvant treatment with bone targeted therapy.

P55

Osteoblasts Alter Steroidogenesis in CRPC Cells Under Steroid Deprived Conditions

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Background: Androgen-deprivation therapy (ADT) is the first line therapy for metastasized prostate cancer (PC), and is initially effective in the majority of the patients. Unfortunately, relapse of castration resistant PC (CRPC), often as osteoblastic bone metastasis, is inevitable. Today, there is no cure for CRPC, and the mechanisms associated with metastatic bone disease and hormonal and metastatic progression remains elusive. The aim of the study was to elucidate the hormonal regulation of the osteoblastic response of CRPC.
Methods: Under steroid deprived conditions, a cell culture model based on conditioned medium from osteoblasts and the CRPC cell lines was used to simulate the bidirectional interaction of the osteoblastic situation of CRPC *in vitro*. A human androgen signature array comprising 92 genes was used to detect differentially expressed genes in the CRPC cells upon osteoblast stimulation. Long term effects were investigated after 21 days of co-culture, at the time when mineralization is detected in osteogenic CRPC cells. RTqPCR was used to detect changes on the transcriptional level. Proliferation assays were assessed using BrdU incorporation.
Results: Under steroid deprived conditions, osteoblasts induced an altered steroidogenesis towards increased testosterone and estrogen synthesis in CRPC cells, by upregulating genes encoding for the enzymes CYP11A1, HSD3B1, AKR1C3 and CYP19A1, in accordance with increased ERβ expression. Osteoblasts also suppressed DHT activity by upregulating genes mediating the glucuronidation of DHT, and by downregulation of SRD5A. Moreover, CRPC cells substituted steroids, or other factors, sufficient to stimulate osteoblastic proliferation under steroid deprived conditions. Interestingly, ERβ expression in osteoblasts was significantly upregulated in response to CRPC stimulation, whereas the effect on ERα was minimal.
Conclusions: We here show that osteoblasts induced a changed pattern in the expression of steroidogenic enzymes in CRPC cells, which closely mimics the altered steroidogenesis described in CRPC tumors. This suggests that osteoblast may support castration resistance in the bone compartment. The altered steroidogenesis in the osteoblast-CRPC interaction may also enable osteoblasts to supply themselves with sufficient steroid stimulation during ADT.

P56

Parathyroid Hormone-related Protein (PTHrP) Potentiates Myeloid-derived Suppressor Cells (MDSCS) with in the Bone Marrow via Osteoblast-derived Interleukin (IL)-6 and Vascular Endothelial Growth Factor (VEGF)-A

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Bone is an essential partner for tumor progression, supplying a variety of cells critical for favorable microenvironment. Among bone marrow (BM)-derived cells, MDSCs, expressing CD11b and Gr1 markers, suppress the host immune response and infiltrate tumor tissue to promote tumor growth and angiogenesis, whereas regulation of MDSCs in the BM of tumor hosts remains unclear. Prostate cancer cell lines with altered levels of PTHrP were used to show that subcutaneous prostate cancer-derived PTHrP correlates with MDSC recruitment in tumor tissues and also with tumor microvessel density. MDSCs derived from the BM of mice harboring subcutaneous tumors highly expressing PTHrP and co-implanted with prostate cancer resulted in greater tumor growth and angiogenesis than MDSCs from low PTHrP tumors, suggesting PTHrP contributes to pre-metastatic microenvironment in the BM. The angiogenic effect was associated with MDSC-derived matrix metalloproteinase (MMP)-9. However, as MDSCs do not express receptors for PTHrP, subsequent studies elucidated the mechanism of MDSC activation. Osteoblasts were examined for their role in PTHrP-dependent MDSC activation. Conditioned media from saline- or PTHrP-treated primary calvarial osteoblasts were collected and added to $CD11b^{+}Gr1^{+}$ BM cells *ex vivo*, followed by quantitative PCR for *Mmp9* (a marker for MDSC activation). Additionally, mice were treated with recombinant PTHrP (1-34) 12 hours prior to flow cytometric analyses and sorting of $CD11b^{+}Gr1^{+}$ BM cells. PTHrP significantly increased Mmp9 expression and activating phosphorylation of Src family kinases (SFKs) in MDSCs. Furthermore, $CD11b^{+}Gr1^{+}$ BM cells were isolated and treated with osteoblastic cytokines including receptor activator of nuclear factor κB ligand (RANKL), IL-6, VEGF-A, and C-C chemokine ligand (CCL)-2. Interestingly, VEGF-A and IL-6 increased Mmp9 expression, which was reversed by PP2, a pharmacological inhibitor of SFKs. There was no alteration in gene expression for other markers of MDSC activation including integrin β1, CCL2, CXCR-2, or CXCR-4. In summary, tumor-derived PTHrP circulates and stimulates osteoblasts to release IL-6 and VEGF-A, contributing to activating phosphorylation of SFKs in MDSCs and subsequent expression of MMP-9. These data suggest that tumors positively regulate the bone marrow microenvironment via PTHrP expression to promote tumor angiogenesis and immune suppression.

P57

Combined Use of Samarium-153-EDTMP and Zoledronic Acid Therapy for Bone Pain Palliation in Non Breast and Non-prostate Primaries

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Introduction: There is paucity of literature describing efficacy and utility of combined used of Samarium-153-ethylenediaminetetramethylenephosphonic acid (Sm153-EDTMP) and zoledronic acid in pain palliation due to metastatic malignancies other than breast and prostate primaries. Samarium is indicated for the treatment of painful bone metastases, whereas the bisphosphonate zoledronic acid is indicated for the prevention of skeletal events. Combination of these two treatment modalities has been demonstrated to be beneficial in breast and prostate cancer. Aim of the present study was to evaluate the effectiveness of combined samarium-153 and zoledronic

acid therapy in clinically aggressive malignancies excluding the breast and prostate cancer.
Material and Method: Study population: Fifty-four patients (38 male: 16 female) with pain due to bone metastases, without effective control via conventional therapy were included after informed consent. Their mean age was 52.9±10.5 years (19-85 years). Therapeutic intervention: After proper clinical and laboratory evaluation, included patients were treated with 4 mg zoledronic acid intravenously on recruitment. Within 7 days of the zoledronic acid therapy they were treated with Sm153-EDTMP at a dose of 1 milicurie/kg body weight intravenously after ensuring adequate hematological parameters.
Result: All 54 patients showed good tolerability to zoledronic acid infusion with none developing any acute adverse effects traditionally attributed to bisphosphonates like flu like syndrome or musculoskeletal pain. Reduction in pain resulting in reduction in analgesic requirement was reported by 40 patients. Onset of analgesia was rapid; with all the 40 patient experiencing various extend of pain relief with in 24 hour of infusion. No analgesic effect was noted in the remaining 14 patients.
Sm153-EDTMP therapy was well tolerated by all the 54 patients with no immediate adverse effect. Reduction in pain as assessed by reduction in pain scale score resulting in reduction in analgesic requirement was reported by 53 patients, where as one patient did not obtain any benefit. Onset of analgesia was rapid among the responders and this includes 13 out of 14 patients who have not responded to zoledronic acid infusion.
Conclusion: Combined treatment with the zoledronic acid and Sm153-EDTMP is an effective way to palliate pain in clinically aggressive malignancies with good safety and efficacy.

P58

Clusterin Inhibition Using OGX-011 Synergistically Enhances Zoledronic Acid Activity in Osteosarcoma

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Despite recent improvements in therapeutic management of osteosarcoma, ongoing challenges in improving the response to chemotherapy warrants new strategies still needed to improve overall patient survival. Among new therapeutic approaches, zoledronic acid represents a promising adjuvant molecule to chemotherapy to limit the osteolytic component of bone tumors. However, zoledronic acid triggers the elevation of heat shock proteins (Hsp), including Hsp27 and clusterin (CLU), which could enhance tumor cell survival and treatment resistance. We hypothesized that targeting clusterin (CLU) using siRNA or the antisense drug, OGX-011, will suppress treatment-induced CLU induction and enhance zoledronic acid-induced cell death in osteosarcoma (OS) cells.
The combined effects of OGX-011 and zoledronic acid were investigated *in vitro* on cell growth, viability, apoptosis and cell cycle repartition of zoledronic acid-sensitive or zoledronic acid-resistant human cell lines (SaOS2, U2OS, MG63 and HOS).
In OS cell lines, zoledronic acid increased levels of HSPs, especially CLU, in a dose- and time-dependent manner by mechanism including increased HSF-1 transcription activity. The OS resistant cells to zoledronic acid exhibited higher CLU expression level than the sensitive cells. Moreover, CLU overexpression protects OS sensitive cells to zoledronic acid-induced cell death. OGX-011 suppressed treatment-induced increases in CLU and synergistically enhanced the activity of zoledronic acid on cell growth and apoptosis. These biologic events were accompanied by decreased expression of HSPs, Akt, and HSF-1 transcriptional activity. These results indicate that zoledonic acid-mediated induction of CLU can be attenuated by OGX-011, with synergistic effects on delaying progression of osteosarcoma.

P59

The Upregulation of the Micrornas-30 Family in Osteotropic Human Breast Cancer Cells Decreases Progression of Skeletal Lesions in Animals

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Breast cancer cells preferentially invade and grow as secondary tumors in bone where they induce osteolytic lesions. The deregulated expression of a set of genes and/or of microRNAs (miRs) in a subset of primary breast tumor cells may confer them the potential to disseminate and successfully invade and survive in the bone marrow. MiR-30s family (miR-30s) is a family of 5 miRs: miR-30a, b, c, d and e sharing the same 'seed region' and thus targeting the same genes. Their higher expression levels in hormone-dependent and well differentiated breast tumors versus hormone-independent tumors (Iorio M. *et al*., *Cancer Res* 2005) and their lower expression levels in lymph node metastases versus primary tumors suggest a role for miR-30s during metastatic dissemination of breast carcinomas (Rosenberg A. *et al*., *J Pathol* 2009). We have observed that the expression levels of miR-30s were strongly down-regulated in a breast cancer cell line (MDA-B02) which highly and specifically metastasizes to bone, indicating that miR-30s may interfere with the dissemination of breast cancer cells to bone. MDA-BO2 cells were first transduced with the retroviral vector pmiR-Vec containing the genomic DNA sequence of the miR-30b, d cluster and transduced cells were then inoculated to nude mice. We observed (1) a 50% decrease in the extent of osteolytic bone lesions and a concomitant decrease of skeletal tumor burden, (2) a reduced tumor volume when mice are implanted subcutaneously with these transduced cells, and (3) a down-regulation of Runx2, CTGF, CX43, integrin beta3 and CDH11 expression in miR30b,d over-expressing cells. Forced expression of all of the 5 miR-30s or of solely miR30c-b in MDA-B02 cells was next achieved. MiR30c-b are the two miRs from the family which generate only the mature -5p arm and not the -3p arm during the enzymatic processing of miR-30s in MDA-B02 cells. When these cells over-expressing the 5 mir-30s or miR30c-b were iv injected in mice, the extent osteolytic lesions was substantially decreased compared with the parental MDA-B02 cells, confirming the negative regulatory role of all the miR-

30s in bone metastasis formation. CTGF, CX43 and CDH11 being over-expressed in bone metastases from breast cancer patients (Bellahcène A. *et al*., *Breast Cancer Res Treat* 2007), we therefore suggest that the targeting of these genes with miR-30s might interfere with the progression of breast cancer skeletal lesions.

P60

Does PMCA2 Promote the Skeleal Metastasis of Breast Cancer Cells?

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Overexpression of the plasma membrane calcium-ATPase 2 (PMCA2) allows breast cancer cells to evade apoptosis induced by high intracellular calcium levels. Furthermore, high levels of PMCA2 staining correlated with worse survival in a large tissue microarray cohort. We hypothesized that high levels of PMCA2 could allow metastatic breast cancer cells to survive in the bone microenvironment, characterized by high extracellular calcium concentrations. The current study tested this hypothesis by determining whether PMCA2 expression levels could modulate skeletal metastasis in 4T1 mouse mammary carcinoma cells. 4T1-luc2 cells were engineered with reduced or enhanced PMCA2 expression. As expected, the level of PMCA2 expression correlated with increased proliferation and reduced apoptosis in 4T1-luc2 cells treated with extracellular calcium *in vitro*. We then implanted the cells into the mammary fat pads of BALB/c mice, and used bioluminescent imaging to track their growth and metastasis *in vivo*. Although we failed to detect any changes in the number of skeletal metastases that developed, the volume of osteolytic tibial lesions, measured *ex vivo* by microCT, correlated with the level of PMCA2 expression. These studies suggest that PMCA2 promotes the growth of breast cancer cells in bone.

P61 **S5**

MicroRNA-34c Inversely Couples the Biological Functions of the Bone-specific Transcription Factor RUNX2 and the Tumor Suppressor P53 in Osteosarcoma

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Abstract selected for oral presentation. See Speaker Abstracts.

P62

Elevated Expression of FGFR2 in Breast Cancer Cells Enhances Proliferation Rate and Bone Resorption *In Vivo* in an Intratibial Bone Metastasis Model

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Fibroblast growth factor receptors (FGFRs) are known to participate in the formation and growth of primary breast tumors. However, their role in breast cancer induced bone metastasis is not well understood. In this study we aimed to characterize the roles of FGFR1 and FGFR2 in breast cancer induced bone metastasis using an *in vivo* bone metastasis mouse model.
FGFR1 or FGFR2 expression was silenced in Shionogi 115 mouse breast cancer cell line with lentiviral transfection of shRNA constructs. Silencing of the FGFR expression in the cell lines sh-FGFR1 (low expression of FGFR1), sh-FGFR2 (low expression of FGFR2) and sh-lacZ (control) was confirmed by qRT-PCR and western blot analysis. Cells ($1x10^9$) were inoculated intratibially into 4-week-old nude mice and the tumors were excised and examined after a 8-week growth period by x-ray, micro-CT and immunohistochemical analysis. Tumor size was analyzed from the x-ray images and osteolysis was quantified by measuring the osteolytic areas inside the bone.
The level of FGFR1 expression was low in sh-FGFR1 tumors and FGFR2 was the dominant receptor in these tumors. In sh-FGFR2 tumors decreased expression of FGFR2 was associated with increased FGFR1 expression. The tumors in the sh-FGFR1 group were largest and their osteolytic effect was pronounced. These tumors also had most resorption in the cortical bone based on the micro-CT analysis. The tumors in the sh-FGFR2 group had less osteolysis and their size was smaller when compared to control and sh-FGFR1 tumors. The proliferation rate (evaluated by p-HisH3 staining) in sh-FGFR1 tumors was higher than in the control and sh-FGFR2 group, which may partly explain their large size.
In summary, intratibial S115 tumors expressing high levels of FGFR2 were larger and caused more severe bone lesions than tumors expressing FGFR1. We conclude that FGFR2 is important for bone metastasis of breast cancer.

P63

Localized Sclerotic Bone Response Demonstrated Reduced Nanomechanical Creep Properties

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Sclerosis development is a common occurrence in slow growing or benign osteolytic lesions. However, there is lack of knowledge on the mechanical changes associated with sclerotic bone response.
In this study, localized sclerotic response in an immunocompetent model of Walker 256 breast carcinoma in SD rats showed a healing flare, with subsequent increase in new reactive

bone formation and mineralization. Sclerotic rat femurs (SCL) had significant increases in bone mineral density (BMD), bone mineral content (BMC), bone volume fraction (BV/TV), bone surface density (BS/TV), trabecular number (Tb.N) and a significant decrease in trabecular separation (Tb.Sp) and structural model index (SMI) as compared to control rat femurs (SHAM). Significantly reduced creep responses were observed for both trabecular and cortical bone in sclerotic bones (Figure 1) while no significant difference were observed in elastic modulus (E) and hardness (H) values. Therefore, we conclude that viscoelastic creep property using nanoindentation would serve as a more sensitive indicator of localized bone remodeling than the elastic properties. Although enhanced microstructural changes and increased BMD contributes towards increased bone strength, reduced viscoelasticity can contribute towards increased microcrack propagation and therefore a reduced toughness. Thus, our results indicated that sclerotic response of bone metastasis would cause increased strength but reduced toughness.

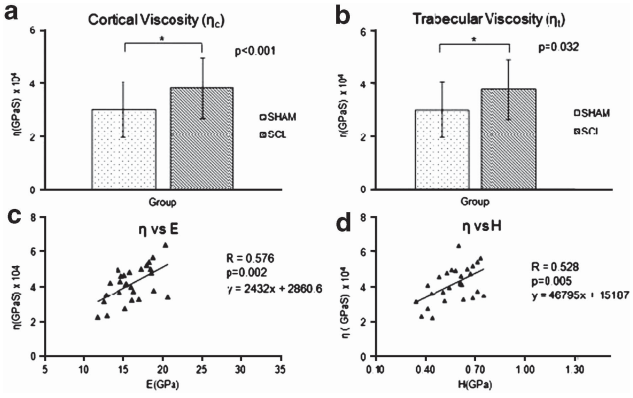

Figure 1 (**a**) cortical viscosity of sham and sclerotic left femurs, with * indicating p <0.001 and (**b**) trabecular viscosity of sham and sclerotic femur with * indicating p=0.032, (**c**) Correlation between viscosity and E and (**d**) Correlation between viscosity and H.

P64

Breast Cancer Cells Exert Inhibitory Effects on Bone Cell Mechanical Properties, Proliferation and Differentiation

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Metastatic cancer cells were found to inhibit osteoblastic cells in terms of proliferation, differentiation and morphology. However, there is no prior study on mechanical property changes of bone cells affected by tumor, which could in turn affect bone cells mechanosensing and tissue level adaptation. Thus in this study, atomic force microscopy (AFM) indentations on primary bone cells exposed to 50% conditioned medium from Walker 256 carcinoma cell line (W) or primary tumor (T) (derived from W256 tumor in SD rat) were carried out. It was shown that bone cell elastic modulus in W and T groups decreased significantly by 46.4% and 65.5% respectively, compared to control (Figure 1). The stiffness change could be positively associated with the cytoskeleton changes and inhibited bone cell differentiation with tumor conditioning. This is the first study to demonstrate that there was a significant reduction in stiffness of bone cells exposed to tumor conditioned medium, which could in turn affect its mechanosensing properties. It also demonstrated that there was a stronger sustained inhibitive effect on bone cells viability and differentiation by primary tumor cells than the original cell line used. The overall inhibition on bone cell viability and functionality by tumor could contribute to the net loss of bone in bone metastasis.

P65

Effects of Mechanical Stimulation in the Bone-mimetic Phenotype and Migration of Prostate Cancer Cells

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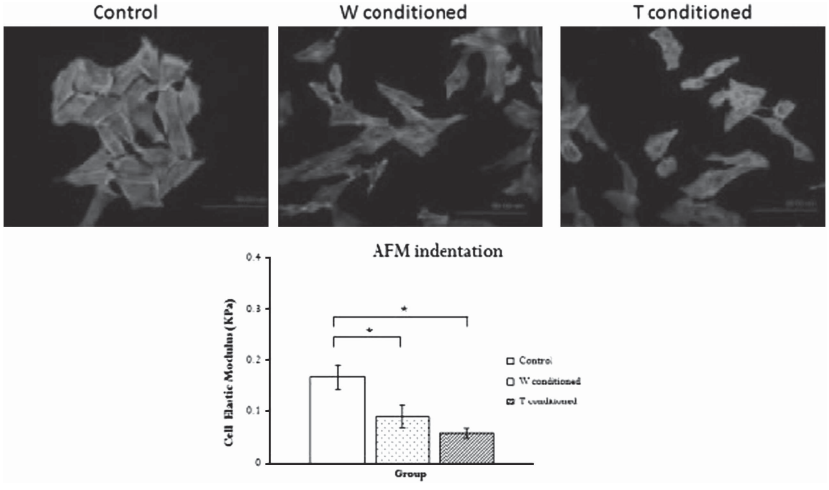
Recent studies have described pro-metastatic changes in prostate tumors cells such as the adquisition of a bone-



[P64] Figure 1

mimetic phenotype. The osteomimetic phenotype might explain the ability of prostate cancer cells to establish in bone. In bone tissues, mechanical stimuli modulate the survival and proliferation of osteocytes and osteoblast cells. However, it is unknown the effect of mechanical stimuli on prostate cells and its consequences on cancer progression in bone metastases.
To test these effects, we use the fluid flow technology to induce mechanical stimulation on bone and cancer cells. We observed that gene expression of the bone-related factors Runx2, OPG, PTHrP and its receptor, PTHR and the pro-angiogenic factor, vascular endothelial factor receptor type 2, were increased after mechanical stimuli on PC3 and LNCaP prostate cancer cell lines. Moreover, our results show an increase of LNCaP migration compared to colon cancer cells (HT 29) after mechanical stimulation. These findings demonstrate that mechanical stimuli enhance pro-metastatic properties. The modulation of mechano-transduction signaling could be a new therapeutic target to suppress bone metastases development.

P66

Effects of Zoledronic Acid and Denosumab on Human Vγ9Vδ2 T-cell-mediated Cell Death of Rank-expressing Breast and Prostate Cancer Cells

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Introduction: Aminobisphosphonates such as zoledronic acid (ZOL) inhibit osteoclast-mediated osteolysis by blocking farnesyl pyrophosphate synthase (FPPS) activity, leading to accumulation of isopentenyl pyrophosphate (IPP), a phosphoantigen for anticancer gamma-delta T cells (Vγ9Vδ2). Indeed, interleukin 2 (IL-2) + ZOL stimulates expansion of Vγ9Vδ2 T cells from human peripheral blood mononuclear cells (hPBMCs). ZOL also induces IPP accumulation and secretion by breast cancer cells, resulting in activation and chemotaxis of Vγ9Vδ2 T cells to breast tumors. Denosumab (Dmab), a fully human monoclonal antibody directed against RANKL, inhibits osteolysis by blocking RANK/RANKL interaction. RANKL also mediates the migration of RANK-expressing breast and prostate cancer cells. In the current study, we compared the effects of ZOL and Dmab on (1) the expansion of Vγ9Vδ2 T cells and (2) Vγ9Vδ2 T-cell-cytotoxicity against human RANK-expressing breast and prostate cancer cells.
Material and Methods: hPBMCs were obtained from healthy donors. Expansion of Vγ9Vδ2 T cells in hPBMCs and RANK expression by tumor cells were evaluated by flow cytometry and western blotting, respectively. IPP accumulation was measured by mass spectrometry.
Results: We first found that IL-2 + ZOL (1-10 μM) but not IL-2 + Dmab (0.001-0.1 mg/mL) caused expansion of Vγ9Vδ2 T cells *in vitro*. In addition, Dmab did not interfer with Vγ9Vδ2 T-cell expansion induced by IL-2 + ZOL, using antibody concentrations which did inhibit RANKL- and macrophage colony-stimulating factor-induced osteoclast differentiation from hPBMCs. Moreover, we found that Dmab inhibited RANKL-induced migration of RANK-positive human breast and prostate cancer cells. ZOL (1-10 μM, 1h) caused high IPP accumulation in T47D and DU145 but not B02 and PC3 cells. RANKL-stimulated T47D and DU145 cells were targeted for IPP-dependent cytotoxicity by Vγ9Vδ2 T cells after ZOL, but not Dmab, pretreatment. B02 and PC3 cells were not targeted under any of these conditions. Moreover, Dmab pretreatment (0.01 or 0.1 mg/mL) neither induced nor blocked Vγ9Vδ2 T-cell cytotoxicity against RANKL-stimulated T47D or DU145 cells induced by ZOL pretreatment.
Conclusion: These data suggest that breast and prostate cancer cells producing high IPP levels after ZOL treatment are most likely to respond to Vγ9Vδ2 T-cell-mediated immunotherapy. Dmab had no immunomodulatory effects at concentrations that did inhibit osteoclast differentiation.

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***In Vivo* Phosphoantigen Levels in Bisphosphonate-treated Human Breast Tumors Trigger Vγ9Vδ2 T-cell Antitumor Cytotoxicity Through ICAM-1 Engagement**

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Nitrogen-containing bisphosphonates (N-BPs) such as zoledronate (ZOL) and risedronate (RIS) inhibit osteoclast-mediated bone resorption and interfere with cancer cell functions by blocking the activity of a key enzyme in the mevalonate pathway, farnesyl pyrophosphate synthase (FPPS). FPPS inhibition leads to intracellular accumulation of isopentenyl pyrophosphate (IPP) and triphosphoric acid I-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) mevalonate metabolites. In addition, both IPP and ApppI are recognized as tumor phosphoantigens by Vγ9Vδ2 T cells, a subset of human T cells that exhibits anticancer activity. Thus, the anti-tumor immunity of Vγ9Vδ2 T cells provided cell-cell contacts through unrelated TCR cell surface expressed by Vγ9Vδ2 T cells and cell surface receptors expressed by tumor cells. Here, we investigated whether the potency of human Vγ9Vδ2 T cells to kill cancer cells depends on intracellular IPP/ApppI levels in N-BPs-treated different human breast cancer cells *in vitro* and *in vivo*. Furthermore, we examined whether the expression level of adhesion molecule-1 (ICAM-1) on breast cancer cells correlates with the cytotoxicity by Vγ9Vδ2 T cells *in vivo*. In this study, using RIS or ZOL we found a strong correlation between Vγ9Vδ2 -T cell anticancer activity and intracellular levels accumulation of IPP/ApppI in bisphosphonates-treated breast cancer cells *in vitro*. Additionally, following RIS or ZOL treatment of immunodeficient mice bearing subcutaneous human breast tumors xenografts, human Vγ9Vδ2 T cells infiltrated and inhibited growth of tumors that produced high IPP/ApppI levels, but not those expressing low IPP/ApppI levels. Moreover, we found that Vγ9Vδ2 T-cell cytotoxic activity in mice treated with RIS or ZOL did not only depend on IPP/ApppI accumulation in tumors but also on expression of human tumor cell surface receptor ICAM-1 and the release of this receptor from tumor detected in mice serum, which triggered the recognition of N-BP-treated breast cancer cells by Vγ9Vδ2 T cells

in vivo. These findings suggest that N-BPs can have an adjuvant role in cancer therapy, especially by activating Vγ9Vδ2 T-cell cytotoxicity in patients with breast cancer that expresses high levels of ICAM-1 and produces high IPP/ApppI levels after N-BP treatment.

P68

Metabolomics Identifies Potential Biomarkers of Multiple Myeloma Development and Progression

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Multiple myeloma is a malignancy of plasma cells, which grow at multiple foci in the bone marrow, secrete monoclonal immunoglobulins, and induce bone disease, hypercalcemia, anemia, and renal failure. This frequent and still incurable cancer originates from monoclonal gammopathy of undetermined significance (MGUS), a premalignant expansion of plasma cells that behave benignly despite the presence of most myeloma-specific genetic abnormalities. Indeed, development and progression of multiple myeloma are believed to rely on vicious interactions with the bone marrow environment, offering a paradigm to investigate the bone-cancer relationship. A better knowledge of such interplay, still elusive, would help identify prognostic markers, pathomechanisms, and therapeutic targets for future validation.To achieve an unbiased, comprehensive assessment of the extracellular milieu during multiple myeloma genesis and progression, we performed a metabolomic analysis of patient-derived peripheral and bone marrow plasma by ultra high performance liquid and gas chromatography followed by mass spectrometry. Multivariate analysis of peripheral metabolic footprinting successfully discriminated active disease from health or premalignant conditions. Among myeloma patients, significant changes in the peripheral metabolome were found to be associated with abnormal renal function. Noteworthy, both central and peripheral metabolic scores significantly correlated with tumor burden. The bone marrow plasma metabolome successfully discriminated active myeloma from premalignant conditions, MGUS and smoldering myeloma, or remitting disease. Independent comparative analyses of distinct disease vs control groups consistently identified a set of myeloma-associated metabolic alterations. Among these, increased levels of the C3f-derived peptide, HWESASLL, and loss of circulating lysophosphocholines emerged as hallmarks of active disease. *In vitro* tests on myeloma cell lines and primary patient-derived cells revealed a previously unsuspected direct trophic role exerted by lysophosphocholines on malignant plasma cells. Altogether, our data demonstrate that metabolomics is a powerful approach to study the complex interactions of multiple myeloma with the bone marrow environment. This novel strategy holds potential to identify unanticipated markers and pathways involved in development and progression of multiple myeloma.

P69

Breast Carcinoma Cells Modulate Functional Properties of the Human Bone Marrow Niche *In Vitro*

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Introduction: The soil for systemic metastasis in breast cancer is thought to be localized in the bone marrow. In the marrow space, hematopoietic stem and progenitor cells (HSPC) and metastatic tumor cells probably compete for a functional unit defined as the stem cell niche. The interaction with mesenchymal stromal cells (MSC) is mediated by chemoattractants, matrix- and membrane bound growth factors and a cell adhesion signalling network. Methods: Primary MSC, an immortalized MSC cell line (SCP-1), breast cancer cell lines (MCF-7, MDA-MB-231) and the non-carcinogenic breast epithelial cell line MCF-10A were used in different in-vitro cell culture models, employing contact and non-contact cultures. Moreover, an assay to study competitive cell adhesion has been established to recapitulate micrometastases.
Results: The secretion of various cytokines of MSC was modulated in direct and indirect cocultures with MCF-7 and MDA-MB-231 cells. One important molecule which plays a critical role for the homing and retention of HSC in the niche is stromal derived factor-1 (SDF-1). Conditioned medium of both tumor cell lines caused a significant SDF-1 downregulation at the mRNA as well as the protein level to about 40% of the controls. In contrast, MCF-10A non-carcinogenic cells had no or even an increasing effect on the SDF-1 secretion of MSC. As a functional consequence of changed SDF-1 levels, the HSPC migration potential to tumor cell conditioned medium was found to be decreased to 46% (MCF-7) and 54% (MDA-MB-231) compared to controls, whereas the motility to MCF-10A conditioned medium was not affected. In a competitive cell adhesion assay, competition with MCF-7 cells caused a reduction in HSPC adhesion to the MSC layer. Direct cell-cell interactions within the bone marrow niche mediated by surface molecules are affected by invaded tumor cells as well. In both MCF-7 and MDA-MB-231/MSC cocultures, we detected a significant upregulation of MCAM (CD146) on MSC, which plays a critical role for HSC adhesion and stemness in the stromal compartment but could also be involved in the seed of tumor cells in the marrow environment. Summary. The phenotypic and functional changes induced in MSC by breast carcinoma cells suggest a possibly relevant modulation of the HSC niche. If confirmed by *in vivo* studies, the 'hijacking' of the niche environment in the bone marrow by tumor cells may represent a potential avenue for the development of targeted therapies.

P70

Potential Use of Rank Expression On Circulating Tumor Cell in Metastatic Breast Cancer

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Background: Bone is the most common site of metastatic invasion in breast cancer. The TNF ligand superfamily member RANKL is critical for the formation, function and survival of osteoclasts and recently was demonstrated the activation of RANK signaling promotes mammary tumorigenesis in mice. These data highlight the central role of RANK/RANKL/OPG pathway as therapeutic target in bone metastasis management.
The CTCs are considered an appealing biomarker and a powerful tools for investigating the phenotype and the role of this migrating tumor cell. For this reason we investigate the RANK presence on the CTC surface and the modulation of this receptor during Denosumab therapy.
Experimental Design: An automated sample preparation and analysis system for enumerating CTCs (CellSearch) was integrated for detecting RANK-positive CTCs.
Results: 8 bone metastatic breast cancer (MBC) patients with an age 41-78 are currently included. In 8 patients (Table 1) at the first blood draw 5 were CTC-positive and RANK-positive and 3 were CTC-negative, the percentage of RANK-positive CTC varies from 3.6% to 100%. The higher intensity of positive RANK signal are localized in CTC with lower cytokeratin intensity.
Conclusion: For the first time in literature we demonstrated that RANK expression is detectable by immunofluorescence on CTCs in MBC. The RANK-integrated test has potential for monitoring dynamic changes, in addition to CTC count, to evaluate response in patients under Denosumab therapy. Accrual is ongoing, updated data including CTCs evaluation during Denosumab therapy will be presented at the meeting.

Table 1. CTCs count in bone metastatic breast cancer patients using the CellSearch method.

Patients	CTC/7,5ml	RANK+ CTC (n)	RANK+ CTC (%)
1	neg	neg	–
2	6	4	66,7
3	84	3	3,6
4	5	2	40,0
5	neg	neg	–
6	4	4	100,0
7	neg	neg	–
8	6	6	100,0

P71

In Vivo Micro-computed Tomography Can Visualize and Quantify Osteosarcoma Primary Tumor Growth and Pulmonary Metastases Over Time

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Introduction: In osteosarcoma (OS), one important imaging tool to assess the extent of primary tumor growth, metastatic spread (mainly in the patients' lungs), and thus patient prognosis is computed tomography (CT). In the past two decades, this technique has been adapted in order to image small laboratory animals like mice. As such, micro-CT imaging proved its functionality in a number of other cancer types. In our laboratory, we established a method to visualize the presence of single OS tumor cells *ex vivo*, by means of LacZ tagging. However, what exactly occurs during the course of the disease process remains largely unknown, since *in vivo* visualization methods are not yet commonly employed in preclinical OS models. We therefore tested if *in vivo* micro-CT can be employed to monitor the growth of both the primary tumor as well as the pulmonary metastases in a preclinical OS model.
Methods: Female SCID mice received an intratibial injection with LacZ-tagged osteoblastic SAOS-2 or osteolytic 143B OS cells. After growth of the primary tumor, mice were anesthetized and scanned in the Skyscan 1176 *in vivo* microtomography system using the 35 µm setting. Two separate scans were made, one of the chest area and one of the hind limbs. Scan duration per scan was 8-10 minutes, with a dose of ~0.5 Gy. After two weeks, the scans were repeated. As verification, mice were sacrificed immediately after the second scan, and their lungs were excised, X-Gal stained, air-dried, and scanned again at high (9 µm) resolution.
Results: In mice injected with SAOS-2 cells, mineralized foci could be observed inside the primary tumor mass, as well as in the pulmonary metastases. The size of the smallest detectable metastasis was 0.5 mm. In the second scan, the mineralized foci became more pronounced. In 143B tumors, bone destruction at the proximal tibia could be visualized in detail. X-Gal stained and re-scanned lungs showed a perfect match between X-Gal staining and micro-CT-detected metastatic sites *ex vivo*.
Conclusions: Micro-computed tomography can be used to monitor both primary and distal OS tumor growth *in vivo*, and reveals detailed 3D information of micro-metastasis distribution *ex vivo*. Future challenges will be to increase the contrast between tumor tissue and normal tissues, e.g. by using gold-labeled antibodies directed against specific tumor markers, and thus to be able to identify and monitor even smaller metastases over time.

P72

Maldi IMS Identifies Protein Signatures that Vary Between Normal and Tumor-bearing Bone

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While much is known about the later stages of tumor-induced bone disease, less is known about the earlier stages of tumor establishment in bone and the resulting changes in protein expression in the tumor microenvironment. This is partly due to the limited tools for comprehensive imaging and detailed protein analysis. Matrix-Assisted Laser Desorption/Ionization Imaging Mass Spectrometry (IMS) allows for protein analysis from tissue sections while preserving spatial localization, allowing for a semi-quantitative, non-biased analysis of protein signatures. Protein signatures can be correlated with other imaging modalities through co-registration to provide additional anatomical detail and qualitative measurements, which together can provide a comprehensive analysis of the tumor and the surrounding tissue. We hypothesized that IMS would show signatures that varied between the tumor associated and the non-tumor microenvironment, and that specific protein profiles could be associated with tumor tissue, muscle and marrow. For these studies we integrated IMS data with magnetic resonance imaging (MRI), anatomical block face, and histology in an intratibial model of breast tumor-induced bone disease. As expected, mass-to-charge ratio peaks indicate differential expression of proteins that were associated with the tumor bearing limb, including calcyclin, ubiquitin, calgranulin A, hemoglobin, and other proteins. Using histologically defined ROIs, we generated a classification algorithm that could differentiate these cell types and reasoned that it could be used to identify tumor tissue in bone. As anticipated, the software was able to accurately identify tumor tissue, muscle, skin, and bone marrow based on the protein signatures when compared to the other the imaging modalities. Thus far the technique has only been applied to sections with obvious tumor burden, but we intend to test whether this technique will allow us to accurately detect tumor or tumor associated stroma in samples with smaller tumors that are more indicative of early metastatic disease. Importantly, this technique has allowed us to identify proteins that have not previously been associated with tumor-induced bone disease. In conclusion, this technique preserves the structure of the tissue while synergizing with other powerful imaging tools to perform more detailed analyses of the tumor microenvironment than previously possible.

P73

Functional Studies of the TMPRSS2:ERG Rearrangement in Prostate Cancer Bone Metastases

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Prostate cancer (CaP) is one of the most common malignancies that affect men in western countries. Bone metastases are frequent and severe complications of CaP. Recurrent gene fusion, involving the ERG gene and the androgen-regulated TMPRSS2 gene promoter, occurs in over 50% of CaP. We and others have shown the ERG transcription factor, a member of ETS family, is associated with embryonic skeleton development. Interestingly, part of potential ERG-target genes identified using high-throughput DNA microarray analysis have been shown involved in bone metastases development, suggesting possible ERG transcriptional regulations in CaP bone metastases derived.

To evaluate the implication of ERG protein in CaP metastases formation, we first asked whether a part of ERG-target genes identified in our previous microarrays studies, such as Metalloproteinase-9 (MMP9) and Osteopontin (OPN), who were known to be involved in bone metastases processes, were regulated also by ERG in CaP. By using PC3c cells line (derived from PC3 cell line) which stably TMPRSS2:ERG fusion gene, we confirmed the up-regulation of endogenous MMP9 and OPN in CaP cells. Concomitantly, chromatin immunoprecipitation (ChIP) experiments revealed an ERG specific binding to both promoters in PC3c cell clones. MMP9 and OPN immunostaining were co-localized with ERG in human CaP samples. These results indicate that these genes are regulated by ERG transcription factor in CaP.

To test the hypothesis of the implication of ERG in bone metastases development, we performed intra-tibial injection of PC3c cell clones that stably expressed TMPRSS2:ERG fusion gene in SCID mice. X-ray and micro-CT scan analysis showed an dramatic increased of osteoblastic lesions induced by PC3c/TMPRSS2:ERG cells compared with PC3c fusion negative control cells. These results show, for the first time, the possible implication of TMPRSS2:ERG expression as a stimulator of osteoblastic lesions in CaP bone metastasis formation. Certainly MMP9 and OPN overexpression could not fully explain the phenotype, we are therefore performing transcriptomic studies to identify genes expressed by the stable cell clones and associated with the osteoblastic phenotype.

P74

Bone Rigidity Regulates Invasive Oral Squamous Cell Carcinoma Gene Expression

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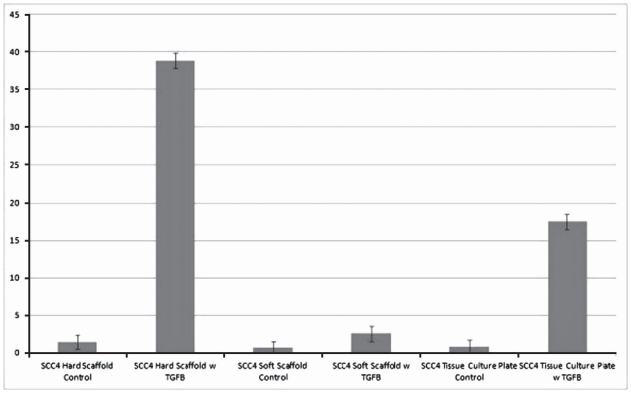
Oral Squamous Cell Carcinoma (OSCC) is an aggressive cancer type that makes up approximately 90% of all oral cancers. In the oral cavity, OSCC presents in two states; erosive and invasive. Despite recent improvements in treating this disease, about half of all patients diagnosed with OSCC die within 5 years. It has been shown that the high mortality rate of OSCC correlates strongly with mandibular invasion. However, factors responsible for mediating the erosive or invasive patterns of OSCC have yet to be identified. Through micro-array analysis, and confirmed by qPCR, we found that OSCC over-expresses parathyroid hormone-related protein (PTHrP), similar to other tumors that metastasize to bone. Furthermore, other groups have confirmed PTHrP expression in feline OSCC samples and in some human mandibular biopsies, and that Gli2, a hedgehog transcription factor, is over-expressed in OSCC patients by immunohistochemistry. Since our previous studies have indicated that PTHrP is regulated by Gli2, transforming growth factor beta (TGF-β) and matrix rigidity, we reasoned that these factors may also regulate mandible invasion in OSCC. We hypothesized that hard matrices promote PTHrP expression and thus mandibular invasion. To address this, we seeded SCC4, an OSCC that expresses PTHrP, onto 2D polyurethane scaffold of varying rigidities. Our preliminary data demonstrated that PTHrP increased on the rigid matrices. To determine if this was controlled in a TGF-β and Gli dependent manner we treated cells with two known mediators of PTHrP, the PTHrP activator TGF-β or the Gli antagonist, Gant58, and measured PTHrP expression by real-time PCR. These experiments demonstrated that TGF-β increased PTHrP expression of SCC4 grown on hard scaffolds by almost 40 fold, while SCC4 cells grown on the soft scaffolds had significantly lower PTHrP expression than both SCC4 cells grown on hard scaffolds and standard tissue culture plates (data shown). In addition, we report that Gant58 reduced PTHrP expression by 3.39 fold. Taken together, our data suggest that OSCC invasion into the mandible is mediated by similar mechanisms as other cell types that metastasize to bone. Furthermore, this research suggests a TGF-β and PTHrP dependent mechanism in the regulation of OSCC cells, and that inhibitors of these pathways may be therapeutic targets for reducing mandibular invasion in OSCC patients.



TGF-β and matrix rigidities mediates PTHrP expression. PTHrP expression of TGF-β treated SCC4 cells increase when grown on hard scaffolds by almost 40 fold, while SCC4 cells grown on soft scaffolds had significantly lower PTHrP expression than both SCC4 cells grown on hard scaffolds and standard tissue culture plates.

P75

Tumour Stroma Cooperates with Cancer Cells in Suppressing Bone Formation and Stimulating Bone Resorption in Osteolytic Bone Metastasis

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The interaction between cancer cells and stroma is essential for growth progression in primary and metastatic tumours. Prostate cancer (PCa) and mammary cancer (MCa) cells preferentially metastasize to bone inducing an osteolytic or osteoblastic response. The aim of this study was to decipher the stroma-specific expression profile in xenograft models of osteolytic bone metastasis.
Human osteolytic PCa (PC-3) and MCa (MDA-MB-231) cells were xenografted in the tibia of immunodeficient mice. RNA isolated from tumour-bearing bones, consisting of a mixture of mouse (stroma compartment) and human (cancer cell compartment) transcripts, was analysed by RNA sequencing. Differential gene regulation was validated by RT-qPCR with mouse- and human-specific probes. The vast majority of the differentially regulated stromal genes were common to both PCa and MCa xenografts and the direction of regulation was concordant for both cancer types. Up-regulation of Periostin, Asporin, Fscn1, Sparcl1, Mcam and Pdgfrb was common to the stroma of osteolytic and osteoblastic bone metastasis and of primary, human PCa and MCa tumours. BMP/Wnt antagonists and semaphorins were among the stromal genes specifically up-regulated in osteolytic lesions. Some BMP/Wnt antagonists, such as Dickkopf (Dkk) 1, Noggin and Chordin, were expressed by both cancer cells and stroma, whereas others, such as Dkk2, Dkk3 and Sclerostin, exclusively by stroma. Semaphorin 3B, 3F, 4C, 4G, 6C and 7A were expressed by cancer cells and additionally induced in stromal cells.
The transcriptome analysis of the bone stroma reaction to osteolytic cancer cells suggests that one component is a universal, tissue- and cancer type-independent, response, similar to the injury/wound healing response. Conversely, there is an additional component specific of the stromal response to osteolytic cancer cells. This includes genes encoding factors either stimulating bone resorption or inhibiting bone formation. Semaphorin 3B and 7A are shown for the first time to participate in pathological bone resorption. Previously, we have demonstrated that inhibition of bone formation by cancer cell-derived noggin contributes to osteolysis. Here we show that stromal cells may further amplify this process by expressing an extended list of BMP/Wnt antagonists.

P76

Effect of Zoledronic Acid on Bone Mineral Density in Premenopausal Women Receiving Neoadjuvant or Adjuvant Therapies for Breast Cancer: the ProBONE II Study

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Purpose: Bone mineral density (BMD) evaluations have shown that adjuvant chemotherapy or endocrine therapy for early breast cancer is associated with accelerated BMD loss and increased fracture risk. In recent studies, zoledronic acid (ZOL) increased BMD in premenopausal and postmenopausal women with breast cancer, and improved disease-free survival (DFS) in some patient subsets compared with no ZOL. The purpose of the current study was to investigate the effect of adjuvant treatment with ZOL on BMD in premenopausal women with early breast cancer treated with chemotherapy or endocrine therapy.
Methods: In this randomized, double-blind, placebo-controlled study, 71 patients receiving adjuvant chemotherapy and/or endocrine therapy were randomly assigned to also receive ZOL (4 mg IV q 3 months) or placebo for 24 months. The primary endpoint was change in BMD at lumbar spine (LS) at 24 months relative to baseline. Secondary endpoints included change in femoral neck and total femoral BMD, course and change in bone turnover marker levels, assessment of potential correlations between BMD and bone turnover, development of metastases, pathologic fractures, and safety and tolerability.
Results: At 24 months, LS BMD substantially increased (+3.13%) with ZOL, and decreased (-6.46%) with placebo relative to baseline (P<.001, between-group comparison). Femoral neck and total BMD also increased with ZOL, versus decreases with placebo at 24 months relative to baseline (P<.001, between-group comparisons). By month 3, mean bone marker levels decreased (-65% for C-telopeptide of type I collagen [CTX] and -61% for N-terminal propeptide of type 1 procollagen [P1NP], relative to baseline) with ZOL, with significant (P<.001) between-group differences in levels of both bone markers at 24 months versus baseline. Overall, ZOL was well tolerated, and only 1 case of osteonecrosis of the jaw was reported.
Conclusions: This study demonstrates that early initiation of ZOL is well tolerated and preserves BMD and reduces bone turnover biomarker levels in premenopausal women with early breast cancer undergoing chemotherapy or endocrine therapy.

P77

Mechanosensitive TRP Channels Are Required for RANKL-independent Ca^{2+} Signaling in Osteoclastogenesis

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Bone remodeling and maintenance require a fine balance between bone formation of osteoblasts and resorption of osteoclasts and various skeletal disorders cause by imbalanced differentiation and activities of these cells. RANKL (receptor activator of NF-κB ligand) induces Ca^{2+} oscillations and activates NFATc1 (nuclear factor of activated T cells 1) during osteoclast differentiation. Although Ca^{2+} oscillations play a key role for osteoclastogenesis, the molecular identification of Ca^{2+} influx via mechanosensitive calcium channels located on the plasma membrane for the generation of Ca^{2+} oscillation are not well known. In the present study, we investigated the expression and functional role of mechanosensitive TRP (transient receptor potential) channels, TRPC3, TRPC6, TRPM7, and TRPV1, on Ca^{2+} oscillations and osteoclastogenesis in RAW264.7, a mouse bone marrow derived monocytes, and bone marrow macrophage (BMM) cells. RANKL-independent Ca^{2+} oscillations were induced by the overexpression of TRPC3 and TRPC6, and agonists of TRPM7 and TRPV1. Activation of these channels had effects on the expression of NFATc1, PLCγ1, and IP_3Rs, and $TRAP^+$ cell formations. These results suggest that mechanosensitive TRP channels are essential for RANKL-independent Ca^{2+} signaling in osteoclastogenesis.

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Effects of Zoledronic Acid on Hormone Levels in Premenopausal Women with Breast Cancer Receiving Neoadjuvant Or Adjuvant Chemotherapy and Endocrine Therapy: ProBONE II Study

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Background: Accelerated loss in bone mineral density (BMD) may occur soon after initiation of adjuvant therapy for hormone-receptor-positive (HR+) breast cancer (BC), and correlates with changes in hormone levels. Adding zoledronic acid (ZOL) to adjuvant treatment for BC can preserve or improve BMD and delay disease recurrence (especially in women expected to have low estrogen levels). However, the effects of ZOL on endocrine hormone levels are currently unclear.
Methods: ProBONE II assessed the course of key endocrine hormones (estradiol, parathyroid [PTH], follicle-stimulating [FSH], anti-Muellerian [AMH], inhibins A and B, sex hormone binding globulin, and total testosterone) in premenopausal women with HR+ BC during 24 months of neoadjuvant or adjuvant treatment with chemotherapy and/or endocrine therapy, and ZOL (4 mg q 3 months) or placebo. Safety was continuously monitored.
Results: Patients (N = 70; mean age = 43 years [range, 23-51 years]) had a predominant diagnosis of T1 N0-1 M0 BC. Adjuvant chemotherapy was primarily standard anthracycline-cyclophosphamide followed by taxane; adjuvant endocrine therapy involved a gonadotropin-releasing hormone analogue (84.3%) and tamoxifen (94.3%). Estradiol levels reached a nadir after 3 months in the placebo group and 9 months in the ZOL group. In both groups, FSH levels increased by month 3

and returned to near baseline by treatment end; in contrast, inhibins A and B decreased by month 6 and remained low throughout. Levels of AMH decreased by 57% and 71% by month 6 in the placebo and ZOL groups, respectively, with continued decrease on-study in the ZOL group (vs remaining constant in the placebo group). Testosterone and PTH levels tended to be slightly higher in ZOL-treated patients versus placebo. The most frequent treatment-emergent adverse events were consistent with the known profiles of ZOL and adjuvant therapy.
Conclusions: Hormonal effects of 2 years of adjuvant treatment in this study were consistent with earlier reports of chemotherapy and endocrine treatments. Adding ZOL (4 mg q 3 months) did not affect the changes in hormone levels that accompany chemotherapy and/or endocrine therapy in HR+ BC, suggesting that the adjuvant benefits observed with ZOL in some patient populations are mediated through nonhormonal mechanisms.

P79

Axitinib and Crizotinib Combination Therapy Inhibits Bone Loss in a Mouse Model of Castration Resistant Prostate Cancer (CRPC)

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Background: CRPC is the leading cause of cancer-related death in men in the United States and Europe. The primary cause of mortality and morbidity in these patients is due to bone metastasis. Bone metastasis causes local disruption of normal bone remodeling with an increase in osteoblastic and some osteolytic lesions. These lesions eventually lead to skeletal fractures, spinal cord compression, intractable bone pain and decreased survival. Recently, a dual inhibitor of c-MET and VEGFR2 was shown to have efficacy on bone lesions as well as survival in human patients. In this study we tested 2 approved small molecule drugs (Axitinib-VEGFRi and Crizotinib-cMETi) that target these pathways in a combination trial in mice.
Methods: Two cohort of 40 NOD-SCID-gamma (NSG) mice (castrated and non-castrated) were used in the study. Mice were anesthetized with ketamine : medetomidine and injected with VCaP-Luc cells (106) orthotopically into the left tibia. Mice were monitored weekly for tumor growth using an IVIS scanner. Animals were randomized into 4 groups (vehicle, Crizotinib alone, Axitinib alone, Crizotinib and Axitinib in combination). Animals were imaged weekly by Faxitron to monitor bone remodeling. After 8 weeks of dosing animals were euthanized and both tibias extracted for microCT (mCT) imaging.
Results: VCaP cells in intact animals caused extensive remodeling of bone with severe osteoblastic and osteolytic lesions. The osteoblastic lesions were more predominant and were sometimes seen to extend beyond the shaft of the tibia into the surrounding tissue. In contrast, castrated animals osteolytic lesions were more prominent throughout the study period indicating the role of androgens in the bone phenotype of the disease. Treatment with Crizotinib alone reduced the osteolytic lesions seen in castrated animals. Axitinib alone reduced the osteoblastic lesions in the intact animals. Combination therapy with Axitinib and Crizotinib had a remarkable effect in inhibiting the tibial remodeling by VCaP cells with a regression of both osteoblastic and osteolytic lesions. These results show that c-MET and VEGF inhibition by the two Pfizer drugs can be very beneficial for treatment of metastatic bone disease in CRPC and that the drugs act on two different stages of the disease.

P80

Effect of Zoledronic Acid on Bone Metabolism in Patients with Bone Metastases from Prostate or Breast Cancer: The Zotect Study

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Introduction: The prospective, single-arm, open-label ZOTECT study was designed to assess the effect of zoledronic acid (ZOL) on bone-marker levels and potential correlations with disease outcomes in bisphosphonate-naive patients with bone metastases.
Methods: Patients with bone metastases from prostate cancer (PC; n = 301) or breast cancer (BC; n = 99) who have not received bisphosphonates for ≥ 6 months were enrolled at 98 sites in Germany (from May 2006 to July 2008). Patients received ZOL (4 mg) intravenously every 4 weeks for 4 months, with a final follow-up at 12 months. The primary endpoint was change in bone marker levels at 12 months relative to baseline. Secondary assessments included skeletal-related event (SRE) rate, pain, quality of life (QoL), and prostate-specific antigen (PSA) levels. Endpoints were assessed using summary statistics by visit/tumor type and Kaplan-Meier analyses.
Results: ZOL treatment significantly decreased bone-marker levels versus baseline (amino-terminal propeptide of type I collagen [P1NP], C-terminal cross-linking telopeptide of type I collagen [CTX]; $P < 0.0001$), and this decrease was maintained through the final 1-year follow-up visit. Baseline P1NP and CTX levels correlated with the extent of bone disease ($P < 0.0001$, each) and on-treatment decreases in marker levels. Skeletal disease burden and bone-marker levels were similar between PC and BC patients, and ZOL did not significantly influence osteoprotegerin/receptor activator of nuclear factor-kappaB ligand levels. During the 12-month period, only 13 SREs occurred in 11 patients. On-treatment bone-marker level changes did not correlate with SRE rate, pain scores, or QoL. Mean PSA levels were lower at study end (120 days; 92.5 µg/L) than at baseline (168.5 µg/L; Wilcoxon signed-rank test $P = 0.27$). In general, ZOL was well tolerated and adverse events were consistent with its established safety profile.
Conclusions: This study confirms that ZOL therapy significantly reduces bone turnover (measured as P1NP and CTX levels) in patients with bone metastases from PC or BC.

P81

Proteomic Signaling in Bone Metastases: the Role of the Tumor/microenvironment Interaction

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Bone colonization is a main cause of cancer associated pain and mortality. While some progress has been made in understanding bone metastasis pathophysiology, this has not translated to successful therapies that have significantly reduced the mortality of bone metastasis. We used a proteomics approach to identify novel treatment targets for bone metastasis. We mapped activated kinase pathway interconnections in the bone microenvironment from 160 bone metastasis samples derived from carcinomas and sarcomas. The overall goal is the identification of molecules involved in the tumor/host cross talk as targets for new molecular targeted therapies. Specimens: 136 bone metastasis frozen samples from different types of primary carcinomas and 24 from primary sarcomas were collected at the Istituto Ortopedico Rizzoli, IRCCS, Bologna, Italy.

Each specimen was divided in 2 pieces: 1 was formalin-fixed and paraffin-embedded to evaluate the tumor percentage by H&E staining and 1 was lysed using Covaris Adaptive Focused Acoustic™ technology to quantify the level of 88 post-translationally modified cell signaling kinases by Reverse Phase Protein Microarray.

Metastases originating from different primary tumors showed similar levels of cell signaling across tissue types for the majority of proliferation, invasion and adhesion pathway proteins analyzed, suggesting that the bone microenvironment influences the signaling profiles of the metastatic tumor.

Spearman's rho rank comparison analyses were performed to determine and clarify the network linkages within the bone metastasis microenvironment. Results showed a highly significant number of interactions intersecting with ERα, Serotonin, TNFα, TNFR1 and MMPs in the bone metastasis signaling network, regardless of the primary tumor of origin. Outcome data were available for 143 bone metastasis samples and were used for survival analysis. The data set was equally divided into a discovery and a validation set. Eleven end-points in the discovery set (Ezrin Y353, IL6, MMP11, Serotonin, TNFR1, HSP90, p53 S15, WNT5 a/b, AMPKα S485 and Akt) and 6 in the validation set (DKK1, RANK, TNFR1, AMPKα S485, CREB S133 and PDGFRβ) showed a statistically significant ($p \leq 0.05$) Kaplan-Meier correlation with survival, using the median of the expression as the cut point. Results were confirmed by an independent bioinformatic analysis using a support vector machine learning model.

P82

Galectin 3 and MMP-9 Relation with Spontaneous Osteoclastogenesis in Bone Marrow from Advanced Breast Cancer Patients

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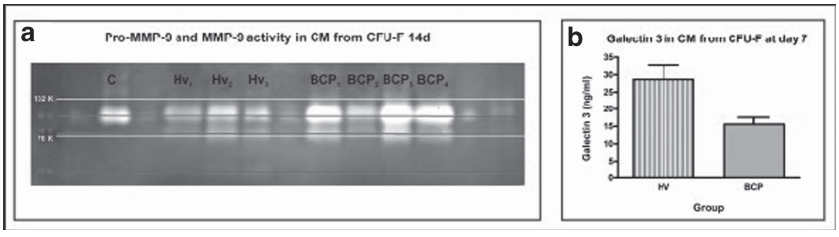
Background: The bone osteolytic metastasis is one of the worst complications for advanced breast cancer patients (BCP). Bone imbalance towards osteoclastogenesis seems to be crucial for bone metastasis development. Most of the mechanisms that regulate bone formation and bone resorption are related with mesenchymal stem cells (MSC) from bone marrow (BM). We have previously observed that MSC from untreated advanced BCP (ductal infiltrative carcinoma, stage IIIb, without bone and/or BM metastasis and without any bone metabolic disease) have lower cloning efficiency for colony forming units-fibroblast (CFU-F) and lower osteogenesis capacity than MSC from healthy volunteers (HV). We have also shown that BCP develop spontaneous osteoclastogenesis from BM hematopoietic precursors *in vitro*. So, bone imbalance occurs even before bone metastasis appearance. Galectin 3 (Gal3) released by hematopoietic and stromal cells, mostly under inflammatory conditions, inhibits osteoclastogenesis. Its cleavage by metalloproteinases such as MMP-9, produced by stromal cells, not only lets osteoclastogenesis process to proceed but also favors angiogenesis and tumor progression.

Methodology: mononuclear cells from BM of BCP (n=8) and HV (n=8) were cultured to obtain CFU-F. Medium was changed at day 7 and the culture was stopped at day 14. Condition



[P82] MMP-9 and Galectin3 as regulators of spontaneous osteoclastogenesis in bone marrow of advanced breast cancer patients. (**a**) Example of zymography in conditioned medium of CFU-F 14 days (d) for Pro-MMP-9 and MMP-9 activity (C=control, Hv=healthy volunteer, Bcp=breast cancer patient). (**b**) Galectin 3 levels in conditioned medium from CFU-F 14d. Values are expressed as X±SE (Unpaired t-test with Welchs Correction, $P<0.05$).

medium (CM) from day 7 and 14 were harvested and stored until use. ELISA for Gal3 was assayed in CM from 7 and 14 days (d). Zymography for MMP-9 activity was made over CM from 14d.
Results: 7d CM from BCP have lower levels of Gal3 than those from HV. Values are expressed as ng/ml (X±SE): 28.70±4.06 and 15.56±1.98 for BCP and HV respectively (Unpaired t-Test with Welchs correction; $p<0.05$). MMP-9 gelatinolytic activity is higher in CM from CFU-F 14d from BCP than HVs. Values of semi-quantification are expressed as relative units: 1.72±0.33 and 0.82±0.07 for BCP and HV respectively (Unpaired t-Test with Welchs correction; $p<0.05$).
Conclusions: Lower levels of Gal3 may not be able to inhibit osteoclastogenesis. In BCP higher levels of MMP-9 promote osteoclatogenesis directly and also by Gal3 cleavage. Finally, Gal3 and MMP-9 are involved in bone imbalance towards bone resorption in addition to other factors such as RANKL, OPG, Dkk-1, MIF, GM-CSF and ICAM-1 as we previously reported.

P83

Targeting of αv Integrins Reduces Malignancy of Bladder Carcinoma

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Low survival rates of metastatic cancers, including bladder cancer, emphasize the need for a drug that can prevent and/or treat metastatic cancer. In several solid cancers, αv integrins are involved in essential processes for primary tumor growth and metastasis formation and targeting of αv integrins has been shown to decrease angiogenesis, tumor growth and metastasis. In this study, the role of αv integrin- that is highly expressed in bladder cancer- and its potential as a drug target in bladder cancer was investigated. αv integrin was knocked down in the bladder cancer cell lines UM-UC-3luc2 and RT-4. In parallel, these cell lines were treated with an αv integrin antagonist. Upon αvkd and treatment, cells showed reduced malignancy *in vitro*, as illustrated by decreased proliferative, migratory and clonogenic capacity. The E-cadherin/N-cadherin ratio increased, indicating a shift towards a more epithelial phenotype. This shift appeared to be largely dependent of the EMT-inducing transcription factor *SNAI2*, but not *TWIST* and *SNAI*. The expression levels of the self-renewal cancer stem cell marker *NANOG*, but not *POU5F1* (*Oct-4*) decreased as well as the number of cells with high activity of the putative cancer stem cell marker $ALDH^{hi}$. In line with these observations, knock down or treatment of αv integrins resulted in decreased metastatic growth in preclinical *in vivo* models as assessed by bioluminescence imaging. In conclusion, we show that αv integrins are involved in migration, EMT and maintenance of the cancer stem cell marker $ALDH^{hi}$ in bladder cancer cells. Targeting of αv integrins might be a promising approach for treatment and/or prevention of metastatic bladder cancer.

P84

Correlation of Conventional vs Experimental Biomarkers of Bone Turnover and Metastasis with Skeletal Related Events in the Triumph Study

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Background: Despite variability in patient (pt) risk of skeletal related events (SREs) from bone metastases (BM), all pts are treated using the same dose and schedule (q3-4 wk) of IV bisphosphonate (BP). To better tailor treatment, novel markers of individual SRE risk are required. TRIUMPH is an ongoing clinical trial evaluating q12 wk IV BP therapy for 1 year, following >3 months of standard q3-4 wk BP, in women with low risk BM [defined by the bone resorption marker C-telopeptide (CTx) levels <600 ng/L]. This sub-study evaluated whether novel biomarkers could predict SRE risk in this cohort.
Methods: TRIUMPH enrolled 71 pts. Pt serum at baseline, 6 and 12 wks post-entry were analyzed for CTx and bone-specific alkaline phosphatase (BSAP) as per study protocol. Urine N-telopeptide (NTx) levels and serum levels of transforming growth factor (TGF)-β, activinA, procollagen type I amino-terminal propeptide (P1NP), and bone sialoprotein (BSP) levels were also assessed by ELISA. Biomarker levels were correlated with; time to development of BM, previous SREs, and SREs post-study entry using linear regression analysis. Changes in levels of biomarkers from baseline to 6 or 12 wks were used to calculate odds ratios of coming off study as per protocol (due to either CTx>600 ng/ml or SRE) or of SRE alone using logistic regression analysis.
Results: Baseline CTx and NTx were elevated in pts who went on to develop SREs, but this was not statistically significant. Baseline activinA trended towards total number of prior SREs ($p=0.07$). Baseline TGF-β correlated with duration of BM ($p=0.004$). Change in activinA (baseline to wk 6) was the only biomarker that predicted coming off study early ($p=0.043$). Results of other biomarkers and their changes from baseline to wk 12 will also be presented.
Conclusions: This study highlights the need to further question the role of bone turnover markers for driving treatment decisions around de-intensification of BP therapy and highlights the need for novel markers of SRE risk. As activinA levels were associated with the incidence of SREs and with coming off study early, future studies in breast cancer pts assessing activinA as a predictor of SRE risk associated with breast cancer BM is warranted.
This study was supported by grants from the Ontario Institute for Cancer Research with funding from the Government of Ontario, and from the Ontario Chapter of the Canadian Breast Cancer Foundation.

P85

Effects of De-escalated Bisphosphonate Therapy on Bone Turnover or Metastasis Markers and their Correlation with Risk of Skeletal Related Events – a Biomarker Analysis in Conjunction with the Reform Study

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Background: Despite variability in individual skeletal related event (SRE) risk from bone metastases (BM), all patients (pts) are treated using a similar dose and schedule of IV bisphosphonate (BP). The REFORM trial was a pilot randomised study evaluating the efficacy of de-escalated (q12 wk) versus standard (q3-4 wk) pamidronate in maintaining C-telopeptide (CTx) levels in the low risk range (<600 ng/L) in patients with BM from breast cancer. We analyzed biomarkers of bone turnover and BM behaviour in these pts to determine their correlation with SRE risk.
Methods: Serum & urine were collected from enrolled pts at baseline and at 12 wks. Samples were assessed for urinary N-telopeptide (uNTx), serum procollagen type I amino-terminal propeptide (P1NP), transforming growth factor (TGF)-β, activinA and bone sialoprotein (BSP) by ELISA. Levels were correlated with number of SREs using linear regression analysis. Changes in biomarkers from baseline to 12 wks were used to calculate odds ratios for coming off study (due to either elevated CTx or SRE) or having an SRE using logistic regression analysis.
Results: Similar SRE rates at 1 yr were seen in both arms (n=2/arm). Mean CTx levels decreased slightly from baseline to wk 12 in the q3-4 wk group (240±50 to 206±46 ng/L), and slightly increased in the q12 wk treated group (263±65 to 313±71 ng/L), but was not statistically significant (p=0.8). Mean activinA levels slightly increased in both treatment arms from baseline to wk 12 (730±93 to 875±148 pg/ml in q3-4 wk vs 445±35 to 582±61 pg/ml in q12 wk group) but did not reach statistical significance (p=0.1). Levels of TGF-β from baseline to 12 wks were similar in both groups (p=0.8). Mean CTx levels at wk 12 were statistically different between pts who had SREs vs those that did not (615±72 (n=4) vs 190±26 ng/L (n=19), p<0.0001). Mean activinA levels at wk 12 also trended to be higher in pts who had SREs than those that did not (1069±358 (n=3) vs 681±83 pg/ml (n=18), p=0.12). Results of NTx, BSP and P1NP will also be presented.
Conclusions: CTx predicted and activinA trended to predict SRE risk in these cohorts. However the non-significant trends in increasing CTx in de-escalated BP treatment, together with the observation that activinA levels are similar regardless of dosing regimen, suggest that analysis of conventional and experimental biomarkers of SRE risk requires further examination in other larger patient cohorts comparing de-escalated therapy.

P86

FHL2 Silencing Reduces Wnt Signalling and Osteosarcoma Tumorigenesis

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Osteosarcoma is the most common primary malignant bone tumor occuring in children and young adults. Despite chemotherapeutic treatments, the development of metastatic lesions and resistance to chemotherapy remain responsible for the failure of treatments and poor survival rate of patients. A role of Wnt signalling in osteosarcoma development is supported by the finding that several Wnt ligands, receptors and coreceptors are highly expressed, while Wnt inhibitors are downregulated in osteosarcoma cells. The transcriptional cofactor LIM-only protein FHL2 (four-and-a-half LIM domain protein 2) may act as an oncoprotein or as a tumour suppressor depending on the tissue context. We previously showed that FHL2 acts as an endogenous activator of mesenchymal cell differentiation into osteoblasts through activation of Wnt/β-catenin signaling. The implication of FHL2 in primary bone cancer progression and tumorigenesis is however unknown. In this study, we determined the role of FHL2 in osteosarcoma. Western blot analysis revealed that FHL2 is overexpressed in human and murine osteosarcoma cells, suggesting a role of FHL2 in osteosarcoma tumorigenesis. Using shRNA transduced cells which exhibit a decrease in FHL2 expression by 50-60 % compared to parental cells, we found that FHL2 silencing in aggressive K7M2 murine osteosarcoma cells decreased β-catenin transcriptional activity and reduced cell proliferation. Moreover, FHL2 silencing markedly decreased invasion and migration of K7M2 osteosarcoma cells *in vitro*. We then tested whether this anti-invasive effect may impact tumorigenesis in a mouse model. To this goal, shControl- and shFHL2-transduced murine K7M2 cells were injected in BALB/C mice and tumor growth was determined. We found that FHL2 silencing reduced tumor size compared to control. Consistent with the anticancer activity that we found *in vitro*, FHL2 silencing reduced tumor volume by about 2-fold compared to control tumors. Finally, we investigated whether FHL2 silencing may impact osteosarcoma cell invasiveness. We found that mice injected with shFHL2-infected K7M2 cells developped less lung metastasis than shControl-transduced K7M2 cells. Both the number and size of the metastases were markedly reduced by FHL2 silencing. Our results demonstrate for the first time that FHL2, a Wnt activator in osteosarcoma cells, acts as an oncogene in osteosarcoma and suggest that FHL2 may be a potential target for therapeutical intervention in primary bone cancer.

P87

An Experimental Dog Model of Primary and Metastatic Prostate Cancer for *In Vivo* Imaging and Therapeutic Research

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Rodent models of prostate cancer have proven valuable in studies of this disease. However, prostate cancer is a complex, multifactorial process, and despite advances provided by rodent models, they are unable to recapitulate all features of human prostate cancer, especially bone metastasis. The dog is the only other large mammal that commonly develops spontaneous prostate cancer. The dog prostate shares a variety of anatomic and functional similarities with men. Spontaneous prostate cancer in dogs is similar to that of men, including osteoblastic metastases in bone, a feature which most rodent models do not share. We developed an experimental dog model of prostate cancer that more fully represents human prostate cancer and will be invaluable in pre-clinical studies of diagnostic and therapeutic regimens. Adult male 5-6-year-old beagle dogs (n=12) were immunosuppressed with cyclosporine A (12-40 mg/kg/day) and implanted with Ace-1 canine prostate cancer cells (3-6x10E7 cells) using ultrasound-guided biopsy techniques. The Ace-1 cell line (developed by the Rosol lab) is an immortalized canine prostate cancer line from a spontaneous prostate cancer that forms osteoblastic bone metastases in nude mice after intracardiac injection (LeRoy *et al*., *Prostate* 66: 1213-22, 2006; Thudi *et al*., *Prostate* 68:1116-25, 2008; Thudi *et al*., *Prostate* 71:615-25, 2011). Ace-1 tumors grew in all dogs in the prostatic capsule and parenchyma until euthanasia at 4-6 weeks and prostate volume increased from 15 to 22 cc. The Ace-1 xenografts formed carcinomas that invaded the prostatic parenchyma, capsule, and vessels. Gross metastases occurred in the lungs and regional lymph nodes in 50% of the dogs. Positron emission tomography (PET) scanning was performed in two dogs. The first dog was imaged using 5 mCi of 11C-choline for 60 minutes and 5 mCi of 18F-choline for 60 minutes. No tumor tissue was identified. The second dog was imaged using 5 mCi 11C-methionine and 5 mCi 18F-fluorodeoxyglucose (FDG). The PET scan using 11C-methionine detected the primary tumor in the prostate gland and distant metastatic sites, include bone metastases. The dog model of prostate cancer has great potential for advancing studies in chemotherapy, imaging using radioactive tracers for novel molecular targets, surgical therapy, local ablative therapy, studies on growth, local invasion, and metastasis, and investigations on tumor microenvironment in a host that naturally develops prostate cancer.

P88

Beneficial Effects of Combined Therapy Using Halofuginone and Zoledronic Acid on Breast Cancer Metastases to Bone and Normal Bone Remodeling

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Breast cancer frequently metastasizes to the skeleton causing bone destruction. We have shown that halofuginone (Hfg), a plant alkaloid derivate, 1) reduces osteolytic bone metastasis through inhibition of TGF-β and BMP signaling; and 2) induces bone loss by increasing bone resorption and decreasing bone formation. To further evaluate the mechanism for Hfg-induced bone loss, we determined if zoledronic acid (ZA) treatment could 1) prevent Hfg-induced bone loss in normal mice and 2) be used as combination therapy in mice with breast cancer bone metastases.

Female-nude mice (n=15) were treated with Hfg (1 or 5 μg/d, i.p.) for 4 wks. Hfg (5 μg) significantly decreased total body BMD (20±3 vs 15±2 g/cm^2, p<0.01), as well as spine, tibia, and femur. μCT analysis of the hindlimbs showed reduced trabecular bone volume (0.12±.01 BV/TV vs 0.04±.01 p<0.001) in mice treated with Hfg (5 μg). This was associated with decreased osteoblast (15±3 vs 30±2 2 mm^{-1}, p<0.001) and increased osteoclast number compared to vehicle (6±2 vs 3±2 mm^{-1}, p<0.001). Dynamic histomorphometry demonstrated that Hfg also significantly reduced mineralizing surface and bone formation rate. Next, Hfg (5 μg/d) and ZA [5 μg/kg/3x/wk] alone or combined were administrated to female-nude mice over 4 wks. Mice treated with Hfg alone had lower total body BMD, but concomitant treatment with ZA completely prevented the Hfg-induced bone loss at the total body (40±4 vs 10±2 g/cm^2, p<0.01) as well as spine, tibia and femur. We next tested the effect of Hfg (5 μg/d) and ZA (5 μg/kg/3x/wk) alone or combined in nude mice bearing MDA-MB-231 breast cancer bone metastases. Hfg or ZA alone significantly reduced osteolytic area compared with vehicle (1.9±.9 vs 3.5±.2 mm^2, p<0.001 and 1.3±.4 vs 3.5±.2 mm^2, p<0.001, respectively). Combined treatment with Hfg and ZA was significantly more efficient at reducing osteolytic lesions area than either treatment alone (0.37± .1 mm^2, p<0.001).

In conclusion, Hfg reduced breast cancer bone metastases in mice, and this effect is enhanced when Hfg is combined with ZA. Further, ZA prevented Hfg-induced bone loss in mice. These data suggest that Hfg should be used in combination with anti-resorptive agents such as ZA to prevent bone loss. Hfg has completed Phase II trials in sarcoma patients and could rapidly be brought to the clinic for the treatment of patients with breast cancer bone metastases. However, since Hfg induces bone loss, it should be combined with anti-resorptive therapy.

P89

Discoidin Domain Receptor-1 (DDR1) Blockade Prevents Tumor Cell Survival and Bone Metastastatic Activity

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Collagen receptor DDR1 is receptor-tyrosine kinase involved in tumor-matrix interactions and is required for a variety of cellular functions such as cell survival and invasion, implicated in bone metastases.

In this report we investigated the effects of a highly-specific anti-human DDR1 blocking antibody (ab). Collagen-induced hyperphosphorylation of DDR1 was abrogated by ab treatment *in vitro*. Interestingly, etoposide or TRAIL-induced apoptosis in lung cancer cells, measured by PARP cleavage, was exacerbated after treatment with anti-DDR1 blocking antibody, even in anti-apoptotic conditions mediated by collagen type I, indicating that DDR1 phosphorylation was required for DDR1-mediated prosurvival activity. Moreover, ab treatment severely impaired collagen-induced chemotactic ability and invasiveness of tumor cells *in vitro*. To evaluate the relevance of these findings, we first overexpressed DDR1 in a DDR1 non-expressing lung cancer cell line. These cells showed an increased DDR1 hyperphosphorylation as compared to mock-transduced cells. We inoculated these cells in athymic nude mice in the left cardiac ventricle (i.c.). At day 15 postinoculation, a dramatic increase in bioluminescence was observed in the hind limbs of mice inoculated with DDR1-overexpressing cells. The number of single-cell-derived colonies (SCDC) after bone marrow 'flushing' was also increased indicating that DDR1 levels conferred an overt ability of bone homing.

Next, we tested the anti-metastatic activity of DDR1 blocking antibody (ab). Tumor burden and osteolytic lesions were evaluated by bioluminescence imaging (BLI), X-Ray and μCT image analysis, after i.c. in athymic nude mice. At day 7, BLI showed a dramatic decrease in skeletal tumor burden in ab injected mice. More importantly, a marked reduction in osteolytic lesions assessed by X-Ray imaging and μCT scans was also detected at day 14 ($p<0.001$). To evaluate whether these effects were the result of impaired bone homing, we are currently performing an ab treatment after i.c. inoculation of lung cancer cells. We are also evaluating the effect of ab-treatment to explore its possible contribution in bone colonization.

Thus, these data indicate that DDR1 promotes tumor cell survival in early tumor-bone engagement during skeletal homing. Overexpression of DDR1critically fuels osseous colonization increasing the appearance of osteolytic lesions. These data implicate DDR1 as a novel therapeutic target involved in bone metastasis.

P90

APC/EPCR Signaling Promotes Bone Metastasis in Lung and Neuroblastoma Tumors

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Endothelial protein C receptor (EPCR) is a single transmembrane receptor widely expressed in endothelial cells where it exerts cytoprotective and anticoagulant activities. It is also expressed in lung tumor cells but its function has not been characterized. The aim of this study was to elucidate the function of EPCR activity upon interaction with its natural ligand activated protein C (APC) in bone metastases. Comparative transcriptomic analysis upon APC incubation of EPCR expressing ADC cells revealed an enriched function for apoptotic-related genes. The survival pathways AKT and ERK were rapidly activated in response to APC in a dose-dependent manner. Moreover, the proapoptotic effect induced by staurosporine was reduced upon APC incubation as assessed by PARP cleavage. This effect was abrogated by the use of EPCR blocking antibodies indicating that APC/EPCR triggers a prosurvival signaling pathway in lung cancer cells. To explore the implication of these findings, we assessed the role of EPCR in a model of lung cancer metastasis to bone. Intracardiac inoculation (i.c.) of EPCR knock down cells (shEPCR) dramatically reduced osteolytic lesions ($p<0,01$) and tumor burden ($p<0,001$). Similarly, the use of a blocking antibody preventing APC/EPCR interaction showed similar antimetastatic activity leading to low tumor burden ($p<0,01$) and osteolytic lesions ($p<0,001$) as assessed by bioluminescence imaging, X-ray image analysis and microCT scans. Consistently, overexpression of EPCR in lung cancer cells also increased the osseous prometastatic activity, an effect that was further attenuated by anti-ECPR blocking antibody *in vivo*. Moreover, forced expression of EPCR in a non-expressing neuroblastoma cell line was associated with an overt increase in metastasis *in vivo*.

Thus our results indicate that EPCR/APC signaling confers prometastatic activity by increasing bone homing and facilitating cell survival within the bone compartment. Taken together, these results suggest that EPCR participates in a common mechanism conserved in tumors of different origin and constitutes a potential therapeutic target in bone metastasis.

P91

Characterisation of a Novel Orthotopic Mouse Model of Multiple Myeloma and Therapeutic Response by Quantitative MRI

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Bone marrow provides the primary niche supporting the proliferation of tumour cells in the plasma cell malignancy multiple

myeloma (MM). Although MRI approaches of interrogating MM are becoming more established in the clinic, there is a paucity of imaging strategies used to interrogate disease response in orthotopic pre-clinical models *in vivo*. In this study we investigated a) the effectiveness of MRI methods to non-invasively quantify disease and assess the therapeutic response, and b) the sensitivity of diffusion weighted (DW) imaging as an early biomarker of treatment response compared to standard morphological imaging, in a novel intratibial MM model.

Model Characterisation: Mice were inoculated with MM-luciferase cells via direct intratibial injection. Osteolytic lesions were confirmed by histological and μCT analysis. MR and bioluminescence imaging confirmed tumour growth throughout the murine skeleton, emulating clinical presentation. Over 9 weeks, average intra-leg-bone signal volume, derived from serial T_2-weighted MR imaging, showed a strong association with Ig levels in the murine blood serum.

Therapeutic Response: Bortezomib (BZB 1.0 mg/kg i.p.), and the novel aminopeptidase inhibitor tosedostat (TSD 75 mg/kg i.p.), were administered at week 5 for 4 weeks. Representative images of femur and tibia from positive control, BZB and TSD treated mice are shown in Figure 1A. Both treatments caused a highly significant decrease in intra-bone signal. The BZB group was not significantly different to the negative control group. Measures of Igλ in blood sera (Figure 1B), CD138+ human myeloma cell counts by flow cytometry, and histological staining with H&E and anti-CD138 antibodies (Figure 1C) confirmed the quantitative MRI response data. DW images were acquired prior to and 48 hours after treatment with BZB, using a home-built single leg MRI coil (Figure 2A). The mean ADC significantly increased from 802 ± 60 to 1157 ± 27 $x10^{-6}$ cm^2 s^{-1}, even though tumour volume, measured by T_2-weighted MRI, remained unchanged (Figure 2B).

These data suggest that the intratibial MM model is sensitive to the clinically approved, standard of care, anti-MM agent BZB, and the novel agent, TSD, and that MRI provides a non-invasive quantifiable measure of therapeutic efficacy, correlating with the gold-standard measures of disease. In addition, early increase in tumour ADC may give a more specific, and clinically translatable biomarker of therapeutic response in this model.

P92

miR-326 Is a Novel Biochemical Marker of Bone Metastasis in a Lung Cancer Model

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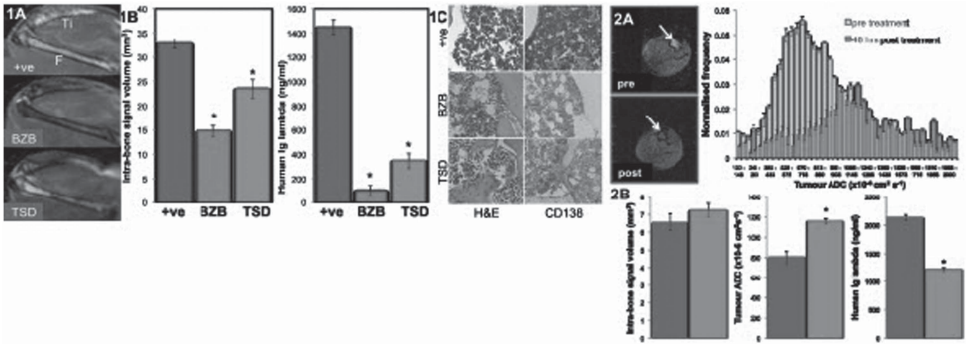
Bone metastasis of non-small cell lung cancer (NSCLC) is an incurable condition that often remains undetected until advanced stages. Patients diagnosed with NSCLC benefit from the use of antiresorptive agents such as bisphosphonates (ZA) that delay the apparition of skeletal related events. The diagnosis is usually performed with bone scintigraphy screening and confirmed by radiography and/or computed tomography or magnetic resonance. During the clinical course, response to treatment that relies on serial radiographs to evaluate bone changes is often confronted by limitations. This is mainly due to the slow detectable changes, and the confounding appearance of lesions containing mixed and/or osteosclerotic areas. Thus non-invasive methods would be useful in the clinical practice for early detection and monitoring treatment response. Emerging evidence suggest that miRNAs (miR) associate with tumor and metastasis progression. Moreover, miR are stabilised and released to the extracellular milieu into microvesicles which can be detected in the circulation. The aim of this study was to identify novel serum markers and investigate their relevance when compared to clinical serum biomarkers during the development of metastases. Tumor burden and osteolytic lesions were evaluated by bioluminescence imaging (BLI), X-Ray and μCT image analysis, after intracardiac inoculation of metastatic A549M1 in athymic nude mice. Animals were treated with ZA or the vehicle. We measured biochemical markers TRAP5b, BGP, PINP and CTX and we performed screening of miR in serum associated with tumor burden and response to ZA treatment. We identified miR-326 that showed robust correlation with BLI (r=0.603, p<0.001) only in control animals. In contrast, PINP was highly associated with BLI in vehicle (r=-0.723 and p<0.001) and ZA-treated mice (r=-0.608 and p<0.001) as well as with tumor burden in



[P91] Figure 1 (**A**) T_2-weighted images showing hyperintensity from (+ve) tumour involvement compared to BZB and TSD treatment in the femur (F) and tibia (Ti). (**B**) Mean tumour volume of signal within leg bones (left), and mean serum Igλ (right), calculated for each treatment group (n=6-9). (**C**) Histological staining from (+ve) control mice showing a high infiltration of CD138+ cells with loss of normal architecture. BZB and TSD treatment restored normal architecture and eliminated CD138+ cells. Figure 2. (**A**) DW images (b=700) prior to and 48 hours post BZB treatment, showing a reduction in tumour signal intensity. The normalized frequency histogram clearly shows an acute, treatment-induced increase in the distribution of ADC values (n=3). (**B**) Mean tumour volume remained constant, mean ADC increased significantly, coincident with a significant decrease in serum Igλ post BZB treatment (all data are mean ± 1 s.e.m., *p<0.05).

both groups as assessed by X-ray imaging. As a consequence, a robust correlation between miR-326 and PINP (r=-0.59 and p<0.001) and BGP (r=0.529, p<0.005) was detected in vehicle treated animals. Moreover, miR193b was the only miR that correlated with BGP, CTX and PINP only in ZA-treated animals. In conclusion, miR-326 and PINP showed strong correlation with tumor burden in untreated animals, whereas monitoring treatment response to ZA could be better achieved with miR193b. Thus, miR326 represents a potential novel biochemical marker for monitoring bone metastatic progression.

P93

Treatment of Metastatic Osteosarcoma by Combinatorial Oncolytic Adenovirus Promotes Autophagic Cell Death

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Osteosarcoma is the most common malignant bone tumor of childhood and adolescence. Despite multimodal management, 70% of patients with metastatic disease at diagnosis will relapse.

Autophagic cell death is a type of programmed cell death that is an alternative to apoptosis. Therefore, it is very likely that cells that are resistant to apoptosis would display permeable autophagic pathways. This study was designed to assess the combination of the oncolytic adenovirus Delta-24-RGD with cisplatin (CDDP) and explore its mechanism of action *in vitro* and *in vivo*. In order to characterize the therapeutic potential of Delta-24-RGD in pediatric osteosarcoma, we first performed *in vitro* studies using 4 different pediatric osteosarcoma cell lines that have been established from patients with metastatic disease. All the cell lines were susceptible to the Delta-24-RGD infection ranging from 60 to 100% of infected cells. Delta-24-RGD showed cytopathic effect and replication capacity in all cell lines. Viability assays showed that CDDP antitumoral activity was synergistically enhanced by combination with Delta-24-RGD, lowering the IC50 in at least two logs of concentration. Treatment with CDDP resulted in G2-M cell cycle arrest that was overcome by the combination with Delta-24-RGD, indicating that addition of the virus sensitizes these cells to the drug antitumor effect. Of importance, combination treatment of Delta-24-RGD with CDDP resulted in autophagic cell death as shown by electron microscopy and several autophagic biochemical markers including LC3 conversion. Using an orthotopic model, an overt decrease in tumor burden was detected by X-ray imaging, PET analysis, and histological examination using combinatorial treatment as compared to each single regimen alone. Similar results were observed in a model of lung osteosarcoma metastases after intravenous injection, using quantitative image analysis in histological sections.

Thus, these findings demonstrate that combination of the oncolytic adenovirus Delta-24-RGD with CDDP results in synergistic cytotoxicity through autophagic cell death. Our data suggests that exploiting autophagic cell death could provide new approaches to manage pulmonary metastatic disease in osteosarcoma.

P94

Everolimus Restrains the *In Vitro* Osteoclastogenic Activity of Brest Cancer Cells

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Background: Generation of osteolytic lesions by skeleton-invading cancers are known to derive from osteoclast (OC) hyperactivity primarily promoted by tumor cells including breast cancer (BC), which secrete osteoclastogenic factors within the metastatic sites. Since the mammalian target of rapamycin (mTOR) pathway is functional in OCs, we explored whether or not the mTOR inhibitor Everolimus is also effective in inhibiting the pro-osteoclastogenic ability of cancer cells in a model of BC-mediated bone resorption.

Methods: MDA-231-MB cells, namely a BC line, were incubated with different amounts of Everolimus and assessed by MTT assay to define its IC20 to be used as sublethal dose in BC cell cultures. Both Everolimus-pretreated and untreated MDA-231-MB cells, as well as their relative culture media, were investigated by real-time PCR and ELISA respectively for the expression of OC-stimulating factors, as RANKL, M-CSF, TNF-α and IL-1β. Then, OC-committed peripheral blood mononuclear cells (PBMCs) were cultivated with conditioned media from either Everolimus-pretreated or untreated MDA-231-MB cells, and mature OCs were counted by light microscopy two weeks later. Parallel cultures of OC-differentiating PBMCs were performed on experimental bone substrates and measured in their capability to produce erosion pits with respect to normal OCs.

Results: Everolimus' IC20 was assessed at 3 ng/ml. MDA-231-MB cells showed no expression of RANK-L, whereas their basal secretion of M-CSF, TNF-α and IL-1β was significantly inhibited by treatment with Everolimus, both at mRNA and protein level. In addition, OCs differentiated in presence of medium from BC cells were shown to exert higher bone resorption than control OCs. However, when these cells were cultivated on dentin slices with surnatants from Everolimus pre-treated BC cells, their bone resorbing capability was significantly suppressed as both number of pits and size of erosive lacunae.

Conclusions: Our findings support the hypothesis that BC cells secrete several osteoclastogenic factors, but RANKL, that are capable of reinforcing both differentiation and function of OCs, whereas their treatment with Everolimus abrogates this capacity. Although new data are needed to better define the osteoclastogenic factors released by BC cells, our results suggest that this mTOR inhibitor could be useful in restraining the progression of skeletal metastases in BC.

P95

3-D Bone-marrow Model to Study Multiple Myeloma-microenvironment Interactions

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Introduction: It has become increasingly evident that hematological malignancies are not driven solely by alterations in hematopoetic cells, but also rely on alterations in, and feedback from, mesenchymal stromal cells within the bone marrow. Our work has aimed at understanding the bidirectional interaction between MM cells and human bone marrow-derived mesenchymal stromal cells (hMSCs) by developing an *in vitro* bone marrow model to study myeloma growth within the bone. Methods: We developed a 3D *in vitro* model using hMSCS, HUVECS, and luciferase-labelled multiple myeloma cell lines. Cells were labeled with cell tracker dyes or expressed fluorescent proteins. These were grown in co-cultures on cylindrical water-based silk fibroin scaffolds (5 mm diameter by 3 mm height) with pores of 500-600 microns in diameter. Confocal imaging was used to spatially visualize cellular interactions and bioluminescence was used to quantify MM1S cell growth in response to bortezomib over a 1 month period. Results: Scaffolds seeded with GFP-LUC-MM1S cells and hMSCS allowed for culture of MM1S in 5 nM bortezomib for up to 1 month and demonstrated stromal-induced protection of MM1S which models minimal residual disease in patients (MRD). 3D culture with stromal showed greater protection from bortezomib than 2D culture with stroma. MM1S was protected from bortezomib-induced death by stroma in both 2D and 3D culture over a short time period (48 hours), but, after a 2 week period, this protection was only found in 3D and in 2D no MM1S remained (with or without stroma). MM1S were found to adhere to stromal cells and HUVECS more often than to silk scaffolds alone. Conclusion: The different responses of MM1S to therapies when in 2-D or 3-dimensional surfaces suggests that a 3D culture environment with bone-marrow stromal cells may represent a more realistic model of drug resistance and minimal residual disease than 2D or monoculture models.

P96

Walker 256/B Malignant Breast Cancer Improves Angio-architecture and Disrupts Hematological Parameters in A Murin Model of Tumor Osteolysis

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This study was designed to assess femur angioarchitecture and hematological effects of Walker 256/B malignant breast cancer cells in a rat model of tumor osteolysis. Tumor osteolysis was induced in femur of 8 rats by *in situ* inoculation of Walker 256/B malignant cells. Six other rats were sham-operated and served as control. 20 days after surgery, rats were euthanized, and femurs were collected than radiographed. Angioarchitecture [mean lumen diameter (MLD), wall thickness (WTh), Vessel number, volume, and separation (VNb, VV, and VSp respectively)] was studied by histomorphometry at 2 different positions (P1: diaphysis, and P2: metaphysis) of the operated femora. Some hematological parameters were also assessed. Walker 256/B induced marked tumor osteolysis, with cortical perforation and trabecular destruction, associated increase in bone vascularization (increases of VNb and VV and decrease of VSp). Angioarchitecture of W256/B rats was disorganized and showed large MLD and lower WTh. These effects were more prominent in P2. When compared to Sham group, significantly decreases at levels of red blood cell (RBC), hemoglobin (Hb), hematocrit (Ht), and white blood cell (WBC) were observed in W256/B rats. These results suggest that Walker 256/B cells – induced tumor osteolysis, improve hypervasculature especially near the tumoral foci (P2) associated hematological disruption. Besides, tumor vessels showed abnormal (enlarged and thinner) and disorganized morphology.

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Mapping the Early Tumour Cell Colonization of the Bone Metastasis Niche in a Prostate Cancer Model

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Rationale and Hypothesis: Skeletal metastasis occurs in ~70% of patients with advanced prostate cancer. It has been suggested that metastasis-initiating cells gain a foothold in bone by homing to a metastasis niche. The nature of the niche is unknown but is likely to contain bone resident cell populations including osteoblasts and osteoclasts. We have modelled the arrival of cells in the skeleton using human tumour xenografts in athymic mice. In the mouse tibia, the distribution of osteoblasts on endocortical bone surface is non-uniform and we hypothesize that studying co-localization of individual tumour cells with resident cell populations will reveal the identity of critical cellular components of the niche. Objectives: To map the process of prostate cancer cell colonising the bone metastatic niche and evaluate their interaction with the niche in a xenograft model. Methodology: The human prostate cancer cell line PC3 (Parental) was transfected in house with a luciferase gene (PC3-NW1) and labelled with a fluorescent cell membrane dye (Vybrant DiD). Male Balb/C nude mice received a single intracardiac injection of labelled cells (10^5/injection) and the presence of growing tumours was monitored by an *in vivo* imaging system (IVIS). Cohorts of animals were culled and the presence of DID positive tumour cells were mapped in the tibiae by multiphoton microscopy. Bone histomorphometry was used to determine the topography of resident cell populations within the tibiae. Findings: With the IVIS imaging, PC3-NW1 cells proved a valuable prostate cancer bone metastasis model, with tumours being present exclusively in bone in ~70% of mice. Seven days following tumour cell injection, individual

PC3-NW1 cells were found engrafted in the tibiae in close proximity (<50 μm) to bone surfaces. Multiphoton analysis showed there were 2 folds more tumour cells associated with the lateral endocortical bone surfaces (15.3 cells/mm bone) than the medial surface (5.6 cells/mm bone). Bone histomorphometry demonstrated significantly more osteoblasts on the lateral compared to the medial endocortical surfaces. Conclusions: The preferential colonisation of prostate cancer cells to osteoblast-rich bone surfaces suggests that the metastatic niche involves cells of the osteoblast lineage. The use of multiphoton microscopy provides the capability to study prostate cancer cell colonization of bone in detail and to further determine specific cellular interactions within the metastatic niche.

P98

Osteoclast-like Transdifferentiation of Malignant Plasma Cells: Role of 1,25(OH)2 Vitamin D(3)

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Introduction: Recent evidences suggest that multiple myeloma plasma cells (MMPCs) may acquire the osteoclast (OC)-like phenotype and directly participate to the bone resorption in myeloma bone disease (MBD). Since OCs derive from monocyte-macrophages, the MMPCs OC-like transdifferentiation implies both morphological and functional overlaps between the lymphoid and myeloid lineages. To this regard, it has been demonstrated that MMPCs acquire morphology of monocyte-like cells by treatment with both 1,25(OH)2VitaminD3 (VitD) and the transcription factor CCAAT/enhancer binding protein α (CEBPα). Thus, here we investigated the effect of VitD on the bone resorbing activity of OC-like MMPCs.
Methods: RPMI8226 and U266 myeloma cells were incubated with VitD and assessed by MTT assay to define its IC20 to be used as sublethal dose in MMPCs cultures. Both VitD-pretreated and control RPMI8226 and U266 were investigated by flow cytometry for CD33 expression and by real-time PCR for CEBPα and PU.1 as markers of myeloid commitment. Furthermore, RPMI8226 and U266 cells were cultivated on calcium phosphate discs and measured in their bone resorption capability by calculating both number and size of the erosion pits.
Results: IC20 was assessed at 10 ng/ml. After VitD stimulation, both RPMI8226 and U266 cells acquired elongated morphology and expressed CD33. Moreover, the stimulation of RPMI8226 and U266 cells with VitD increased the mRNA expression of CEBPα and PU.1, namely the transcription factors involved in the myeloid and osteoclast commitment. In parallel, incubation of MM cells with VitD triggered the transcription of several molecules of OC differentiation including alpha(v)beta(3) integrin, v-ATPase and the macrophage colony-stimulating factor (M-CSF) receptor. Finally, VitD pretreatment of MMPCs cultured on bone substrates in presence of M-CSF and RANKL, resulted in higher resorption activity as compared with control MMPCs.
Conclusions: Our results support previous observation on the ability of VitD to prime the lymphoid-myeloid transdifferentiation of MMPCs postulating their OC-like activity. This effect was revealed in our work by the expression of MCSF-R, alpha(v)beta(3) integrin and v-ATPase whose mRNA up-regulation paralleled the increased bone resorption following the VitD treatment.

P99

The Role of Molecular Chaperones in Metastasis

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Molecular chaperones are essential for the function of multiple 'client' proteins, particularly oncoproteins. Adverse tumour microenvironmental conditions (e.g. hypoxia, glycolytic metabolism), have been linked to chaperone activation, metastasis and drug resistance.
Highly metastatic human MDA-MB-231 and MDA-MB-435 tumour cells were profiled for chaperone expression and response to inhibitors. HSP90, HSP70, GRP78 and GRP94 were abundant, whilst small chaperones (HSP27 and αB crystallin) were variable. Chaperone inhibitors 17-AAG or NVP-AUY922 differentially affected cellular functions. MDA-MB-231 proliferation was relatively resistant to 17-AAG (due to low DT-diaphorase levels) but was sensitive to NVP-AUY922. MDA-MB-231 chemotaxis was inhibited by both compounds, and reduced in hypoxia. Basal HSP27 was minimal, but robustly induced following HSP90 inhibition and in hypoxia.
MDA-MB-435 cell proliferation was sensitive to 17-AAG and NVP-AUY922 although chemotaxis was unaffected; furthermore, hypoxia enhanced chemotaxis. HSP27 was again up-regulated by HSP90 inhibition and hypoxia. αB crystallin was higher in a metastatic subline and further induced by HSP90 inhibition and hypoxia. Interestingly, both HSP27 and αβ crystallin were also induced by 3D spheroid culture and this was again associated with reduced response to chaperone inhibitors. Thus, upregulation of small (ATP-independent) chaperones under certain conditions may reduce the efficacy of HSP90 inhibitors. MDA-MB-231 orthotopic xenografts responded to 17-AAG, but bone metastases did not. The reduced sensitivity of bone metastases (or indeed growth enhancement according to some reports) may be due to the hypoxic nature of the marrow microenvironment.
Stress-inducible chaperones were explored further as potential confounders of response to inhibitors in 3D spheroids and under hypoxia. Stable knockdown of GRP78 or 94 by shRNA had no discernable phenotypic effects *in vitro* or *in vivo*. However, since knockdown induced compensatory reciprocal induction, dual depletion is required. Overall, it is clear that individual chaperones constitute a highly interdependent survival system allowing tumour cells to successfully achieve metastasis and circumvent therapeutic inhibition.

P100

Raman Spectroscopic Study of Radiotherapy-induced Bone Damage

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We show that Raman spectroscopy provides valuable qualitative information on the quality of irradiated bone tissue and can

be an important supplement to X-ray based measurements in management of cancer patients undergoing radiotherapy. Spectroscopy reports on important bone chemical composition parameters, including the crystallinity and carbonate content of bone mineral, the amount of mineral compared to collagen and the state of collagen cross-linking. These parameters have been shown to be correlated with standard biomechanical parameters that are measures of biomechanical competence, such as hardness, plasticity and Young's modules. Importantly, bone composition parameters in irradiated tissue may deviate from their values in healthy tissue even when computed tomography reports normal or near-normal bone mineral content or bone volume fraction. One of the major complications of radiotherapy is osteoradionecrosis, which occurs because of radiation-induced damage to osteogenic cells, resulting in blunting of the remodeling process. In a murine tibia model of osteoradionecrosis we show that composition abnormalities persist for at least twelve weeks after irradiation. In this mouse model, Raman spectroscopy reports both bone mineral and collagen cross-link abnormalities caused by radiation. We hypothesize that pathological cross-links formed by radiation damage to collagen are poorly resorbed during remodeling, so that new tissue is formed on a defective scaffold. Our findings help define the mechanism of action by which incomplete bone regeneration occurs. Equally importantly, this study shows that Raman spectroscopy is capable of elucidating the radiotherapy-induced changes in both bone mineral and matrix. Spectroscopy may allow us to evaluate the effects of new irradiation protocols and radioprotective agents on bone chemical composition. *In vivo* Raman spectroscopy has been demonstrated in a longitudinal study of fracture healing in rats in our laboratory. Measurement validation studies of bone Raman spectroscopy in human subjects are underway. At present, Raman spectroscopy can be used for *in vivo* longitudinal studies of radiotherapy in rodent models and may prove useful in human subjects in the future.

P101

Linking Obesity with Bone Marrow Inflammation and Metastatic Prostate Cancer: Role of Adipocyte- and Macrophage-derived Cathepsin K

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Obesity and inflammation are major contributors to development of aggressive prostate cancer (PCa) with higher recurrence and higher mortality rates. Bone is a primary site of metastasis of PCa and obese and overweight men have a three-fold higher risk of progression to metastatic disease compared to normal-weight men receiving the same treatment. With age and obesity, a shift in bone marrow composition occurs toward formation of fat cells, parallel with increased osteoclast function and resulting bone loss. Growing evidence also suggests, that obesity invokes inflammatory changes in the bone marrow, but impact of those changes on growth and aggressiveness of metastatic prostate tumors is not known. Highly involved in regulation of bone metabolism is a cysteine protease cathepsin K (CTSK), protease predominantly expressed in osteoclasts and macrophages, but also a marker of obesity, whose levels are significantly increased in adipose tissue of obese mice and human subjects. We have shown recently that CTSK is involved in macrophage-regulated inflammatory pathways that drive progression of PCa tumors in the skeleton. Herein, utilizing CTSK knockout and diet induced obesity models we investigated how adipocyte- and macrophage-derived CTSK contributes to bone marrow inflammation and subsequently affects the tumor growth in bone. Our results indicate that mice deficient in CTSK have fewer bone marrow adipocytes and exhibit reduced levels of inflammatory markers (e.g., CCL2, COX2, IL-6) in their bone marrow. PCa tumor growth and progression in the bone are impaired in the absence of CTSK. Importantly, CTSK knockout mice appear to be resistant to diet-induced bone marrow adiposity. In line with these findings, bone marrow stromal cells from CTSK-deficient mice have reduced capability to form mature adipocytes *in vitro*. Diet-induced obesity accelerates progression of intratibial PCa tumors in wild type mice. In addition, interactions of PCa cells with bone marrow adipocytes *in vitro* results in increased growth, invasiveness, and metabolic changes that induce tumor cell survival. Collectively, these results indicate that obesity invokes inflammatory state in the bone marrow that drives metastatic disease, and that macrophage- and adipocyte-derived CTSK is a potential player in this process. Studies to selectively target CTSK in bone marrow macrophages and adipocytes are currently being initiated to unravel the molecular mechanisms behind these findings.

P102

Comparative Effects of Estrogen Depletion by Aromatase Inhibitors or Ovariectomy on Bone Mineral Density in Female Mice

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Estrogen has a protective effect against bone resorption in premenopausal women and estrogen deficiency causes osteoporosis. Aromatase inhibitors (AI) are first line of treatment in patients with estrogen receptor positive breast cancer and deplete estrogen concentrations. AIs accelerate bone loss in breast cancer patients.

Previously, we showed that depleting estrogen by ovariectomy (OVX) in female nude mice decreased BMD at total body, spine, tibia and distal femur sites 28 weeks after surgery. Unexpectedly, the AI letrozole (5 mg/kg/day) for 16 weeks increased BMD at total body and tibia sites in similarly aged mice. Bone marrow cultures (BMC) revealed increased colony forming unit-fibroblast (CFU-F), osteoblast (CFU-OB) and TRAP+ mutinucleated cells (MNC) in ovariectomized mice and in mice treated with letrozole.

Since T cells were previously described to mediate effects of estrogen depletion on osteoclastic bone resorption, we studied the effects of estrogen depletion by OVX or letrozole in immunocompetent mice. Four week old female immunocompetent BALBc mice were randomized to letrozole (5 mg/kg/day) or vehicle for 28 weeks. BMD was measured every 4 weeks. At 32 weeks, letrozole-treated mice had higher BMD

at the distal femur, but lower BMD at the spine compared to vehicle. This was associated with a greater number of CFU-F, and CFU-OB in BMC. In contrast, OVX mice had decreased BMD at the total body, spine and tibia sites, but there was no difference in CFU-F, CFU-OB or TRAP+ MNC compared to sham mice.
To determine if combined effects of OVX+letrozole would have greater effects on BMD, 4 week old female immunocompetent BALBc mice were treated with OVX+letrozole or OVX+vehicle for 28 weeks. At 32 weeks, OVX+letrozole treated mice had lower BMD compared to OVX+vehicle at all sites. OVX+letrozole treated mice exhibited a greater number of CFU-F, CFU-OB and TRAP+ MNC compared to OVX+vehicle. Collectively, our data showed that estrogen depletion by different modalities led to high bone turnover states but different BMD. Mice treated with letrozole alone had higher BMD, while OVX mice had lower BMD, compared to their controls. These effects differed partially based on the age of the mice, rather than immune status. The combination OVX+letrozole decreased BMD greater than either alone. These data indicate that in order to study effects of breast cancer treatments on bone metabolism, OVX+AI are necessary to completely deplete estrogen in mice.

P103

Initial Clinical Experience with Targeted Radiofrequency Ablation of Malignant Spine Lesions Using a Novel Bipolar Navigational Device

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Purpose: Report initial clinical experience in targeted radiofrequency ablation (t-RFA) of malignant lesions of the spine using a novel bipolar RF ablation system, purpose built for minimally invasive procedures in the axial skeleton.
Material/Method: 35 spinal lesions in 31 patients were included. Lesion etiology and location included a variety of metastatic malignant solid tumors and multiple myeloma in T2- S1, sacral ala and ileum. The STAR Tumor Ablation System includes a robust articulating, navigational bipolar electrode containing two active thermocouples (TC) positioned to permit real time monitoring of the peripheral edge of the ablation zone. Treatment is controlled by adjusting power while monitoring TC temperature in-situ. Pre-op planning used CT and thermal distribution curves. Cement augmentation of the compromised vertebra via the same guiding cannula was performed when required. In some cases, post-procedural contrast enhanced magnetic resonance imaging (MRI) was performed.
Results: Procedures were performed safely with no complications or thermal injury. Post-operative imaging confirmed discrete ablation zones were achieved, which were consistent with intra-operative thermal monitoring of the ablative zone by TCs. All patients reported considerable pain relief and significantly reduced disability post treatment.
Conclusion: The STAR Tumor Ablation System, the first purpose-built, bipolar RF device for targeted ablation of spinal malignant lesions, functioned in a safe and effective manner. Malignant lesions, regardless of location were easily accessed. Proximal and distal TCs allowed accurate monitoring of temperature inside the vertebral body to avoid complications of nearby vital structures. High viscosity cement could be delivered after ablation via the same guiding cannula in compromised vertebra.

P104

An Anti-CD115 Monoclonal Antibody Targeting Both Tumor Cells and Myeloid Cells Involved in Cancer Progression: Inhibition of Osteoclasts and M2-polarized Macrophages

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Tumor progression is promoted by tumor-associated macrophages and metastasis-induced bone destruction by osteoclasts, both cell types depending on the CD115-CSF-1 pathway for their differentiation and function. Progression of many epithelial cancers (notably from breast, ovary, cervix and endometrium) is associated with increased expression of CD115 and CSF-1 by tumor cells. We show in a mouse tumor model that treatment with a monoclonal antibody (mAb) blocking host CD115 decreases tumor growth and expression of the F4/80 macrophage marker in tumors. In another model of breast cancer metastasis-induced osteolysis, the anti-mouse CD115 mAb inhibits bone destruction and the serum osteoclast marker TRAP5b. We have generated a monoclonal antibody to human CD115, TG3003, which inhibits the receptor function through a non-ligand competitive mode of action. TG3003 is a humanized IgG1 with antibody-dependent cell cytotoxicity (ADCC) towards CD115-positive tumor cells. *In vitro*, TG3003 partially blocked osteoclast differentiation and osteoclastic bone resorption. When present during monocyte-macrophage differentiation, the anti-CD115 mAb inhibited the M2 polarization of macrophages and their secretion of M2-polarizing factors. The mAb did not show significant cytotoxic activity towards normal human primary CD115-positive monocytes and did not kill CD115-high dendritic cells by ADCC *in vitro*, which should minimize toxicity *in vivo*. Through its multiple intervention points on cancer targets, TG3003 represents a promising candidate for the treatment of solid tumors associated with bone metastases.

P105

Osteoclast Cytomorphometry and Activity Are Mediated by α-tocopherol Acetate in Tumor Osteolytic Rats

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Vitamin E (VE) has shown improved efficacy of chemotherapy as an adjuvant in skeletal metastases (SM). In this study, the effects of vitamin E supplementation (VES) on osteoclast

(OC) resorbing activity and cytomorphometry were investigated in rats with breast cancer SM. Twenty-four aged male rats (2 months old) were randomized into 3 groups: 6 were Sham operated; 9 were injected in the right hind limb with Walker 256/B cells (W256 group); and 9 were injected as above and supplemented with VE (45 mg/kgBW) (W256VE group). Twenty days after surgery, bones were radiographed and scored. Bone mass (BV/TV) and some microarchitectural parameters (Tb.Th, Tb.Sp, Tb.N, OV/BV, OS/BS) were assessed. Some conventional histodynamic parameters of OC (ES/BS, N.Oc/B.Ar, N.Oc/B.Pm, and Oc.S/BS) were measured. Cellular and nuclear form factors (FFC and FFN) were measured for OC populations. The nuclear-cytoplasmic ratio (N/C) was also determined. W256 group exhibited osteolytic lesions in the operated femora. Walker 256/B induced trabecular perforation and decreased BV/TV associated with significant increases in OC numbering (N.Oc/B.Ar and Oc.N/B.Pm) and activity (ES/BS and Oc.S/BS). While FFN remain unchanged, FFC and N/C ratio increased in W256 group. W256VE showed less osteolytic lesions. In fact, VES to cancerous rats had alleviative effects on bone loss with cytoinhibition rate reaching 41%. Moreover, disruption of bone microarchitecture and OC activity in W256VE group decreased. VES reduced the malignant Walker 256/B-induced enhanced OC resorbing activity. The protective effect of VE may be due to modulation of OC cytomorphometry and subsequently their activity.

P106

Implication of ERRα in the Formation of Osteoblastic/Osteolytic Lesions Induced by Human Prostate Cancer Cells

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Up to 80% of patients dying from prostate carcinoma have developed bone metastases (mixed or osteoblastic) that are incurable and responsible for a massive deregulation of normal bone physiology. Estrogen receptor related receptor alpha (ERRα) is an orphan nuclear receptor. It is involved in skeletal development and we have shown in breast cancer its implication as an inhibitor of bone metastases [via the regulation of osteoprotegerin (OPG)]. Because ERRα is also expressed in prostate carcinomas, we investigated here its role in the development of bone metastasis from prostate cancer.

We have selected two prostate cancer cell lines, the PC3 and a subpopulation of the parental PC3 cell line (namely PC3c) that induced pure osteolytic and mixed lesions, respectively. As judged by immunohistochemistry, ERRα is expressed in bone metastases from animals inoculated with PC3 or PC3c cells. PC3 cells overexpressing ERRα (WT-PC3) stimulated tumor growth after subcutaneous injection, which was associated with an increase of VEGF and TGFβ1 expression. On the other hand, intra-tibial injection of WT-PC3 clones stimulated osteolysis, but surprisingly bone destruction was combined with new bone formation, as 70% of the metastatic limbs had mixed lesions compared with control (CT-PC3). Pro-osteolytic factors such as TGFβ1, MCP1, Runx2 and Cathepsin K were found to be up-regulated in WT-PC3 clones compared with CT-PC3 clones. The expression of pro-osteoblastic factors Wnt10b and ET1 was also stimulated, which may explain the occurrence of bone formation in these skeletal lesions. Similarly, the intra-tibial injection of PC3c cells overexpressing ERRα (WT-PC3c, mixed model) led to abnormal bone formation and was associated with the up-regulation of ET1 and Wnt10b. Moreover a slight stimulation of bone nodules number (calvaria culture) was observed when cells were treated with conditioned medium extracted from WT-PC3 and WT-PC3c clones, compared with CT-PC3 and CT-PC3c. In conclusion, we showed that ERRα promotes both osteolysis and osteosclerosis in animal models of prostate cancer bone metastasis.

P107

Role of miR-203 in Skeletal Metastasis of Prostate Cancer Cells

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Prostate cancer cells predominantly metastasize to bone and the complex crosstalk between prostate cancer cells and osteoblasts (bone-forming cells) and osteoclasts (bone-destroying cells) results in an increase in tumour growth and worsening of bone disease. An understanding of the mechanisms by which prostate cancer cells metastasize to bone can both identify the aggressive fraction of prostate cancers earlier in the life cycle of the cancer, resulting in earlier intervention, and reveal new therapeutic approaches for the treatment of skeletal metastases. Short non-coding RNAs called microRNAs play a significant role in the development of various cancers and the microRNA miR-203 has previously been shown to be expressed at lower levels in bone metastatic cell lines and tissue samples. We hypothesized that miR-203 is involved in the crosstalk between prostate cancer and bone cells.Using a panel of prostate cancer cell lines that are non-cancerous, non-metastatic, or predisposed to metastasize to specific tissues including bone, we quantitated expression of miR-203 and potential target genes by real-time PCR. Bone metastatic prostate cancer cells were stably transfected with miR-203, and effects on cellular function and gene expression assessed. A doxycycline-inducible miR-203 expression system was also established in bone metastatic prostate cancer cells. We observed that miR-203 is expressed at a significantly lower level in the bone metastatic prostate cancer cell lines. We also observed that miR-203 expression is inversely correlated to a number of genes regulating osteoblast function, including DKK1, PAR1 and Runx2, suggesting that miR-203 may play a role in osteomimicry. Stable over-expression of miR-203 in the bone-metastatic prostate cancer cell line PC3 resulted in a reduction in proliferation and the acquisition of an epithelial phenotype, associated with greater adherent properties and differential expression of osteoblastic factors and tumour-associated miRNAs. miR-203 expressing cells also

exhibit greater senescence as indicated by senescence-associated β-Galactosidase activity. MiR-203 over-expression by doxycycline induction resulted in a reduced secretion of active MMP-9 by the prostate cancer cells. Our study has identified distinct functional effects of miR-203 that may suppress the development of the osteoblastic metastases associated with prostate cancer.

P108

Differential miRNA Expression in Human Stem/progenitor Cells of Osteotropic Prostate Cancer Is Indicative of Epithelial Plasticity and an Invasive, Mesenchymal Phenotype

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Increasing evidence suggests that a rare aldehyde dehydrogenase-positive (ALDHhigh)/α2high/CD44+ subpopulation of tumor cells has the unique ability to initiate and maintain primary tumor growth and tumor progression. Previously, we reported that the ALDHhigh subpopulation in human prostate cancer cells is enriched for cancer stem cells (CSCs). ALDHhigh cancer cells display, in addition to tumor-initiating ability, strong metastasis-initiating properties in preclinical models of tumor progression and bone metastasis *in vivo* (van den Hoogen *et al.*, *Cancer Research* 2010).
In this study we have investigated and compared the miRNA expression profiles of CSCs (ALDHhigh/α2high/CD44+) vs non-tumorigenic/non-metastatic (ALDHlow) subpopulations in two osteotropic, human prostate cancer cell lines (PC-3M-Pro4, C4-2B). ALDHhigh and ALDHlow subpopulations were isolated by vital cell sorting using the ALDEFLUOR kit.
The ALDHhigh subpopulations in both tested prostate cancer cell lines readily formed colonies as assayed with a single-cell based clonogenic assay and were found to be higly invasive when compared to the ALDHlow subpopulation. Our data indicated that 42.6% of 162 miRs tested in PC-3M-Pro4 cells were downregulated, 9.3% were upregulated and there was no significant difference in 48.1%. Furthermore 76 miRs were differentially expressed between PC-3M-Pro4 and C4-2B cells and 24 showed a similar result. Strikingly, the miR profile of the ALDHhigh cells is supportive of an invasive, mesenchymal phenotype and a number of common predicted target genes have now been identified with TargetScan v.6.1 for further evaluation based on the common most downregulated miRs.
Functional analysis indicated that overexpression of the most downregulated miR lead to a reduction of 30% in the size of the ALDHhigh subpopulation of cells as well as of different prostate cancer stem/progenitor-like cell markers.
We are currently investigating the link between Mesenchymal to Epithelial transition (MET) effectors and the subsequent upregulation of different microRNAs identified in our profile paralleled by a downregulation of different target genes and stem/progenitor-like markers. Our data suggest that the differential miRs expression in the ALDHhigh vs ALDHlow subpopulation of osteotropic prostate cancer cell lines (PC-3M-Pro4 and C4-2B) could play an important role in maintaining the stem/progenitor-like cell phenotype and in differential response to different MET effectors.

P109

Quantitative Assessments of Bone Remodelling 12 Months After Cessation of Zoledronic Acid in Breast Cancer Patients in the Azure Study

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Background: The phase III AZURE trial evaluated adjuvant zoledronic acid (ZA) in 3360 stage II/III breast cancer patients. Patients receive standard adjuvant treatment for breast cancer +/- 19 doses of ZA 4 mg over 5 years. Although this schedule is anticipated to result in an increase in bone mineral density (BMD), and has reduced fracture rates to date, the potential for long term suppression of bone remodelling and adverse effects on bone quality needs assessment. One aim of the 5 year AZURE Bone Health Follow-on study is to evaluate the effects of 5 years ZA on bone remodelling using a newly developed quantitative bone scan (QBS) approach. This is less invasive than bone biopsy and reports on both whole skeleton and regional bone.
Methods: Following completion of treatment on AZURE, 229 patients (123 ZA and 106 control) have undergone bone health evaluation by DXA scan and bone turnover markers. 37 of these (19 ZA and 18 control) are also participating in the QBS sub-study. QBS involves intravenous injection of 99mTc bound to a diphosphate tracer (MDP or HMDP), followed by gamma camera whole body bone scans at multiple times up to 4 hours and six consecutive blood samples. The technique yields values for 99mTc whole skeleton plasma clearance (Kbone) by 2 methods, the modified Brenner and Patlak Plot methods. The latter method additionally yields values for regional skeletal plasma clearance.
Results: At baseline (follow-on study), QBS assessment showed that bone turnover was significantly suppressed in the ZA group compared with the control group, both in the whole skeleton and in specific skeletal regions. Of 37 patients who underwent baseline QBS assessment, 30 returned for a 12 month follow up assessment. Of the original cohort, 3 have since been diagnosed with relapse, 3 did not wish to continue on the study and sufficient blood could not be obtained in 1 patient. The % change from baseline in whole skeleton Kbone and regional Kbone for the remaining patients, 12 months after cessation of zoledronic acid will be reported.
Discussion: Baseline results indicated significant differences in the bone health of breast cancer survivors who have received ZA for 5 years compared to standard treatment alone. Here we report whether that effect remains 12 months after cessation of ZA and to what extent there are regional differences.

P110

Myeloma as a Model for Cell Metastasis

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Multiple myeloma (MM) is a plasma cell dyscrasia characterized by the presence of multiple myelomatous 'omas' throughout the skeleton, indicating that there is continuous trafficking of tumor cells to multiple areas in the bone marrow niches. MM may therefore represent one of the best models to study cell trafficking or cell metastasis. The process of cell metastasis is described as a multistep process, the invasion-metastasis cascade. This involves cell invasion, intravasation into nearby blood vessels, passage into the circulation, followed by homing into predetermined distant tissues, the formation of new foci of micrometastases, and finally the growth of micrometastasis into macroscopic tumors. Here, we describe our *in vivo* model system to examine cell dissemination or cell metastasis to the bone marrow. We describe cell autonomous factors and factors related to the bone marrow niche including the role of mesenchymal stromal cells and endothelial progenitor cells. We use live confocal imaging and *in vivo* flow cytometry to determine time to dissemination to the bone marrow from a localized tumor site (plasmacytoma). Based on this model, we examine many interactions of myeloma with the bone marrow niches including the role of hypoxia in regulating cell dissemination and epithelial-mesenchymal like transition in myeloma. We also show that specific inhibitors can delay tumor dissemination to the bone marrow including novel CXCR7 inhibitors and CXCL12 inhibitors. We also examine mesenchymal stromal cell interaction with myeloma cells through exosome transfer between the cellular compartments. These model systems help us examine mechanisms of cell dissemination that are cell-autonomous or niche-mediated. Despite the significant advances in the treatment of MM, better therapeutic agents that target this metastatic cascade are urgently needed.

P111

The Combination of PTH and Zoledronic Acid Increases Tumor Cell Homing to the Bone Marrow

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Modifying the hematopoietic stem cell niche by PTH affects the number of prostate cancer cells that home to the bone marrow. Furthermore, zoledronic acid diminishes bone metastases formation, at least in part by suppressing the release of growth factor from the bone matrix by resorbing osteoclasts. Since both PTH and zoledronic acid are approved therapies for the treatment of osteoporosis we aimed to determine the effect of the combination on cancer cell homing to the bone marrow. To test this, immune deficient mice were treated with PTH+ zoledronic acid, cancer cells were injected intracardially and the number of human cancer cells (MDA-MB-231) that homed to the bone marrow was determined by quantitative PCR. PTH+zoledronic acid resulted in a significant increase in the number of human cancer cells that were detected in the bone marrow compared to PTH alone, zoledronic acid alone or neither (CT: 5.2±1.3 vs PTH+zoledronic acid: 18.4±2.1 MDA cells/106 murine bone marrow cells). This was associated with the expected changes in bone mineral density and bone histomorphometry. To examine whether the effect was due to an effect on prenylation, pravastatin, an HMGCoA reductase inhibitor, was used. The combination of PTH with pravastatin failed to cause an increase in human cells homing to the bone marrow (n=8/group, p=ns). This suggests that the simultaneous activation of osteoblasts and inhibition of osteoclasts has a homing promoting effect. Injection of CD34+ human stem cells in mice pretreated with the combination, resulted in a significant increase in the number of human stem cells homing to the bone marrow as was the case with cancer cells. Preliminary experiments failed to show a significant effect on the number of hematopoietic stem cells with the combination, presumably due to the relatively short treatment. A multiplex analysis of cytokines affected by the combination therapy revealed a significant increase in MCP-1 (31%, $p<0.05$) and IL-12 (35%). The mRNA expression of both molecules however was diminished by 42% and 33% respectively. This suggests that the combination therapy results in an increase in the amount of homing promoting cytokines that is not due to an increase in their mRNA expression.
In summary, the combination of PTH and zoledronic acid increases homing of cells to the bone marrow by modifying the cytokine environment. This effect could be taken advantage of in stem cell transplantation and bone marrow transplantation.

P112

Spontaneous Bone Metastasis in a Preclinical, Orthotopic Breast Cancer Model of Minimal Residual Disease (MRD): the Effect of TGF-β Signalling Inhibition

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The skeleton is the preferred site for breast cancer metastasis leading to incurable and painful metastatic bone disease. Minimal residual disease (MRD) or micrometastasis is a major clinical problem in breast cancer that occurs without clinicopathological signs after surgical removal or treatment of primary tumours. Invasive lobular carcinoma (ILC) represents a highly metastatic subtype of disease. Transforming growth factor β (TGF-β) appears to have a dual (pro and anti-tumour) role in carcinogenesis and tumour progression. The aim of this study was to develop a mouse model of ILC and MRD disease and to investigate the role of TGF-β signalling in invasion and bone metastasis by blocking it with TGF-β receptor inhibitor 1 (SD208). A mouse model carrying somatic inactivation of E-cadherin and p53 genes was developed using keratin14-driven Cre/loxP system [Derksen *et al.*, *Cancer Cell* 2006] leading to ILC with bone involvement. KEP/luc+ breast cancer cells were orthotopically implanted into mammary fat pads of immunodeficient mice and surgically removed after 4 weeks. Distant relapse was monitored weekly using sensitive bioluminescence

reporter imaging. During *in vivo* study 60 mg/kg/d of SD208 was applied as continuous and curative treatment in animal groups. *In vitro* experiments revealed that ≥1 μM concentrations of SD208 completely blocked TGF-β signalling (CAGA - luciferase reporter) and stimulated the proliferation of KEP/luc+ cells. After surgical removal of the orthotopically-implanted tumour, bone metastasis readily formed within 2-3 weeks, while no detectable visceral metastases were found. Both treatments with SD208 resulted in increased orthotopic and metastatic tumour burden.
In conclusion, we developed an orthotopic mouse model of breast cancer bone metastasis, which closely resembles the clinical situation, thus allowing the monitoring of the multistep metastatic cascade. Importantly, the majority of the distant metastases developed in bones, without lung or other soft tissue involvement (major advance over 4T1 model). Our data show that, in this preclinical model, the anti-tumour effects of TGF-β prevail.

P113

Targeting The Hedgehog Pathway to Inhibit Osteosarcoma Growth Through Dual Effects on Tumor and Microenvironment Cells

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Hedgehog (Hh) signaling is recognized to contribute to the development and progression of many cancers through both cell-intrinsic and paracrine mechanisms. Recent data suggest that Hh-targeted therapeutics exert pluripotent effects on host microenvironment cells and interrupt the 'vicious cycle' of tumors, osteoblasts (OB), osteoclasts (OC), bone marrow stromal cells (BMSC) in bone metastatic breast cancers.
The 5-year survival rate for metastatic osteosarcoma (OS), the most common primary bone cancer, is under 30%, highlighting the need for novel and targeted treatments. We have demonstrate that mice deficient in the tumor suppressor ARF have enhanced rates of bone turnover, mimicking the adolescent growth period in which OS is prevalent. By crossing ARF-/- mice with those expressing Tax, an HTLV-1 oncogene that results in osteolytic tumors, we developed a model of high penetrant, spontaneous OS that recapitulates many aspects of human disease. In this model, suppression of bone turnover with the bisphosphonate zoledronic acid prevented the development of OS, suggesting that enhanced OC activity may stimulate OS growth. In agreement, we found that non-tumorigenic Tax+Arf-/- OB had increased expression of RANKL compared to OPG, while Tax+Arf-/- OS cells further increased their RANKL expression and osteoclastogenic ability.
Compared to normal osteoblasts and mesenchymal stem cell precursors, Tax+Arf-/- OS cells have increased expression of Hh pathway genes and exhibit increased susceptibility to Hh inhibitors (SMO antagonists). In particular, Tax+Arf-/- OS cells express very high levels of the ligand Sonic Hedgehog (SHH). SHH or conditioned media from Tax+Arf-/- OS cells enhanced the OC activity and BMSC cell production of pro-tumorigenic growth factors. Furthermore, the increased OB and OC differentiation and activity present in Tax+Arf-/- cells could be abrogated with SMO inhibitors. We have established an OS cell line from Tax+Arf-/- mice (TAN) with the ability to form mineralized tumors upon intratibial injection. We hypothesize that treatment of TAN OS bearing mice with SMO antagonists will decrease OS growth due to both tumor cytotoxic effects and direct effects on host microenvironment cells that abrogate the pro-tumor microenvironment.

P114

Let-7 Micrornas Regulate Cell Proliferation in Multiple Myeloma

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Backround: MicroRNAs (miRNAs) play a pivotal role in tumorigenesis, due to their ability to target mRNAs involved in the regulation of cell proliferation, survival and differentiation. In particular, the Let-7 miRNA family members have been described to act as tumor suppressors, as demonstrated both in solid cancer and hematologic malignancies. However, the role of Let-7 in multiple myeloma (MM) has not been studied.
Method: Circulating miRNA profiling has been performed in MM patients compared to healthy individuals using TaqMan human miRNA profiling, and validated by qRT-PCR. Exosomes were collected from both normal and MM peripheral blood, using ultracentrifugation; and further studied by using electron microscopy and immunogold labeling for the detection of CD63 and CD81. Exosomes were then evaluated for their miRNA content, by qRT-PCR. Gain- and loss-of functions studies were performed on MM cell lines (MM.1S; U266), using Let-7-mimic and Lin28B siRNA, respectively. Scramble probe-transfected cells were used as control. Effects of Let-7 and Lin28B on signaling cascades have been evaluated by western blot.
Results: We identified a MM specific signature characterized by down-regulation of miRNA-15a, -19b, -21, let-7b, let-7c and over-expression of miR-720 ($P < 0.001$). Moreover, the same miRNA signature was found in the circulating exosomes, suggesting that circulating miRNAs may be transported by exosomes. The Let-7 family members were significantly decreased in peripheral blood of MM patient compared to healthy individuals, suggesting that Let-7 family could be down-regulated in MM cells. We then performed qRT-PCR in MM primary cells; and found that the Let-7 family is significantly down-regulated in MM primary cells, especially for Let-7b and c (5 fold change, $P< 0.05$). Over-expression of Let-7b and Let-7c in MM cells (U266; MM1S) transfected decreased cell proliferation. The RNA binding protein Lin28B is known to regulate the Let-7 family: we therefore performed Lin28-loss of function studies which led to up-regulation of the Let-7 family members, in MM transfected cells. Lin28B-knockdown cells presented with reduced cell proliferation, supported by down-regulation of c-Myc and K-Ras.
Conclusion: This data indicate that miRNA play an important role in the MM biology; providing the basis for the development of new miRNA-based target therapies and biomarker in this disease.

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