

Insights into Clinical and Biologic Aspects of Tendon Biology, Mechanics and Repair

Jo A. Hannafin, MD PhD, Hospital for Special Surgery, Weill Medical College of Cornell University, New York, NY

The talk will be comprised of three distinct sections:

1. Review of common tendon injury encountered in the clinical situation, the clinical significance of those injuries and current approaches to treatment. This will provide the basic scientist with an overview of the clinically relevant questions that currently exist in tendon injury and repair.
2. A discussion of currently available in vivo small and large animal models for the study of tendon disease and repair.
3. A detailed scientific review of the mechanical (tendon overload and stress deprivation) and biologic factors (the role of aging, vascularity and matrix metalloproteinases) in the pathogenesis of tendon disease and response to treatment.

This will set the stage for presentations to follow in the areas of tendon development and cellular responses to mechanical and biologic stimuli to be presented by Drs. Schweitzer, Banes and Wang.

Indentation Testing to Determine Bone Fracturability

Paul K. Hansma, PhD

Department of Physics, University of California, Santa Barbara, CA 93106-9530

Advances in fundamental understanding of bone fracture have led to the possibility of determining bone fracturability from a new method of indentation: Reference Point Indentation, RPI. A primary goal of the field is to establish the scientific basis for the method and to conduct laboratory testing to illuminate the differences between more and less easily fractured bone. Though bone is a hierarchically structured material with levels of hierarchy all the way from nanometers to meters, it is becoming clear that the distance range from 100 nm to 100 μ m is critical. Indentations with a 90 degree conical indenter with depths of order 100 μ m are sufficient to open and propagate microscopic cracks in this critical distance range¹. A Reference Point Indentation, RPI, instrument can perform indentation measurements in this critical distance range not only in the laboratory, but also on living patients^{1,2}. Scientists and physicians are beginning to investigate the relation between these new indentation measurements and more established mechanical testing. For example, there is an inverse correlation between R curve analysis of crack propagation and a new parameter, Indentation Distance Increase, IDI, measured with a Reference Point Indentation instrument¹. The Reference Point Indentation instrument measures IDI by cycling a conical indenter tip into the bone multiple times (typically 20 times at 2 Hz) at the same location^{2,3}. The IDI is defined to be the difference between the indentation distance of the final cycle and the indentation distance of initial cycle. The observed correlation can be explained by the hypothesis that both the R curve analysis and the IDI measure the susceptibility of the bone to crack propagation: if cracks propagate more easily the slope of the R curve will be less and the IDI will be greater. Other laboratory studies^{3,4,5} have established other correlations between Reference Point Indentation measurements and established mechanical testing. A frontier is developing methods for using Reference Point Indentation on horses, mice and other animals. Seminal work with RPI from the laboratories of Mary Bouxsein demonstrating differences between different mouse strains⁶, Tamara Alliston quantifying the degradation of stratified cartilage material properties in osteoarthritis⁷, and Michelle Dickenson showing the changes in bone properties between bone hydrated in vivo, hydrated in vitro and dried in vitro⁸ will be discussed.

1. *J. of Bone and Mineral Research*, accepted **2010**, available online: DOI 10.1002/jbmr.73, first author Diez-Perez

2. *Rev. Sci. Instrum.* **2009**, *80*, 54303-54303-6

3. *Rev. Sci. Instrum.* **2008**, *79*, 64303-64303-8

4. *Rev. Sci. Instrum.* **2009**, *80*, 65108-65108-3

5. *Polymer Testing* **2010**, *29*, 159–163

6. *ASBMR 31st annual meeting 2009* abstract A09002126

7. *ASBMR 31st annual meeting 2009* abstract A09001339

8. *ASBMR 31st annual meeting 2009* abstract A09002151

Adolfo Diez-Perez M.D., Ph.D., Hospital del Mar, Autonomous University of Barcelona, Barcelona, Spain

Osteoporosis is defined as a decrease in bone strength, and this results from the integration of bone density and bone quality. Bone mineral density is a common surrogate used clinically although their performance in predicting the mechanical resistance of bone to fracture is limited. Bone quality is assessed in several ways but the different approaches only measure one of the elements (microarchitecture, geometry, microdamage, etc.) of this elusive concept. Moreover, these methods are not suitable for a wide use in clinical practice.

Bone tissue mechanical properties are deemed a key component of bone strength but their assessment requires invasive procedures. We have validated a new instrument, the Tissue Diagnostic Instrument (TDI), for measuring these tissue properties *in vivo*. The TDI performs Bone Microindentation Testing by inserting a probe assembly through the skin covering the tibia and, after displacing periosteum, applying 20 indentation cycles at 2 Hz each with a maximum force of 11 N. Recently we published the study of 27 women with osteoporosis-related fractures and 8 controls of comparable ages. Measured Total Indentation Distance (46.0 ± 14 vs. 31.7 ± 3.3 microns, $p=0.008$) and Indentation Distance Increase (18.1 ± 5.6 vs. 12.3 ± 2.9 microns, $p=0.008$) were significantly greater in fracture patients than in controls. Areas under the Receiver Operating Characteristic, ROC, curve for the two measurements were 93.1% (95% CI: 83.1, 100) and 90.3% (95% CI: 73.2, 100) respectively. Interobserver CV ranged from 8.7 to 15.5% and the procedure was well tolerated.

Our method has several advantages. Measures the tibia because there is a wide flat bone surface in their anterior face that permits a quasi-perpendicular indentation. Periosteum is easily removable here and the procedure is totally painless by using local anesthesia. However, several questions need to be addressed in the near future. Since we measure the tibial diaphysis, we assess a purely cortical bone. Whether their microindentation properties are representative of bone tissue mechanical strength in other bone compartments or anatomical areas needs to be explored. We need normal reference values and the assessment of the technique performance in other populations and clinical conditions. Predictive ability of the TDI has to be evaluated in longitudinal cohort studies. The response of bone to different treatments or pathological situations, one of the main limitations of bone densitometry, has to be measured.

Finally, if TDI is able to demonstrate precision, reproducibility, discriminant ability, sensitivity to change and a good prediction of future fracture, either in basal condition or, more importantly, after exposure of the bone tissue to a drug, TDI might be the main surrogate for fracture occurrence. In this way, we could assess individual fracture risk with high predictive ability. Furthermore, expensive studies on bone response to treatment, requiring large population samples and long periods of follow up with clinical fractures as the main outcome, might be replaced by shorter studies in a limited number of individuals.

As of today, we hypothesize that Bone Microindentation Test, by inducing microscopic fractures, directly measures bone mechanical properties at the tissue level. The technique is feasible for use in clinics with good reproducibility. It discriminates precisely between cases with and without fragility fracture and may provide clinicians and researchers with a direct *in vivo* measurement of bone tissue resistance to fracture.

Sclerostin Mode of Action

M. Kneissel

Novartis Institutes for BioMedical Research

Osteocytes selectively express *SOST*, which encodes the secreted bone formation inhibitor sclerostin [1,2]. Transgenic mice overexpressing *Sost* exhibit reduced bone mass [3,4], while constitutive *Sost* knockout mice have a progressive high bone mass phenotype [5-7]. Sclerosteosis (MIM269500) and van Buchem disease (MIM 239100) patients display lifelong bone overgrowth due to lack of sclerostin [1]. This is in sclerosteosis patients due to mutations in *SOST*, whereas van Buchem disease patients lack a non-coding region downstream of the gene that contains an enhancer element implicated in adult *SOST* bone expression [1,3]. Animal studies, suggest that suppression of sclerostin levels by PTH treatment and by mechanical loading mediates part of bone anabolism induced by either principle [8,9]. Sclerostin was originally believed to act as a bone morphogenetic protein (BMP) antagonist based on its amino acid sequence similarity to members of the DAN/cerberus family of cystine knot-containing secreted glycoproteins, [1,10]. *In vitro* studies revealed however that sclerostin binds to low-density lipoprotein receptor-related protein (LRP) 5 and 6 which are co-receptors for secreted Wnt ligands [11,12]. Currently, it is thought that sclerostin passes through the osteocytic canalicular network to the bone surface where it inhibits osteoblastic canonical Wnt/beta-catenin signaling, which is implicated in bone mass regulation [13]. Consistent with this hypothesis decreased binding of sclerostin to mutated forms of LRP5, which are causative for a high bone mass phenotype, has been demonstrated [14,15]. However, *in vivo* proof for this hypothesis is lacking, especially since recent findings suggest that *Lrp5* does not mediate Wnt signaling in bone but does control pre-osteoblast proliferation indirectly by inhibiting serotonin synthesis in the duodenum [16]. Our recent studies in *Lrp5; Sost* double knockout mice indicate that *Sost* at least requires *Lrp5* dependent pathways, since relative net bone gain is blunted in *Lrp5; Sost* double compared to *Sost* single mutant mice [17]. Future studies will need to address whether the remaining bone gain in the double mutant mice is related to functional compensation by *Lrp6*, which is up-regulated in the bones of these animals. Interestingly, we and others identified recently LRP4 as a novel additional interaction partner for sclerostin [18, 19]. Functional studies indicate that LRP4 acts as a facilitator of sclerostin-mediated inhibition of bone formation [19]. Furthermore, mutations in LRP4 impairing this facilitator function are associated with bone overgrowth in humans [20]. It is currently unknown whether the assumption is correct that sclerostin exerts its action by targeting osteoblasts as a paracrine factor. It may well also affect osteocyte function in an autocrine manner. In line with this possibility it was described that loss of *Sost* function in mice results in decreased osteocyte apoptosis [7]. Moreover, we recently found that osteocytic Wnt/beta-catenin signaling is required for normal bone homeostasis [21] and for full bone anabolism induced by *Sost* loss-of-function [22]. Finally it can currently not be excluded that the sclerostin entering into circulation [23-25] might have additional roles.

While the exact molecular mechanisms by which sclerostin exerts its actions on bone remain to be elucidated, findings to date indicate that sclerostin inhibition might provide an effective osteoporosis therapy. Inhibition of sclerostin by an antibody increases both the extent of bone forming surfaces and osteoblastic bone matrix synthesis at all skeletal envelopes in pre-clinical animal models [26-28]. Consistently, preliminary data from a blinded, placebo-controlled, dose-escalating single-dose study in healthy postmenopausal women revealed that a single anti-sclerostin antibody injection increased bone formation markers and bone mineral density [29]. Notably current data suggest that bone anabolism induced by sclerostin inhibition is not associated with a concomitant increase in bone resorption and that it is not linked to elevated

bone remodeling. In contrast, a reduction in osteoclast number was described at least after short-term treatment [26]. Data obtained from mice with altered *Sost* expression levels and from sclerostin deficient patients have not uncovered yet a role for *Sost* in regulation of osteoclastogenesis or bone resorption [1,4-6]. Therefore, a putative direct or indirect impact of sclerostin on bone resorption requires further investigation. Future studies will reveal whether inhibition of this presumed local Wnt/beta catenin signaling antagonist is safe or whether de-repression of Wnt/beta catenin signaling in bone has oncogenic potential [30]. Finally, while sclerostin function in adult bone mass regulation is established, novel putative roles are currently explored such as involvement in fracture healing [31], in osteoimmunology [32] and in developmental [33], cartilage [34], periodontal [35], kidney [36], and vascular biology [37].

References:

1. van Bezooijen, R.L. et al. (2005) SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. *Cytokine Growth Factor Rev.* 16, 319-327
2. Poole, K.E. et al. (2005) Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *Faseb J.* 19, 1842-1844
3. Winkler, D.G. et al. (2003) Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *EMBO J* 22:6267–6276
4. Loots, G.G. et al. (2005) Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. *Genome Res.* 15, 928-935
5. Li, X. et al. (2008) Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *J Bone Miner. Res.* 23, 860-869
6. Kramer, I. et al. (2009) PTH induced bone mass gain is blunted but not abolished in SOST overexpressing and deficient mice. *J. Bone Miner. Res.* 25: 178-189
7. Lin, C. et al. (2009) Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-catenin signaling. *J. Bone. Miner. Res.* 24, 1651-1661
8. Kramer, I. et al. (2009) Does SOST suppression mediate parathyroid hormone (PTH) bone forming action? *Trends Endocrinol. Metab.* 21(4):237-44
9. Moester, M.J.C. et al. (2010) Sclerostin: current knowledge and future perspective. *Calcif. Tiss. Int.* DOI 10.1007/s00223-010-9372-1
10. ten Dijke, P. et al. (2008) Osteocyte-derived sclerostin inhibits bone formation: its role in bone morphogenetic protein and Wnt signaling. *J. Bone Joint Surg. Am.* 90, 31-35
11. Li, X. et al. (2005) Sclerostin Binds to LRP5/6 and Antagonizes Canonical Wnt Signaling. *J. Biol. Chem.* 280, 19883-19887
12. Semenov, M. et al. (2005) SOST Is a Ligand for LRP5/LRP6 and a Wnt signaling inhibitor. *J. Biol. Chem.* 280, 26770-26775
13. Johnson, M.L. and Kamel, M.A. (2007) The Wnt signaling pathway and bone metabolism. *Curr. Opin. Rheumatol.* 19, 376-382
14. Semenov, M.V. and He, X. (2006) LRP5 Mutations linked to high bone mass diseases cause reduced LRP5 binding and inhibition by SOST. *J. Biol. Chem.* 281, 38276-38284
15. Balemans, W. et al. (2008) The binding between sclerostin and LRP5 is altered by DKK1 and by high-bone mass LRP5 mutations. *Calcif. Tissue Int.* 82, 445-453
16. Yadav, V.K. et al. (2008) Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. *Cell* 135, 825-837
17. Kramer, I. et al. (2009). Sost Exerts its' action via Lrp5 dependent and independent pathways to control bone formation in vivo. *J. Bone. Miner. Res.* 24 Suppl 1
18. Choi, H.Y. et al. (2009) Lrp4, a novel receptor for dickkopf 1 and sclerostin, is expressed by osteoblasts and regulates bone growth and turnover in vivo. *PLOS1* 4, e7930
19. Leupin, O. et al. (2009) LRP4 is a novel osteoblast and osteocyte expressed specific facilitator of sclerostin-mediated inhibition of in vitro bone formation. *J. Bone Miner. Res.* 24 Suppl 1

20. Piters, E. et al. (2010) Identification and characterization of 2 missense mutations in the LRP4 gene causing increased bone mineral density. *Bone* 46, Suppl.1
21. Kramer, I. et al. (2010) Osteocyte Wnt/beta-catenin signaling is required for normal bone homeostasis. *Mol. Cell. Biol.* 30:3071-3085
22. Kramer, I. et al. (2009). Sost deficiency dependent bone gain is blunted in osteocyte specific beta-catenin mutant mice. *J. Bone. Miner. Res.* 24 Suppl 1
23. Mödder, U.I. (2010) Regulation of circulating sclerostin levels by sex steroids in women and in men. *J. Bone Miner. Res.* 2010 May 17. Epub ahead of print
24. Gaudio, A. et al. (2010) Increased sclerostin serum levels associated with bone formation and resorption markers in patients with immobilization-induced bone loss. *J. Clin. Endocrinol. Metab.* 95:2248-53.
25. Mirza, F.S. (2010) Serum sclerostin levels negatively correlate with parathyroid hormone levels and free estrogen index in postmenopausal women. *J. Clin. Endocrinol. Metab.* 95:1991-1997
26. Li, X. et al. (2009) Sclerostin Antibody Treatment Increases Bone Formation, Bone Mass, and Bone Strength in a Rat Model of Postmenopausal Osteoporosis. *J. Bone. Miner. Res.* 24, 578-588
27. Eddleston, A. (2009) A short treatment with an antibody to sclerostin can inhibit bone loss in an ongoing model of colitis. *J. Bone Miner. Res.* 24:1662-1671
28. Ominsky, M.S. (2010) Two doses of sclerostin antibody in cynomolgus monkeys increases bone formation, bone mineral density, and bone strength. *J. Bone Miner. Res.* 25:948-959.
29. Padhi, D. et al. (2007) Anti-sclerostin antibody increases markers of bone formation in healthy postmenopausal women. *J Bone Miner Res* 21 Suppl 1
30. Kansara, M. et al. (2009) Wnt inhibitory factor 1 is epigenetically silenced in human osteosarcoma, and targeted disruption accelerates osteosarcomagenesis in mice. *J Clin. Invest.* 119:837–851
31. Agholme, F. (2010) Sclerostin antibody treatment enhances metaphyseal bone healing in rats. *J Bone Miner. Res.* May 17. [Epub ahead of print]
32. Schett, G. and Sieper, J. (2009) Inflammation and repair mechanisms. *Clin. Exp. Rheumatol.* 27; 4 Suppl 55:S33-S35.
33. Collette, N.M. (2010) Genetic evidence that SOST inhibits WNT signaling in the limb. *Dev. Biol.* 342:169-179.
34. Van Bezooijen, R.L. (2009) Sclerostin in mineralized matrices and van Buchem disease. *J. Dent. Res.* 88:569-574.
35. Jäger, A. et al. (2010) Localization of SOST/sclerostin in cementocytes in vivo and in mineralizing periodontal ligament cells. *J. Periodontal. Res.* 45:246-254
36. D. Cejka, D. et al. (2010) Sclerostin and dickkopf-1 serum levels in dialysis patients and healthy volunteers. 37th European Calcified Tissue Symposium, June 26 – 30, Glasgow, UK PP408
37. van Bezooijen, R.L. (2007) SOST expression is restricted to the great arteries during embryonic and neonatal cardiovascular development. *Dev. Dyn.* 236:606-612.

The Role of the Wnt Pathway in Mediating the Effects of Mechanical Loading

Alexander G Robling

Introduction

The search for pathways involved in load-induced bone formation was advanced considerably several years ago, when genetic mapping studies among families with osteoporosis pseudoglioma (OPPG) revealed that the low density lipoprotein receptor related protein 5 (LRP5) had important functions in the mammalian skeleton. Mice engineered with a loss-of-function mutation in *Lrp5* exhibit low bone mass, but the weight-bearing portions of the skeleton bear a greater deficit in bone mass than the non weight-bearing portions. Those observations led us to test the hypothesis that *Lrp5* is important in mechanical signaling. When subjected to *in vivo* ulnar loading, we found an 88 to 99% reduction in load-induced bone formation among *Lrp5*^{-/-} mice, compared to wild-type controls.

Shortly after the discovery of families harboring loss-of-function mutations in LRP5, other investigators identified a series of missense mutations in LRP5 that result in abnormally high bone mass (HBM). These mutations result in very high bone mass, reminiscent of osteosclerosis-type disorders. In light of the strong effects of *Lrp5* loss-of-function mutations on mechanotransduction, we investigated whether the *Lrp5* gain-of-function (HBM) alleles might also alter mechano-responsiveness in the bone tissue. Specifically, we asked whether normal expression of one of two different *Lrp5* HBM mutations would enhance load-induced bone gain and/or prevent disuse-induced bone loss.

Materials and Methods

Mice

Lrp5 HBM knock-in mice were engineered by replacing a portion of intron 2 through a portion of intron 4 with targeting constructs that harbored either the G171V (equivalent to residue 170 in the mouse) or the A214V (equivalent to residue 213 in the mouse) within exon 3, using homologous recombination. Both *Lrp5* knock-in mutants (and their WT relatives) were on a mixed genetic background of 129S1/SvIMJ and C57Bl/6J.

Ex vivo strain gauging and in vivo axial loading of the tibia

Four 18 week-old male mice of each *Lrp5* genotype (WT, A214V, G171V) were used for strain measurements under dynamic axial compressive loading. A single element strain gage was applied to the posteromedial surface of the midshaft tibia, which was subsequently mounted between molded knee and ankle fixtures that secured the tibia in the vertical direction. The fixtures were mounted to a Bose 3200ELF mechanical testing system for load application. Strain and force was recorded during a 10-cycle loading bout of comprising increasing force (4.5-8.0 N), which allowed computation of a force:microstrain factor.

For *in vivo* loading, 18-wk old mice were anesthetized under isoflurane and had their right hindlimb (knee to foot) placed in molded loading cups that secured the tibia. A sinusoidal wave form at 2 Hz, 120 cycles, was applied with peak force adjusted for each genotype to achieve 2130 $\mu\epsilon$. Mice were given three bouts of loading with a day of rest between each bout. Intraperitoneal fluorochrome labels were given to facilitate histomorphometric measurements of new bone formation. The right and left tibias were harvested and processed for fluorochrome histomorphometry at 3 different diaphyseal locations (proximal, midshaft, and distal).

Tail suspension and Botox-induced disuse of the lower limb

Sixteen 10 week old female mice from each genotype (WT, A214V, G171V) were used for the tail suspension studies (8 ground control and 8 suspended). All mice were individually housed and a tail harness was used to suspend the experimental mice. Control mice were unencumbered in their cage. Mice were suspended for 24 days prior to sacrifice.

Twenty-four 16 week old male mice from each genotype (WT, A214V, G171V) were used for the unilateral Botox studies. The right hindlimb musculature (quads, hamstrings, calves, tibialis anterior) was injected with 20 μ L of Botox (Allergan, Irvine, CA). The left hindlimb musculature was injected with 20 μ L of saline and served as an internal control. These injections were repeated one week later and the mice were sacrificed 22 days after the first injection.

Mice in the tail suspension and Botox studies were radiographically scanned using pixiMUS II, just prior to intervention, and again at sacrifice. Standard microCT measurements were collected on the dissected and fixed femurs after sacrifice.

Results and Conclusion

Tibial loading:

The tibial loading model significantly increased periosteal bone formation parameters in the loaded limb compared to non-loaded limb at the proximal and midshaft locations, but not at the distal location. The A214V mutant mice had a significantly greater load-induced periosteal bone formation response (MAR, MS/BS, and BFR/BS) compared to WT mice. The G171V mutants, however, were not significantly different from WT mice in their periosteal response to loading. Endocortically, the WT and A214V mutants failed to show a measureable response to loading, but the G171V mutants exhibited significantly increased MAR, MS/BS, and BFR/BS. These data indicate that the G171V mutant mice have a lower strain threshold for initiating endocortical bone formation, compared to WT mice.

Disuse:

In the Botox experiment, we observed a significant decrease BMC in the treated (paralyzed) femur among WT mice (-22%; $p < 0.05$), but both HBM groups were unaffected (3-5% loss, NS). μ CT analysis of the distal femur trabecular bone, however, revealed roughly equivalent bone loss (3-5% decrease in BV/TV, $p < 0.05$), regardless of genotype. In the hindlimb suspension experiment, WT mice lost a significant amount of lower limb BMC (-4.2%; $p < 0.05$), whereas the A214V mice actually gained BMC (+5.0%; $p < 0.05$) and the G171V mice maintained their BMC (-0.4%; NS) throughout the suspension period. Similar to the Botox experiment, μ CT analysis of the distal femur trabecular bone, revealed roughly equivalent bone loss (5-10% decrease in BV/TV, $p < 0.05$), regardless of genotype.

Our data support the hypothesis that the HBM-causing mutations in the Lrp5 receptor (1) confer increased mechanoresponsiveness to the bone tissue in cases of increase mechanical stimulation, and (2) provide protection from disuse-induced bone loss, particularly in the cortical bone compartment.

Tendon Stem Cells and Tendon Repair

James H-C. Wang, Department of Orthopaedic Surgery, University of Pittsburgh

Tendons are dense connective tissues that enable joint movement by transmitting muscular forces to bone. Because it bears large mechanical loads, tendon injury is common, especially in athletic settings. Once injured, tendon healing often results in formation of scar tissue, which has inferior mechanical strength and is therefore susceptible to re-injury at the repair site. In fact, restoration of normal structure and function of injured tendons represents one of the most challenging areas in orthopaedic medicine. In recent years, cell therapy has been used on animal models in an attempt to improve the structure and function of injured tendons. However, only limited success in terms of improving structure and function of injured tendons has been achieved, mainly due to lack of characterization of tendon cells. In this lecture, I will present our work on characterization of rabbit tendon cells. I will show that in addition to tenocytes, the residential cells within tendons, a new population of cells exists, referred to as tendon stem cells (TSCs). TSCs exhibit distinct properties compared to tenocytes, including differences in cell marker expression, proliferative and differentiation potential, and cell morphology in culture. In the second part of my presentation, I will present our recent research on the effects of platelet-rich plasma (PRP) on TSCs. Our data show that PRP treatment promotes differentiation of TSCs into tenocytes that are activated as evidenced by high proliferation rates and collagen production capability. The lecture will close with our perspectives on future research directions in the areas of tendon stem cell mechanobiology and TSC-therapy for injured tendons.

Recent Advances in Mechanotransduction and Where We Stand in Tendon.

^{1,2,3} Banes, A.J., Qi, J., ^{1,3} Sumanasinghe, R., ³ Tsuzaki, M, ⁴ Banes, A.N., ⁵ Dmochowski, J., and ³ Wall, M. Joint Department of Biomedical Engineering¹ UNC, NCSU, Chapel Hill, NC, Curriculum of Applied Sciences and Engineering², UNC, Chapel Hill, NC, Flexcell Intl. Corp³, Hillsborough, NC., College of Veterinary Medicine⁴ NCSU, Raleigh, NC, School of Medicine⁵, Chapel Hill, NC.

The field of cytomechanics and elucidation of the mechanisms of mechanotransduction have evolved greatly in the past 5 years. Strong support for Ingber's theory of tensegrity has emerged. Na and Wang have shown that mechanical forces can drive COS cell signaling and Src activation much faster than can a ligand such as EGF. There are competing data from Tzima to support the idea that shear stress may activate endothelial cells differently than substrate strain through Pecam-1 and VEGF. The lack of primary cilia in the etiology of polycystic kidney disease by Nauli and the lack of response to shear stress in osteoblasts without primary cilia by Malone and Jacobs point to the idea that cells require the primary cilium but have multiple mechanisms to respond to physical forces. Recent reports by Farnum, Poole and Qi and Banes show that tenocytes have primary cilia that are needed for a proper mechanical stimulus response. Tenocytes respond to substrate strain and shear stress by signaling, activating pathways and strengthening their matrix. Butler's group has shown the relevance of stem cell addition to matrix mechanically conditioned in vitro then returned to the patellar tendon in vivo. However, the magnitude of strain can have adverse affects or act in a positive manner as has been shown for chondrocytes, tenocytes and endothelial cells. Effector ligands such as cytokines, anabolic steroids, norepinephrine and even ATP can modulate tenocyte response to strain. There are likely differences in overall cell responses to strain that depend on the level of cell-cell connectivity and individual vs group, ie syncytial responses. Are cell-cell connections of greater importance than matrix-integrin connections in responses? There is a need to define better markers for tendon cell expression, recognize the multiple cell types in various tendons as well as the contribution from pericytes, stem cells, the vasculature and innervation in these responses.

NMR Measurement of Bone Quality

D.P. Nicolella, Q. Ni, T.L. Bredbenner, L.M., Havill Southwest Research Institute

The problem of increased risk of skeletal fractures due to bone mass loss in aging or disease is a major clinical problem with associated estimated health care costs of nearly \$17 billion in the US [1]. It has been estimated that 40%-46% of all women over the age of 50 and 13%-22% of all men over the age of 50 will suffer a fracture as a result of bone loss [2]. With the number of persons aged 60 years or older projected to almost triple by the year 2050 [3], the aging of the general population will lead to a significant increase in the at-risk population for fractures. As such, the number of world-wide fractures will likely increase from 1.26 million, as estimated in 1990, to 2.6 million by 2025 [4]. Notwithstanding the economic burden, non-vertebral fractures are a significant cause of morbidity and mortality in the aging population [5-7]. Thus, concerted efforts are needed to not only identify those at risk of bone fractures, but also to identify treatment strategies that can maintain the health of the skeleton with age.

Age-related increase in the incidence of skeletal fractures results from interactions among a variety of factors including impaired balance and reflexes, reduced bone mineral density, changes in bone geometry, porosity, and architecture, physicochemical properties of bone's mineral and organic phases, and accumulation of damage in bone tissue [8-12]. The latter five factors are collectively referred to as "*bone quality*." It is becoming increasingly evident that bone mass alone cannot account for variation in observed fracture risk and that more accurate fracture risk prediction will only be possible through incorporation of measures of bone quality [13]. To improve our ability to predict fracture risk by including measures of bone quality, we must first understand the mechanisms through which various measures of bone quality act to control bone mechanical properties and, ultimately, bone strength. This is not currently well understood.

To understand the full range of determinants of fracture risk, bone must be analyzed at each of the hierarchical levels of its organization. Bone has a complex hierarchical structure that resembles a composite at various length scales ranging from nanometers to nearly meters [14, 15]. The basic building blocks in a bone are nanometer-sized platelet-shaped crystals of carbonate apatite or hydroxyapatite (HA), which are arranged in parallel layers with a collagen matrix to form a mineralized collagen fibril [14]. At the next hierarchical level the mineralized collagen fibrils are organized into various structures with different property characteristics. In lamellar bone, the mineralized collagen fibrils are ordered into arrays in which the fibril axes and the crystals are aligned into a three-dimensional structure resembling a nanometer-sized composite exhibiting anisotropic elastic and fracture properties. Well-organized lamellae of different thicknesses and fibril orientations form the structure in an osteon, including a Haversian canal and numerous lacunae located at lamellae interfaces. In contrast, disorganized fibril arrangements and orientations constitute the structure in interstitial bone tissue. At the next hierarchical scale, bone structure is comprised of a microstructure of osteons and interstitial lamellae that are separated by cement lines. At the macroscopic or continuum scale, bone can be considered a porous solid whose elastic and fracture properties depend on bone mass, porosity, and pore size distribution. Bone microstructure evolves as damaged bone is remodeled to form damage-free bone tissues.

Bone tissue is 25% water. Water is not only present in the microscopic pores as free mobile water (~18% by volume), it also exists within the extracellular matrix of bone tissue as tightly or loosely bound water (~7% by volume). Previous studies have suggested that the removal of water from the extracellular matrix, in addition to that removed from the void spaces within bone tissue, affects the mechanical properties of bone. The objective of this investigation was to

determine the porosity, mobile, and bound water distribution, and, for the first time, attempt to determine the loosely and tightly bound water in cortical bone *in vitro* using a non-destructive low-field NMR technique and to characterize how changes in bound water within bone tissue are related to bone mechanical properties.

We found no age-related differences in either bone porosity or the ratio of bound to mobile water between young and old baboon bone. However, we did find a significant difference in loosely bound water between the young and old bone. Furthermore, it appears that this loosely bound water may play an important role in bone mechanical properties, particularly in the tensile yield and post yield behavior of bone tissue. In our previous study, we found that bound water plays an important role in human bone mechanical properties [16]. Since the ratio of bound water to mobile water is much higher in this sample of baboon bone than in human bone, it is reasonable to assume that the loosely bound water within the bone matrix plays an important function in bone mechanical properties. However, a more detailed characterization of the bound water (such as the fraction of loosely bound or tightly bound water) is needed to assess the mechanical integrity of bone tissue.

References:

- [1] Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the united states, 2005-2025. *J Bone Miner Res*, 2007; 22:465-75.
- [2] Kanis JA, Johnell O, Oden A, Sembo I, Redlund-Johnell I, Dawson A, De Laet C, Jonsson B. Long-term risk of osteoporotic fracture in malmo. *Osteoporos.Int.*, 2000; 11:669-674.
- [3] United Nations World population prospects: The 2008 revision. 2009.
- [4] Gullberg B, Johnell O, Kanis JA. World-wide projections for hip fracture. *Osteoporos.Int.*, 1997; 7:407-413.
- [5] Melton LJ, 3rd. Adverse outcomes of osteoporotic fractures in the general population. *J Bone Miner Res*, 2003; 18:1139-41.
- [6] Kanis JA, Oden A, Johnell O, De Laet C, Jonsson B, Oglesby AK. The components of excess mortality after hip fracture. *Bone*, 2003; 32:468-73.
- [7] Johnell O, Kanis JA, Oden A, Sernbo I, Redlund-Johnell I, Petterson C, De Laet C, Jonsson B. Mortality after osteoporotic fractures. *Osteoporos Int*, 2004; 15:38-42.
- [8] Schaffler MB, Choi K, Milgrom C. Aging and matrix microdamage accumulation in human compact bone. *Bone*, 1995; 17:521-525.
- [9] Burr DB, Forwood MR, Fyhrie DP, Martin RB, Schaffler MB, Turner CH. Bone microdamage and skeletal fragility in osteoporotic and stress fractures. *J.Bone Miner.Res.*, 1997; 12:6-15.
- [10] Schaffler MB, Jepsen KJ. Fatigue and repair in bone. *International Journal of Fatigue*, 2000; 22:839-846.
- [11] Akkus O, Polyakova-Akkus A, Adar F, Schaffler MB. Aging of microstructural compartments in human compact bone. *J.Bone Miner.Res.*, 2003; 18:1012-1019.
- [12] Diab T, Condon KW, Burr DB, Vashishth D. Age-related change in the damage morphology of human cortical bone and its role in bone fragility. *Bone*, 2005.
- [13] van der Meulen MC, Jepsen KJ, Mikic B. Understanding bone strength: Size isn't everything. *Bone*, 2001; 29:101-4.
- [14] Fratzl P, Gupta HS, Paschalis EP, Roschger P. Structure and mechanical quality of the collagen-mineral nano-composite in bone. In: *J Mater Chem*; 2004. p. 2115-2123.

- [15] Weiner S, Traub W, Wagner HD. Lamellar bone: Structure-function relations. *J.Struct.Biol.*, 1999; 126:241-255.
- [16] Nyman, J.S., Ni, Q., Nicolella, D.P., Wang, X. Measurements of mobile and bound water by nuclear magnetic resonance correlate with mechanical properties of bone. *Bone*, 2008; 42:193-9

Skeletal Abnormalities in *Mecp2* Deficient Animals: A Model of Mineralization Deficits in Rett Syndrome

*R. D. O'Connor*¹, *M. Zayzafoon*², *M. C. Farach-Carson*¹, ***N. C. Schanen***³. ¹*Department of Biological Sciences, University of Delaware, Newark, DE, USA,* ²*Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA,* ³*Laboratory for Human Genetics, Nemours/A.I. duPont Hospital for Children, Wilmington, DE, USA.*

Rett Syndrome (RTT), a neurodevelopmental disorder, is most often caused by inactivating mutations in the X-linked gene encoding a regulator of epigenetic gene expression, methyl CpG binding protein, MeCP2. Clinical data show that along with neurological defects, females with RTT frequently have marked decreases in *Bone Mineral Density* (BMD) beyond that expected from disuse atrophy. Our work with a *Mecp2* null mouse model, *Mecp2*^{-yBIRD}, revealed differences between the wild-type and *Mecp2*^{-yBIRD} null mice, with diminished skeletal size and significantly shorter femurs. Histological studies revealed growth plate shortening as well as trabecular bone deficiency apparent in the primary spongiosum of *Mecp2* null femurs by 21 days of age, prior to the onset of neurological symptoms. Additionally, the trabeculae in the primary spongiosum of 60 day old *Mecp2*^{-yBIRD} mice were abnormally shaped and hypercellularity was noted in the marrow space. Both histological and histomorphometrical analyses have shown reductions in the cortical bone parameters of *Mecp2* null mice. It does not appear that these decreases in bone are owed to a primary effect on osteoclasts, as osteoclast numbers were comparable between wild-type and null animals. Dynamic histomorphometric analysis was consistent with a deficiency in mineralization, suggesting disruption in formation in this model in adolescent and young adult animals. We speculate that *Mecp2* deficiency leads to a primary dysregulation of genes critical for regulation of bone growth, differentiation and mineral homeostasis that leads to a direct effect on bone cell health. The primary effect on bone cells, combined with other risk factors, place patients with RTT at marked increased risk for pathological fractures due to low BMD.

Histomorphometry in Translational Research

Don Kimmel, Osteoporosis Research Center, Creighton University

This presentation is based on a quarter century of research surrounding agents and conditions with promise for treating osteoporosis. All were tested in both animals and humans. Histomorphometry and densitometry were the essential measuring methods to evaluate their performance in both species. Fluorochrome-based histomorphometry was used to reveal tissue level mechanisms. The nine selected instances discussed here are: 1) estrogen; 2) SERMs; 3) bisphosphonates; 4) RANK Ligand inhibitors; 5) PTH; 6) Strontium ranelate; and 7) Vitamin D-like compounds. Though the rat was uncanny in its ability to predict human outcomes in the first seven, two notable misses were: 8) bone gain during BP therapy, 9) inhibition of PTH response by prior BP therapy.

The rat skeleton slows its bone elongation rate suitably to be an acceptable model of the adult human skeleton. Furthermore, at steady state, adult rats remodel cancellous, but not cortical bone. The best cancellous bone sampling site is that around the knee joint. Rats exhibit OVX-related site-specific bone loss with an associated elevation in formation rate that abates after 3-6 months. Metaphyseal cancellous bone disappears more quickly than vertebral body cancellous bone, while yellow marrow sites do not lose bone in a reasonable timeframe. Cortical bone loss occurs more slowly. Bone mass changes in OVX rats can be observed with densitometry and histomorphometry. Turnover changes can be observed with histomorphometry and serum/urine markers of bone turnover.

The menopausal transition in humans is characterized by accelerated bone loss. This bone loss is associated with an increased turnover rate, both of which abate somewhat at five+ years after last menses. Estrogen replacement therapy (ERT) prevents osteoporotic fracture. ERT prevents bone loss and suppresses the menopause-related increased in turnover. Site-specific bone mass changes are best observed with densitometry of spine, hip, and wrist. Turnover changes can be observed with serum/urine markers of bone turnover and histomorphometry of transilial bone biopsy specimens.

The parallels between rats and humans for estrogen deficiency and bone behavior were established first and are striking. Removal causes bone loss in both. Removal accompanied by ERT experiences no loss. Removal is accompanied by a rise in formation rate. Removal accompanied by ERT allows no rise in turnover rate. The histomorphometric findings with estrogen were always inhibition of estrogen-depletion bone loss, accompanied by reduced bone formation rate. Histomorphometry never measures resorption directly, leaving one always deducing the status of resorption from bone mass and bone formation rate data. While bone resorption biomarkers provide some help, they have never achieved the same status in rats as humans.

The bone research field concentrated on what it understood and could control. Human studies showed that: 1) estrogen depletion causes bone loss; 2) ERT stopped menopause-associated bone loss; 3) ERT reduces fracture risk; 4) ERT suppresses the rise in turnover. The same occurred in rats. **The first logical hypothesis was that any agent that suppressed turnover would slow post-menopausal bone loss. Secondly the idea was tacitly advanced that such testing could be carried out in rats.**

The estrogen experience convinced the bone field that the rat gave the same answers as humans to these questions. Just as important, in the late 1980's, with the pharmaceutical

industry still taking a pharmacology-based approach to drug development, this paradigm encouraged companies to enter the osteoporosis business, because the logic was easily understood (i.e., reduce turnover, save bone, reduce fracture risk).

The three most prominent types of agents with this type of potential are SERMs, bisphosphonates, and RANK Ligand inhibitors. SERMs were billed as “non-estrogenic” estrogen. BPs and RANK Ligand inhibitors were definitely non-estrogenic. In rats, early experiments showed that non-hormonal agents that prevent increased turnover block bone loss. Multiple labs and investigators continue to have similar findings.

Only one SERM has been brought successfully to market for bone in the US. Raloxifene (RLX) achieved success when being “estrogen-light” for bone, with a reduction in spine fracture risk, was good enough to garner FDA approval. RLX had mild-to-moderate effects on bone (blocking ~50% of post-OVX bone loss in a dose-effect fashion), but its effect on the uterus was one-of-a-kind. When tested in the rat, all SERMs show less uterine hypertrophy than ERT. This is probably also true, but seldom noted in humans. RLX had a generally insignificant hypertrophic effect on the uterus of the OVX rat (agonist mode), and a substantial reduction in uterine weight in the intact rat (antagonist mode), consistent with its SERM nature. The unique part for RLX was that there was no dose-effect relationship for hypertrophy in the OVX rat. No other SERM ever matched this profile... no other SERM ever got to market. More potent SERMs that inhibited bone loss in OVX rats more strongly than RLX also had a dose-effect relationship for uterine hypertrophy. Published work with idoxifene and levormeloxifene, two that failed in Phase III due to uterine prolapse, does not include extensive rat work. When post-tested in OVX rats, both were much more uterotrophic than RLX (or lasofoxifene). Lasofoxifene is more bone efficacious, but also modestly more uterotrophic than RLX in OVX rats. It also had uterine issues in humans. Though bazedoxifene is similar to RLX in both uterus and bone, its clinical data showed insignificant fracture risk reduction. No SERM ever achieved significant anti-hip fracture efficacy. There were as many as a half dozen. All except RLX and bazedoxifene failed due to uterine issues. The OVX rat was great for both bone and uterine effects of SERMs in humans.

Four bisphosphonates (BPs) are marketed for osteoporosis and bone metastases in the US. All are nitrogen-containing BPs with a unique mechanism of action, inhibition of farnesyl diphosphate synthase, that sets them apart from their ancestors, etidronate and clodronate. The NBPs reached ERT’s standard for osteoporosis therapy by reducing both spinal and hip fracture risk. They also reduced hypercalcemia and bone metastases in an unprecedented way. Their bone specificity suggested that side effects, unlike with SERMS, could be limited to bone and tissues touched directly by dosing. The four NBPs, when dosed in efficacious amounts and with full compliance, have essentially the same effect, 70% reduction of vertebral and 50% reduction in hip fracture risk. NBPs were more than “mineral poisons.” Their extended bone residence time eventually gave rise to the idea of intermittent dosing that has been carried all the way to yearly, with no decline in efficacy.

Rat data predicted initial bone residence of any BP that reaches the skeleton, on bone surfaces. It predicted burial at formation sites, rapid removal at resorption sites, and slow removal at neutral sites as long as they undergo no remodeling. The human data are consistent with those observations.

Etidronate induced mineralization defects in rats that reversed upon ending treatment. Etidronate’s mineralization defect was discoverable only by histomorphometry with

fluorochrome labeling, and was seen very early in rats. Histomorphometric studies showed that etidronate-treated humans also had intermittent mineralization defects. Rat safety studies with histomorphometry were crucial in differentiating the four NBPs from etidronate, permitting their introduction into humans. Human data with NBPs showed bone gain with reduced turnover. The bone gain was apparent by densitometry. The turnover reduction in humans was noticed first by turnover markers and later by histomorphometry. Human histomorphometry with the NBPs was done mainly for safety reasons.

Rat data for all four NBPs predicted similar outcomes, prevention of post-OVX bone loss combined with inhibition of bone formation. The rat data predicted the order of potency in humans: zoledronate>ibandronate>risedronate>alendronate. When NBP treatment of osteopenic, OVX rats was initiated, further loss was halted, but no gain occurred; reduction in turnover rate was noted. The bone mass changes were found by both histomorphometry and densitometry. The turnover changes were found first by histomorphometry and later by serum/urine biomarkers. Rat data predicted that intermittent dosing with equal cumulative amounts to those found efficacious in daily dosing, would have equal efficacy for bone mass gain and turnover reduction. The OVX rat was great for bone and safety effects of BPs in humans.

The OVX rat data did **not** predict the gain in bone mass seen with BP treatment in humans. While this has not been formally explained, the early short-term burst is generally attributed to the fact that adult humans have copious amounts of cancellous and cortical bone remodeling that creates a detectable “remodeling space.” A decline in initiation of new remodeling units, combined with continued infilling of in-process, partially empty ones (filling of remodeling space) is the first explanation. Compared to humans, rats have relatively minimal amounts of cancellous bone remodeling and no cortical remodeling. Accordingly, they would have difficulty showing this type of phenotype. The longer term rise in humans is partially attributed to increased levels of secondary mineralization. Humans also have a longer timeframe for observing secondary mineralization than do rats.

There is less extensive OVX rat work with denosumab, a RANK Ligand inhibitor, than with the above agents. Existing data from both pharmacologic studies and knockout mice prove that it (or osteoprotogerin) blocks bone loss in the OVX rat while suppressing formation rate in small animals. The human data concur. Denosumab was approved a few months ago as a treatment for osteoporosis.

One anabolic agent, PTH(1-34) has been successfully marketed for osteoporosis in the US. A second similar agent (PTH(1-84))exists in Europe. When given intermittently daily, PTH increases bone mass in the spine and hip, decreasing fracture risk at both sites. PTH forced a deeper understanding of the term “anabolic agent.” Many outside (and inside) the bone field still believe that increasing bone mass constitutes anabolic activity. When most tissues increase their mass, be it through increase in cell number or increase in extracellular tissue quantity, it is anabolism. Not so for bone. The mechanism is important. Since the bone field has examples of drugs that increase bone mass through only an anti-resorptive mechanism, the term anabolic for bone, now requires evidence of stimulation of bone formation. Increased serum/urine bone formation markers is the minimum. Some mistakenly accept increased individual cell activity (mineral apposition rate). This is insufficient, as the main requirement for an anabolic agent is to increase the quantity of fluorochrome-double-label surface. PTH and related molecules have met this requirement most consistently, across multiple laboratories and experiments. The sclerostin antibody appears to be a second, though it has not been in wide clinical use at this time, as compared to PTH.

Rat work with PTH long preceded its use as a bone anabolic agent in humans. Rat studies were eventually done across many labs, and confirmed by multiple investigators, long before its use in people. The controversy over effects of intermittent vs. continuous treatment nagged at the field. Histomorphometry was crucial in establishing its phenotype by proving the existence of larger amounts of double label surface. PTH was the agent of choice for early rebuilding strategies that were tested in osteopenic, OVX rats. Early PTH rat work head-to-head with BPs and estrogen, documented the superior ability of PTH to build bone in a mode where bone formation was stimulated. PTH treatment was also used to prove the principle that bone added by “anabolic therapy,” would disappear after discontinuation of treatment. All proved true in people. The question was how efficacious PTH would be when used pharmacologically and whether it would ultimately be safe for adult humans. It caused osteosarcoma in standard rat carcinogenicity studies that require use of juvenile rats, but did not do so in adult rats. It has been safe in adult humans. The adult OVX rat was exceptional for bone and safety effects of PTH in humans.

There are two instances of agents where rat histomorphometry has been less conclusive as to what is happening and how it is happening: strontium ranelate and the vitamin D-like agents. Both inhibit bone loss in the OVX rat. Claims abound that both are anabolic. However, repeated studies have produced no overwhelming and uniform data in the form of increased fluorochrome double labeled surface, as seen with PTH. Both represent success of the OVX rat, as human data from strontium ranelate and D-like compounds have also not produced profound evidence in support of anabolism. The inability of histomorphometry to directly document anti-resorptive activity makes things difficult. In OVX rats, it appears possible that D-like compounds enhanced calcium economy, *indirectly* reducing remodeling rate. In the presence of an efficacious BP, D-like compounds did not further suppress resorption; bone formation rate appeared somewhat higher than with BP monotherapy. The adult OVX rat was excellent for bone effects of strontium and D-like compounds in humans.

The majority of published reports in humans suggest that persons taking BPs for significant time periods before trying PTH have a less vigorous response to PTH than untreated people. There is controversy on this point among the recorded human studies. Most OVX rat studies suggest that prior BP treatment does **not** affect the response to an anabolic agent. There are possible reasons for this disparity: 1) the dose of PTH used in rats is relatively greater than the one approved for people, enabling animal dosing to “overpower” BP-related inhibition; 2) the pre-treatment period of BPs used in rats is insufficient to mimic properly the BP buildup that occurs in people taking BPs for multiple years; 3) the small animal may not properly mimic the distribution and redistribution of BP that occurs in the adult human skeleton with its relatively large amount of cancellous and cortical remodeling; 4) the response to PTH may involve both a direct stimulation of osteoblast activity and an indirect effect by increasing remodeling with associated positive bone balance at each remodeling unit. The rat has relatively less remodeling than does the adult human, meaning that most of its response may be through the direct osteoblast route. BPs reduce remodeling rate in adult humans. If remodeling is a significant route for PTH-stimulated formation, since it is inhibited in adult humans with prior BP treatment, it might make sense that it would be more frequent to observe inhibition of PTH response in adult humans than in OVX rats.

Activation Frequency and Erosion Depth: Meaning and Measurement

Robert S. Weinstein, M.D.

Division of Endocrinology and Metabolism, Center for Osteoporosis and Metabolic Bone Diseases, Department of Internal Medicine, Central Arkansas Veterans Healthcare System and the University of Arkansas for Medical Sciences, Little Rock, AR.

Bone remodeling or turnover is carried out by a battalion of juxtaposed osteoclasts (at the front) and osteoblasts (bringing up the rear), comprising temporary anatomical structures known as basic multicellular units (BMUs). In cortical bone, the BMUs drill tunnels or “cutting cones” through the compact tissue while in spongy, cancellous bone; they usually gouge across the trabecular surface forming a serpentine trench. Bone turnover begins by conversion of a quiescent skeletal surface to a remodeling site, a process referred to as activation. The activation frequency (Ac.f) represents the probability that a new remodeling cycle will be initiated at any point on the cancellous perimeter and is calculated by dividing the bone formation rate (BFR = double-labeled perimeter + $\frac{1}{2}$ single-labeled perimeter \times mineral appositional rate) by the average amount of bone formed by a team of osteoblasts per activation event or the wall width (Ac.f = BFR/wall width). The measurement is more than just the rate of BMU origination as it corresponds to the product of the frequency of BMU origination and BMU width, rate of progression and lifespan. Ac.f is the best available 2-dimensional histological index of **the intensity of bone remodeling or turnover** and as such, represents the number of battalions or teams currently in play.

Origination of a BMU involves proliferation of new blood vessels needed to bring recruited resorbing cells to the remodeling site and retraction of the flat, pavement-like cells that cover quiescent perimeters to expose the mineralized bone. The recruited cells become multinucleated osteoclasts, which attach to the newly exposed bone with a ring of contractile proteins sealing off a subosteoclastic resorption compartment. Lysosomal enzymes, hydrogen ions, and collagenase are secreted through the microvilli of the ruffled underside of the osteoclasts and begin to excavate a resorption cavity or bay. Osteoclasts are motile cells, capable of resorbing more than just the cavity within which they are identified. After an osteoclast digs a cavity, it may detach from bone and move on to a new resorption site. When the osteoclasts have moved away, osteoblasts are drafted to reconstitute the previously resorbed cavity with new bone. In any established BMU, both events are happening at the same time; bone formation begins to occur while bone resorption advances. Intermediate between the end of bone resorption and the beginning of bone formation is the reversal phase, when mononuclear phagocytes smooth out the jagged erosion bays. During this phase, the old bone is coated by a thin layer of cement substance, a collagen- and mineral-poor matrix rich in glycosaminoglycans, glycoproteins and acid phosphatase, to which the new osteoblasts attach. During normal bone remodeling, new osteoblasts assemble only at sites where osteoclasts have recently been eroding bone; a phenomenon referred to as coupling. The arrival of the osteoblasts in the right place at the right time and in sufficient numbers is likely due to simultaneous production of osteoblasts and osteoclast in the bone marrow, release of osteotropic substances from resorbed bone and chemotaxis by the cement substance. When the osteoblasts completely reconstitute previously resorbed cavity, the turnover is referred to as balanced.

Treatment with an antiresorptive drug will reduce the Ac.f, stop the addition of new stress risers caused by erosion cavities, and increase the resistance to fracture long before changes in bone mineral density (BMD) are detectable. With additional administration of antiresorptive therapy, the remodeling space will contract “reversing” some temporary bone loss in a few months and, over years, there will be a substantial reduction in the rate of bone loss. However, the determination of the Ac.f does present some issues. Ac.f is usually calculated from a small sample and single skeletal site (a transilial bone biopsy). Sometimes, fluorescent labels may be missing. In addition, even without an increase in the bone formation rate, the Ac.f will increase if the wall width decreases, as it does with age. Furthermore, interpretation of the Ac.f becomes complicated when an anabolic therapy thickens a trabecular profile by adding bone to a previously completed bone structural unit, as occurs with the intermittent administration of parathyroid hormone.

The concept of erosion depth is more important than the problems associated with its measurement. Erosion depth is cumbersome to measure at best but does represent a clear **index of osteoclast vigor**, which is otherwise only rarely apparent from bone histomorphometry. Erosion cavities have an adverse effect on cancellous bone strength disproportionate to the decrease in bone mass that the cavities represent. Furthermore, loss of bone strength due to perforation of trabecular profiles becomes more likely as the erosion depth increases. The final depth of erosion has a wide frequency of distribution but substantial evidence indicates that it decreases with age more noticeably in women than in men. Difficulty with the measurement occurs when erosion cavities have unusual shapes that defy efforts to reconstruct the original bone perimeter. In addition, erosion cavities may be few and inconspicuous in a specimen obtained from an elderly patient with osteoporosis and selection of only the cavities ideal for measurement may cause considerable bias in the measurement. Additional measurements of bone resorption such as erosion area, depth, width, bottom length, and cavity count should be considered but use of the erosion perimeter (2D) or erosion surface (3D) should be avoided. The eroded surface is composed of the osteoclast surface (about 1% of the bone surface in humans) plus the reversal surface (this histological representation of the reversal phase covers about 9% of the bone surface in humans) and, therefore, is often an unfaithful index of bone resorption. The reversal surface increases with defective or delayed bone formation, as occurs with glucocorticoid excess, and thus has little to do with the current amount of bone resorption. Moreover, most antiresorptive drugs decrease the ability of osteoclasts to erode bone, reduce the BFR, and actually increase the reversal surface, thereby, lengthening the so-called erosion surface.

References:

1. Weinstein RS, Manolagas SC. Apoptosis and Osteoporosis. *Am J Med* 108:153-164, 2000.
2. Weinstein RS: Chapter 2: Understanding Bone Histomorphometry: Sampling, Evaluation, and Interpretation. In ES Orwoll (ed) *Atlas of Osteoporosis*, Springer, Philadelphia, PA. 2009:13-19.

3. Parfitt AM: Chapter 5: Skeletal Heterogeneity and the Purposes of Bone Remodeling. In Marcus R, Feldman D, Nelsen DA, Rosen CJ (eds) Osteoporosis 3rd edition, Elsevier-Academic Press, San Diego, CA 2008:71-89.
4. Recker R, Lappe J, Davies KM, Heaney R. Bone remodeling increases substantially after menopause and remains increased in older osteoporosis patients. *J Bone Miner Res* 19:1628-1633, 2004.
5. Sato K, Byers PD. Quantitative study of tunneling and hook resorption in metabolic bone disease. *Calcif Tissue Int* 33:459-466, 1981.
6. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR histomorphometry nomenclature committee. *J Bone Miner Res* 2:595-610, 1987.
7. Weinstein RS, Roberson PK, Manolagas SC. Giant Osteoclast Formation and Long-Term Oral Aminobisphosphonate Therapy. *N Engl J Med* 360:53-62, 2009.
8. Eriksen EF, Mosekilde L, Melsen F. Trabecular bone resorption depth decreases with age: differences between normal males and females. *Bone* 6:141-146, 1985.
9. Cohen-Solal ME, Shih M-S, Lundy MW, Parfitt AM. A new method for measuring cancellous bone erosion depth: application to the cellular mechanisms of bone loss in postmenopausal osteoporosis. *J Bone Miner Res* 6:1331-1338, 1991.
10. Allen MR, Erickson AM, Wang X, Burr DB, Martin RB, Hazelwood SJ. Morphological assessment of basic multicellular unit resorption parameters in dogs shows additional mechanisms of bisphosphonate effects on bone. *Calcif Tissue Int* 86:67-71, 2010.
11. Hernandez CJ. How can bone turnover modify bone strength independent of bone mass? *Bone* 42:1014-1020, 2008.

Remodeling Oversuppression and the Handling of Missing and Single labels

David W. Dempster

Regional Bone Center, Helen Hayes Hospital, West Haverstraw, New York and
Department of Pathology, College of Physicians and Surgeon of Columbia
University, New York, NY, USA

The clinical significance of long-term therapeutic inhibition of bone turnover is unknown. Given the impracticality and ethical problems associated with long-term fracture trials we will be constrained to rely more and more heavily on surrogate markers of bone strength and quality. The iliac crest biopsy will continue to play an important role in this endeavor. The use of tetracycline labels and the measurement of the dynamic parameters of bone formation that they afford allows direct and quantitative assessment of bone turnover rate in the bone biopsy. The theory and practice of administering and measuring tetracycline labels were developed 50 years ago by Frost and others. Usually, a double label is given during the weeks immediately preceding the biopsy with a typical sequence being 3 days ON, 10-12 days OFF and 3 days ON. The biopsy is taken 5 days after the last labeling day. When unstained sections are viewed under polarized light, the labels that were incorporated at sites of new bone formation can be visualized and quantified.

There are three categories of labels depending on the prevailing rate of new bone formation: double, single or absent. When bone formation rate is normal or elevated and there is no inhibition of mineralization, both double and single labels are usually present and the quantification of dynamic parameters is straightforward. The extent of double and single labels can be measured to obtain the mineralized perimeter (Md.Pm) and the average distance between the double labels can be measured and divided by the inter-label interval to obtain the mineral apposition rate (MAR). The bone formation rate (BFR) can then be calculated as the product of Md.Pm and MAR.

Excluding conditions with mineralization defects, when the bone turnover rate is low it is not uncommon to find only single labels and perhaps no labels in the biopsy. In this situation the quantification of dynamic parameters becomes more complicated and there is no general consensus on how it should be done. Some authors (1,2) suggest that if only single labels are present in a biopsy, MAR should be assigned a value that approximates the lowest measurable value in the population under study. This allows BFR to be calculated and datasets do not become imbalanced by the exclusion of samples with only single labels from group means. Sometimes there are no double labels present in the cancellous envelope but there are some in the intracortical or endocortical envelopes. In that case, the measured value for MAR in one of these envelopes could be used to calculate BFR in cancellous bone (1). Other authors elect to exclude subjects with only single labels from the calculation of BFR. This has been the case in several studies involving potent antiresorptive agents.

Our group recently compared the two most common methods (i.e., imputation for MAR or exclusion) for dealing with the single label phenomenon in alendronate-treated subjects (3). We compared the values obtained using both methods in 46 subjects who were either treated with

alendronate (ALN) or were treatment-naïve (Rx-naïve). Following tetracycline labeling biopsies were obtained from 46 postmenopausal women, aged 66.1 ± 1.3 years, with low bone mass and/or fractures who had been treated with ALN ($n=24$) for 6.6 ± 0.6 years or from 22 treatment-naïve subjects ($n=22$). MAR, Md.Pm and BFR were quantified using 2 methods: by imputing a value of 0.3 microns/day for subjects with only single labels and a value of 0 for subjects with no labels or excluding these subjects from the calculation of group mean values. MAR and BFR were significantly lower with the imputation method in the ALN group, whereas there were no differences between the results obtained by the 2 methods in the Rx-naïve group. Furthermore, MAR was significantly lower in ALN-treated versus Rx-naïve by the imputation method. We concluded from this study that the method used to evaluate MAR has a profound effect on the results for MAR and BFR and can lead to a different conclusion regarding the effects of ALN on MAR. We recommended that the method used be clearly stated in all publications and that, preferably, results obtained by both methods be presented.

The intent at the 2010 Sun Valley meeting is to discuss this issue with the panel and attendees in the hope of reaching a consensus recommendation on how to deal with the issue of single and missing labels should be handled in future clinical studies. This recommendation will have important implications for the interpretation of future studies of potent antiresorptive agents and evaluating the clinical significance of long-term therapeutic inhibition of bone turnover.

References

1. Foldes J, Shih MS, Parfitt AM. Frequency distributions of tetracycline-based measurements: implications for the interpretation of bone formation indices in the absence of double-labeled surfaces. *J Bone Miner Res.* 1990;5:1063-7.
2. Hauge E, Mosekilde L, Melsen F. Missing observations in bone histomorphometry on osteoporosis: implications and suggestions for an approach. *Bone.* 1999;25:389-95.
3. Dempster D, Zhou H, Bostrom M, Nieves J, Cosman F, Lindsay R. To impute or not to impute: That is the question. *J Bone Miner Res* 2009;24, Suppl. 1:S (abstract)

Raman Spectroscopic Measures of Bone Quality and Function

Michael D. Morris, Department of Chemistry, University of Michigan, Ann Arbor, MI

Raman spectroscopy provides mineral and matrix composition information that are important inputs into bone quality (1). Raman spectroscopy can be performed on cells and fresh tissue as well as on fixed and embedded specimens and even on human subjects and live animals. Cell or tissue cultures can be followed over time.

With standard Raman microscopy instruments the spatial resolution is 0.5-1 micron. Low definition mapping and higher definition imaging are possible. In maps or images contrast is based on band intensities or on derived parameters such as band intensity ratios. With fiber optic probes non-invasive Raman spectra can be obtained on humans or animals at depths below the skin exceeding 1 cm, and in favorable cases 2 cm. Low definition two- and three-dimensional mapping has been demonstrated. However, validation of Raman markers of bone composition is just beginning (2), while validation is well-advanced in Fourier transform infrared (FTIR) spectroscopy. Additionally, the differing physics of Raman scattering and infrared absorption means that relative band intensities differ between the two methods of vibrational spectroscopy, especially for the mineral components.

Non-invasive spectroscopy and imaging are attractive for animal studies (3), because they allow measurements on the same animals over time. Our laboratory is currently investigating applications to autograft osseointegration and fracture healing in rodents. The same techniques can be used to monitor, for example, the effects of pharmaceuticals or diet on changes in composition. It is necessary to use custom-made fiber optic probes designed to account for the extensive turbidity of tissue. With such probes, low resolution mapping and even Raman tomography have been demonstrated (3). There have been brief preliminary reports of non-invasive bone Raman spectra from cadavers and even a live subject, but systematic studies have not yet appeared.

We have also shown that there are composition differences in the undamaged tissue from hip replacement subjects who suffered an osteoporotic fracture and tissue from the same site of controls (cadaveric) matched for gender, age and bone volume fraction who died from causes unrelated to osteoporosis or other bone disorders. As part of a larger study of several physical/chemical techniques, an ongoing study is testing the hypothesis that Raman spectra contain fracture risk as well as diagnostic information.

With polarized light Raman microspectroscopy can provide independent orientation and orientation distribution information on bone mineral and bone matrix orientation (5,6). Excised specimens are needed, but no special instrumentation is required. Such information supplements the composition information available from Raman spectroscopy and may be especially important for evaluation of biomechanical properties of bone. However, Raman spectroscopy probes the exposed surface of the tissue specimen only, while biomechanical properties are determined by bone architecture and composition at all scales and throughout the depth of the tissue.

References:

1. Morris, M.D., in *Emerging Raman Applications and Techniques in Biomedical and Pharmaceutical Fields*, Matousek, P. and Morris, M.D., Eds., Springer, Berlin, 2010.
2. Gourion-Arsiquaud, S. et al. 2009 *J Bone Miner Res* 24(7):1271-9.
3. Schulmerich, M.V. et al. 2009, *Appl. Spectrosc* 63(3):286-295.
4. Schulmerich, M. V. et al. 2008, *J Biomed Optics* 13(2):020506.
5. Kazanci, M. et al. 2006, *J Struct Biol* 156(3):489-496.
6. Raghavan, M. et al 2010, *J Biomed Optics* 15(3):037001.

Osteocyte-Independent Mechanotransduction of Interstitial Fluid Flow

Ronald Y. Kwon, Diana R. Meays, Alexander S. Meilan, Natalie L. Kardos, John A. Frangos

Bone contains a porous network of canaliculi that has been shown to facilitate substantial and rapid transcortical interstitial fluid flow (IFF) [1]. This fluid flow originates from leaky venous sinusoids in the intramedullary cavity and is driven radially outward through cortical bone by a transmural pressure gradient between the endosteal vasculature and the lymphatic drainage at the periosteal surface [1-3]. Under mechanical bending or compressive loads, pressure gradients are created that drive fluid from areas of compression to areas of tension which rapidly accelerate fluid at rates of the order of 6 milliseconds [4-5]. High impact exercise such as running and jumping will drive rapid fluid flow, and with associated relaxation phases after loading, results in highly oscillatory flow.

It has been hypothesized that changes in IFF due to intraosseous pressure changes influence bone remodeling [6-9]. Numerous investigations into flow effects on osteoblasts in vitro have shown that osteoblasts exposed to flow exhibit increased prostaglandin E₂ and nitric oxide release [10-11]. Fluid shear stress potently stimulates nitric oxide production in pre-osteoclasts as well [12].

It has been speculated that skeletal adaptation to mechanical loading involves IFF stimulation of osteocytes. Recently, Tatsumi and co-workers generated mice possessing a diphtheria toxin (DT) receptor transgene driven by the DMP1 promoter (DMP1-DTR), allowing for inducible osteocyte ablation by administration of DT [13]. While these authors found that osteocyte ablation conferred resistance to bone loss upon hindlimb suspension (HLS), mechanotransduction upon reloading was normal, giving rise to the intriguing possibility that loading-induced IFF may be sensed by cells other than osteocytes.

We recently developed a microfluidic system for modulating femoral intramedullary pressure (ImP) in alert mice, and showed that the generation of dynamic ImP significantly increased IFF within lacunae and protected against bone loss in mice subjected to HLS [14]. Using this system, we investigated the effects of osteocyte ablation on IFF-induced adaptation. 16wk F wildtype (WT) and transgenic (Tg) DMP1-DTR mice were subjected to HLS for 14d. One limb was exposed to dynamic ImP/IFF (3min/d, 5Hz, peak flow rate: 5 μ L/s); the other limb served as a sham control. Mice were administered DT (10 or 50 μ g/kg) 1d prior to HLS and a booster 7d later. BMD and structural indices were quantified at the lesser trochanter using pQCT and uCT [2]. Osteocyte ablation was confirmed by observing empty lacunae (~30%) in H&E-stained sections from Tg mice. In both WT and Tg mice, we observed significant gains in BMD, trabecular volume fraction (BV/TV), cortical thickness (Ct.Th), and cortical area (Ct.Ar) in limbs exposed to flow compared to sham controls (Table 1). In addition, a significant increase in trabecular thickness (Tb.Th) was observed in Tg mice. Interestingly, relative gains (i.e., flow-no flow) in all parameters were greater in Tg mice, indicating that osteocyte ablation did not affect, or even enhanced skeletal adaptation to flow. In particular, rBMD and rTb.Th were significantly different between WT and Tg mice administered 10 or 50 μ g/kg DT (Table 1).

Taken together, osteocyte ablation does not abrogate skeletal adaptation to dynamic ImP/IFF, suggesting that this response occurs independently of flow-induced stimulation of osteocytes. One cellular target of ImP/IFF may be osteoclastic resorption. Support for this comes from in vitro observations of shear-induced nitric oxide production in pre-osteoclasts, possibly leading to autocrine inhibition of resorption. The role of osteocytes in mechanotransduction in bone remains to be defined.

In addition, osteocyte ablation may enhance the response to dynamic ImP/IFF, perhaps by altering the function and/or number of other types of cells within bone.

References:

- [1] Montgomery, R.J., Sutker, B.D., Bronk, J.T., Smith, S.R. and Kelly, P.J. (1988). Interstitial fluid flow in cortical bone. *Microvasc Res* 35: 295-307.
- [2] Kelly, P.J. (1983). Pathways of transport in bone, Williams and Wilkins.
- [3] Dillaman, R.M., Roer, R.D. and Gay, D.M. (1991). Fluid movement in bone: theoretical and empirical. *J Biomech* 24 Suppl 1: 163-177.
- [4] Piekarski, K. and Munro, M. (1977). Transport mechanism operating between blood supply and osteocytes in long bones. *Nature* 269: 80-82.
- [5] Qin, Y.X., Kaplan, T., Saldanha, A. and Rubin, C. (2003). Fluid pressure gradients, arising from oscillations in intramedullary pressure, is correlated with the formation of bone and inhibition of intracortical porosity. *J Biomech* 36: 1427-1437.
- [6] Reich, K.M., Gay, C.V. and Frangos, J.A. (1990). Fluid shear-stress as a mediator of osteoblast cyclic adenosine-monophosphate production. *J Cell Physiol* 143: 100-104.
- [7] Reich, K.M. and Frangos, J.A. (1991). Effect of Flow on Prostaglandin-E2 and Inositol Trisphosphate Levels in Osteoblasts. *American Journal of Physiology* 261: C428-C432.
- [8] Hillsley, M.V. and Frangos, J.A. (1994). Bone tissue engineering: the role of interstitial fluid flow. *Biotechnol Bioeng* 43: 573-581.
- [9] Turner, C.H., Forwood, M.R. and Otter, M.W. (1994). Mechanotransduction in bone: do bone cells act as sensors of fluid flow? *FASEB J* 8: 875-878.
- [10] Johnson, D.L., McAllister, T.N. and Frangos, J.A. (1996). Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts. *Am J Physiol* 271: E205-208.
- [11] McAllister, T.N. and Frangos, J.A. (1999). Steady and transient fluid shear stress stimulate NO release in osteoblasts through distinct biochemical pathways. *J Bone Miner Res* 14: 930-936.
- [12] McAllister, T.N., Du, T. and Frangos, J.A. (2000). Fluid shear stress stimulates prostaglandin and nitric oxide release in bone marrow-derived preosteoclast-like cells. *Biochem Biophys Res Commun* 270: 643-648.
- [13] Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K. (2007). Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab.*;5:464-75.
- [14] Kwon RY, Meays DR, Tang WJ, Frangos JA. Microfluidic enhancement of intramedullary pressure increases intersitital fluid flow and inhibits bone loss in hindlimb suspended mice. *J Bone Miner Res.* 2010 Feb 23.

WT (n=15)	Tg+10ug/kg DT (n=11)	Tg+50ug/kg DT (n=5)	One-way ANOVA
-----------	-------------------------	------------------------	------------------

Parameter (flow – no flow)	rBMD (mg/ccm)	15.7±4.0**	18.9±6.9*	48.2±17.0*	p<0.05
	rBV/TV (%)	5.8±1.0***	7.0±1.5***	7.0±1.3***	NS
	rTb.Th (um)	0.3±3.1	13.0±3.0**	19.2±1.6***	p<0.01
	rCt.Th (um)	10.9±3.7*	16.2±3.7**	17.3±6.5	NS
	rCt.Ar (mm ²)	0.07±0.02**	0.09±0.02**	0.09±0.03	NS

Table 1. Osteocyte ablation does not affect or enhances skeletal adaptation to dynamic IFF. Values (presented as mean±SE) are relative differences between limbs exposed to dynamic IFF vs. contralateral controls (i.e., flow – no flow) for WT and Tg mice administered 10 or 50ug/kg DT. For WT mice, no differences in relative values were observed for mice administered 10 or 50ug/kg DT, thus for statistical analysis these groups were combined. *, **, or *** indicate p<0.05, p<0.01, or p<0.001 obtained using a one-sample t-test with an assumed zero mean. One-way ANOVA revealed a statistically significant difference between groups (i.e., WT, Tg+10ug/kg DT, and Tg+50ug/kg DT) for rBMD and rTb.Th.

Issues in Modern Histomorphometry: 50 Years Later

Robert R. Recker

Osteoporosis Research Center, Creighton University

This session was occasioned by recent developments in human bone histomorphometry associated with drug interventions that cause marked reduction in bone remodeling. We titled it, “Issues in Modern Histomorphometry: 50 Years Later”, to honor the milestone publication by Dr. Harold Frost in 1960. In the *Henry Ford* bulletin, he published a single case of a 57 year old male in which he examined a lower extremity surgical specimen that had tetracycline labels in it from pre-surgical tetracycline administration for infection. This was the first attempt at calculating dynamic remodeling from a fluorochrome tissue-time marker, tetracycline. Subsequently, in the 60s and early 70s, it became apparent that the only practical sampling location for living human bone histomorphometry was the transiliac bone biopsy described by Bordier in 1964. In the ensuing decade or so, the stereological theories, the histological measurements, and the calculations were worked out in order to provide information on variables such as activation frequency, mineral apposition rates and bone formation rates. In the process, we learned much about bone remodeling, and bone biology in general, in humans and vertebrate animals.

With the advent of remodeling suppression therapy in humans with osteoporosis, we began to see biopsies that had little or no tetracycline label. This created difficulties in interpretation. These have resulted in planning for the current session which will try to establish reasonable consensus for a number of questions. These include; how to express and interpret bone forming surface in the absence of label in standard sections areas, what is the value of extended label searches in specimens, how to calculate mineral apposition rate, and activation frequency. Other questions include what is sufficient section area sampling, and cortical bone sampling. All of these, and more, have become very important in the past two decades. This session will have an overview plus three presentations, all directed at answering a list of questions provided for the audience. In addition, there will be nearly two hours for open discussion with the panelists with audience participation. The object is to come to consensus regarding the questions posed by animal and human biopsies obtained in the presence of treatment with agents that suppress remodeling, and to refine our understanding of the strengths and limitations of human and animal bone histomorphometry. The following is a list of questions that will be discussed:

1. How should we express and interpret forming surface (MS/BS) in the absence of label in the standard section area?
2. Is an “extended label search” worth doing in the absence of label in the standard section area? If label is found, how should it be expressed?
3. How should mineral apposition rate (MAR) be expressed in the absence of double label in the standard section area?
4. Should MAR ever be imputed?
5. Should MAR be measured and reported if found only on extended label search?
6. What is an adequate sample of double label width measurements for a reliable estimate of MAR?
7. How should we interpret the calculation of activation frequency? Is there a problem with the assumptions required? Is it a valid expression of remodeling rate?
8. What is an adequate section area sample? 40mm^2 ?
9. Should we examine cortical bone in human biopsies? What variables?

10. What is the confidence in extrapolations from a transiliac biopsy (1/14,000 of the skeleton) to the entire skeleton?
11. Can we define “anabolic” in histomorphometric terms?
12. What is the histomorphometric definition of “over-suppression” of remodeling (or “excessive remodeling)?
13. Can we measure “erosion depth” reliably in human biopsies, and is it valuable to do so?
14. Is the “obliquity correction” required for width measurements?

Changes in Bone Structure During Growth and Aging

Sundeep Khosla, M.D. Mayo Clinic, Rochester, Minnesota

Using both conventional and high resolution quantitative computed tomography (QCT) imaging as well as mouse studies, our group has focused on better defining bone structural changes during growth and with aging. In adolescence, the most common site of fracture is the distal forearm, with peak incidence at the pubertal growth spurt (1). Our previous study in Rochester, MN revealed that the incidence of forearm fractures increased by 32% in boys and 56% in girls over the past 30 years (2). Since 25-50% of adult bone mass is accumulated during the pubertal growth spurt, adolescents today may be at increased risk of osteoporotic fracture later in life.

While previous studies have used DXA to assess changes in bone mass during growth, DXA measurements are confounded by bone size and are unable to differentiate cortical from trabecular bone. Moreover, standard peripheral QCT (pQCT) has an in vivo resolution of ~400 μm and thus cannot assess bone microarchitecture or evaluate bone strength. Thus, we studied healthy 6 to 21 year-old girls ($n = 66$) and boys ($n = 61$) using high-resolution pQCT (HRpQCT, voxel size, 82 micrometers) at the distal radius (3). Subjects were classified into 5 groups by bone-age: Group I (pre-puberty, 6-8 yrs), Group II (early puberty, 9-11 yrs), Group III (mid-puberty, 12-14 yrs), Group IV (late puberty, 15-17 yrs) and Group V (post-puberty, 18-21 yrs). Compared to Group I, trabecular parameters (bone volume fraction, trabecular number and thickness) did not change in girls, but increased in boys from late puberty onwards. Cortical thickness and density decreased from pre- to mid-puberty in girls, but were unchanged in boys, before rising to higher levels at the end of puberty in both sexes. Total bone strength, assessed using micro-finite element models, increased linearly across bone age groups in both sexes, with boys showing greater bone strength than girls after mid-puberty. The proportion of load borne by cortical bone, and the ratio of cortical to trabecular bone volume, decreased transiently during mid- to late-puberty in both sexes, with apparent cortical porosity peaking during this time. This mirrors the incidence of distal forearm fractures in prior studies. These findings thus demonstrated that regional deficits in cortical bone may underlie the adolescent peak in forearm fractures. Whether these deficits are more severe in children who sustain forearm fractures or persist into later life warrants further investigation.

In parallel studies, we used HRpQCT imaging to define, in a relatively large ($n = 602$) population-based sample of women and men spanning a broad age range (21 to 97 years), sex and age effects on bone microarchitecture at the wrist (4). We found that relative to young women (age 20-29 years), youngmen had greater trabecular bone volume/tissue volume (BV/TV, by 26%, $P = 0.001$) and trabecular thickness (TbTh, by 28%, $P < 0.001$) but similar values for trabecular number (TbN) and trabecular separation (TbSp). Between ages 20 and 90 years, cross-sectional decreases in BV/TV were similar in women (-27%) and in men (-26%), but whereas women had significant decreases in TbN (-13%) and increases in TbSp (+24%), these parameters had little net change over life in men (+7% and -2% for TbN and TbSp, respectively, $P < 0.001$ vs. women). However, TbTh decreased to a greater extent in men (-24%) than in women (-18%, $P = 0.010$ vs. men). These findings demonstrated that while decreases with age in trabecular BV/TV are similar in men and women, the structural basis for the decrease in trabecular volume is quite different between the sexes. Thus, over life, women undergo loss of trabeculae with an increase in TbSp, whereas men begin young adult life with thicker trabeculae and primarily sustain trabecular thinning with no net change in TbN or TbSp. Since decreases in TbN have been shown to have a much greater impact on bone strength as compared to decreases in TbTh, these findings may help explain the lower life-long risk of

fractures in men, and specifically, their virtual immunity to age-related increases in distal forearm fractures.

In cross-sectional (5) and longitudinal (6) studies using QCT at multiple sites (spine, hip, wrist, tibia), we further demonstrated that cortical bone loss begins in middle life in women and around age 70 years in men, concomitantly with, and probably due to, the previously documented menopausal and late-life decreases in sex steroids in each sex, respectively. However, in both cross-sectional and longitudinal studies, we found that trabecular bone loss at multiple sites begins in both sexes in the 3rd decade, during sex steroid sufficiency. Thus, while trabecular bone loss is accelerated by sex steroid deficiency (e.g., menopause in women), a substantial proportion of trabecular bone loss over life is independent of changes in sex steroids.

In recent mouse studies (7), we have attempted to better define the role of estrogen deficiency in age-related trabecular versus cortical bone loss. While female mice do not have the equivalent of a menopause, they do undergo reproductive senescence. Thus, to dissociate effects of aging versus estrogen deficiency on age-related bone loss, we sham operated, ovariectomized, or ovariectomized and estrogen replaced female C57/BL6 mice at 6 months of age and followed them to age 18-22 months. Lumbar spines and femurs were excised for analysis. Six month old intact control mice were sacrificed to define baseline parameters. Compared to young mice, aged/sham mice had a 42% reduction in lumbar spine bone volume/total volume (BV/TV), and maintaining constant estrogen levels over life in ovariectomized/estrogen-treated mice did not prevent age-related trabecular bone loss at this site. By contrast, life-long estrogen treatment of ovariectomized mice completely prevented the age-related reduction in cortical volumetric BMD and thickness at the tibial diaphysis present in the aged/sham mice. These data thus demonstrate that, in mice (as in humans), age-related loss of cortical bone in the appendicular skeleton is potentially related to estrogen deficiency, whereas trabecular bone loss, while accentuated by estrogen deficiency, is largely independent of estrogen. Further studies in rodents and in humans are needed to define the cause(s) of trabecular bone loss over life independent of changes in sex steroid levels.

References:

1. Bailey DA, Wedge JH, McCulloch RG, Martin AD, Bernhardson SC 1989 Epidemiology of fractures of the distal end of the radius in children as associated with growth. *J Bone Joint Surg* **71-A**:1225-1231.
2. Khosla S, Melton LJ, III, Dekutoski MB, Achenbach SJ, Oberg AL, Riggs BL 2003 Incidence of childhood distal forearm fractures over 30 years: A population-based study. *JAMA* **290**:1479-1485.
3. Kirmani S, Christen D, van Lenthe GH, Fischer PR, Bouxsein ML, McCready LK, Melton LJ, Riggs BL, Amin S, Muller R, Khosla S 2009 Bone structure at the distal radius during adolescent growth. *J Bone Miner Res* **24**:1033-1042.
4. Khosla S, Riggs BL, Atkinson EJ, Oberg AL, McDaniel LJ, Holets M, Peterson JM, Melton LJ, III 2006 Effects of sex and age on bone microstructure at the ultradistal radius: a population-based noninvasive in vivo assessment. *J Bone Miner Res* **21**:124-131.
5. Riggs BL, Melton LJ, III, Robb RA, Camp JJ, Atkinson EJ, Peterson JM, Rouleau PA, McCollough CH, Bouxsein ML, Khosla S 2004 Population-based study of age and sex differences in bone volumetric density, size, geometry, and structure at different skeletal sites. *J Bone Miner Res* **19**:1945-1954.
6. Riggs BL, Melton LJI, Robb RA, Camp JJ, Atkinson EJ, McDaniel L, Amin S, Rouleau PA, Khosla S 2008 A population-based assessment of rates of bone loss at multiple

- skeletal sites: evidence for substantial trabecular bone loss in young adult women and men. *J Bone Miner Res* **23**:205-214.
7. Syed FA, Mödder UI, Roforth M, Hensen I, Fraser DG, Peterson JM, Oursler MJ, Khosla S. Effects of chronic estrogen treatment on modulating age-related bone loss in female mice. *J Bone Miner Res* (In press).

Probing Alterations in Bone Structure and Composition in Osteoporosis using Synchrotron-Based Imaging

Lisa M. Miller, Brookhaven National Laboratory

Both quantity and quality are determining factors in the strength of bone. During the growth process, inherent defects in the mineralization process may arise which can compromise the quality of bone tissue. These defects may originate at one or more different hierarchical levels, from the macro- to the nanoscale. Regardless of their cause, changes in collagen deposition or mineralization can lead to altered biomechanical properties, such as reduced bone stiffness, hardness or strength. In our ongoing work, synchrotron-based imaging methods are being combined with laboratory-based techniques to directly correlate bone's chemical, structural, and mechanical properties in osteoporosis and treated bone. Specifically, Fourier transform infrared imaging (FTIRI) is used to image chemical properties such as mineralization, crystallinity, and collagen cross-linking. X-ray microdiffraction provides a complementary measure of mineralization and crystallinity whereas x-ray computed tomography is used to generate three-dimensional images of bone architecture with a spatial resolution as small as 30 nm. In this talk, examples will be presented that involve applications of synchrotron-based infrared and x-ray imaging to the study of (1) trabecular and cortical bone in osteoporosis, (2) microdamage in bisphosphate-treated bone, (3) the effects of strontium ranelate on osteoporotic bone, and (4) the effects of risedronate on mineralizing osteoblasts. The goal of this work is to understand how bone chemistry and structure contribute to bone quality in order to improve the diagnosis and treatment of bone disease.

Histone Deacetylases are Crucial for Skeletal Development

Jennifer J. Westendorf, David F. Raziolo, and Meghan E. McGee-Lawrence, Mayo Clinic, Rochester, MN

Histone deacetylases (Hdacs) are enzymatic components of large multi-protein complexes that remove acetyl groups from lysine side chains of histones and other protein substrates. They contribute to epigenetic programming and regulation of gene expression during development and throughout life by interacting with transcription factors, such as Runx2. Pharmaceutical Hdac inhibitors (HDIs) including valproate and suberoylanilide hydroxamic acid (SAHA; vorinostat, Zolinza™) have already demonstrated clinical success as treatments for epilepsy, bipolar disorder, and cancer, and research into the usage of these and other HDIs for treating a wide variety of diseases or clinical conditions (e.g., rheumatoid arthritis, traumatic brain injury, cystic fibrosis) is ongoing; however, the physiological effects of HDIs on bone structure and function are not well understood. In a four-week in vivo study with male C57Bl/6 mice, we found that SAHA (100 mg/kg/d) decreased trabecular bone volume fraction and trabecular number in the distal femur. In a second study, we found that conditional deletion of Hdac3 in osterix-expressing osteoblasts also reduced trabecular bone volume and trabecular number. Adipocyte volume was increased in the Hdac3 conditional knockout mice, but not in the SAHA-treated mice. Microarray analyses of osteoblasts from Hdac3 CKO mice revealed that numerous developmental signaling pathways, including the Wnt pathway, were affected by Hdac3-deficiency. Together these results demonstrate that Hdac inhibition, and in particular Hdac3 suppression, is detrimental to skeletal health. Clinical use of SAHA and other Hdac inhibitors to treat cancer, epilepsy or other conditions may compromise skeletal strength.

IBMS Sun Valley Workshop: Musculoskeletal Biology
August 1 – 4, 2010

