XIIth Congress of the
International Society of
Bone Morphometry

October 16-19, 2012

Sheraton Minneapolis West
Minneapolis, Minnesota

www.conference.ifas.ufl.edu/isbm
Welcome to the XIIth Congress of the International Society of Bone Morphometry!

ISBM is a non-profit organization that has its origins in a series of workshops on bone morphometry, the first of which was held in Ottawa in 1973. The core mission of ISBM is to emphasize the importance of traditional morphometric techniques in the field of bone research. ISBM also embraces new technologies that result in refinements and advances in bone morphometric analyses, and facilitates education and training in all aspects of bone morphometry. Many preclinical and clinical studies utilize bone morphometry as an essential technique to assess changes in bone structure and levels of bone formation and resorption in response to a variety of influences such as hormones, cytokines, growth factors, mechanical loading, and bone-active drugs. Bone morphometry is also essential for skeletal phenotyping of knockout and transgenic mice.

The scientific program consists of invited talks by prominent investigators in the areas of osteoporosis treatments, bone imaging, bone cancer, bone fracture healing, bone biomechanics, and bone implants. These talks will be followed in each session by oral presentations of the most highly-scored abstracts. Poster presentations will also be available for viewing throughout the Congress. Ample time will be available for discussions of the findings of the investigators. The Congress will conclude with a practical training session on proper use of bone morphometric techniques, primarily in rodent models.

The social program consists of an opening reception before the keynote address, an evening excursion to the legendary Mall of America for interested attendees, and a dinner cruise on beautiful Lake Minnetonka aboard the Queen of Excelsior. The amiable, intimate environment of the ISBM Congress is conducive to interactions among scientists that promote research collaborations.

This Congress would not be possible without the generosity of our sponsors and exhibitors. Special thanks are in order for Ms. Holly Paszko, the Congress Coordinator, for her outstanding organizational efforts. Thanks very much for attending, and I hope that you find the ISBM Congress to be a rewarding educational experience, and enjoyable as well!

Thomas J. Wronski
Professor and President, ISBM
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Officers and Board Members of the International Society of Bone Morphometry

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Congress Agenda

Tuesday, October 16, 2012

4:00pm-8:00pm  Pre-Conference Registration Open (Calhoun)
4:00pm-7:00pm  Poster Presenter Setup (Minnesota Ballroom)
6:00pm-7:15pm  Opening Reception (Poolside)
7:30pm-8:30pm  Keynote Address: Robert Recker, M.D.
                Bone Morphometry in Evaluation of Osteoporosis Therapies (Minnesota Ballroom)

Wednesday, October 17, 2012

7:30am-5:00pm  Registration Open (Calhoun)
7:30am-10:30am Poster Presenter Setup (Minnesota Ballroom)
7:30am-8:30am  Morning Refreshments / Poster Viewing (Minnesota Ballroom)
8:30am-10:00am Session 1 (Minnesota Ballroom)
                Chairs: Roland Baron and Masaki Noda

8:30am-9:00am  Invited Speaker: Roland Baron, D.D.S., Ph.D.
                Osteoporosis Treatment: Challenges and Mechanisms of Action of Emerging
                Therapies

9:00am-9:15am  Wei Yao: Reversing Bone Loss by Directing Mesenchymal Stem Cells to the Bone

9:15am-9:30am  Chaoyang Li: Sclerostin Antibody Enhanced Modeling-based Bone Formation in
                Both Rats and Cynomolgus Monkeys

9:30am-9:45am  Rachelle Johnson: Conditional Deletion of Gp130 in Osteoblasts and Osteocytes
                has Divergent Effects on Trabecular and Cortical Bone

9:45am-10:00am Stephen Tonna: EphrinB2 is Required for Support of Osteoclast Formation by
                Osteoblasts and Chondrocytes

10:00am-10:30am Mid-Morning Refreshment Break / Poster Viewing (Minnesota Ballroom)
10:30am-12:00pm Session 2 (Minnesota Ballroom)
                Chairs: Michaela Kneissel and David Rowe

10:30am-11:00am  Invited Speaker: David Rowe, M.D.
                Recent Advances in Imaging of Bone and Cartilage

11:00am-11:15am  Seung-Hyun Hong: An Update on Automated 2D Bone Histomorphometry

11:15am-11:30am  Andreas Roschger: Combination of Quantitative Backscattered Electron Imaging
                with Confocal Laser Microscopy: A Powerful Tool for the Evaluation of Bone Matrix
                Mineralization Kinetics

11:30am-11:45am  Donald Kimmel: Quantitative Comparison of Human and Mouse Trabecular Bone
                Ultrastructure by 3D Imaging

11:45am-12:00pm  Philip Salmon: Comparison of High with Low Resolution Micro-CT Morphometry of
                Murine Trabecular Bone to Assess how Segmentation Methods Influence Analysis
                Results

12:00pm-12:15pm  Presentation of ISBM Travel Awards for Young Investigators

12:15pm-1:30pm  Boxed Lunch (Poolside) & Poster Viewing (Minnesota Ballroom)
### Wednesday, October 17, 2012

<table>
<thead>
<tr>
<th>Time</th>
<th>Session/Invited Speaker</th>
<th>Topic</th>
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</thead>
<tbody>
<tr>
<td>1:30pm-2:00pm</td>
<td>Invited Speaker: <strong>Laurie McCauley, D.D.S., Ph.D.</strong></td>
<td>Bone Cancer: Dissecting the Role of Myeloid Lineage Cells in Prostate Cancer Skeletal Metastasis</td>
</tr>
<tr>
<td>2:00pm-2:15pm</td>
<td><strong>Urszula Iwaniec</strong></td>
<td>Low Calcium Intake Increases 4T1 Breast Cell Tumor Growth in Bone and Metastasis to Lung in Mice</td>
</tr>
<tr>
<td>2:15pm-2:30pm</td>
<td><strong>Francesca Salamanna</strong></td>
<td>Electrochemotherapy in Bone Metastases</td>
</tr>
<tr>
<td>2:30pm-2:45pm</td>
<td><strong>Susanta Hui</strong></td>
<td>Assessment of Bone Mineral Kinetics Following Exposure to Therapeutic Radiation</td>
</tr>
<tr>
<td>2:45pm-3:00pm</td>
<td><strong>Russell Turner</strong></td>
<td>Effects of Marrow Transplantation on Bone Architecture and Turnover</td>
</tr>
</tbody>
</table>

**Afternoon Refreshment Break / Poster Viewing** *(Minnesota Ballroom)*

<table>
<thead>
<tr>
<th>Time</th>
<th>Session/Invited Speaker</th>
<th>Topic</th>
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</thead>
<tbody>
<tr>
<td>3:30pm-5:00pm</td>
<td>Invited Speaker: <strong>David Burr, Ph.D.</strong></td>
<td>Morphometric and Biomechanical Assessment of Skeletal Fragility</td>
</tr>
<tr>
<td>4:00pm-4:15pm</td>
<td><strong>Floor Lambers</strong></td>
<td>Three-Dimensional Fluorescence Imaging of Microdamage Events in Cancellous Bone</td>
</tr>
<tr>
<td>4:15pm-4:30pm</td>
<td><strong>Robert Ritchie</strong></td>
<td>Multi-Scale Characterization of the Fracture Resistance of Human Cortical Bone and its Biological Degradation Due to Aging and Disease</td>
</tr>
<tr>
<td>4:30pm-4:45pm</td>
<td><strong>Elizabeth Zimmermann</strong></td>
<td>Multi-scale Study of Deformation and Fracture in Bone at Physiological Strain Rates</td>
</tr>
<tr>
<td>4:45pm-5:00pm</td>
<td><strong>Jean-Paul Roux</strong></td>
<td>Association of Trabecular Bone Score (TBS) with Mechanical Behavior of Human Lumbar Vertebrae</td>
</tr>
</tbody>
</table>

5:00pm Adjourn

5:30pm-6:00pm Shuttle Bus to Mall of America for interested attendees

Participants on their Own for the Evening

### Thursday, October 18, 2012

<table>
<thead>
<tr>
<th>Time</th>
<th>Session/Invited Speaker</th>
<th>Topic</th>
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<tbody>
<tr>
<td>7:30am-5:00pm</td>
<td>Registration Open <em>(Calhoun)</em></td>
<td></td>
</tr>
<tr>
<td>7:30am-8:30am</td>
<td><strong>Morning Refreshments / Poster Viewing</strong> <em>(Minnesota Ballroom)</em></td>
<td></td>
</tr>
<tr>
<td>8:30am-10:00am</td>
<td>Invited Speaker: <strong>David Dempster, Ph.D.</strong></td>
<td>Histomorphometry in Bone Disorders: Animal Models and Clinical Studies</td>
</tr>
<tr>
<td>9:00am-9:15am</td>
<td><strong>Yanfei Linda Ma</strong></td>
<td>Cortical Histomorphometric Analyses of Teriparatide Effects in Postmenopausal Women with or Without Long-term Alendronate Therapy</td>
</tr>
<tr>
<td>9:15am-9:30am</td>
<td><strong>Robert Recker</strong></td>
<td>Cortical Bone Behavior in Post-Menopausal Osteoporotic Women Given Hormone Replacement Therapy and/or Alendronate</td>
</tr>
<tr>
<td>9:30am-9:45am</td>
<td><strong>Maria-Grazia Ascenzi</strong></td>
<td>Differences in Cortical Micro-structure between Hypo- and Hyper- Parathyroidism in Women</td>
</tr>
<tr>
<td>Time</td>
<td>Event</td>
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<tr>
<td>9:45am-10:00am</td>
<td>Tanja Sikjaer: PTH (1-84) Replacement Therapy in Hypoparathyroidism: Effects on Bone Metabolism and Structure</td>
<td></td>
</tr>
<tr>
<td>10:00am-10:30am</td>
<td>Mid-Morning Refreshment Break / Poster Viewing (Minnesota Ballroom)</td>
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<tr>
<td>10:30am-12:00pm</td>
<td>Session 6 (Minnesota Ballroom) Chairs: Chris Hernandez and Regis O’Keefe</td>
<td></td>
</tr>
<tr>
<td>10:30am-11:00am</td>
<td>Invited Speaker: Regis O’Keefe, M.D., Ph.D. Morphometric and Molecular Assessment of Bone Fracture Repair</td>
<td></td>
</tr>
<tr>
<td>11:00am-11:15am</td>
<td>Philip Salmon: Micro-CT Quantification of Changes in Bone and Callus in a Rat Closed Fracture Healing Model</td>
<td></td>
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<tr>
<td>11:15am-11:30am</td>
<td>Max Villa: 2-Photon Microscopy for Live Animal Imaging of Bone Regeneration</td>
<td></td>
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<tr>
<td>11:30am-12:00pm</td>
<td>Session 7 (Minnesota Ballroom) Chairs: Mary Bouxsein and Don Kimmel</td>
<td></td>
</tr>
<tr>
<td>1:30pm-3:00pm</td>
<td>Invited Speaker: Mary Bouxsein, Ph.D. Non-Destructive Assessment of Bone Mass and Quality</td>
<td></td>
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<tr>
<td>2:00pm-2:15pm</td>
<td>Mohammed Akhter: Osteocyte Lacunar Properties in Humans and Rats</td>
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<tr>
<td>2:15pm-2:30pm</td>
<td>Nadja Fratzl-Zelman: OI Type I Quantitative and Qualitative Mutations Studied by Bone Histomorphometry, Quantitative Backscattered Electron Imaging and Synchrotron X-ray Scattering</td>
<td></td>
</tr>
<tr>
<td>2:30pm-2:45pm</td>
<td>Reinhold Erben: Estrogen Deficiency and Cortical Porosity in Female Rats Revisited</td>
<td></td>
</tr>
<tr>
<td>3:00pm-3:30pm</td>
<td>Vanessa Yingling: Effects of Dietary Fat on Bone Histomorphometry</td>
<td></td>
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<tr>
<td>3:30pm-5:00pm</td>
<td>Invited Speaker: D. Rick Sumner, Ph.D. Applications of Morphometry to the Study of Bone Implants and Dental Disorders</td>
<td></td>
</tr>
<tr>
<td>4:00pm-4:15pm</td>
<td>Ignacio Aguirre: High Doses of Zoledronic Acid Induce Osteonecrosis of the Jaw-like Lesions in Rice Rats (Oryzomys Palustris) with Periodontitis</td>
<td></td>
</tr>
<tr>
<td>4:15pm-4:30pm</td>
<td>Rachna Parwani: Comparison of Bone Implant Contact Measurements Obtained using High Resolution Micro-Computed Tomography and Backscatter Scanning Electron Microscopy</td>
<td></td>
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<tr>
<td>4:30pm-4:45pm</td>
<td>Maria Sartori: Long-term in vivo Experimental Investigations on Nanostructured HA-based Bone Substitutes</td>
<td></td>
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<tr>
<td>4:45pm-5:00pm</td>
<td>Francesca Salamanna: Long-term Results Following a Cranial Hydroxyapatite Prosthesis Implantation in a Large Skull Defect Model</td>
<td></td>
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<tr>
<td>5:00pm</td>
<td>Adjourn</td>
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<tr>
<td>5:45pm</td>
<td>Board Buses</td>
<td></td>
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<tr>
<td>6:30pm-9:30pm</td>
<td>Dinner Cruise on the Queen of Excelsior on Lake Minnetonka</td>
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<tr>
<td>9:30pm-10:15pm</td>
<td>Board Buses and Return to the Sheraton Minneapolis West</td>
<td></td>
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**Friday, October 19, 2012**

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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<tbody>
<tr>
<td>7:00am-11:00am</td>
<td>Registration Open <em>(Calhoun)</em></td>
</tr>
<tr>
<td>7:00am-8:00am</td>
<td>Morning Refreshments <em>(Minnesota Ballroom)</em></td>
</tr>
<tr>
<td>8:00am-9:00am</td>
<td>Thomas J. Wronski, Ph.D., and Reinhold Erben, M.D., D.V.M.: Practical Bone Histomorphometry in Rodent Models <em>(Minnesota Ballroom)</em></td>
</tr>
<tr>
<td>9:00am-11:00am</td>
<td><strong>Practical Training:</strong></td>
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<td>- Sectioning bone samples with a microtome</td>
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<td></td>
<td>- Identification of bone cells in histologic sections</td>
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<tr>
<td></td>
<td>- MicroCT imaging of bone samples</td>
</tr>
<tr>
<td>11:00am</td>
<td>Adjourn</td>
</tr>
</tbody>
</table>
Poster Directory

1. **Early Detection of Pathologic Changes in the Mice’s Knee Arthritis Induced by Collagen Injection Using Phase-Contrast X-Ray Microscopy** (Bone Imaging) -- **Han Sung Choi**, Kyung Hee University Hospital, Seoul 130-702 Korea-Republic of (KOR), Phone: 8229588275, Email: hsg3748@hanmail.net

2. **Automated 3D Bone Histomorphometry** (Bone Imaging) -- **Seung-Hyun Hong**, University of Connecticut, Storrs, CT 06269-2155 United States, Phone: 860-486-3654, Email: shhong@engr.uconn.edu

3. **Changes of Trabecular Bone Micro Architecture in Rats with Spinal Cord Injury** (Other Bone/Cartilage Disorders: Mechanisms And Treatments) -- **Akira Minematsu**, Kio University, Nara-ken 635-0832 Japan, Phone: 81745541601, Email: a.minematsu@kio.ac.jp

4. **Risendronate Alone or in Combination with Glucosamine for Osteoarthritis. An Histological and Histomorphometric Study** (Other Bone/Cartilage Disorders: Mechanisms And Treatments) -- **Fernando María Muñoz Guzón**, Universidad de Santiago de Compostela, Lugo 27002 Spain, Phone: 0034982820920, Email: fernandom.munoz@usc.es

5. **Effects of Bisphosphonates on Bone Mass in Growing Rats** (Osteoporosis: Treatments) -- **Kathleen Neuville**, Univ. of Florida, Gainesville, FL 32610 United States, Phone: 352-294-4045, Email: kneuville@ufl.edu

6. **Silk Fibroin Prosthesis Evaluation for Anterior Cruciate Ligament Reconstruction** (Other Bone/Cartilage Disorders: Mechanisms and Treatments) -- **Maria Sartori**, Rizzoli Orthopaedic Institute, Bologna 40136 Italy, Phone: 390516366558, Email: maria.sartori@ior.it

7. **Bone Quality Assessment in Lung Transplant Patients by Using Rib Specimens** (Other Bone/Cartilage Disorders: Mechanisms And Treatments) -- **Louis-Georges Ste-Marie**, CHUM - Universite de Montreal, Montreal H2X 1P1 Canada, Phone: 514-890-8000 ext. 35708, Email: recherche-osteo.chum@sympatico.ca

8. **Bone Histomorphometry Findings in Patients with Chronic Kidney Disease – Preliminary Results** (Other Bone/Cartilage Disorders: Mechanisms and Treatments) -- **Inari Tamminen**, University of Eastern Finland, Kuopio 70211 Finland, Phone: 358294451111, Email: inari.tamminen@uef.fi

9. **Bone Recovery in Ovariectomized Mice Following Lactation** (Osteoporosis: Treatments) -- **Andrea Thompson**, Lexicon Pharmaceuticals, The Woodlands, TX 77381 United States, Phone: 281-863-3367, Email: athompson@lexpharma.com
XIIth Congress of the International Society of Bone Morphometry
Congress Abstracts

Listed alphabetically by presenting author. Presenting author names appear in **bold**.
High Doses of Zoledronic Acid Induce Osteonecrosis of the Jaw-Like Lesions in Rice Rats (Oryzomys Palustris) with Periodontitis

J. I. Aguirre1,2, D. B. Kimmel3, A. M. Leeper1, K. G. Neuville1, M. Jorgensen4, L. Kesavalu5 and T. J. Wronski1

1Department of Physiological Sciences, University of Florida (UF), Gainesville, FL, USA
2Animal Care Services, UF, Gainesville, FL, USA
3Osteoporosis Research Center, Creighton University, Omaha, NE, USA
4Cell & Tissue Analysis Core, McKnight Brain Institute, UF, Gainesville, FL, USA
5Department of Periodontology and Oral Biology, College of Dentistry, UF, Gainesville, FL, USA

This research was supported by the National Institute of Dental and Craniofacial Research (NIH/NIDCR) NIH grant R03DE018924-01A1.

Although osteonecrosis of the jaw (ONJ) is temporally-associated with the use of nitrogen-containing bisphosphonates (N-BP), a cause/effect relationship has not yet been established. We hypothesize that N-BP-associated ONJ is a two-stage process in which: a) risk factors initiate pathologic processes in the oral cavity that lead to a supranormal rate of hard tissue necrosis, and b) powerful anti-resorptives reduce the rate of removal of necrotic bone sufficiently to allow its net accumulation in the jaw. To test this hypothesis, we used the rice rat model of periodontitis. At age 28 days, rats (n=15/group) were placed on a high sucrose and casein diet to exacerbate the development of periodontitis. Animals were injected SC biweekly with vehicle or alendronate (ALN, 15μg/kg), or IV once monthly with vehicle, a low (LD; 8 μg/kg) or a high dose (HD; 80 μg/kg) of zoledronic acid (ZOL) and sacrificed after 6, 12, 18, and 24 wks. The doses of ALN and LD-ZOL are equivalent to osteoporosis doses, whereas HD-ZOL is equivalent to an oncologic dose. Mandibles and maxillae were analyzed to determine the effects on the: a) progression of periodontitis, b) integrity of alveolar bone, c) status of bone resorption and formation, d) vascularity, and e) osteocyte viability. We found that HD-ZOL, but not ALN or LD-ZOL, induced ONJ-like lesions in mandibles of rice rats after 18 and 24 wks of treatment. These lesions were characterized by areas of exposed necrotic alveolar bone, osteolysis, a honey comb-like appearance of the alveolar bone, presence of bacterial colonies, and periodontal tissue destruction. In addition, inhibition of bone formation, a paradoxical abolition of the antiresorptive effect of HD-ZOL, increased osteocyte necrosis/apoptosis, and decreased blood vessel number were found after 18 and/or 24 wks. Our study suggests that HD-ZOL exacerbates the inflammatory response and periodontal tissue damage in rice rats, inducing bone lesions that resemble N-BP-associated ONJ. Therefore, rice rats treated with high doses of ZOL appear to be a promising animal model for studies of the pathogenesis and treatment of ONJ.

Contact Information: Jose Aguirre, University of Florida, 1600 SW Archer Rd, Building 206, Room B2-003 Gainesville, FL 32610, Phone: 352-294-4038, Email: aguirrej@ufl.edu
Osteocyte Lacunar Properties in Humans and Rats

M. P. Akhter¹, D. B. Kimmel¹, R. R. Recker¹ and T. J. Wronski²

¹Osteoporosis Research Center, Creighton University; Omaha, NE, USA
²Department of Physiological Sciences, University of Florida; Gainesville, FL, USA

Estrogen deficiency in humans may increase osteocyte (Ot) apoptosis and reduce Ot lacunar (Ot.Lc) density. The ovariectomized (OVX) rat mimicks bone mass, microarchitectural, and turnover changes in transmenopausal women. Our purpose is to compare Ot.Lc properties and their behavior in the estrogen deficient state in humans and rats. Though synchrotron radiation (SR) microscopy achieves ~0.7µm pixel resolution (PR), resolving whole lacunae in 3D images of intact mouse bone, current laboratory 3D-imaging devices have difficulty. We report Ot.Lc volume (Ot.LcV) and density (Ot.LcD) in humans before and after the onset of estrogen deficiency and in adult OVX rats, using laboratory-acquired 3D images (MicroXCT-200; Xradia, Inc.; Pleasanton, CA) that attains SR-like resolution.

Biopsy specimens were obtained from opposite ilia of a healthy 48yo woman before (PRE) and at one year after (POST) her last menses, then embedded undecalcified in plastic. Thirteen week-old female rats were OVXd or Sham-OVXd (N=4 each) and necropsied 22wks later. L2 vertebrae (LV2) were fixed in 70% ethanol and embedded in plastic. A portion of each iliac specimen was trimmed to 2X2X8mm and scout-scanned at 5µmPR with MicroXCT-200. Similar-sized sagittal samples of each rat LV2 were scout-scanned beginning in the central trabecular region 0.5mm distal to the cranial growth plate.

For humans, twelve trabecular sub-volumes of interest (sub-VOIs) were selected from the scout scan and scanned non-destructively at ~0.97µm PR; ten more sub-VOIs were scanned at ~0.6µm PR. For rats, two sub-VOIs from each LV2 specimen were scanned at ~0.6µm PR. The 3D images from each sub-VOI were analyzed (Avizo 7; VSG; Bordeaux, FR) for Ot.LcD and Ot.LcV. Ot.Lc were defined as discrete bone tissue voids >50 and <500µm³.

Ot.LcD was 34-71% lower, respectively, in estrogen-replete and estrogen-deficient humans than in comparable rats. Ot.LcV was 26-86% lower in rats than in comparable humans (Table). Ot.LcD was 38% lower in OVX rats than in Sham-OVX rats. In humans, Ot.LcV was 9-15% lower in the POST than in the PRE specimen. Ot.LcD was 66% lower in OVX rats as compared to Sham-OVX rats. Ot.LcD was 23% lower in the POST than in the PRE specimen.

These data suggest that Ot.LcV is lower, but Ot.LcD is higher in vertebral body trabecular bone of rats than in iliac trabecular bone of humans. These data also suggest that both lacunar density and volume in trabecular bone decrease with the onset of estrogen deficiency in both rats and humans. Bone tissue with smaller, less numerous lacunae may have reduced ability to intercept propagating microcracks, increasing its intrinsic fragility in a way not detected by current measurements. Our data not only may explain an additional portion of increased bone fragility related to estrogen-deficiency, but also indicate that rats are yet again, an accurate model of post-menopausal bone behavior. MicroXCT-200 is a promising instrument for studying bone features seen only with submicron PR.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Units</th>
<th>PR</th>
<th>Sham-OVX</th>
<th>O VX</th>
<th>PRE</th>
<th>POST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ot.LcD</td>
<td>mm³</td>
<td>0.6µm</td>
<td>44795±15390</td>
<td>15163±7770°</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ot.LcV</td>
<td>µm³</td>
<td>0.6µm</td>
<td>136±38</td>
<td>84±9°</td>
<td>171±121</td>
<td>156±108°</td>
</tr>
<tr>
<td>Ot.LcD</td>
<td>mm³</td>
<td>0.97µm</td>
<td>-</td>
<td>-</td>
<td>13063±1583</td>
<td>10033±2187°</td>
</tr>
<tr>
<td>Ot.LcV</td>
<td>µm³</td>
<td>0.97µm</td>
<td>-</td>
<td>-</td>
<td>183±10</td>
<td>156±14°</td>
</tr>
</tbody>
</table>

Mean±SD; different from Sham-OVX or PRE (° P<.05; a - P<.10); PR- pixel resolution

Contact Information: Mohammed Akhter, Creighton University, 601 North 30th Street #5740, Omaha, NE 68131, Phone: 402-280-5019, Email: akhterm@creighton.edu
Differences in Cortical Micro-structure between Hypo- and Hyper-Parathyroidism in Women

Maria-Grazia Ascenzi, Jaya Gill, Laureen Klimecky, Qian Zhang, Hua Zhou, John Bilezikian and David Dempster

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Purpose: To compare the effect of hypoparathyroidism (HPTH, parathyroid (PTH) level < 10pg/mL) and primary hyperparathyroidism (PHPT, PTH level > 55 pg/mL) on cortical bone micro-structure of women. The study was motivated by the increased heterogeneity of collagen orientation with PTH treatment of osteoporotic patients (Ascenzi et al. J Bone Miner Res 2012; 27:702).

Scope: The cortical micro-structure was assessed on transiliac crest biopsies of 24 pre- and post-menopausal women (Rubin et al. Bone 2010; 46:190; Dempster et al. J Clin Endocr Met 1999; 84:1562), to identify tissue factors of fracture risk, independent from bone mineral density.

Methods: Bone biopsies were embedded, cut, and prepared as described in Dempster et al, J Clin Endocr Met 1999; 84:1562. We employed circularly polarized light microscopy (CPL) to investigate at x160 the Haversian system of one cortex per section in one section per biopsy (Ascenzi et al. J Bone Miner Res 2012; 27:702). CPL birefringence, bright or extinct, signifies the specific orientation range of collagen type I and of carbonated hydroxyapatite (Ascenzi et al. J Struct Biol 2003; 141:22). We measured with Metamorph software cortical width, cortical porosity, average birefringent brightness, area of bright birefringence, number of osteons, osteon area, osteon diameter, and percent of single bright lamellae. The scale of birefringence was set from 0 (black) to 1 (white) with the bright signal set from 0.5 to 1. The microscopist was blinded to the disease state. A t-test was used to test for statistical significance between group means; p < 0.05 was considered significant. The data are presented as mean ± standard error.

Results:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-menopausal</th>
<th>Post-menopausal</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPTH (n=7)</td>
<td>PHPT (n=7)</td>
<td>HPTH (n=5)</td>
</tr>
<tr>
<td>Age</td>
<td>43 ± 1.9</td>
<td>43 ± 2.1</td>
</tr>
<tr>
<td>Cortical porosity (%)</td>
<td>4.04 ± 2.27</td>
<td>14.40 ± 2.69a</td>
</tr>
<tr>
<td>Average birefringent brightness</td>
<td>0.39 ± 0.05</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Area of bright birefringence (%)</td>
<td>24.84 ± 9.39</td>
<td>15.24 ± 3.96</td>
</tr>
<tr>
<td>Single bright lamellae (%)</td>
<td>75.79 ± 1.74</td>
<td>78.77 ± 1.05c</td>
</tr>
</tbody>
</table>

a=0.01; b=0.003; c=0.04 are the p-values of significant differences between PHPT and HPTH groups.

Discussion and Conclusions: The significant micro-structural differences between HPTH and PHPT are specific to either pre- or post-menopausal state. In pre-menopausal women, the higher cortical porosity for PHPT is indicative of higher remodeling rate, which may increase skeletal fragility; and the higher percentage of single bright lamellae in the pre-menopausal group for PHPT indicates that the heterogeneity of collagen orientation is higher in comparison to HPTH. In the post-menopausal women, the larger average birefringence brightness and percent area of bright birefringence in PHPT, which correspond to a larger percentage of collagen oriented at large angles with respect to the longitudinal direction, may be associated with increased fracture risk (Ascenzi et al. J Bone Miner Res 2012; 27:702). We are currently investigating the determinants (e.g. age, disease duration) of the disease-specific micro-structural characteristics.

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Osteoporosis Treatment: Challenges and Mechanisms of Action of Emerging Therapies

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The therapeutic options for the treatment of osteoporosis have so far comprised mostly anti-resorptive drugs, in particular bisphosphonates and more recently RANKL antibodies (denosumab). The efficacy of these drugs has, however, been limited by the fact that they lead to a low turnover state where bone formation decreases with the decrease in bone remodeling activity, closing the anabolic window. Novel anti-resorptives (odanacatib) may have a different profile with effective inhibition of bone resorption associated with minimal reduction in bone formation and bone turnover and, possibly even some cortical stimulation of bone formation. Furthermore alternative osteoporosis drugs, i.e. bone anabolics, have recently reached the market or clinical development. They are based on a different biology and the perspectives they offer for our therapeutic armamentarium are both promising and challenging. The two main osteo-anabolic pathways identified as of today are parathyroid hormone (PTH), the only anabolic drug currently on the market, and activation of canonical Wnt signaling through inhibition of the endogenous inhibitors sclerostin or dickkopf1 (Dkk1). Each approach is based on a different molecular mechanism but most recent evidence suggests that these two pathways may actually converge, at least in part. Whereas recombinant human PTH (rhPTH) treatment is being revisited with different formulations and attempts to regulate endogenous PTH secretion via the calcium sensing receptor (CaSR), antibodies to sclerostin and Dkk1 are currently in clinical trials and may prove to be even more efficient at increasing bone mass, possibly independent of bone turnover. Each of these anabolic approaches has its own limitations and safety issues, but offer good prospects for the anabolic therapy of osteoporosis.

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Wrong Evidence-Based Medicine, Osteoporosis and Breast Cancer

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Background: Researchers looked at women aged 50 to 79 taking a combination of conjugated equine estrogens 0.625 mg and medroxyprogesterone acetate 2.5 mg. This confirmed the long held assumption that HRT prevents osteoporotic fractures. HRT was widely prescribed to women to relieve the menopausal symptoms. Researchers found that women who took the hormones had an increased risk of developing breast cancer.

Aim: Searching for new answers in the link between breast cancer, osteoporosis and targeted breast cancer drugs.

Method: We have performed a bibliography review in a worldwide basis and from our own experience

Results: While the exact cause of breast cancer is not known, the risk of developing it increases with age. Breast cancer risk is strongly related to age, with 81% of cases occurring in women aged 50 years and over. Breast cancer is the most common cancer in women. The lifetime risk of developing breast cancer is 1 in 8 for women. Risk factors associated with the disease could be viruses, environmental factors or others acting on breast cell. Women in developed countries are at increased risk of breast cancer compared with women from less developed countries. A large part of this variation can be explained by the fact that women in developed countries have fewer children on average and a limited duration of breastfeeding, it is said. But in reality reproductive factors that influence breast cancer risk do not explain it. Female breast cancer incidence rates vary considerably, with the highest rates in Europe and the lowest rates in Africa and Asia. Breast cancer is one of the few cancers where incidence rates are higher for more affluent women and there is a clear trend of decreasing rates from least to most deprived groups. Postmenopausal osteoporosis usually affects women over the age of 60. The leading cause of osteoporosis is a lack of estrogens in women and opposite drugs. Osteoporosis, affects 1 in 2 women, is now three times more common that breast cancer. Bisphosphonates may contribute to fewer breast cancers. The cell cycle consists of four phases. DNA and RNA viruses have been shown to be able to cause cancer and referred to as carcinogens.

Conclusions: The natural lack of estrogen does not decrease breast cancer incidence. Targeted breast cancer therapies are to be studied. Environmental factors could play any role but viruses can attack cells in different phases and that could explain the different breast cancer types. And there is the track to go up in this matter.

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Non-Destructive Assessment of Bone Mass and Quality

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Osteoporosis is defined as "a skeletal disorder characterized by compromised bone strength leading to an increased risk of fracture", thereby underscoring the key role of bone strength in understanding fracture risk. Whereas low bone mineral density (BMD) is among the strongest risk factors for fracture, a number of clinical investigations have demonstrated the limitations of BMD measurements in assessing fracture risk and monitoring the response to therapy. These observations have brought renewed attention to the array of factors that influence bone strength, and have motivated development of new approaches to assess these factors. The ability of a bone to resist fracture (or "whole bone strength") depends on the amount of bone (i.e., mass), the spatial distribution of the bone mass (i.e., shape and microarchitecture), and the intrinsic properties of the materials that comprise the bone. This presentation will review current approaches for assessing components of bone strength (e.g., geometry, microarchitecture), as well as whole bone bone macro- and micro-architecture, and prediction of whole bone strength using finite element analysis. Clinical studies showing age- and sex-specific differences in these novel outcomes, as well as their relationship to fracture risk will be discussed

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Morphometric and Biomechanical Assessment of Skeletal Fragility

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“Skeletal fragility” is a poorly defined and abstract concept meant to characterize bone’s ability to resist fracture. Bone can be strong but fragile; therefore we define skeletal fragility as the amount of energy required to fracture the bone. Energy to fracture includes components of strength, stiffness, and post-yield displacement that all contribute to bone’s ability to resist fracture. The measurement of fragility is purely a mechanical one, but is determined by a combination of structural and tissue properties. Adding to the complexity, bone is arranged hierarchically, with morphological components from the organ level down to the molecular interacting with each other in complex ways that ultimately determine its mechanical competence. Therefore, the assessment of why bone may be fragile, that is, the mechanism of fragility, is multifactorial, and must include measurements of bone mass, geometry/architecture, microdamage accumulation, turnover rate, mineralization, and collagen structure and cross-linking. Each of these factors operates within a normal range, and when they become abnormal – whether above the normal range or below it – they can contribute to fragility. These morphological entities do not exist independently, and adaptive compensations among them can offset the fragility created when one or more is out of balance. This dynamic interaction ultimately protects our bone from fracture for as long as possible. But it also leads to the inevitable conclusion that skeletal fragility cannot be assessed by one component alone, eg bone mineral density, and that the development of additional measurements that can be used clinically to assess fragility must be a research priority.

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Early Detection of Pathologic Changes in the Mice’s Knee Arthritis Induced by Collagen Injection Using Phase-Contrast X-Ray Microscopy

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Purpose: Early detection of pathologic changes in the knee arthritis of mice allows us to make early diagnosis in many articular involved diseases and injuries. Therefore, the objective of this study was to find out whether it could be possible to evaluate early pathologic changes of arthritis in mice’s knee.

Scope: The phase-contrast x-ray microtomography (PCXMT) is a new imaging technique with high resolution power. However, PCXMT has some limitations for using in the clinical fields. The scope of this study is to investigate the possibility of clinical use of PCXMT.

Methods: The authors used 15 male DBA1 mice weighing 20-25 g (8 weeks old), and were divided into two groups; 5 mice in the control group without collagen injection, and 10 mice in the study group that was injured by intraarticular collagen injection into the knee joint. The study group was divided into 2 subgroups according to the image taken time. One day after induction of collagen-induced arthritis (CIA) by type II collagen, knee images of the five mice of the study group and 5 mice of the control group were taken using phase-contrast x-ray microtomography (PCXMT) under anesthesia. The rest mice’s knee images of study group were taken using PCXMT on the 6 day after induction of CIA. The images were compared in the shapes and in mean volumes of the haversian canals measured on the basis of the number of bright objects using Image-Pro Plus® program. ANOVA was conducted using SPSS package 13.0 for any statistical work and the significant statistical difference was in any case of p-value<0.05.

Results: In anatomical findings of the microtomogram, there were no significant differences between the groups. The average volume of haversian canals in control group was 76536 pixels but the average volumes of haversian canals in mice of one day after and 6 day after CIA groups were significantly decreased into 69846 and 56748, respectively (p<0.05).

Conclusion: The average volume of haversian canals were decreased in CIA groups. CIA was developed in most of the mice that exhibited this type of change, which in turn suggests that such a change may exhibit possible findings for preceding stages of CIA and other type of articular injuries.

Recommendation: If further studies are conducted on various diagnostic clues, it is with no doubt many breakthrough researches on unknown pathogeneses of diverse arthritis can be successfully accomplished.

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Histomorphometry in Bone Disorders: Animal Models and Clinical Studies

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Histomorphometric analysis of human iliac crest bone biopsies and selected bones from animal models has proved to be invaluable in elucidating the pathogenesis of metabolic bone disorders and the mechanism of action of drugs used to treat them. In one carefully prepared sample, histomorphometrists can collect information on bone mass, structure, cellular activity and material characteristics of the matrix. No other single technique provides as much and as diverse information. In the author's laboratory, the technique has primarily been used to study osteoporosis, primary hyperparathyroidism, hypoparathyroidism and Paget's disease in humans and animal models have complemented the studies on osteoporosis and Paget's disease. Ovariectomized rats and mice proved to be particularly useful models of postmenopausal osteoporosis. The mechanism of bone loss due to estrogen deficiency was shown to be similar to that in women (1) and the changes in cancellous bone structure also faithfully reproduced those seen in human osteoporosis with osteoclast-mediated conversion of plates to rods and disconnection of the rods. This model also proved useful in studying the effects of experimental treatments for osteoporosis, for example, by establishing the ability of parathyroid hormone to improve trabecular connectivity (2,3), an attribute that was later confirmed in humans (4). Antiresorptive agents, such as estrogen and the bisphosphonates also were shown to have similar effects in rat and mouse models to those seen in humans (5). However, some animal models reproduce some, but not all features of human bone disease. For example, we found that the glucocorticoid, prednisolone, inhibited bone formation in rats but did not induce bone loss (6). For a model of Paget's disease, we employed mice with a mutation in the sequestosome 1 gene. This by itself did not cause Paget's-like features to develop in bone, but, when combined with expression of a measles virus protein, Pagetic-like lesions, with large, hypernucleated osteoclasts and woven bone were seen (7,8).

REFERENCES

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A Girl with Fractures and Increased Cortical Porosity

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Cortical porosity increases with advancing age and is believed to be a factor in skeletal fragility in the elderly, but it is not known to contribute to skeletal fragility in children. We studied a child aged 10½ years who had sustained four fractures in the previous 8 years (including a fracture of the femoral neck). She was phenotypically normal, with no signs of osteogenesis imperfecta. Her bone density was normal (lumbar spine z-score -0.7) and there was no biochemical evidence of metabolic bone disease. She had been diagnosed with epilepsy at the age of 3 and treated with valproate until age 7 without any recurrence of seizures. As the cause of her skeletal fragility was not clear we investigated further with a tetracycline-labeled transiliac bone biopsy.

The bone was processed in a standard undecalcified fashion and histomorphometric measurements performed using the Osteomeasure system. Standard nomenclature was used and the results compared to age-related normal subjects [1]. We also undertook micro-computed tomography (micro-CT) analysis of the biopsy specimen to measure 3-dimensional bone parameters and assist with visualisation of the bone structure.

On histomorphometry and micro-CT analysis, the bone biopsy showed marked cortical porosity at 22.4% and 24.2% respectively (normal 1-17%), and decreased trabecular bone volume at 11% and 10.6% (normal 16-32%). On histomorphometry there was no mineralization defect and tetracycline-based dynamic measurements were normal. The cortical osteons appeared active. After sustaining a further fracture at age 11 the patient was treated with intravenous pamidronate infusions over the next two years. She had no further fractures over this period, but histomorphometric and micro-CT analysis of a repeat bone biopsy age 13 showed persistence of marked cortical porosity (18.7% and 18.2%) with some increase in trabecular bone volume (17% and 15.1%).

In this unusual paediatric case, bone fragility was associated with a marked increased cortical porosity, perhaps due to delayed corticalization. It is uncertain whether this has a genetic basis, is developmental, or is an unusual drug association (valproate). The bisphosphonate therapy seemed to have little impact on the cortical porosity. Although the fracture rate has apparently slowed, this is a feature common to most types of childhood osteoporosis.

This case suggests a novel mechanism for skeletal fragility in children. It also reminds us that quantitative bone histomorphometry can identify tissue level abnormalities that cannot be detected by standard imaging and biochemical tests.


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Estrogen Deficiency and Cortical Porosity in Female Rats Revisited

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It is known that estrogen deficiency leads to increased cortical porosity in humans. Earlier studies in ovariectomized (OVX) rats did not provide firm evidence in favor or against estrogen deficiency-induced cortical porosity in rats. However, to our knowledge this question has never been addressed in a systematic fashion, using 3-dimensional analysis techniques. Therefore, it was the aim of this study to reassess the effects of ovariectomy on cortical porosity in the rat, using high resolution μCT technology. We performed two experiments for this study. For experiment I, 3-month-old Fischer 344 rats were sham-operated (SHAM) or OVX. SHAM and OVX groups were killed at 3 and 12 months post-OVX. To assess a possible age-related modulation of OVX-induced cortical porosity, we performed an additional experiment II in 8-month-old rats. For experiment II, SHAM and OVX rats were killed 4 months postsurgery. Cortical porosity in the tibial midshaft was assessed by 3D μ-CT analysis in a stack of 231 slices at 3.5 μm spatial resolution. After μCT scanning, the bones were embedded in methylmethacrylate, and 20 μm-thick microground cross-sections from the identical region were prepared. To optimize thresholding for determination of cortical porosity, the μCT images were compared to the matching histological sections. Ovariectomy induced cortical bone thinning in young and aged rats, relative to SHAM controls. However, neither in young nor in aged OVX rats estrogen deficiency led to increased cortical porosity at the tibial midshaft. Our data show that cortical porosity does not increase in young or aged estrogen deficient OVX rats. Therefore, OVX rats do not mimic the increase in cortical porosity observed in postmenopausal osteoporosis, most likely because rats lack true Haversian intracortical remodeling.

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OI Type I Quantitative and Qualitative Mutations Studied by Bone Histomorphometry, Quantitative Backscattered Electron Imaging and Synchrotron X-Ray Scattering

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Osteogenesis Imperfecta type I (OI-I) is the mildest form of OI, a heritable bone fragility disorder that is usually caused by dominant mutations in one of the two genes that encode collagen type I alpha chains, COL1A1 and COL1A2. OI-I can result from stop or frameshift mutations in COL1A1, leading to haploinsufficiency (OI-I HI) and consequently to formation of a reduced quantity of structurally normal collagen, or from mutations affecting a glycine residue in the triple helical domain in one of the two alpha chains (OI-I TH). The latter mutations lead to the generation of structurally aberrant collagen chains that may be incorporated into the bone matrix. An abnormal high bone matrix mineralization has been observed in all OI cases investigated so far and it is not clear whether different matrix defects cause different mineralization patterns.

We have previously evaluated bone biopsies from children with OI-I (age: 2.0-14y; OI-I HI: n=13, OI-I TH: n=6) and 19 age-matched controls. Neither histomorphometry nor bone matrix mineralization as evaluated by quantitative backscattered electron imaging (qBEI), revealed any statistical difference between the two OI-I groups. Now, we have measured bone crystal size in a subset of this cohort (age: 2-11y) with OI-I HI (n=5), OI-I TH (n=5) and controls (n=6) by using an x-ray scattering setup with a beam diameter of 10µm at the synchrotron BESSY, Berlin. The thickness parameter of mineral particles (T) was obtained from small angle x-ray scattering data and the mineral volume fraction was calculated from the mean calcium content of the bone matrix determined by qBEI. The combination of these two quantities allowed calculating the true particle width (W) of the mineral crystals. Additional information on mineral crystal length was obtained for some cases by wide-angle x-ray-diffraction (WAXD).

The qBEI results showed that the mineral volume fraction was about 13% higher in both OI genotype groups as compared to controls (p=0.0002). W did not vary with genotype, but increased with age (p<0.002), irrespective of genotype. Interestingly, W and the mineral volume fraction increased proportionally with age in controls as well as in both OI-I groups, which indicates that the number of particles per volume remains constant in the process. WAXD measurements showed that particle length in OI was rather smaller than in controls.

Conclusion: Bone from OI-I patients (both HI and TH) have a larger mineral content but the same particle width W. Therefore, the high mineral density in OI is not due to elevated particle size but rather to increased particle density. We suggest that in OI-I, independently of the mutation type, dysfunctional osteoblasts secrete an altered organic matrix that mineralizes by following abnormal mineralization kinetics. The fact that there was no difference between the two mutation types suggests the occurrence of a common bone cell defect downstream of the collagen mutation.

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Automated 3D Bone Histomorphometry

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Although bone histomorphometry has been a standard for measuring the static and dynamic parameters in distinctly specified regions of bone tissue, it is very labor intensive and costly requiring long hours of highly skilled technicians. Measurements are subjective and vary from person to person. When the evaluation is carried out on 2D bone sections, bone tissue needs to be sectioned consistently within the center region of the bone, which often is not possible. Most importantly the evaluation of a 2D bone section cannot be representative of the physiological activities of the whole bone. While the quantitative parameters of bone histomorphometry remain valid, we believe that future bone histomorphometry should be done in 3D.

Specifically, we argue that 3D bone histomorphometry overcomes the following two limitations of the 2D method: 1) Measurements obtained using 2D histomorphometry could be misleading because signals pertaining to z-axis cannot be captured in 2D, for example, eroded surfaces are not generally visible in 2D, and 2) Due to sectioning, incised shape of the labels in the sectioned bone may appear as a plane rather than a thin line near the surface of the bone in 2D image, which then will lead to ambiguous interpretations.

Advanced 3D imaging techniques have been developed and are currently being widely used including confocal microscopy, multi photon microscopy and micro-CT. Micro-CT enables us to image the 3D structure of a bone and microscopy can image 3D fluorescence signals. Although confocal microscopy cannot image bone structure (DIC), 2 photon microscopy (TPM), one of the subclass of multi photon microscopy, with the second harmonic generator (SHG) can be used to acquire a stack of optical fluorescence and bone section images. The stack of images can be used to construct a block of a 3D image and one can develop a 3D image analysis platform by extending the established 2D image analysis method.

For a preliminary study, we investigated whether a 100 µm thick femur with single label can be scanned with TPM and SHG. The surface ratio of a mineralized label fluctuates up to 300% as the depth of the section changes. In the past, we have experienced that selecting the section to be analyzed is important in 2D histomorphometry. We have seen that even static quantification from 2D histomorphometry differs from the 3D static quantification output from the micro-CT. This measurement difference could be due to the difference between 2D and 3D quantification processing. We discover that the 2D BV/TV measurements vary more than 200% as the depth changes in our preliminary study—a clear evidence demonstrating the limitations of 2D analysis. We believe that a 3D histomorphometry will significantly improve accuracy of signal measurements and it will remedy the problem of unidentified label/cell activity that may occur from sectioning bones in 2D histomorphometry.

We are currently developing a rapid, yet comprehensive and cost effective 3D automated way of processing bone histomorphometry. Our effort can also produce a new guideline for carrying out 3D histomorphometric analysis. We also aim at making this guideline ensure comparing analysis outcomes between different laboratories possible.

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As the possibility of personalized medicine based on genomic sequencing becomes a technical reality, the impact of each coding loci on human health needs to be assessed. The mouse knock out phenotyping projects (KOMP) being carried out in Europe and the US is an attempt to make this assessment in mice under highly controlled genetic and phenotyping conditions. Unfortunately a meaningful evaluation of the skeleton is currently not included in the effort because the µCT and histomorphometric tools that could provide this information cannot be applied in a rapid and cost effective manner. At the 11th Congress of this Society, we presented a method for generating dynamic histomorphometry from frozen sections of non-decalcified mouse bone to demonstrate the feasibility of this approach. Since that time we have expanded the workflow with the goal of providing an analysis, which could serve as the platform for KOMP as well as other mouse based studies affecting skeletal health. The key developments that make this approach possible include:

1. Completion of the sample-sectioning steps within 3-5 days after animal sacrifice using a tape based stabilization of formaldehyde fixed, frozen and non-decalcified tissue samples.

2. A non-autofluorescent tape that transmits all the inherent fluorescent signals that are incorporated into the sample to reflect a biologic activity (mineralization lines), fluorescent enzyme activity (alkaline phosphatase and TRAP), or GFP reporter signals.

3. Use of fiduciary beads placed adjacent to the histological section to align the images derived from the multiple microscope scanning steps.

4. Highly automated scanning epifluorescent microscope with image processing algorithms to generate and manipulate almost 500 image files from a single experiment.

5. A computer processing algorithm and heuristic rules have been develop that are implemented on a 24 node cluster computer that can complete the analysis in about 30 min.

6. An image and data management database to allow the user to associate the measurements of an individual sample with the acquisition and computer interpretation images.

The workflow currently generates traditional static and dynamic bone histomorphometric measurements and it utilizes expression of AP and TRAP as surrogates for osteoblasts and osteoclasts. The process takes about two working weeks to perform and requires about 20 hours of technician time to complete. Full experiments have been performed to establish standard values in C57Bl/6, CD1 and SV129 mice in males and females at 8 and 16 weeks of age in the distal femur and vertebra. Comparisons have been made in mice with different transgenic and knock out phenotypes and animals subjected to various physiological effects that alter bone turnover. The absolute values and variance between the control mice across experiments has been very reproducible suggesting that our processing steps have become stable. The protocol performs equally well in rats, although the relative size of the bones is a challenge for managing rat image files, which are far bigger than mouse image files.

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Assessment of Bone Mineral Kinetics following Exposure to Therapeutic Radiation


Objective: There are an increasing number of long-term cancer survivors as a result of successes in treatment modalities. These patients may present accelerated bone loss and increased fracture risk, ultimately impacting long-term morbidities. However, understanding the effects of therapeutic radiation on calcium kinetics and bone remodeling are limited. We present the influence of clinically relevant localized radiation on the murine femur and tibia in order to elucidate the skeletal consequences of this treatment protocol. The 16 Gy dose employed by this study simulates the effects of a therapeutic pelvic radiation dose of 60 Gy (2 Gy/fraction) used to treat endometrial or rectal cancers.

Experimental design: BALB/C mice (12-14 weeks old) were irradiated (IR) with 16 Gy targeting the hind limbs (tibia and femur). Twelve days later, tetracycline (37.5mg/kg s.q.) was administered and calcein (37.5mg/kg) was injected s.q. 17d after this. Mice were euthanized five days later and proximal tibiae were harvested for dynamic histomorphometry. Undemineralized bone sections were processed for UV microscopy. Mineral apposition rate (MAR; um/d) was quantitated by measuring the distance between parallel vital labels and dividing the resultant means by the number of days between administrations. Contralateral tibiae / femurs were fixed and processed for microCT, to estimate cancellous bone volume (BV/TV). Limbs were then processed for H&E staining to determine osteoblast surface (Ob.S.) and osteoclast number (Oc.N). A group of age-matched mice were injected with 45Ca and treated by the same irradiation protocol. Post-irradiation bone turnover was evaluated by 45Ca excretion every 3 days for 30 days.

Results: IR reduced tibial MAR by 15.8% compared with control. Femoral Ob.S exhibited an 83.4% decline, while Oc.N increased by 321%. Femora BV/TV measured by microCT showed a 53% decrease, whereas tibial BV/TV revealed a 25% reduction. 45Ca excretion decreased by 46%, 44%, 38%, 20%, 25%, 6% at days 12, 14, 17, 21, 24, 29 respectively, with a 33% average change during the same 12-29 day period examined with MAR.

Discussion: 16Gy IR increased Oc.N and reduced MAR. Early release of systemic 45Ca (12-18d) was substantially higher compared to the later study period (MAR: 20-29d). While excreted calcium radioisotope represents systemic skeletal kinetics and histomorphometric parameters are limited to specific sites, the direction of change is consistent. Marrow cellularity declines with a corresponding increase in marrow fat (data not shown). Marrow mesenchyme may have differentiated towards adipogenesis at the expense of osteoblastogenesis. Increased osteoclastogenesis may indicate recruitment of circulating osteoclast progenitors responding to apoptotic bone and marrow populations. The time course of changes in marrow and bone surfaces, reduced MAR and increased excretion of Ca2+ may shed light on the dynamics of early changes in these envelopes.

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Low Calcium Intake Increases 4T1 Breast Cell Tumor Growth in Bone and Metastasis to Lung in Mice

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Breast cancer metastasizes to bone in the majority of patients with advanced stages of disease. We tested the hypothesis that increased bone turnover, due to inadequate dietary calcium, 1) accelerates tumor growth once cancer is established within the bone microenvironment and 2) increases metastasis from bone to other organs. Seven-month-old intact female Balb/c mice were placed on an adequate Ca (5 g/kg diet, n=8) or low Ca (80 mg/kg diet, n=10) diet, injected in the tibia with vehicle or 1,000 4T1 breast cancer cells, and sacrificed 21 days later. Tibiae with muscles intact were excised, placed in 70% ethanol, and scanned (Scanco μCT 40) for evaluation of bone. In vivo bioluminescent imaging of 4T1 breast cancer cells in which the luciferase gene has been stably integrated revealed that 4T1 cells metastasize from bone to lung. Therefore, lungs were removed and stained with India ink for quantification of tumor. Extensive osteolysis and equally dramatic osteosclerosis in the form of woven bone extending from the periosteal surface into muscle were observed in both groups injected with 4T1 cells. Severity of lesions was scored blinded from 0 (no osteolysis/osteosclerosis) to 5 (extensive osteolysis/osteosclerosis) using 3-dimensional images of entire tibiae. Mice fed low Ca exhibited significantly (P<0.05) higher tibial lesion scores (3.3 ± 0.4; mean ± SE) than mice fed adequate Ca (1.4 ± 0.2). Furthermore, tumor burden in the lungs of mice fed the low Ca diet was significantly higher (4.2 ± 0.8) than in lungs of mice fed the adequate Ca diet (2.3 ± 0.6). These results show that low calcium diets may exacerbate tumor growth in bone and result in subsequent metastasis to other organs.

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Conditional Deletion of Gp130 in Osteoblasts and Osteocytes has Divergent Effects on Trabecular and Cortical Bone

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Glycoprotein 130 (gp130) is a cytokine co-receptor that transduces intracellular signals in response to IL-6, IL-11, leukemia inhibitory factor (LIF) and oncostatin M (OSM). These cytokines stimulate osteoblastic RANKL expression, osteoclast formation and bone formation, but surprisingly, global deletion of gp130 resulted in enhanced osteoclast formation, while the effect of gp130 deletion on bone formation was complicated by neonatal lethality and systemic defects. To determine the role of gp130 in the osteoblast lineage, gp130 was deleted conditionally from early stages of osteoblast differentiation (Osx1-Cre) to late stage osteoblasts/osteocytes (DMP1-Cre) in C57BL/6 mice. Efficient gp130 recombination was verified by real-time PCR.

MicroCT and histomorphometry on 12-week old male mice revealed a significant reduction in trabecular bone volume (~50%) in tibiae, femora and vertebrae of Osx1 gp130f/f and DMP1 gp130f/f mice compared with appropriate Cre+ wt/wt controls. Both low bone mass phenotypes were accompanied by significant reductions (between 22-51%) in the histomorphometric parameters of trabecular number, mineralizing surface and bone formation rate, indicating that late-stage osteoblast/osteocytic gp130 signaling is essential for normal bone formation. Although gp130-binding cytokines stimulate osteoclast formation, osteoclast number, surface and size were not changed in either KO, suggesting that while osteoblastic gp130 is critical for bone formation, it is not required for normal osteoclast formation. Even in 3-day old Osx1 and DMP1 gp130 KOs, no differences in osteoclast numbers were detected, indicating compensation for the lack of gp130 to support osteoclast formation in the context of normal development and remodeling.

In contrast to reduced trabecular bone, cortical analysis by microCT revealed a significant increase in cortical thickness (4 & 12%), marrow volume (18 & 29%) and periosteal surface area (10 & 17%) in Osx1 and DMP1 gp130 KOs, respectively, with no change in bone length, indicating that osteoblastic gp130 may function in dramatically different ways in trabecular and cortical bone.

In conclusion, while osteoblastic gp130 is not required for osteoclast formation, its deletion may allow for osteoblast and osteocyte compensatory signaling to support osteoclast development. Furthermore, the similarity between Osx1 and DMP1 gp130 KO phenotypes indicates that gp130 signaling in late osteoblasts and osteocytes, but not early osteoblasts, is critical for normal bone formation.

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Quantitative Comparison of Human and Mouse Trabecular Bone Ultrastructure by 3D Imaging

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Osteocyte (Ot) cell processes are believed to facilitate intercellular communication throughout mineralized bone. These cell processes are found in canaliculi (Cn), channels that meander through mineralized bone, allowing processes of neighboring Otcs to contact each other through tight junctions. Cn are very small and connect to Ot lacunae, that are themselves of relatively infrequent distribution in mineralized bone. Studying their properties thus requires combining large-volume inspection capability with high spatial resolution. 3D imaging with quantification of Cn properties has been produced by FIB/SEM (focused ion beam-coupled scanning electron microscopy). However, FIB/SEM is limited in its inspection volume, making it challenging to sample adequately the extensive interlacunar bone volume. The initial purpose of this report is to image Cn in murine trabecular bone in 3D and quantify the morphology of Cn in interlacunar mineralized trabecular bone. This result is achieved by 3D X-ray microscopy (3DXRM), a large-volume, high-resolution, non-destructive imaging technique in murine and human bone, facilitating a quantitative comparison of mouse and human bone 3D ultrastructural properties.

The right distal femur of an intact 12 week old intact female mouse was obtained at necropsy. A transilial bone biopsy sample was taken from the ilium of a 64yo woman with post-menopausal osteoporosis. Both were fixed in 70% ethanol and embedded undecalcified in methyl methacrylate. Specimens measuring ~0.15mmX 0.15mmX0.15mm were prepared from the trabecular bone region of each sample. Each was investigated using an UltraXRM-L200 laboratory X-ray microscope operating with 150nm pixel resolution (Xradia; Pleasanton, CA USA), that provides non-destructive 3D imaging of mineralized bone. Three volumes of interest (sub-VOIs; ~0.09mmX0.09mmX0.09mm) within mineralized bone, were selected from each specimen and visualized in 3D. The reconstructed 3D images containing both Ot lacunae and the surrounding interlacunar matrix bearing Cn (images to be shown in presentation) were subsequently analyzed using Avizo 7 (VSG Group; Bordeaux, FR), quantifying Total Volume (TV), Void Volume (VV), Lacunar Volume (LcV), non-lacunar Volume (nLcV), and Canalicular Volume (CnV).

Individual lacunae were of a similar shape and volume (~160-180µm³) in murine and human bone. CnV/nLcTV in mouse trabecular bone compared well to that in murine cortical bone¹. We conclude that; a) 0.70-3.14% of interlacunar bone appears to be occupied by canaliculi; and b) CnV/nLcTV in may be greater in trabecular bone of the human ilium than in the mouse distal femur. Additional quantitative work and 3D images from these and similar specimens will be shown.


Table 1
Quantitative Ultrastructure of Mouse and Human Bone

<table>
<thead>
<tr>
<th>Feature</th>
<th>Units</th>
<th>Mouse</th>
<th>Human</th>
</tr>
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<tbody>
<tr>
<td>TV</td>
<td>µm³</td>
<td>14280</td>
<td>2083</td>
</tr>
<tr>
<td>VV</td>
<td>µm³</td>
<td>524.0</td>
<td>217.4</td>
</tr>
<tr>
<td>LcV</td>
<td>µm³</td>
<td>359.2</td>
<td>157.0</td>
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<tr>
<td>nLcV</td>
<td>µm³</td>
<td>13920.8</td>
<td>1926.0</td>
</tr>
<tr>
<td>CnV</td>
<td>µm³</td>
<td>164.7</td>
<td>60.4</td>
</tr>
<tr>
<td>CnV/nLcV</td>
<td></td>
<td>0.0118</td>
<td>0.0314</td>
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</tbody>
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Microdamage in cancellous bone is associated with reductions in mechanical performance and may stimulate bone resorption. Traditionally, microdamage visualization in bone is performed through en bloc staining and two-dimensional imaging with optical microscopy. Two-dimensional measurements, however, cannot provide information regarding the number and size of microdamage events. Three-dimensional measures of microdamage can measure the number and size of microdamage events and can also potentially allow for spatial correlations with finite element models to determine the relationship between tissue stress/strain and the formation of microdamage. The aim of the current study is to use 3D imaging to determine how loading mode (apparent tension v. compression) influences microdamage event number and size.

Cancellous bone cores (8 mm diameter, 15 mm length, bone volume fraction 3-12%) from fourth lumbar vertebral bodies of 10 donors are stained for pre-existing microdamage (caused in vivo or during specimen preparation) using xylenol orange, subjected to either tensile (n=10) or compressive (n=9) loading to apparent yield, and then stained with calcein to detect microdamage caused by loading. Bone and microdamage is visualized in three-dimensions using serial milling to achieve an image resolution of 0.7 x 0.7 x 5.0 µm. Serial milling is a fully automated technique that involves repeatedly milling away the top 5 micrometers of a specimen and collecting images of the newly revealed block face. For each specimen the following measures are determined: bone volume (BV), total damage volume (DV), damage volume per damage site, surface area per damage site, and aspect ratio per damage site (damage surface/damage volume).

Specimens loaded in tension show six times more microscopic tissue damage (DV/BV, 4.2 ± 2.3%, mean ± SD) than specimens loaded in compression (0.7 ± 0.3%, p<0.01). The number of damage sites does not differ significantly between groups, but the average volume of each damage site is five times greater in specimens loaded in tension (1.5 × 10^6 µm^3) than in compression (0.28 × 10^6 µm^3, p<0.01). Also, the mean aspect ratio of each damage site is significantly greater in compression (0.6 ± 0.04) than tension (0.4 ± 0.08, p<0.01), suggesting that microdamage formed by compression is more crack-like and microdamage formed by tension more diffuse.

We have demonstrated a tool for examining the size and morphology of individual microdamage events in large (> 4mm) specimens at an in-plane resolution of less than 1 micrometer. Our findings provide the first experimental demonstration that the amount and morphology of microdamage formed in cancellous bone differs considerably between apparent compression and tension. Our findings are consistent with finite element models that have suggested that there is a greater amount of tissue yielding (i.e. damage) when bulk specimens of cancellous bone are loaded in tension as compared to compression.
Sclerostin Antibody Enhanced Modeling-based Bone Formation in Both Rats and Cynomolgus Monkeys

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Bone formation on previously quiescent surfaces occurs through a process known as modeling-based bone formation (MBF), which differs from the resorption-coupled formation that occurs during bone remodeling (remodeling-based bone formation, RBF). The robust anabolic effects of sclerostin antibody (Scl-Ab) on bone formation were previously reported in several animal models and humans. However, its underlying mechanisms at the tissue-level were not fully understood. Therefore, the effects of Scl-Ab on modeling- and remodeling-based bone formation were determined in OVX rats and gonad-intact cynomolgus monkeys.

Eleven-month-old female SD rats at 5 months post ovariectomy received s.c. injection with either vehicle or Scl-Ab 25 mg/kg, 2x/week for 5 weeks. Four to 5-year-old male cynomolgus monkeys (cynos) received s.c. injection with either vehicle or Scl-Ab 30 mg/kg every 2 weeks for 10 weeks. Histomorphometry was performed on lumbar vertebral cancellous bone in both species and femoral cortical bone in the cynos. MBF and RBF were identified according to the cement lines underneath the mineralizing surfaces and were quantified as percent of total bone surface.

Scl-Ab treatment significantly increased cancellous bone volume (BV/TV: +76% in rats, +50% in cynos) and bone formation on cancellous (MS/BS: +323% in rats, +169% in cynos) and endocortical surfaces (+6-fold in cynos) compared with controls. These increases in bone formation were predominantly modeling-based. At the lumbar vertebra, MBF was significantly increased from 7% in the vehicle group to 63% in the Scl-Ab group in OVX rats and from 0.6% in the vehicle group to 34% in the Scl-Ab group in cynos. At the cyo femoral endocortex, there was also a significant increase in MBF from 7% in the vehicle group to 77% in the Scl-Ab group. No significant changes were observed in RBF between the treatment groups in either species, despite the significant reduction in resorption parameters observed with Scl-Ab. In cynos, these changes were consistent with an extension of duration in bone formation on remodeling surfaces, demonstrated by a greater proportion of RBF that was active from week 2 in cancellous (Scl-Ab 52% vs vehicle 10%) and endocortical (Scl-Ab 72% vs vehicle 4%) surfaces.

These results demonstrate that Scl-Ab markedly increased modeling-based bone formation and reduced bone resorption in both rats and cynos. These results differ from the effects of PTH 1-34 in humans, where the increases in bone formation were reported to occur primarily through increased remodeling. This study demonstrates the unique tissue-level mechanism of sclerostin antibody on bone formation and bone resorption at both cancellous and cortical sites.

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Cortical Histomorphometric Analyses of Teriparatide effects in Postmenopausal Women with or Without Long-term Alendronate Therapy

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Teriparatide (TPTD) treatment increases bone turnover and trabecular bone formation in patients previously treated with potent antiresorptives, such as alendronate (ALN, Stepan et al. 2010). We assessed the cortical effects of TPTD (20 µg/day, sc) treatments in postmenopausal women with osteoporosis previously receiving ALN treatment for a mean duration of 63.6 months or treatment naïve (TN). Forty-five paired tetracycline-labeled transiliac crest bone biopsies obtained from patients at baseline and after 24 months TPTD treatment were evaluated for bone formation activity at the periosteal, endosteal and intracortical osteonal bone surfaces using dynamic histomorphometry. At baseline, double label prevalence in endocortical and periosteal surfaces were 8/29 and 1/29 in the ALN group compared to 8/16 and 6/16 in the TN group. The numbers of specimens without any tetracycline label on endocortical and periosteal surfaces were 11/29 and 17/29 in the ALN group compared to 1/16 and 2/16, respectively, in the TN group. Following TPTD treatment, the frequency of double label increased from baseline for both ALN and TN groups at the endocortical and periosteal surfaces. We found that in the ALN pretreated group, MS/BS values were lower than those in the TN group, at both the periosteal (baseline: 0.61±1.29 vs 1.39±0.96; p= 0.04; post-TPTD: 1.34±1.05 vs 3.94±2.7; p<0.0017), and endocortical surfaces (baseline: 3.16±5.05 vs 6.19±5.07; p=0.06; post TPTD: 4.95±4.03 vs 11±9.57; p=0.0265). In the pooled analysis, percent changes in indices mean of periosteal (MS/BS: 164%; BFR/BS: 282%), and endocortical (MS/BS:71%; BFR/BS:107%) dynamic variables, total cortical area (39%), cortical thickness (34%) and porosity area (67%) increased significantly after 24 months TPTD treatment compared to baseline. These results suggest that 24-month teriparatide treatment increases cortical bone formation activities in patients who were either treatment-naïve (TN) or had lower bone turnover initially due to previous alendronate (ALN) therapy. The increased bone-building activities were associated with thicker cortex, increased cortical area and cortical porosity in both patient groups.

Stepan et al. Osteoporosis Int (2010), 21:2027–2036

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Dissecting the Role of Myeloid Lineage Cells in Prostate Cancer Skeletal Metastasis

Fabiana Soki, Payam Entezami, Serk In Park and Laurie McCauley

The skeleton provides an ideal environment for tumor cells to localize, develop micrometastases and subsequently clinically devastating lesions. Prostate cancer has a high propensity to metastasize to bone and although classically described as 'osteoblastic', bone resorption is a prominent early event, mixed lesions are typical, and ultimately increased immature bone predominates. Numerous prostate cancer cell lines exist for use in animal models. Our laboratory has utilized human PC-3, LNCaP, C4-2B, murine RM-1 and canine ACE-1 cells in well characterized murine implant models including cardiac inoculation, intratibial injection as well as a novel vossicle model. Luciferase tagged cell lines provide the ability to track tumor growth over time in the same animal that correlates well with tumor area determined histomorphometrically on tissue specimens at sacrifice. A primary goal of our research group has been to determine the role of myeloid lineage cells in skeletal metastasis. Myeloid-derived suppressor cells (MDSCs) express CD11b and Gr1 markers via flow cytometric analyses. Tumor derived parathyroid hormone related protein (PTHrP) upregulates CCL2 (a.k.a. monocyte chemotactic protein-1) production by osteoblasts and hence increases myeloid cells in the bone microenvironment of the skeletal metastatic lesion. Prostate cancer cell lines with altered levels of PTHrP were used in a subcutaneous implant model where increased MDSCs were subsequently found in the bone marrow suggesting tumor derived PTHrP supports a pre-metastatic niche. Cells with higher PTHrP levels showed greater tumor growth that correlated with MDSCs. Using a gene targeted macrophage ablation mouse model (Mafia mouse) the role of macrophages in tumor support was further validated. Administration of the pharmacologic agent AP20187 resulted in fas-mediated apoptosis of c-fms positive macrophages. Ablation was validated by flow cytometric analysis of GR1loF4/80+CD115loCD11bhi cells (2.5% in control vs. 0.2% in AP-treated). Tissue sections analyzed for F4/80+ cells via immunohistochemistry further validated the reduction in macrophage lineage cells using an administration regime that did not ablate osteoclastic (TRAP+) cells. A significant reduction in tumor growth was noted in tibiae when macrophages were ablated after intratibial tumor inoculation. These studies utilizing strategies of histomorphometry, imaging and flow cytometric analyses provide new insights implicating myeloid lineage cells in the pathogenesis of prostate cancer skeletal metastasis.

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Changes of Trabecular Bone Micro Architecture in Rats with Spinal Cord Injury

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Purpose: Spinal cord injury (SCI) is one of the factors of immobilization and it is reported that bone mass decreases by especially lower extremity. This study was investigated that changes of trabecular bone micro architecture in rats at 1 and 2 weeks after SCI. Scope: We focused the effect of SCI on trabecular bone micro architecture and investigated the time course changes. Methods: Twenty-two young male Wistar rats were used. Twelve rats were cut the lower thoracic nerve and the remaining rats were sham-operated. They were allowed at libitum feeding and drinking during the experiment. At 1 and 2 weeks after the operation, tibias of the rats were dissected out, respectively. The left tibias were scanned by micro-CT and trabecular bone micro architecture in the region of the proximal tibias was analyzed by bone analysis software. Tissue volume (TV), bone volume (BV), bone mass (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular width (Tb.W), connectivity density (Conn.D), and trabecular bone pattern factor (TBPf) of the bones were measured. In statistical analysis, unpaired t-test was used to find differences in micro architecture between SHAM rats and SCI rats at 1 and 2 weeks after the operation, respectively. A significance level of p=0.05 was set. This study was carried out in accordance with the Guide for Animal Experimentation, Kio University and the Committee of Research Facilities of Laboratory Animal Science, Kio University. Results: BV and BV/TV of SCI rats were significantly lower than those of SHAM rats. In SCI rats, Tb.N, Tb.Sp and Conn.D were significant low and TBPf was significant high, respectively, compared with SHAM rats. Tb.W of SCI rats at 2 weeks were significantly lower than that of age-matched SHAM rats. Conclusions: BV/TV was decreased at 1 week after SCI. This reduction was caused by the decrease of BV, not the increase of TV. It is considered that the reduction of Tb.N, Tb.Sp and Conn.D lead the decline of BV after SCI. Moreover, bone mass is decreased for a short term as 1 week after SCI. This study was suggested that SCI affected the trabecular bone micro architecture such as Tb.N, Tb.Sp and Conn.D for a short term. Recommendation: SCI is one of the bone loss factors. It is considered that investigating the change of bone micro architecture is help to understand the process of the decrease of bone mass and prevent from the bone loss.

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Increased Heterogeneity of Cancellous and Cortical Bone Mineralization Densities in Children with Idiopathic Osteoporosis

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Idiopathic osteoporosis (IOP) in children is diagnosed based on the occurrence of fragility fractures and/or low bone mineral density in otherwise healthy individuals with no secondary cause of osteoporosis. We have previously shown that apparently healthy, fracture-prone children have a great variation in bone volume and turnover as assessed by bone histomorphometry. However, little is known about bone mineralization density distribution (BMDD) in children with IOP. The aim of the present work was to measure BMDD in children with IOP using quantitative backscattered electron imaging (qBEI).

Iliac crest biopsies (n=24, 17 boys; age range 6.7-16.6 years) were obtained from children who were suspected of primary osteoporosis based on vertebral (n=14) or non-vertebral fractures (n=10). Both cortical (Ct.) and cancellous (Cn.) bone were analyzed separately using qBEI. BMDD outcomes were compared to reference BMDD data from healthy children (n=54), and the results were correlated with age and serum levels of vitamin D and PINP (marker for bone formation). Further, correlations with histomorphometric measures of trabecular bone structure (BV/TV, Tb.Th) and bone formation (MS/BS) were performed.

In children with IOP, significantly higher heterogeneity of mineralization was found both in trabecular (Cn.CaWidth +23%, p<0.001) and cortical bone (Ct.CaWidth +15%, p<0.001) compared to reference data. Children with IOP had larger percentages of low mineralized Cn. bone area (Cn.CaLow +35%, p<0.001) and of highly mineralized Ct. bone areas (Ct.CaHigh +82%, p=0.048) than the controls. All other BMDD derived variables including the average (Cn. and Ct. CaMean) and mode calcium concentrations (Cn. and Ct. CaPeak) were not different from normal. None of the BMDD variables correlated with age, serum vitamin D level, or structural bone histomorphometry parameters. Ct.CaMean and Ct.CaPeak were negatively (both R=-0.42), and Ct.CaWidth (R=0.47) and Ct.CaLow (R=0.46, p<0.05) positively correlated with MS/BS. In addition, Cn. and Ct. CaWidth (R=0.42 and 0.46, p<0.05) as well as Cn. and Ct. CaLow were all positively correlated with PINP (R=0.48 and 0.44, p<0.05).

Our findings suggest that the heterogeneity in bone matrix mineralization is distinctly increased in children with IOP. The data on bone formation variables suggest that enhanced and transient variations in bone modeling and remodeling are likely responsible for this BMDD finding in our cohort. However, it remains unknown whether this increased heterogeneity in mineralization contributes to the increased bone fragility in these children with IOP or has to be interpreted as compensatory adaptation to otherwise decreased material property.

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Risendronate Alone or in Combination with Glucosamine for Osteoarthritis: An Histological and Histomorphometric Study

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Introduction: Glucosamine sulfate (GS) and risedronate (RS) act directly on the joint structures affected by osteoarthritis (OA), articular cartilage and subchondral bone respectively. Therefore, these drugs administered individually or in combination, might slow the degenerative process that occurs in the joint during OA. The aim of this study was test the effectiveness of the treatment in an experimental rabbit model of OA.

Material and methods: OA was induced by anterior cruciate ligament transection and partial medial meniscectomy on one knee of 32 New Zealand rabbits. All treatments began 3 weeks later, were orally administered and lasted 8 weeks. Animals were divided into 4 groups: treated with glucosamine (GS), risendronate (RS), a combination of them (RS+GS) and placebo (OA). Operated joints formed OA groups and contralateral joints healthy control (CTRL) groups. After sacrifice, two osteochondral cylinders were obtained from the medial femoral condyle. One was decalcified for paraffin inclusion and the other was directly plastic embedded. Decalcified samples were stained with safranine-O fast green and graded following the guidelines of the OARSI recommendations for a local section of affected cartilage in OA, evaluating the severity of cartilage pathology, chondrocyte pathology and proteoglycan pathology. The calcified sections were processed by the Donath technique and using a computer-based image analysis system, were assessed the cartilage thickness (Cg.Th), subchondral bone thickness (SB.Th) and the presence of surface undulations (FI).

Results: Placebo group (OA) showed a significant increase in Cg.Th and a significant decrease in SB.Th respect to CTRL-OA and no differences in cartilage, chondrocyte and proteoglycan pathology. The RS group showed significant differences respect to OA in FI, Cg.Th and SB.Th and these differences disappear when compared to CTRL-RS indicating a tendency to approach the values to normality. RS+GS had statistical differences in SB.Th respect to OA and no differences with CTRL-RS+GS and there were no significant differences between RS and RS+GS. Comparing groups for cartilage, chondrocyte and proteoglycan pathology in paraffin samples there were not differences in any group. Discussion and conclusions: RS and RS+GS treatment were able to partially reverse the structural effects of swelling of the cartilage, recovering Cg.Th, SB.Th an FI values close to those of healthy joint but there are no differences between treat with only risendronate or with the combination of risendronate and glucosamine. In decalcified samples there were no differences between groups; It could be due to that the treatments had the ability of preserve the external structure but not chondrocytes and proteoglycan structure or may be because the calcified samples better preserve the structure of the articular cartilage and subchondral bone than the decalcified ones.

Acknowledgements:
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Effects of Bisphosphonates on Bone Mass in Growing Rats

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Use of bisphosphonates to treat children and adolescents with osteogenesis imperfecta and osteoporosis has been increasing in recent years. Therefore, it is important to determine whether these drugs adversely affect the growing skeleton. The purpose of this study is to compare the effects of the bisphosphonates alendronate (ALN) and zoledronic acid (ZOL) on bone length and the accumulation of cancellous and cortical bone mass in growing rats.

The experimental animals were male rice rats (*oryzomys palustris*) that were 28 days of age at the beginning of treatment. These rats were maintained on a high sucrose/casein diet for another aspect of the study. Some rats were injected SC biweekly with vehicle or ALN (15 µg/kg) whereas other rats were injected IV monthly with vehicle, or a low dose (8 µg/kg) or high dose (80 µg/kg) of ZOL. The dose of ALN and the low dose of ZOL approximate osteoporosis doses, whereas the high dose of ZOL approximates an oncologic dose. All rats were treated for 18 weeks, after which the left femur and tibia were collected for pQCT and histomorphometric analyses, respectively.

Femoral length was not affected by treatment with ALN and both doses of ZOL. In contrast, treatment with these bisphosphonates markedly increased cancellous bone volume in the secondary spongiosa of the proximal tibial metaphysis. The mean value for vehicle-treated rats was 12.9% compared to 46-55% for the ALN- and ZOL-treated groups. pQCT analyses in the distal femoral metaphysis confirmed these histomorphometric findings. The paucity of double fluorochrome labels in the bisphosphonate-treated rats indicate that the large increase in cancellous bone volume and trabecular BMD was due to the expected inhibition of bone resorption rather than increased bone formation. In the tibial and femoral diaphyses, histomorphometric and pQCT analyses indicated that cortical bone area, width, and BMD were not affected by treatment with ALN and both doses of ZOL.

In summary, treatment of rapidly growing rats with the bisphosphonates ALN and ZOL had no effect on diaphyseal cortical bone mass, but induced a marked accumulation of cancellous bone in the long bone metaphysis. There was little difference in the skeletal response to ALN and ZOL in these growing animals. Therefore, long-term bisphosphonate treatment was not found to have major adverse effects on the growing skeleton.

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Morphometric and Molecular Assessment of Bone Fracture Repair

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Bone regeneration is critical for the maintenance of mobility since skeletal injury occurs in most people at some point during their lives. Fracture management is among the leading costs of medical care. While most bone fractures heal, approximately 10-15% have delayed union or non-union. Impaired healing is increased with aging, disease (diabetes, obesity), environmental exposures (smoking), and with soft tissue injury. Ongoing improvement in morphometric and molecular methodologies enable increased understanding of the molecular, cellular and tissue events necessary for efficient bone healing. The primary event in the process of bone repair is stem cell recruitment, proliferation, expansion, and accumulation at the fracture site; endochondral and intramembranous bone formation and subsequent remodeling are dependent on the initial stem cell response. Thus, a more complete understanding of the sources of stem cells, their relative contributions, and the signals regulating their expansion and differentiation are essential for the development of therapeutic approaches. Potential sources of stem cell progenitors include bone marrow stem cells (BMSCs), periosteum-derived stem cells (PDSCs), systemic circulation-derived stem cells (CDSCs), vascular endothelium-derived pericytes (VEDPs), and muscle-derived stem cells (MDSCs). While evidence suggests that stem cells from all of these sources contribute to repair, periosteal tissues appear to be the primary source of cells. We have utilized murine in vivo femoral and tibial bone-periosteum transplantation models to perform lineage tracing, define the role of various molecular signals involved in stem cell proliferation and expansion, and to quantify and characterize bone formation and the mechanical strength of healing. Molecular understanding of periosteal stem cells has also been compromised by the paucity of these cells in mice that limits in vitro study in primary cell culture. We have also used the transplantation model to expand the periosteal stem cell population and enable the isolation and study of primary murine periosteal stem cells. Our work has defined a critical role for aging, obesity, signals in the cyclooxygenase pathway, and other factors in the stem cell response to injury and bone healing. Another critical area is advance in non-invasive imaging methodologies that serve as a sensitive surrogate and are highly predictive of the functional mechanical strength of healing. This is particularly necessary for the development of more efficient human clinical trials. We have developed a novel algorithm to compute the union across the bone repair site that is termed the union ratio. The semi-automated algorithm uses a computer program to define the bone-callus contact on successive transverse CT sections and experiments demonstrate that this measure is highly predictive of torsional strength. We have used this algorithm to assess healing in patients with non-union and defined their response to anabolic therapies. Ongoing advances in imaging and molecular characterization of fracture healing will have a key role in the development of therapeutic approaches to bone repair.

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Comparison of Bone Implant Contact Measurements Obtained Using High Resolution Micro-Computed Tomography and Backscatter Scanning Electron Microscopy

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The purpose of this study was to determine if high resolution micro-computed tomography (µCT) can be used to obtain accurate Bone Implant Contact (BIC) measurements of a Ti implant in a rat model. Scanning metals using µCT induces streak artifacts at the implant periphery which obscures the bone-implant interface and makes it difficult to quantify BIC. We studied the effects of various scan parameters such as the x-ray beam intensity, aluminum filtering, integration time, frame averaging and number of projections/180° on the streak artifact observed. A model of a Ti rod implanted in a rat femur which mimics a metal implant surrounded by soft tissue was imaged using a high resolution µCT scanner (Scanco 50, Wayne, PA, USA). Previous studies show that in order to accurately quantify BIC using µCT, the measurements need to be made within 10µm of the bone-implant interface (Liu et al. Microscopy 2012). By optimizing the scan parameters we could minimize the streaking observed to within approximately 6 µm of the implant interface. These optimized scan parameters were then used to image six 1mm thick PMMA embedded slab sections of rat femurs with cylindrical 1.5mm diameter Ti metal implants. The images were taken at 1.5µm isotropic voxel size, 90kVp, 88µA. The integration time was set at 600msec with 3400 samples and 1600 projections per 180°. X-ray filtering was done with a 0.5mm thick Aluminum filter and data averaging of 3 was used. BIC calculations were made on the images obtained using a line intersect method. The slabs were then ground down to the thickness corresponding to the region which was imaged using µCT, typically to around 0.5mm and further processed for backscatter scanning electron microscopy (bSEM). The images obtained from bSEM (Hitachi S-3000N, 20KV, 10Pa, Variable Pressure) were also analyzed using the line intersect method. The bSEM images were matched with the images obtained from µCT. The BIC for the µCT was computed as the average BIC value of 3 images, 12µm above and below the matched image to account for slight shifts in the thickness scanned using bSEM. Preliminary results show a high correlation coefficient (r =0.8551, p=0.030) for the BIC values obtained using µCT and bSEM. The equation obtained is $BIC_{µCT} = 1.2291*BIC_{bSEM} + 0.1571$ which indicates that µCT consistently overestimates BIC although the results are highly correlated. It is possible that alternative means of measuring BIC may reduce the bias. Inspection of images suggests that while the ability to recognize small gaps at the bone implant interface was not as good in the µCT images as in the bSEM images, use of high resolution µCT scanning vastly improved the ability to image the interface non-destructively compared to previous generation scanners.

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Bone Morphometry in Evaluation of Osteoporosis Therapies

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The FDA requires that the human randomized clinical trials conducted for registration of a new drug for prevention and treatment of osteoporosis include dynamic histomorphometry performed on transiliac bone biopsies in a significant number of the study subjects, both placebo and treated groups. The rationale for this is to assess bone remodeling, to assess bone cell morphology, to document that bone formed during treatment is normal lamellar bone, to rule out bone pathology such as multiple myeloma, paget’s disease, etc. and to obtain clues to the safety and mechanism of action of a study drug. Basically, the biopsies are designed to document normal function of bone cells and formation of normal bone tissue during treatment with a new agent. The clinical studies of fluoride treatment of osteoporosis probably heavily influenced the decision to obtain dynamic histomorphometry of transiliac bone biopsies since the drug demonstrated a significant “anabolic” effect in the absence of a significant antifracture effect. Transiliac biopsies in fluoride treated patients showed that the new bone was woven bone, and exhibited abnormal trabecular microstructure thus explaining why the anabolic effect of treatment did not result in an antifracture effect.

This experience and subsequent refinement in interpretation of the information that can be gained by iliac bone histomorphometry in human and animal studies has led to its broader use in preclinical development and human testing of candidate drugs for treatment and prevention of human osteoporosis and other bone diseases and conditions. For example, histomorphometry was instrumental in helping us understand the mechanism of action of the “anti-resorptive” agents, bisphosphonates (BPs). The original hypothesis in their development (in the 1960s, as recalled by the author) was that they would reduce resorptive work of osteoclasts and leave bone formation unchanged. The result would be an anabolic effect. However, the early clinical studies did not show much of an anabolic effect. Histomorphometry of iliac crest bone biopsies enlightened us by demonstrating that most of the effect of BP treatment could be explained by suppressing remodeling, both resorption and formation. The bone gain was achieved by filling the remodeling space that was present at the time treatment was introduced. Histomorphometry told us how that was possible, i.e., because the formation phase of remodeling at each remodeling site takes considerable more time for completion than the resorption phase. BPs did not “uncouple” formation from resorption from formation, but allowed filling of the already present, but empty, remodeling space. Further, histomorphometry strengthened the knowledge and understanding that osteoporosis and skeletal fragility is principally due to excessive remodeling above that required to maintain skeletal integrity, and fractures could be prevented by slowing the excessive remodeling.

Bone histomorphometry continues to enlighten us. For example, it has taught us how to use animal (rodent) models, has separated modeling effects from remodeling effects of candidate drugs, and has allowed study of the biology of mechanical loading.

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Cortical Bone Behavior in Post-Menopausal Osteoporotic Women given Hormone Replacement Therapy and/or Alendronate

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The ilium may be useful for evaluating cortical bone behavior in living adult humans. Endpoints describing periosteal (P) and endocortical (Ec) bone formation seem most measurable. Iliac cortical thickness varies significantly according to anatomic location. Quantitation of the Haversian portion is difficult because of an inability to orient the cortex perpendicular to the axis of its Haversian canals during sectioning. Adequate surface extent remains a concern. The purpose here is to evaluate the effects of various osteoporosis treatments on P and Ec bone formation.

Transilial biopsy (TIBx) specimens were obtained following dual fluorochrome labeling during a Phase IV trial involving two years treatment with hormone replacement therapy (HRT), alendronate (ALN, 10mg/d), or their combination. Placebo (PBO) and ALN-treated subjects from other Phase IV ALN trials with similar entry criteria were also included here.

Two unstained 8µm sections from separate levels in each TIBx were evaluated. Total P and Ec surface (mm) was measured from both cortices at 40X. Single [SL] and double [DL]-labeled P and Ec labeled surface (mm) was measured at 100X. Interlabel distance at double label sites was measured at 400X. Mineralizing surface (MS/BS [DLS+0.5*SLS]) and surface-based bone formation rate (BFR) were calculated. Endpoints concerning MS/BS, mineral apposition rate (MAR), and BFR were compared by ANOVA followed by Student-Neuman-Keuls post-hoc testing.

All groups displayed osteoporotic spine BMD with appropriate significant treatment effects. ~31±9mm of surface was measured at both the periosteal and endocortical surface for each TIBx. 25% of all TIBx had P DL; 59% had Ec DL. 45% of all TIBx had no P label; 30% had no Ec label. EMSBS approximated values generally seen for trabecular MS/BS in TIBx from PBO patients. PMSBS was ~five-fold lower than EMSBS in PBO patients.

PMSBS and PBFR were significantly lower in all treatment groups than PBO, and were significantly lower in the HRT+ALN group than in the two monotherapy groups. EMSBS and EBFR were also significantly lower in all treatment groups than PBO, significantly lower in the HRT+ALN group than in the two monotherapy groups, and also lower with ALN than with HRT monotherapy. MAR was not affected significantly by any treatment.

Our data suggest that both HRT and ALN suppress bone formation at both the P and Ec surfaces. ALN monotherapy is more efficacious on these endpoints than HRT monotherapy.

<table>
<thead>
<tr>
<th>Vbl</th>
<th>Units</th>
<th>PBO</th>
<th>ALN 10mg/d</th>
<th>HRT</th>
<th>HRT+ALN</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td></td>
<td>141</td>
<td>58</td>
<td>30</td>
<td>34</td>
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<tr>
<td>Age yrs</td>
<td>yrs</td>
<td>66.6±7.7</td>
<td>66.0±6.8</td>
<td>61.6±7.4</td>
<td>63.6±7.2</td>
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<tr>
<td>BMI</td>
<td>kg/m²</td>
<td>24.7±3.9</td>
<td>25.3±4.0</td>
<td>25.4±4.5</td>
<td>26.4±3.4</td>
</tr>
<tr>
<td>LSBMD g/cm²</td>
<td>0.74±0.09 (90)</td>
<td>0.78±0.10 (57)</td>
<td>0.80±0.08 (30)</td>
<td>0.84±0.08 (34) pah</td>
<td></td>
</tr>
<tr>
<td>PMSBS %</td>
<td>1.49±2.27 (54)</td>
<td>0.43±0.60 (23)</td>
<td>0.63±1.57 (30)</td>
<td>0.25±0.50 (34) p</td>
<td></td>
</tr>
<tr>
<td>EMSBS %</td>
<td>7.48±5.94 (54)</td>
<td>1.24±1.79 (23)</td>
<td>3.39±4.20 (30) pa</td>
<td>0.21±0.68 (34) pha</td>
<td></td>
</tr>
<tr>
<td>PMAR µm/d</td>
<td>0.73±0.28 (20)</td>
<td>0.70±0.29 (7)</td>
<td>0.72±0.37 (5)</td>
<td>0.59±0.36 (3)</td>
<td></td>
</tr>
<tr>
<td>EMAR µm/d</td>
<td>0.60±0.16 (49)</td>
<td>0.54±0.21 (10)</td>
<td>0.58±0.19 (20)</td>
<td>0.47±0.21 (4)</td>
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</tr>
<tr>
<td>PBFR x</td>
<td>0.014±0.026 (34)</td>
<td>0.003±0.005 (15)</td>
<td>0.006±0.017 (24)</td>
<td>0.000±0.001 (24) pa</td>
<td></td>
</tr>
<tr>
<td>EBFR x</td>
<td>0.049±0.041 (51)</td>
<td>0.009±0.013 (18)</td>
<td>0.022±0.027 (26)</td>
<td>0.001±0.005 (31) pha</td>
<td></td>
</tr>
</tbody>
</table>

Mean±SD (N); vs. PBO (p<.05); vs. ALN (p<.05); vs. HRT (p<.05) ; x-mm²/mm/d

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Multi-Scale Characterization of the Fracture Resistance of Human Cortical Bone and its Biological Degradation Due to Aging and Disease

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The structure of human cortical bone evolves over multiple length-scales from its basic constituents of collagen and hydroxyapatite at the nanoscale to osteonal structures at near-millimeter dimensions, which all provide the basis for its mechanical properties. To resist fracture, bone’s toughness is derived intrinsically through plasticity (e.g., fibrillar sliding) at structural-scales typically below a micron and extrinsically (i.e., during crack growth) through mechanisms (e.g., crack deflection/bridging) generated at larger structural-scales. Biological factors such as aging, irradiation and disease can lead to a markedly increased fracture risk, which is often associated with a loss in bone mass (bone quantity). However, these factors can also significantly degrade the fracture resistance (bone quality) over multiple length-scales.

Using a suite of materials science characterization techniques, including in situ small-angle x-ray scattering/wide-angle x-ray diffraction (SAXS/WAXD) to characterize sub-micron to nanoscale structural changes and synchrotron x-ray computed tomography and in situ fracture-toughness measurements in the scanning electron microscope to characterize effects at micron- to macro-scales, we show how age-related structural changes at differing size-scales degrade both the intrinsic and extrinsic toughness of bone. Specifically, we attribute the loss in toughness to increased non-enzymatic collagen cross-linking which suppresses plasticity by fibrillar sliding at nanoscale dimensions and to an increased osteonal density which limits the potency of crack-bridging mechanisms at micron-scales. The link between these processes is that the increased stiffness of the cross-linked collagen requires energy to be absorbed by “plastic” deformation at higher structural levels, which occurs by the process of microcracking. Analogous mechanisms for the embrittlement of bone due to x-ray irradiation are also presented, together with a brief discussion of the effect of bone diseases on bone strength and toughness, such as osteogenesis imperfecta and vitamin-D deficiency.

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Combination of Quantitative Backscattered Electron Imaging with Confocal Laser Microscopy: A Powerful Tool for the Evaluation of Bone Matrix Mineralization Kinetics

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Quantitative backscattered electron imaging (qBEI) is an established method to determine the local distribution of calcium (Ca, weight %) within mineralized tissues such as bone, with a resolution of up to 0.5µm. We combine here for the first time qBEI with confocal laser scanning microscopy (CLSM) to evaluate bone mineralization kinetics in animals by measuring Ca content at different tissue ages.

In specific we analyzed longitudinal (n=9) and transversal (n=9) sections of the femur diaphysis of 16 weeks old mice lacking the bone formation inhibitor sclerostin (Sost-KO mice), and corresponding sections of wildtype mice (n=11 and n=10, respectively). The longitudinal sections were analyzed by qBEI for bone mineralization density distribution (BMDD) of the cortex within the midshaft region. The transversal sections were obtained from animals, which had received alizarin and calcein fluorescent double labeling. Double labeling had been performed with 10 days interval at 8 weeks of age and at 16 weeks of age prior to sacrifice. The undecalcified block-samples were processed for qBEI measurements as described previously. Prior to carbon coating for qBEI imaging the polished sample surface was imaged by reflection and fluorescent CLSM (objective lens 20x, pixel resolution 0.8 µm). By merging the two image types (alizarin and calcein staining) a composite CLSM image was generated, which was superposed to the qBEI image of the identical bone area. Using ImageJ software, the areas between the fluorescent labels were then selected as ROIs and transferred to the qBEI image to determine the mean Ca content between the labels.

Fluorescent labels were observed in the endocortical and subperiosteal envelope. In the endocortical envelope Sost-KO mice had a significantly lower degree of mineralization at the 8 week and 16 week time points (-1.9 %, p<0.001 and -1.5 %, p<0.05, respectively) compared to wildtypes. This was consistent with the BMDD data from the cortical bone in the longitudinal sections with a mean Ca content reduced by 1.9 % (p<0.05). Interestingly, at the subperiosteal bone envelope no such differences were detectable.

Thus, the combined qBEI / CLSM method revealed in sclerostin deficient mice a reduced Ca content in the endocortical envelope at well-defined tissue ages. This suggests altered mineralization kinetics. The fact that mineralization kinetics was not altered in the subperiosteal envelope indicates diverging mineralization dynamics at the different cortical sites. This observation may relate to recent in vitro findings implicating sclerostin in physiologic bone mineralization. In conclusion this example demonstrates that this combination of qBEI with CLSM is a powerful tool to detect alterations in intrinsic mineralization kinetics of the bone matrix.

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Association of Trabecular Bone Score (TBS) with Mechanical Behavior of Human Lumbar Vertebrae

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These authors contributed equally to this work.

# co-owner of the TBS patent and has corresponding ownership shares in medimaps group.

The measurement of areal bone mineral density (aBMD) does not predict at least half of fragility fractures, but assessment of bone microarchitecture may improve this prediction. The trabecular bone score (TBS) is a grey-level measure of texture using a modified version of experimental variogram and can be extracted from DXA images (Pothuaud L. et al., Bone 42, 2008: 775-787). The aim of the current study was to assess whether the TBS is associated with the mechanical behavior of human lumbar vertebrae. Lumbar vertebrae (L3) were harvested fresh from 16 human donors (7 men, 9 women, age: 82 ± 8 yrs for men and 72 ± 11 yrs for women). The antero-posterior and lateral BMC (g) and aBMD (g/cm²) of the vertebral body were measured using DXA (Delphi W, Hologic) and then the TBS was extracted using TBS iNsight software (Medimaps SA, France). The trabecular bone volume (Tb.BV/TV), trabecular thickness (Tb.Th), degree of anisotropy (DA), and structure model index (SMI) were measured using µCT with a 35-µm isotropic voxel size (Skyscan1076). Quasi-static uniaxial compressive testing was performed on L3 vertebral bodies under displacement control (0.5mm/min) to assess failure load (FL, N) and stiffness (STF, N/mm).

The TBS was significantly correlated to Tb.BV/TV, SMI and stiffness (r=0.58, 0.62 and 0.64; p<0.02 for all), borderline not significant with FL but not with BMC or BMD. In bivariate regressions, STF was associated with TBS (r=0.64), lateral BMD (r=0.53) and apBMC (r=0.49) (all p<0.05). FL was associated with SMI (r=-0.56, p=0.03) and lateral BMD (r=0.49, p=0.05) and TBS (r=0.46, p=0.07). Using stepwise regressions, the combination of TBS (first step, p=0.003), Tb.Th (second step, p=0.002) and apBMC (third step, p=0.008) was strongly associated with STF (multiple R=0.89, p<0.001). There was no other significant predictor of bone stiffness.

In conclusion, the TBS was significantly correlated to the most relevant microarchitectural parameters associated with vertebral biomechanical properties (i.e. Tb.BV/TV and SMI). In addition, the combination of TBS, Tb.Th and BMC explained up to 79% of the variability of the stiffness. These initial results suggest that TBS might improve assessment of vertebral strength in combination with standard DXA measurements.

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Recent Advances in Imaging of Bone and Cartilage

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2Computer Science & Engineering Department, University of Connecticut, Storrs, CT, USA

Our objective is to develop a rapidly performed, high content and relatively low cost histology for assessing the cellular basis of skeletal repair whether driven exclusively by the animal itself or when assisted with transplanted donor progenitor cells. The platform employs multiplexed GFP reporter mice in which the fluorescent signal can be interpreted as a cell at a specific stage of differentiation within a lineage utilizing a frozen, non-decalcified histology that has minimal background fluorescence. The GFP signals can be associated with fluorescent markers of cell proliferation and immune-stains for cell identity or function. A highly automated scanning fluorescence microscopy has been adapted to capture a high power image of the entire repair field. Four examples of its use will be discussed:

1. Assessing the lineage and differentiation events that drive tibial fracture repair. The temporal contribution of periosteal derived progenitors to the bone and cartilage elements of the fracture callus can be appreciated. We have come to realize that these cellular lineages are significantly affected by different fixation strategies.

2. Use of a calvarial defect model to assess the differentiation potential of a progenitor cell population whether of mouse or human (adult or hES) origin. A major advance is the adaptation of bone restricted GFP marking of human hES cells using Zn finger based recombineering technology. The model is also useful for assessing the biocompatibility of a scaffold and it’s ability to support osteogenesis from either a host or donor source.


4. Appreciating lineage similarities and differences in callus, fibroarticular, articular and growth plate chondrocytes and their interface with adjacent bone.

Rather than pursue a particular line of basic investigation, we have utilized these models and histological techniques to evaluate the outcome of collaborating tissue engineers and stem cell biologists. Currently we are developing image analysis algorithms and heuristic rules to provide quantitative outcomes of a repair based on the 2D bone histomorphometry we have implemented for trabecular bone. In addition, a centralized database has been developed to store experimental details, radiographic images and histological scans for on line review by approved users irrespective of their physical location. This format solves the issue of data naming and storage on numerous personal computer hard drives that are hard to find or recall.

We believe it is important to provide an unbiased, standardized and potentially quantified evaluation platform against which different trials of a strategy are compared.

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Electrochemotherapy in Bone Metastases

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Purpose

Bone metastases are a frequent complication of cancer, occurring in up to 70\% of patients with advanced breast or prostate cancer and in approximately 15 to 30\% of patients with carcinoma of the lung, colon, stomach, thyroid, or kidney. The purpose of the study was to evaluate if electrochemotherapy (ECT), which consists of local or systemic administration of a cytotoxic agent followed by application of electric pulses (EP) to a tumor, could be employed to treat experimental bone metastases and provide a rational bases for its use in clinical studies.

Scope

Aim of the study was to assess the efficacy of ECT in the treatment of \textit{in vivo} rat mammary adenocarcinoma MRMT-1 bone metastases by histological and microtomographical (µCT) evaluation.

Methods

The study was performed according to the Italian Law on animal experimentation and approved by Ethics Committee of Rizzoli Orthopaedic Institute and by the Italian Health Ministry. The animal model for tumor metastases was a rat model of bone cancer induced by inoculation of MRMT-1 cells in the animals proximal tibia. Seven days post cell inoculation the rats were treated with bleomicyn i.v., bleomicyn i.v. plus electric pulses (ECT), the electric pulses alone (EP) or left untreated. The rats were monitored over a period of 21 days post-implantation by histology and µCT. Bone density (BV/TV, \%), trabecular bone pattern factor (Tb.Pf, mm\textsuperscript{-1}), trabecular thickness (Tb.Th, \textmu m), trabecular number (Tb.N, mm\textsuperscript{-1}), trabecular separation (Tb.Sp, \textmu m) and bone mineral density (BMD in g/cm\textsuperscript{3}) were calculated by using µCT with a three-dimensional (3D) analysis.

Results

The microtomographical evaluation showed that rat tibiae bone metastases treated whit ECT had a significantly higher BV/TV, Tb.N, Tb.Th and BMD than untreated metastases and metastases treated with Bleomicyn or EP alone. As for Tb.Pf, control group and ECT groups had both significant lower values than untreated metastases group and group treated with Bleomicyn or EP alone, indicating better connections of the trabeculae. The only difference between control group (healthy) and ECT group regards the Tb.Sp: ECT treated metastases show a more narrow space between trabeculae.

Conclusions

These results suggest that ECT is feasible and effective in the treatment of rat bone metastases and have laid the bases for the ongoing clinical trials.

Recommendations

Long term effects of ECT treatment of bone metastases and the treatment of late bone metastatic lesions could be the next issues to be investigated.

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Long-term Results following a Cranial Hydroxyapatite Prosthesis Implantation in a Large Skull Defect Model

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Purpose: The purpose of the study was to evaluate the interaction of a commercial hydroxyapatite (HA) custom-made prosthesis implanted in a large skull defect, to assess its osteointegration and its habitability with new formed bone over time.

Scope: The scope of the study was to evaluate the in vivo behaviour of a custom-made HA prosthesis, implanted in a sheep model, 6 and 12 months after cranioplasty.

Methods: The study was performed according to the Italian Law on animal experimentation. Ten sheep underwent craniectomy and reconstruction of the skull defect with a porous HA cranial prosthesis. The animals were divided into two groups: Group A was euthanized after 6 months and Group B after 12 months. At the end of the experimental times each implant was evaluated macroscopically, radiologically and analyzed by micro-CT (Porosity; Pore connection; Pore thickness; Pore separation distribution; Pore separation) histology, histomorphometry (Bone-to-implant contact; Trabecular bone volume; Newly formed bone volume; Trabecular thickness; Trabecular number; Trabecular separation; Material volume; Thickness of fibrous tissue) and microhardness techniques (index of bone microhardness; Bone maturation index).

Results: Histology and histomorphometry showed new bone formation inside the implant at both experimental times; newly formed bone had increased significantly by over 300% between 6 and 12 months. 3D micro-CT analysis showed new bone formation and material remodeling. Microhardness analysis indicated that the mineralization process and the mechanical properties of newly formed bone were not altered.

Conclusions: The hydroxyapatite prosthesis showed its osteoconductivity and good biocompatibility. A low rate of fibrous tissue formation and a high rate of bony regeneration were found.

Recommendations: Surgical removal of cranial bone and optimal reconstruction with a custom-made HA implant is a viable procedure that can be carried out in the same surgical setting to treat various cranial traumas, malformations and diseases.

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Comparison of High with Low Resolution Micro-CT Morphometry of Murine Trabecular Bone to Assess how Segmentation Methods Influence Analysis Results

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Introduction. Micro-CT has become a standard method for the morphometric analysis of trabecular bone in rodent preclinical studies. A central part of micro-CT morphometry is segmentation of the gray-scale reconstructed density maps into binary images, also known as thresholding. In this study, three segmentation techniques were comparatively assessed: global thresholding (GT), Otsu auto-thresholding (OAT) and adaptive local thresholding (ALT). OAT applies an algorithm to the entire gray histogram to identify a global threshold between two principal density phases. ALT finds a threshold with reference to the local gray histogram in a sphere centered at each voxel.

Method. MicroCT imaging was done at pixel sizes of 4.5 um and 11.9 um, on the same set of 24 murine distal femoral bone samples (SkyScan 1172). These were from juvenile female C3H mice in four experimental groups (n=6): sham operated (SHAM), ovariectomised (OVX) and vehicle treated, OVX and alendronate treated (ALEND), and OVX and estradiol treated (E2). Treatment was for 5 weeks by daily oral gavage. Trabecular bone segmentation was by GT, OAT and ALT, with GT using the mean OAT to avoid bias. The effectiveness of each thresholding method was assessed as the similarity of the LR to the HR analysis results. Seven morphometric parameters were comparatively assessed, divided into “structural” parameters BV/TV, BS/BV and Tb.Th and “architectural” parameters SMI, Conn.D, FD (fractal dimension) and DA (degree of anisotropy).

Results. The percentage change (made positive) at LR relative to HR for all parameters (log mean) was similar for the three segmentation methods, at about 8%. For structural parameters the log mean percent change using ALT was 6.8%, lower than the % changes using GT and OAT of 13.5% and 13.2% respectively. However in the architectural parameters the log mean % parameter changes for GT, OAT and ALT were 5.6%, 5.5% and 9.4% respectively. This implies that ALT performed better than the global methods (GT, OAT) in the structural parameters but worse in the architectural parameters. For all 24 mouse bones the linear regression of the LR on HR results was studied for the three segmentation methods. The difference of the gradient from 1 was lower in the structural parameters using ALT compared to GT and OAT, but for architectural parameters the difference was higher with ALT than GT and OAT. The R² of the regressions was very similar for the three segmentation methods. The significance of inter-group differences (OVX-SHAM, OVX-ALEND, OVX-E2) assessed by Student’s T test was also compared. For structural parameters the log mean difference in the p values from LR compared to HR analysis using GT, OAT and ALT were 1.09, 1.32 and 0.90. For architectural parameters the corresponding values were 1.44, 1.63 and 1.57.

Discussion. Overall, adaptive local thresholding (ALT) improved morphometric performance – the ability to preserve results with degraded resolution – for structural parameters, but it performed less well than the global methods (GT, OAT) for architectural parameters. Otsu auto-thresholding performed less well than a fixed global threshold.

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Micro-CT Quantification of Changes in Bone and Callus in a Rat Closed Fracture Healing Model

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Introduction. The preclinical study of fracture healing requires an animal model together with an imaging and analysis protocol. Here micro-CT and quantitative 3D image analysis are used to assess bone healing and callus progression in a rat femur diaphyseal fracture model. During this process “secondary” cortical bone is formed as a condensate at the callus periphery, initially separate from the original “primary” cortical bone.

Method. Twenty seven male Wistar rats, 8-9 weeks old, were fractured at the femur midshaft by guillotine following the insertion of a K-wire from the distal condyle. Groups of rats (n=9) were sacrificed at 2, 4 and 6 weeks following fracture. The femurs were harvested and imaged by micro-CT (SkyScan 1174). Binarisation of the reconstructed datasets was by two methods; first, to auto-delineate the mineralised callus from cortical bone, multi-level Otsu thresholding was applied. Secondly, to segment bone for integrated analysis of the whole structure, double global-adaptive thresholding was used. Furthermore, VOIs were drawn manually to separate secondary cortical bone condensed from the callus, from original cortical bone. Tissue mineral density of the callus region was measured.

Results. The mineralised callus volume decreased steadily by more than tenfold between 2 and 6 weeks post fracture (week 2-4 \( p<1E-6 \), week 4-6 \( p<1E-3 \), week 2-6 \( p<5E-8 \)). Callus thickness also showed a uniform 3-fold decrease over the same period. However parameters of architectural connectivity and complexity of the callus showed little change between weeks 2-4 but substantial change between weeks 4-6 post-fracture. The secondary cortical condensate also decreased in volume steadily and significantly over the 2-6 week post-fracture period. The integrated analysis of bone and callus showed little change in mineralised volume and thickness, but architectural parameters such as fractal dimension and connectivity showed a pronounced and significant change over 2-6 weeks. Thickness histograms of the callus region showed a conspicuous narrowing with time over 2-6 weeks with loss of both the thinnest and thickest callus components. This was confirmed by the thickness SD which declined significantly over the study period. Thus callus thickness SD appeared promising as a parameter to assess callus healing. The density (TMD) histograms also showed graphically the transition from low mineralised callus to cortical like bone.

Discussion. Analysis was divided into (a) measurement of separate components of the callus, primary and secondary cortical bone, and (b) integrated analysis including with thickness and density histograms of the whole integrated structure. Both approaches were similarly successful in tracking changes with high significance in the callus region over the 2-4-6 week post-fracture period. Some analyses such as of the secondary cortical bone required manual delineation of VOIs, although most other analyses were fully automated. Micro-CT analysis distinguished the steady quantitative decline in the callus over 2-6 weeks from the architectural and densitometric callus changes signifying remodelling to cortical bone, which occurred mostly later, in the 4-6 week period.

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Long-term in vivo Experimental Investigations on Nanostructured HA-based Bone Substitutes

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Purpose

The characteristics and properties of bone autograft, making it the gold standard in orthopaedic and dentistry applications, have not yet been matched by available bone substitutes. Synthetic calcium/phosphate materials, like hydroxyapatite (HA), are widely used as bone grafts due to their similarity in the chemical composition of bone mineral matrix, resulting in an excellent biocompatibility, osteoconductivity and osteointegration properties that are suitable for orthopaedics, dentistry and maxillofacial applications.

Scope

The aim of the present study was to acquire better knowledge about three different bone substitutes: a biomimetic nanocrystalline non-stoichiometric magnesium-enriched hydroxyapatite (HA) with two different formulations (SINTlife® Putty and SINTlife® granules) and a nanostructured stoichiometric HA (ENGIpore®), both realized with different biomimetic approaches.

Methods

Bone substitutes were implanted bilaterally in bone defects made in the iliac crest of healthy sheep. Histomorphometric and microhardness assessments were performed 9, 12, 18 and 24 months after surgery to evaluate the histological behavior and in vivo degradation time of tested bone substitutes. The following static and dynamic histomorphometric parameters were evaluated: Bone Volume (BV/TV, %); Residual Material Volume (Mat.V/TV, %); Equivalent Diameter (μm) of granules and their distribution present in SINTlife® Putty and composing SINTlife®; and Biomimetic Matrix Volume (MatrixV/TV, %) of SINTlife® Putty; Mineral Apposition Rate (MAR, μm/day).

Results

The results showed that the biomimetic bone substitutes under evaluation were highly osteoconductive materials able to stimulate the growth of new bone tissue, forming a direct bond with the adjacent healthy bone without any fibrotic or chronic inflammatory reactions.

Conclusions

This long-term preclinical study confirmed the osteoconductive capacity of these different bone substitutes and provided information about the mechanism that leads to degradation of substitutes and new bone tissue ingrowth, which might be suitable for a further improvement of the physical characteristics of tested bone substitutes.

Recommendations

Future studies aimed mainly at the evaluation of these substitutes under suitable mechanical loads are mandatory to provide useful information for further implementation of their morphological properties.

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Silk Fibroin Prosthesis Evaluation for Anterior Cruciate Ligament Reconstruction

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Purpose

The anterior cruciate ligament (ACL) is the knee ligament mostly affected by traumatic lesions, causing mechanical instability, pain and secondary osteoarthritis. The surgical reconstruction based on the autologous tissue transplantation (patellar and semitendinosus tendons) represents the “gold standard” for the ACL reconstruction surgery. However, the laxity of ligament and the morbidity of sampling sites, can have a negative impact on the post-operative rehab and on the complete recovery after the ACL reconstruction.

Scope

The aim of this study was to assess the biocompatibility and integration ability of a silk fibroin textile prosthesis with joint, peri-implant bone tissue and cartilage, and native ACL in a large animal model.

Methods

Ten adult sheep underwent surgical reconstruction of ACL (right knee) through a femoral-tibial tunneling where the silk fibroin textile structure was inserted. The animals were divided in 2 groups of 5 animals each and at the end of the experimental times, 3 and 6 months, the joints were explanted and a semiquantitative Joint Damage score was applied. Then, the joint was analyzed by microtomography (Skyscan 1172) to evaluate the tunnel and bone volume around silk fibroin structure. Thus, bone samples were embedded in resin for qualitative histological evaluations.

Results

Joint damage score showed a significative difference between the operated limb and healthy one, while no significative differences were detected between the two experimental times. Microtomography showed an enlargement of tunnel volume with no differences between 3 and 6 months. Histological evaluation highlighted the formation of a fibro-vascular tissue between the silk fibroin textile structure and bone tissue, in agreement with other preclinical studies. At 6 months a progressive organization of fibro-vascular structures was observed, with morphological characteristics typical of ligamentous tissue.

Conclusions

The obtained results highlight the presence of remodeling phenomena and progressive organization of the structures around the silk fibroin structure, supporting its integration ability with surrounding structures.

Recommendations

Further studies at longer experimental time are necessary to improve these findings and to investigate the progression of the regenerative process.

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Conventional treatment of hypoparathyroidism (hypoPT) with calcium supplements and active vitamin D analogues causes reduced bone turnover and over-mineralized bone.

We studied 62 patients with known hypoPT randomized into 2 groups of treatment with either PTH (1–84) 100 µg/d s.c. or placebo, as an add-on therapy.

We investigated the changes in bone structure and density using µCT in 44 iliac crest bone biopsies (23 on PTH treatment) obtained after 24-wks of treatment. Trabecular tunnelling was evident in 11 (48%) biopsies from the PTH-group, whereas no tunnelling was detected in the placebo group. Patients showing tunnelling had significantly higher levels of biochemical markers of bone resorption (NTX and CTX) and formation (osteocalcin, bone specific alkaline phosphatase, PINP). Compared with placebo, PTH-treatment resulted in lower trabecular thickness (Tb.Th) (p<0.01), and trabecular bone tissue density (p<0.01), whereas connectivity density (CD) was higher (p<0.05) and structural modelling index tended to be lower indicating a change in trabecular architecture from a rod-like to a more plate like structure. The changes in Tb.Th and CD can be explained by the intratrabecular tunnelling.

Cortical porosity tended to be higher in PTH-treated patients, especially in those with tunnelling. Occurrence of tunnelling was not associated with etiology, length of disease, concentration of PTH, ionized calcium, or 1,25-hydroxy vitamin D.

Quantitative computed tomography (QCT) at the spine and hip were performed at baseline and at week 24 in 31 patients. At the lumbar spine (L1+L2), the increase in trabecular vBMD over the study period was significantly (p=0.02) higher in the PTH group (+12.2%) than in the placebo group (-0.7%). On the contrary, total vBMD decreased more in the PTH than in the placebo group at the total hip (-1.83% vs 0.43%, p<0.05), at the trochanteric region vBMD (-3.28% vs 0.46%, p<0.03), and at the femoral neck (-3.3% vs -0.84%, p=0.12). At all three sites, there was a tendency towards an increased trabecular and a decreased cortical vBMD in the PTH treated patients.

The effect of PTH (1–84)-treatment in hypoPT is an increased bone turn-over with a decreased vBMD at cortical sites, and an increased vBMD at the trabecular sites, which is related to morphological changes in the bone microstructure with intratrabecular tunnelling and increased cortical porosity.

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Bone Quality Assessment in Lung Transplant Patients by Using Rib Specimens

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Thoracic fractures are one of the leading complications of cystic fibrosis patients (CF) which seriously undermine their quality of life. Despite its high prevalence, pathogenesis of CF related bone disease (CFBD) remains poorly understood. Given that bone mineral density (BMD) is considered to be a poor predictor of fractures in CF patients, it is reasonable to postulate that alterations in bone quality will likely contribute to the propensity to fractures in this population. In order to verify this assumption, a 2 cm segment of one rib (either the 5th or 6th) routinely removed during lung transplantation, was recovered to perform the following analyses: ex vivo BMD by DXA using the high resolution small animal software, microarchitecture analysis with a high resolution micro-CT system (SkyScan 1176), bone remodelling determination by performing bone histomorphometry (Osteoméasure) and microdamage detection by using calcein staining. In the last 9 months, 15 specimens were collected [7 CF: 31.3±4.0y and 8 non-CF (various non-CF lung diseases): 55.4±1.9y]. Between the two groups, there was no difference in ex vivo BMD (0.16±0.02 vs. 0.18±0.02g/cm²) or trabecular TMD (0.53±0.01 vs. 0.55±0.01g/cm³) and cortical TMD (0.84±0.03 vs. 0.84±0.02g/cm³). However, microarchitecture data in CF patients showed a significant greater BV/TV (8.17±0.9 vs. non-CF: 5.74±0.49%; p=0.03) Tb.N (0.48±0.06 vs. non-CF: 0.31±0.02/mm; p=0.01) and Conn.D (2.76±0.3 vs. non-CF: 1.91±0.31/mm³; p=0.04) whereas Tb.Sp was lower (1.07±0.10 vs. non-CF: 1.61±0.11mm; p=0.003). No major differences in other microstructure indices (SMI, Tb.Th) or in cortical parameters were found between CF and non-CF patients. The differences observed in trabecular bone density and microstructure are, among other factors, due to the younger age of the CF group. With a greater number of CF patients, correlations between bone parameters (density, microstructure, microcracks, bone remodeling) and clinical parameters of CFBD (fractures, BMI, bisphosphonates and glucocorticoids use) as well as biochemical indices will be investigated and data will be presented. This exploratory study has shown the feasibility of using rib specimens to assess bone quality in patients undergoing lung transplantation.

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Applications of Morphometry to the Study of Bone Implants and Dental Disorders

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Morphometric methods used to study the bone-implant interface and surrounding bone architecture and remodeling include undecalcified histology and dynamic histomorphometry, backscatter scanning electron microscopy and micro computed tomography. Each method has certain advantages and disadvantages. For instance, while micro computed tomography is an excellent way to examine the peri-implant bone architecture, metal-induced artifacts preclude direct measurement of bone immediately adjacent to the implant. New instrumentation has reduced the magnitude of this artifact and now permits investigators to estimate bone-implant contact. Backscatter scanning electron microscopy provides an excellent means of directly measuring bone-implant contact and assessing mineralization as well as bone architecture, but is destructive and like histology permits limited sampling of the total volume of tissue. Dynamic histomorphometry has surprisingly received little attention, perhaps because of the difficulty of processing metal-containing sections. Here we describe some recent work showing how dynamic histomorphometry can be used to understand implant fixation in a rat model.

To examine bone turnover following implant placement, female Sprague-Dawley rats underwent either sham ovariectomy (sham-ovx) or ovariectomy (ovx) at 4.5 months and at 11 months received bilateral intramedullary femoral implants. In addition, 5 sham-ovx and 5 ovx rats were sacrificed at 11 months of age and served as baseline controls. Implanted rats were randomized to 4, 8 or 12 week follow-up times. Micro computed tomography was used to assess cortical area and trabecular architecture. Dynamic and static histomorphometry were performed to examine the trabecular and endocortical bone in the distal femoral metaphysis adjacent to the implant and the periosteal surface at the midshaft superior to the implant. Implantation did not affect bone volume in either sham-ovx or ovx rats compared to baseline controls. Implant placement significantly increased mineralizing surface, mineral apposition rate and bone formation rate in both sham-ovx and ovx rats at the trabecular and endocortical surfaces at 4 and sometimes 8 weeks, with a return to baseline values by 12 weeks. At the periosteal surface implant placement increased bone formation at 4 weeks with a return to baseline levels by 8 weeks. Thus, implant placement increases bone turnover without affecting bone volume in sham-ovx and ovx rats.

This approach has also been applied to studies in which the anabolic agent, sclerostin antibody was given systemically to rats after receiving implants. These studies showed that the antibody has the same effects in the peri-implant bone as found in other studies, including increased bone formation rate and decreased erosion surface. In various studies, bone architecture and the remodeling parameters were found to be statistically correlated with the force needed to dislodge the implant from the bone. In sum, these studies shed light on the tissue level mechanisms that account for differences in the mechanical endpoint of implant fixation.

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Bone Histomorphometry and Clinical Aspects in Osteoarthritis and Osteoporosis

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Although an inverse relationship between osteoarthritis (OA) and osteoporosis (OP) has been shown by some studies, other reports supported the co-existence of these pathologies. We aimed at clarifying the relationship between OA and OP by combining both clinical (Harris Hip Score) and structural features (Bone Mineral Density, BMD, and bone histomorphometry). BMD by iDXA (Lunar, GE) and bone quality by histomorphometry were assessed in 80 consecutive patients undergoing hip arthroplasty for osteoporotic femoral fracture (n=20, mean age 79.7) or severe OA with different BMD values (n=60: 20 patients with normal BMD, 20 patients with osteopenic BMD and 20 patients with osteoporotic BMD; mean age 68.4 years). A radiographic evaluation of the pelvis and HHS were also performed in all studied subjects.

During surgery, a double osteotomy of the femoral head was performed and the samples were used for histomorphometry through Bio Quant software (version 7.20.10, Bioquant Analysis Corporation - USA).

Histomorphometrical analysis showed that bone volume fraction (BV/TV) was significantly lower in subjects with femoral neck fracture (19.98±4.72%) than subjects with non-osteopenic OA (31.19±5.47%; P<0.01) or osteopenic OA (28.45±5.77%; P<0.01), respectively. No difference between subjects with OP fractures and those with combined OA and OP (23.58±4.47%) was detected. Moreover, clinical scores tended to be associated with BMD and histomorphometric features; where the HHS score was lower, we also found lower BMD and BV/TV values.

Our data supports evidence indicating impaired bone quality in patients with OA and the absence of the protective effect against OP. The worst bone quality in patients with the lowest HHS and the most surface macroscopic alterations suggests that severe OA can be related to OP especially in older patients. It could be useful to determine the presence of a condition of Poor Bone Quality in patients with severe OA who need surgery, to make an adequate pharmacological and surgical approach.

Furthermore we observed mild osteoarthritic alterations during macroscopic and radiographic evaluation in patients with osteoporotic fractures, indicating a probable protective effect of OP against OA. To confirm this hypothesis it should be necessary to carry out subchondral histomorphometric analysis and bone turnover markers evaluation.

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Bone Histomorphometry Findings in Patients with Chronic Kidney Disease – Preliminary Results

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End-stage renal disease leads often to skeletal complications i.e. disturbances in bone metabolism and remodeling. According to the recent recommendations by KDIGO, the histological classification of renal osteodystrophy should be based on evaluating bone turnover, mineralization, and volume (TMV system) using bone histomorphometry. We studied histological findings in patients with chronic kidney disease (CKD) to characterize the histological subforms of renal osteodystrophy using bone histomorphometry.

Iliac crest bone biopsies were collected from 52 patients with CKD (52.9 ± 12.9 years, 37 males) treated by dialysis. All but one patient underwent tetracycline double labeling prior to biopsy. Trabecular bone was assessed by bone histomorphometry and samples were classified based on TMV system [1, 2]. Age- and gender-matched reference data [3] was used for all parameters except for bone volume, bone formation rate, activation frequency, and mineralization lag time that were compared with normal value range [2]. Extended label search was performed for all samples that had no tetracycline labeling or double labels in the trabecular region of interest.

By bone histomorphometry, bone volume was normal in 14 patients (27%), low in 20 patients (38%), and high in 18 patients (35%). After classifying samples based on TMV system, osteitis fibrosa was found in 14 patients (27%) and mild hyperparathyroid disease in 7 patients (13%). High bone turnover in combination with mineralization defect i.e. mixed uremic disease was found in 14 patients (27%). Adynamic bone disease was shown in 2 patients (4%). None of the patients had frank osteomalacia but low turnover in combination with low mineralization was found in 9 patients (17%). These patients did not have thick osteoid seams (O.Th <2.0 SD). Four patients (8%) had normal turnover but low bone mineralization. One patient (2%) had normal turnover with low trabecular bone volume. One patient (2%) had normal bone biopsy findings.

The histological findings were heterogenous. High bone turnover was common in dialysis patients with CKD. Moreover, the prevalence of mineralizing defect was surprisingly high since more than half of the patients had low bone mineralization. The definite histomorphometric diagnosis is important when considering different treatment options for these patients.

REFERENCES:

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Bone Recovery in Ovariectomized Mice Following Lactation

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Bone is lost during lactation in all species examined and this loss is not prevented by consuming high Ca diets. Feeding rats low Ca diets greatly increases the amount of bone loss during lactation. Following lactation the bone lost is regained, and women undergoing several pregnancies and lactations are not at increased risk for osteoporotic fractures.

We examined post-lactation bone gains in LV5 trabecular bone and femur cortical bone in C57BL/6 mice employing microCT and histomorphometry. To increase bone loss, some mice were fed a 0.1% low Ca diet (LCD) during lactation only and some mice were ovariectomized (OVX) at the end of lactation. Bones were analyzed at zero, 1, 3 and 6 weeks after completing lactation.

For spine trabecular bone, lactation resulted in 25% bone loss (19% to 14%) that was increased with low Ca diet (14% to 4%). By 6 weeks post-lactation, most of the bone lost during lactation was regained with no difference in final bone mass (17%) in control mice and mice fed a low Ca diet during lactation. OVX mice (both control and fed a low Ca diet during lactation) had a final bone mass of \(\sim 13\%\), 24% lower than values in non-OVX mice.

For midshaft femurs, lactation resulted in 21% bone loss (205 to 162 \(\mu\)m) that was greatly increased with low Ca diet (184 to 88 \(\mu\)m). By 6 weeks post-lactation, all of the bone lost during lactation was regained in control mice (206 \(\mu\)m) and mice fed a low Ca diet during lactation (198 \(\mu\)m). OVX had no effect on bone recovery in control mice (196 \(\mu\)m) but slightly reduced bone recovery in mice fed low Ca diet during lactation (174 \(\mu\)m).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>LV5 Tb BV/TV (%)</th>
<th>Femur CtTh ((\mu)m)</th>
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<tr>
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<td>Control</td>
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<td>205 ± 3</td>
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<tr>
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<td>9</td>
<td>OVX + LCD</td>
<td>13.8 ± 0.5</td>
<td>174 ± 3</td>
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Following lactation, mouse bone enters a dramatic anabolic recovery phase to replace bone lost during lactation. Increases in cortical thickness exceed 5 \(\mu\)m/day during the first week of recovery after the extensive bone loss produced by feeding a low calcium diet during lactation. Histomorphometric evaluations of undecalcified sections of the midshaft femur show robust endocortical BFR, with increases in MS and MAR during the recovery phase. Post-lactation skeletal recovery is minimally affected by OVX, suggesting the bone mechanostat is largely independent of estrogen actions. Understanding the pathways involved in this physiological bone recovery might provide insights that could lead to novel anabolic therapies for osteoporosis.

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EphrinB2 is required for Support of Osteoclast Formation by Osteoblasts and Chondrocytes


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In FACS isolated Osx1.Efnb2f/f primary calvarial osteoblasts, the EphrinB2 cytoplasmic domain expression was reduced 90-95% compared with Osx1.Efnb2+/+ cells and reduced 80% in Osx1.Efnb2f/f primary chondrocytes. In FACS-sorted Osx1.Efnb2f/f osteoblasts both early (Runx2, Osx, ALP) and late differentiation markers (OCN, PTHR1, MEPE, SOST) were reduced by 80-90%. Similar findings were obtained when exogenous viral Cre was introduced to Efnb2f/f osteoblasts. These changes are consistent with a 50% reduction in mineralization rate in adult Osx1.Efnb2f/f mice and with impaired expression of late osteoblast differentiation markers induced by specific ephrinB2:EphB4 receptor antagonists (sEphB4 and TNYL). Osx1.Efnb2f/f osteoblasts also demonstrated reduced RANKL expression (50%) and impaired support of osteoclast formation in co-culture with wild-type bone marrow precursors. This indicates that ephrinB2 signaling within the osteoblast lineage is required for appropriate differentiation and mineralization by osteoblasts and to support osteoclast formation.

Reduced support of osteoclast formation was also observed in Osx1.Efnb2f/f neonates, which demonstrated high trabecular bone volume, prominent cartilage remnants, and a 75% reduction in osteoclast numbers close to the growth plate. These features are consistent with mild osteopetrosis that resolved by 12 weeks of age. Electron microscopy revealed that the osteoclasts near the growth plate showed reduced contact with cartilage or bone, did not form ruffled borders or sealing zones and exhibited convoluted nuclei. Contact between osteoblasts and between osteoblasts and the bone surface was also reduced and chondrocytes at all stages of maturation contained more condensed chromatin.

In conclusion, these results indicate that osteoblastic ephrinB2 signaling regulates osteoblast and chondrocyte differentiation and the support of osteoclast formation by both chondrocytes and osteoblasts.

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Effects of Marrow Transplantation on Bone Architecture and Turnover

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Bone marrow transplantation is used to treat aplastic anemia, leukemia, multiple myeloma and osteopetrosis, and has the potential for treatment of skeletal disorders caused by genetic or acquired defects in stem cells. Osteoporosis is a common long-term side effect associated with bone marrow transplantation. However, it is not clear whether marrow transplantation is responsible for the bone loss. In the present studies, we investigated the effects of bone marrow transplantation on cancellous and cortical bone in healthy mice. Eight-week-old female C57BL/6J mice were lethally irradiated with Co60 at 9 Gy (single dose). Following irradiation, 10 million bone marrow cells obtained from littermate female donor mice were injected into the irradiated host mice via tail vein. Mice were sacrificed at baseline and 2 months following marrow transplantation and femur and lumbar vertebra evaluated by microCT and histomorphometry. In addition, bone marrow cells from green fluorescent protein (GFP) expressing mice were transplanted into irradiated normal mice. GFP tracking studies were performed to evaluate reconstitution of hematopoietic and mesenchymal cell populations in vivo. The cell tracking studies revealed that early osteoclast precursors in bone marrow (MCFR+ CD11b+) and immune cells in spleen (B cells, CD8 T cells, CD4 T cells and total spleen cells) were quantitatively replaced by donor cells in host mice. Furthermore, a large proportion of the mesenchymal lineage cells expressed GFP. Significant differences in dynamic indices of bone formation, osteoblast and osteoclast-lined bone perimeter, and marrow cell density in femur (endocortex, distal metaphysis and epiphysis) or vertebra were not detected between untreated and transplanted mice. Cortical bone architecture in the femur diaphysis did not differ with treatment. Also, cancellous bone architecture (BV/TV, trabecular number, thickness and spacing) in distal femur metaphysis and epiphysis did not differ between the untreated and bone marrow transplanted mice. However, BV/TV, connectivity density and trabecular number in lumbar vertebrae were lower in the transplanted mice. In summary, we show that reconstitution of marrow does not result in long-term disturbances in bone turnover and only minor site-specific osteopenia. These findings suggest that the osteoporosis associated with bone marrow transplantation may be primarily due to the underlying disease process or post-transplant treatment modalities such as immune-suppressive drugs.

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2-Photon Microscopy for Live Animal Imaging of Bone Regeneration

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Purpose: We sought to establish a live-animal imaging platform to monitor the progression of tissue engineered bone regeneration in situ. Such a platform would allow in vivo measurement of host cells, donor cells and scaffold interactions.

Scope: Autologous bone is the clinical gold standard for bone grafts, however is severely limited in supply and associated with donor site pain and morbidity. Tissue-engineered bone grafts made from biomimetic scaffolds and autologous cells may regenerate bone with minimal immunogenicity. However, the interactions between donor cells, scaffold and host during bone repair are poorly understood, yet critical for clinical safety and efficacy.

Methods: Adherent bone marrow cells were seeded onto a collagen-hydroxyapatite scaffold and implanted into a 3.5 mm calvarial defect. Host and recipient osteoblasts were visualized with a col3.6 fluorescent reporter. At week 4 and week 6, the calvarial implants were imaged with a 2-photon microscope at the edge and center of the defect area.

Results: At 4 weeks post implantation, fibroblastic host cells formed a woven layer of non-mineralized collagen fibers around the surface of the implant. Underneath the woven layer of host cells, donor osteoblasts appeared to be depositing new mineral around scaffold surfaces. Mineral density increased at 6 weeks, leaving only vertical canals in a cortical-like bone. The scaffold was embedded in the mineral phase, but also showed evidence of resorption. Bone deposited in the scaffold area was mediated by a disparate group of primarily donor cells. Continuous layers of host osteoblasts mediated bone deposition at the host edge. In cross section, the collagen fibers in the scaffold region were much less aligned than host bone.

Conclusions: For the first time, interactions between host, donor and scaffold were continuously observed in vivo during bone regeneration using 2-photon microscopy. Bone regeneration in the scaffold area was primarily donor derived however a host layer on the outermost surface may contribute mediating factors. Donor cell-cell contact was much less than host cell-cell contact in their respective areas of mineralization. 2-Photon microscopy is a powerful tool for probing the cellular dynamics responsible for the mineralization and remodeling of tissue-engineered constructs.

Recommendations: Rich 3D datasets of in vivo spatiotemporal gene expression during bone regeneration are now available with 2-photon microscopy and transgenic reporter lines. However, there is a need for quantitative 3D morphometric analysis and data visualization to make best use of large 2-photon data streams.

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Reversing Bone Loss by Directing Mesenchymal Stem Cells to the Bone

Wei Yao, Min Guan, Junjing Jia, Yuan Lay, Ruiwu Liu, Kit Lam, Jan Nolta and Nancy E. Lane

Aging is associated with a reduction in bone marrow MSC numbers and a deficiency in the supportive bone marrow microenvironment that augments the bone formation process. The decrease in the resident MSC population with osteogenic potential within the bone marrow may be the most important factor responsible for reduced bone formation and the subsequent increase in bone fragility with advancing age. Bone regeneration by the means of induction of osteogenesis from MSCs offers a rational therapeutic option. However, this approach is problematic due to the major obstacle of controlling the MSCs’ commitment, growth and differentiation into functional osteoblasts on the bone surface. Our research group has developed a method to direct the MSCs to the bone surface. We attached a synthetic peptidomimetic ligand (LLP2A) that has high affinity for activated α4β1 integrin on the MSC surface, to a bisphosphonates (alendronate) that has high affinity for bone, to direct the MSCs to bone.

We have performed very extensive preclinical studies on cells or small animals and demonstrating 1). LLP2A has high affinity against α4β1 integrin. When the MSCs transition osteoblasts, the cells that form bone, α4β1 integrin is highly expressed on the cell surfaces. 2). In vitro, LLP2A-Ale increases MSC migration in a Transwell assay system. It increased commitment of MSCs to osteoblast differentiation and increased osteoblast maturation and function, as reflected by increased expression of Runx2 and OC and increased calcified matrix deposition. Furthermore, LLP2A-Ale does not affect either chondrogenic or adipogenic potential of the MSCs. 3). The new hybrid compound, LLP2A-Ale, directs transplanted MSCs to bone and increases the retention of the transplanted MSCs in bone in an in vivo xenotransplantation study. 4). LLP2A-Ale augments bone formation and bone mass in young immune-competent mice. 5). LLP2A-Ale partially prevents trabecular bone loss induced by estrogen deficiency. 6). LLP2A + MSCs reverses bone loss in osteoporotic mice induced by estrogen deficiency. 7). LLP2A + MSCs increases bone formation in aged mice.

LLP2A-Ale augments bone as measured by multiple outcomes in models of healthy young, healthy aging, and estrogen-deficient mice. This is achieved via multiple beneficial effects on bone formation. LLP2A-Ale increases the homing of the transplanted MSCs to bone as well as the endogenous number of osteoblasts at the bone surface and significantly increases the rate of bone formation. These results strongly support LLP2A-Ale as a novel therapeutic option for the treatment of bone loss related to age and hormone deficiency.

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Effects of Dietary Fat on Bone Histomorphometry

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By 2030, over 42% of the Americans will be categorized as obese. Obesity increases the risk of heart disease yet it is commonly thought that the skeletal system benefits from the additional weight and osteoporosis may not be a problem in this population. However, recent studies are starting to counter that argument. Increased periosteal bone formation rates and decreased endocortical bone formation rates were found following a high fat diet compared to a low fat diet. These changes in cortical structure resulted in significantly lower bone strength in the high fat diet group. Modest structural changes (larger total area) did not rescue bone strength. In contrast, mice fed a high fat diet resulted in an increased polar moment of inertia and increased trabecular thickness however when adjusted for the increased body weight there were no significant changes in bone traits. It is difficult to separate the effect of the diet content (high fat versus low fat) from the changes in body weight (gain or loss). Therefore, our focus was to separate the weight gain factor from the fat content of diets. The purpose of this study was to determine the effect of isocaloric (high fat and low fat) diets on cortical bone histomorphometry and trabecular structure.

At 5 weeks of age female Sprague-Dawley rats (n=32) were divided into two groups, based on fat content in their diets. One group (HF) followed a diet with a high fat content (60% fat, 20% carb); while the second group (LF) was fed a diet lower in fat (15% fat, 65% carb) for 6 weeks. The diets were isocaloric and the mice in both groups maintained similar body weights, (HF: 269.4 g and LF: 263.3 g). Calcein injections (10 mg/kg) were given on days 10 and 7 days prior to sacrifice at 11 weeks of age. The left femur was dissected and prepared for histomorphometric analysis. Micro CT scans of the trabecular bone in the distal femur were completed. Differences in kinetic histomorphometry and trabecular micro CT were detected using a Students t-test (p < 0.05). The study was approved by the Institutional Animal Care and Use Committee at Temple University.

The high fat diet (HF) group did have a greater trabecular bone volume, 15.7 (3.9) % compared to the LF group, 12.7 (2.8) % and an increase in trabecular number and a decreased SMI. There were no differences in the cortical morphometry between groups in T.Ar, B.Ar or Ma.Ar but there was a significantly greater anterior cortical width, double label perimeter and percent labeled surface on the periosteal surface. Even though the cross-sectional size and bone area were similar the kinetic histomorphometry suggest that there may be different formation and resorption activity on the periosteal surface. Increased periosteal labels combined with increased percent trabecular bone volume may suggest an increase in bone modeling and remodeling activity in the high fat diet group independent of changes in body weight.

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Multi-scale Study of Deformation and Fracture in Bone at Physiological Strain Rates

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Bone fractures most often occur due to a traumatic injury, such as due to a fall, where the bone is mechanically loaded at a fast strain rate. Despite this, most studies characterizing bone’s fracture resistance have been performed at much slower rates, which prompts the question as to the influence of loading rates on the mechanisms by which bone derives its toughness.

Here we investigate the deformation and fracture of human cortical bone from a single donor subjected to three strain rates representing quasi-static loading (0.0001 s\textsuperscript{-1}), running (0.01 s\textsuperscript{-1}), and a fall (1 s\textsuperscript{-1}). Our approach is to discern the origins of fracture resistance at the multiple hierarchical length-scales characteristic of bone, from the nano-scale dimensions of the mineralized collagen fibril to the microstructural level of the secondary osteons, as each structural length-scale plays a role in developing bone’s deformation and fracture properties. Specifically, in healthy human cortical bone, the bone-matrix structure resists fracture intrinsically by promoting “plastic” (strictly, inelastic) deformation at small (~10-100s nm) length-scales through such mechanisms as fibrillar sliding, and extrinsically at much larger (~10-100s µm) length-scales by developing crack bridges and crack deflection/twist in the path of a growing crack. The issue addressed here is whether such multiple-scale toughening mechanisms persist or are degraded in bone at high strain rates.

To investigate the macroscopic fracture resistance, fracture toughness tests on notched beams were performed at the three strain rates, followed by fracture surface and crack path analyses, respectively, via scanning electron microscopy and 3-D synchrotron x-ray computed micro-tomography at the Advanced Light Source (ALS, Lawrence Berkeley National Laboratory). Our results combined with data in the literature indicate that the fracture toughness of bone gradually decreases by a factor of two as the strain rate increases seven orders of magnitude. The primary microstructural mechanisms of crack deflection/twist responsible for extrinsic fracture resistance occur at both fast and slow strain rates. However, while at slow strain rates, the deflections directly follow the cement lines (i.e., the hypermineralized interfaces of the secondary osteons), the deflected path at high strain rates cuts across the osteon, accounting for changes in toughness.

The smallest length-scales in human cortical bone promote fracture resistance intrinsically by developing “plasticity” mechanisms. Strength testing of unnotched beams indicated higher yield and ultimate strengths at higher strain rates, implying that high rates compromise “plastic” deformation, perhaps due to the time-dependent mechanical response of the collagen. To properly characterize contributions to intrinsic fracture resistance at these small length-scales, \textit{in situ} synchrotron small-angle x-ray scattering and wide-angle x-ray diffraction (SAXS/WAXD) measurements during tensile testing were performed at the ALS to measure strain in the collagen fibril and mineral during deformation. Preliminary SAXS/WAXD data indicate diminished collagen deformation with increasing strain rate.

In conclusion, the hierarchical structure of bone plays a large role in promoting fracture resistance intrinsically through plasticity mechanisms and extrinsically through crack-tip shielding mechanisms at larger micron-scales. Our observations of the potency of these toughening mechanisms at higher strain rates indicate that the extrinsic contributions to the bone toughness at micron length-scales are somewhat degraded due to less crack growth along cement lines, whereas as at sub-micron length-scales, the intrinsic contributions from “plasticity” due to fibrillar sliding are diminished, perhaps due to the time-dependent nature of collagen deformation.

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