

ASSESSMENT OF BONE TISSUE IN PRECLINICAL STUDIES

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EVERY STEP OF THE WAY

DISCLOSURES

Names and the nature of the financial interest or affiliation in commercial firms that I and our immediate families have with any commercial organization that may have a direct or indirect interest in the subject matter of my presentation:

Charles River Laboratories employee

SKELETAL END-POINTS IN PRECLINICAL STUDIES

- Regulatory Requirements
- Rationale
 - - Limitation of standard techniques
 - - Clinical evidence from different class drugs
- In vivo Assessments
 - Biochemical markers of bone turnover/hormones
 - Radiology
 - Bone densitometry – DXA, pQCT, Micro-CT
 - Advance molecular Imaging
- Ex vivo Assessments
 - Histomorphometry
 - Biomechanical strength testing
- Protocol considerations

ABBREVIATIONS

- FDA – Food and Drug Administration
- EMA – European Medicines Agency
- MHLW – Ministry of Health, Labour and Welfare
- PINP – Procollagen Type I amino-terminal propeptide
- CTx-I – Carboxyl-terminal cross-linking telopeptide of Type I collagen
- NTx-I – Amino -terminal cross-linking telopeptide of Type I collagen
- DXA – Dual-energy X-ray Absorptiometry
- pQCT – peripheral Quantitative Computed Tomography
- Micro-CT – MicroComputed Tomography
- NHP – Non Human Primates
- PET – Positron emission tomography

Regulatory Status

REGULATORY STATUS

- US – FDA: Osteoporosis: Nonclinical Evaluation of Drugs Intended for Treatment Guidance for Industry (Draft Guidance June 2016): “nonclinical studies offer the opportunity to provide indirect information about bone quality through the measurement of bone strength, which is determined by both bone mass and bone quality. ... Biochemical markers of bone resorption and formation dual energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT),.... static and dynamic histomorphometry..... Biomechanical testing ”
- Europe – EMA: Guideline on the evaluation of medical products in the treatment of primary osteoporosis (2007): “ Assess bone architecture and bone strength....Bone mass/density measurements, histomorphometry and biomechanical tests. ”
- Japan – MHLW: Guidelines on Clinical Evaluation of Drugs to Treat Osteoporosis (Notification No. 742 of the Evaluation and Licensing Division, PMSB dated April 15, 1999)

REGULATORY STATUS

•Juvenile Toxicology

- US – FDA: Guidance Document: Nonclinical Safety Evaluation of Pediatric Drug Products (2006) Organ systems of concern include skeleton/growth : “It is important that studies include measurement of overall growth (e.g., body weight, growth velocity per unit time, tibial length)”

- Europe – EMA: Guideline on the need for Nonclinical Testing in Juvenile Animals on Human Pharmaceuticals for Pediatric Indications (2008) Organ systems of concern include skeleton/growth:

- “When addressing overall effects on growth and development, studies should include, at a minimum, measurement of growth”

- Japan – MHLW: Guideline on the Nonclinical Safety Study in Juvenile Animals for Pediatric Drugs (2012) Organ systems of concern include skeleton: “organs/functions growth and development...skeletal system... the effects on development and its reversibility.”

REGULATORY STATUS

- **Juvenile Toxicology Recent regulatory requirements**

- Requests for longer studies, combined juvenile toxicology/ chronic studies (dog studies up to 12 months)
- Requests to study skeletal maturation, muscle/bone growth, bone development with bone density and length, bone quality endpoints of bone mass and density, biomechanical properties
- Requests for measures of bone density in vivo and biochemical markers of bone turnover
- Requests to study specific components of the skeleton (ie. growth plates, dentition)

- **Chronic Toxicology Studies**

- **Carcinogenicity**

THERAPIES AFFECTING SKELETON, SKELETAL GROWTH OR DEVELOPMENT

- **Pharmaceuticals with concerns for skeleton or skeletal development**
 - Corticosteroids (McLaughlin *et al.*, 2002)
 - Retinoids and Vitamin A analogues (Johansson *et al.*, 2002)
 - Anti-viral : Tenofovir (Castillo *et al.*, 2002)
 - Anti-diabetics : PPAR- γ agonists (thiazolidinediones, Kumar *et al.*, 2013), FGF21 (Wei *et al.*, 2012).
 - Proton Pump Inhibitor (Cui *et al.*, 2001)
 - Wnt Inhibitors for cancer therapy
 - β 2-Adrenergic agonists (clenbuterol, Bonnet *et al.*, 2005)
 - Coumarin embryopathy (in utero)
- **Therapies for bone diseases or pediatric growth/bone diseases**
 - Bisphosphonates and Bone Anti-resorptive drugs (Boyce *et al.*, 2014)
 - Bone anabolic agents : PTH (Fox *et al.*, 2008), Anti-sclerostin antibodies (Li *et al.*, 2009)
 - Human growth hormone – GH deficiency
 - Corticosteroids – congenital adrenal hyperplasia
 - Aromatase inhibitors – advanced skeletal maturation (Pouliot *et al.*, 2013)
 - Fibroblast growth factor receptor 3 inhibitor - achondroplasia

Biochemical Markers of Bone Turnover

BIOCHEMICAL MARKERS OF BONE TURNOVER

Markers of Bone Formation

- Osteocalcin
- PINP
- Bone specific Alkaline Phosphatase (BAP)

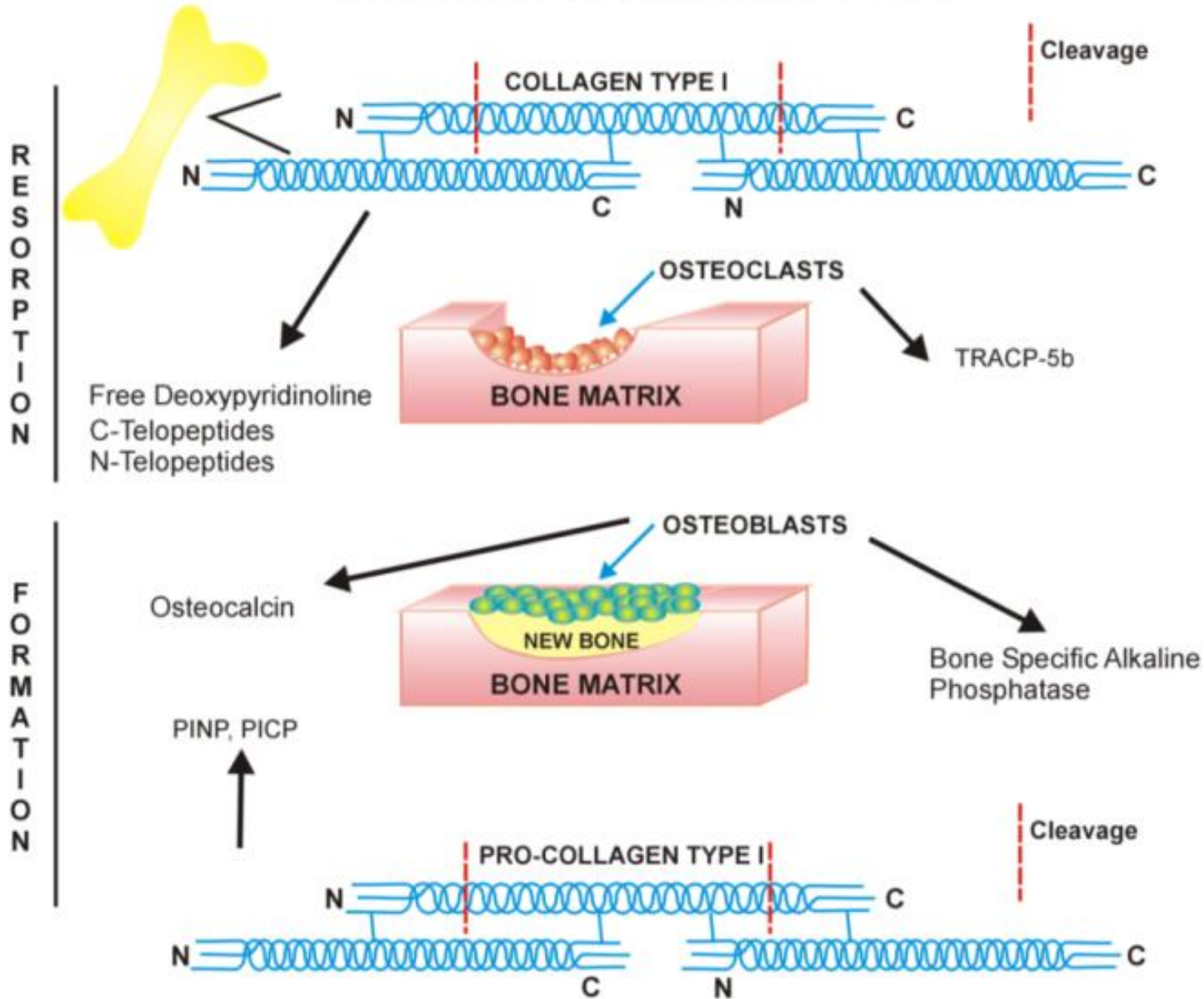
Markers of Bone Resorption

- C-telopeptide (CTX-I)
- N-telopeptide (NTx-I)
- Deoxypyridinoline
- Tartrate-Resistant Acid Phosphate 5b (TRACP 5b)

Reflect bone turnover at the whole skeleton level

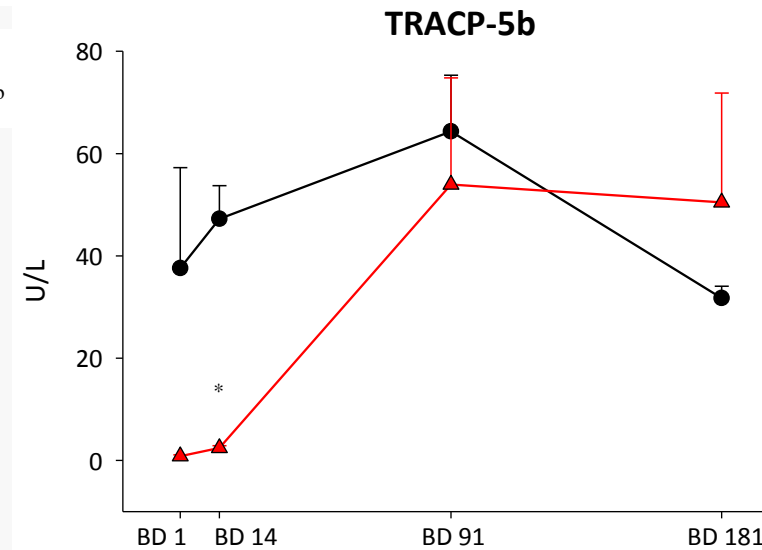
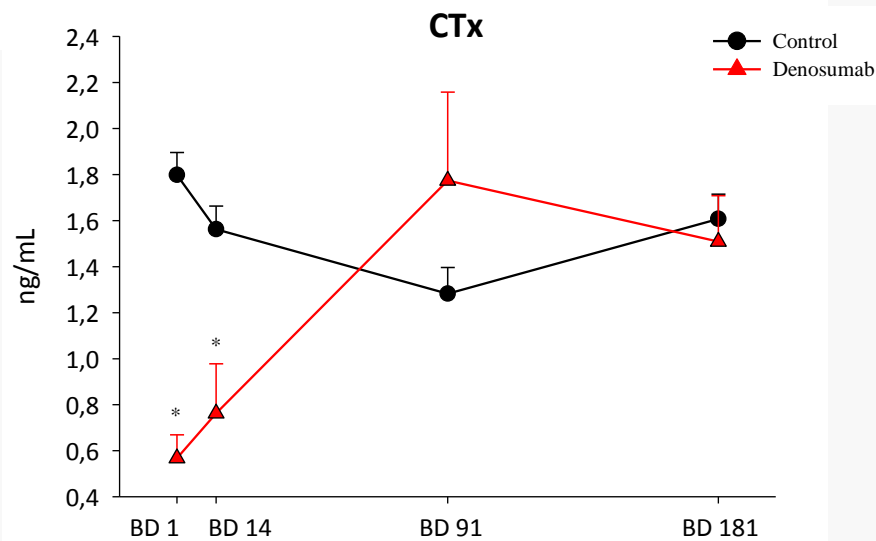
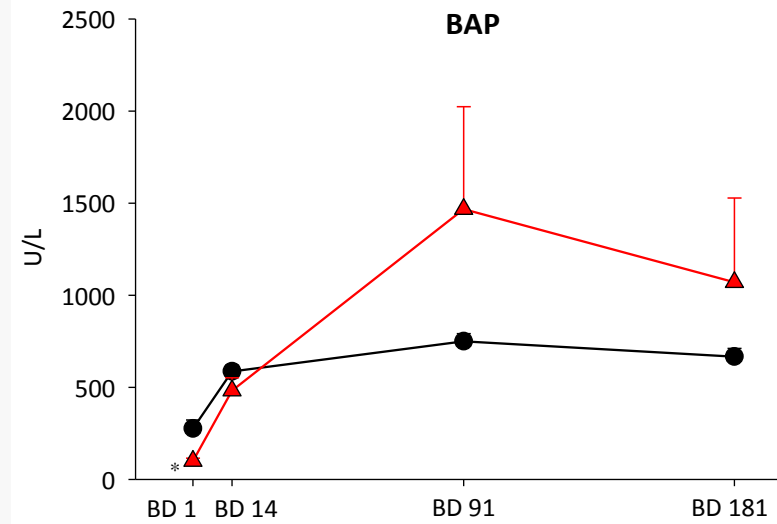
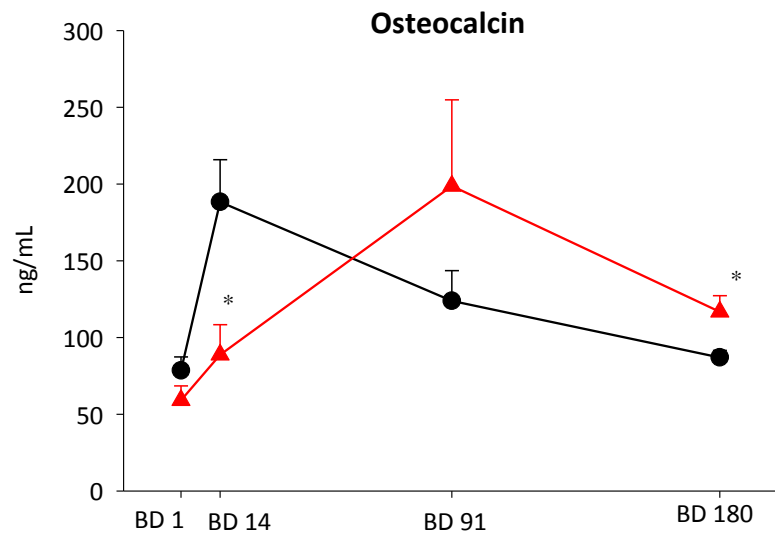
Panel of Markers

SCHEMATIC OF BONE TURNOVER



Gunson, D., Gropp, K.E., Varela, A., 2013. Bone and Joints. In: Haschek, W.M., Rousseaux, C.G., Wallig, M.A. (Eds.), Haschek and Rousseaux's Handbook of Toxicologic Pathology. Elsevier Inc., Academic Press, pp. 2761–2858. With permission.

BIOCHEMICAL MARKERS OF BONE TURNOVER



Mean (SEM), *p ≤ 0.05.

CALCIOTROPIC HORMONES AND SPECIFIC CLINICAL PATHOLOGY PARAMETERS

1,25-dihydroxyvitamin D

25-hydroxyvitamin D

parathyroid hormone

FGF23

calcitonin

calcium/phosphorus balance

BIOCHEMICAL MARKERS OF BONE TURNOVER

Pros

- Real-time, rapid and sensitive read-out of bone turnover activity at the whole skeletal level
- Depending on the target and type of molecule, can be used as a PD marker and a sensitive indicator if ADA affects activity
- All study types

Cons

- Variation depending on the age of the test system
- Window of opportunity
- Diurnal variation and fasting
- Sample volume limitations: blood and urine
- Expensive if multiple markers are measured at several time-points

In vivo Assessments Radiology

IN VIVO ASSESSMENTS - RADIOLOGY

Radiography – long bones, vertebrae, whole body (rodent)

- Identification of skeletal abnormalities, which is particularly important if lesions are focal, small or internal and may not be captured by standard scanning or histology sites
- Permanent and verifiable record of skeletal growth



Modified from Jolette et al. (2006) Defining a noncarcinogenic dose of recombinant human parathyroid hormone 1-84 in a 2-year study in Fischer 344 rats, *Toxicol. Pathol.* 34, pp. 929–940

IN VIVO ASSESSMENTS - RADIOLOGY

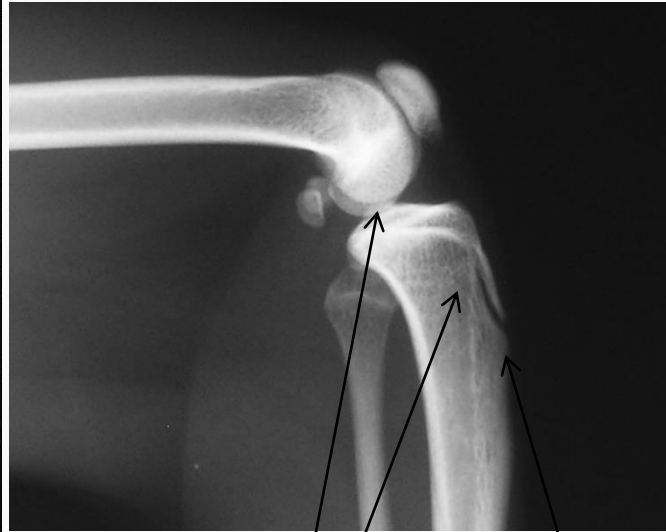
Scoring of growth plate closure

2.0 to 3.5 years



Open growth plate

4 to 5 years



Physeal scar

Open growth plate

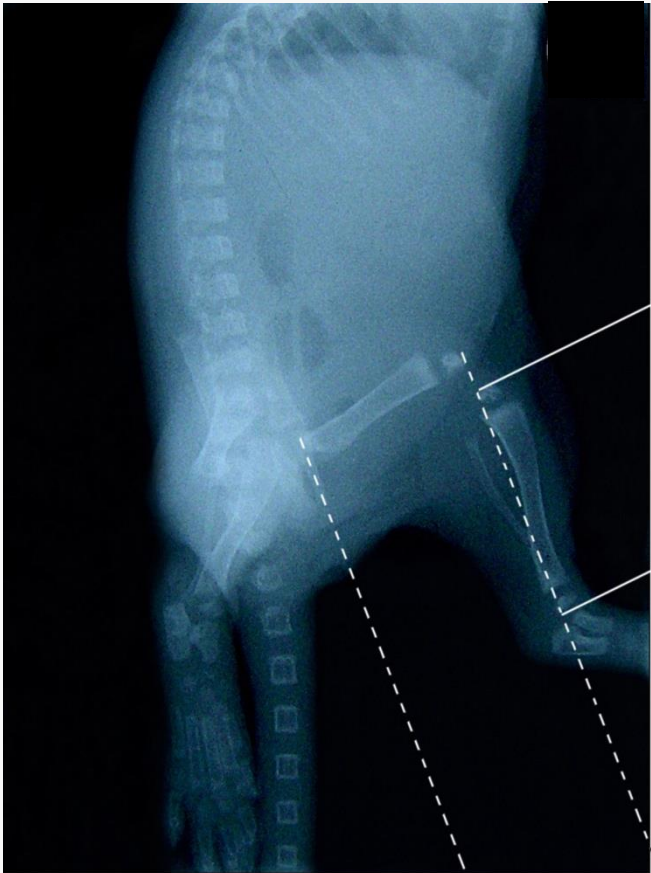
More than 9 years



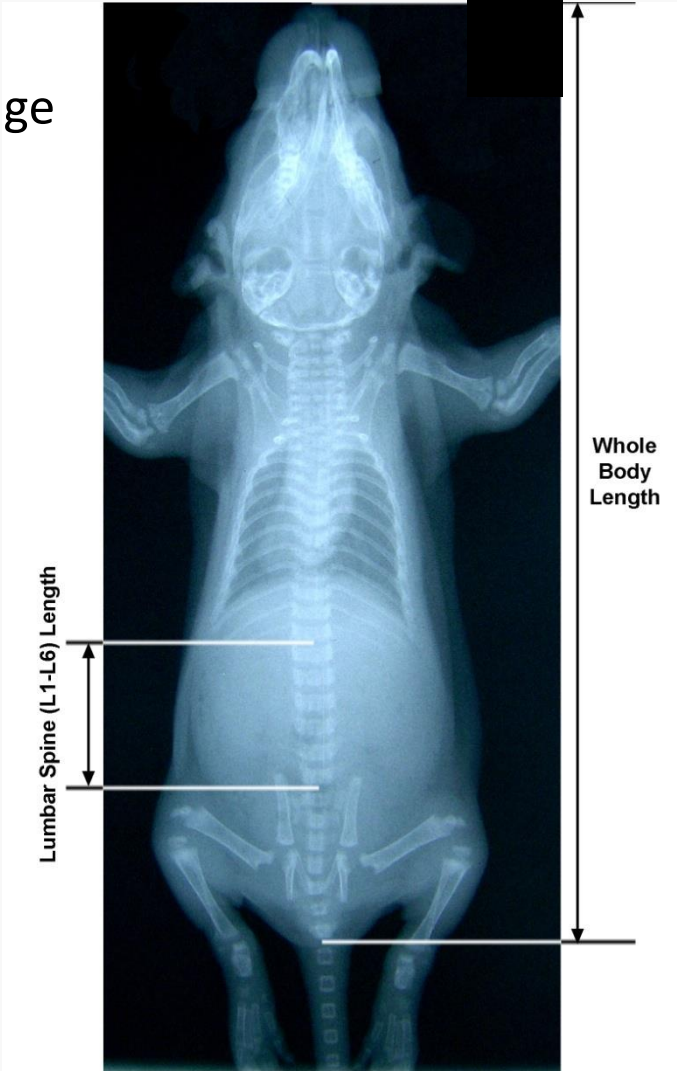
No physis visible

RADIOLOGY – SKELETAL GROWTH

Rat pup 14 days of age

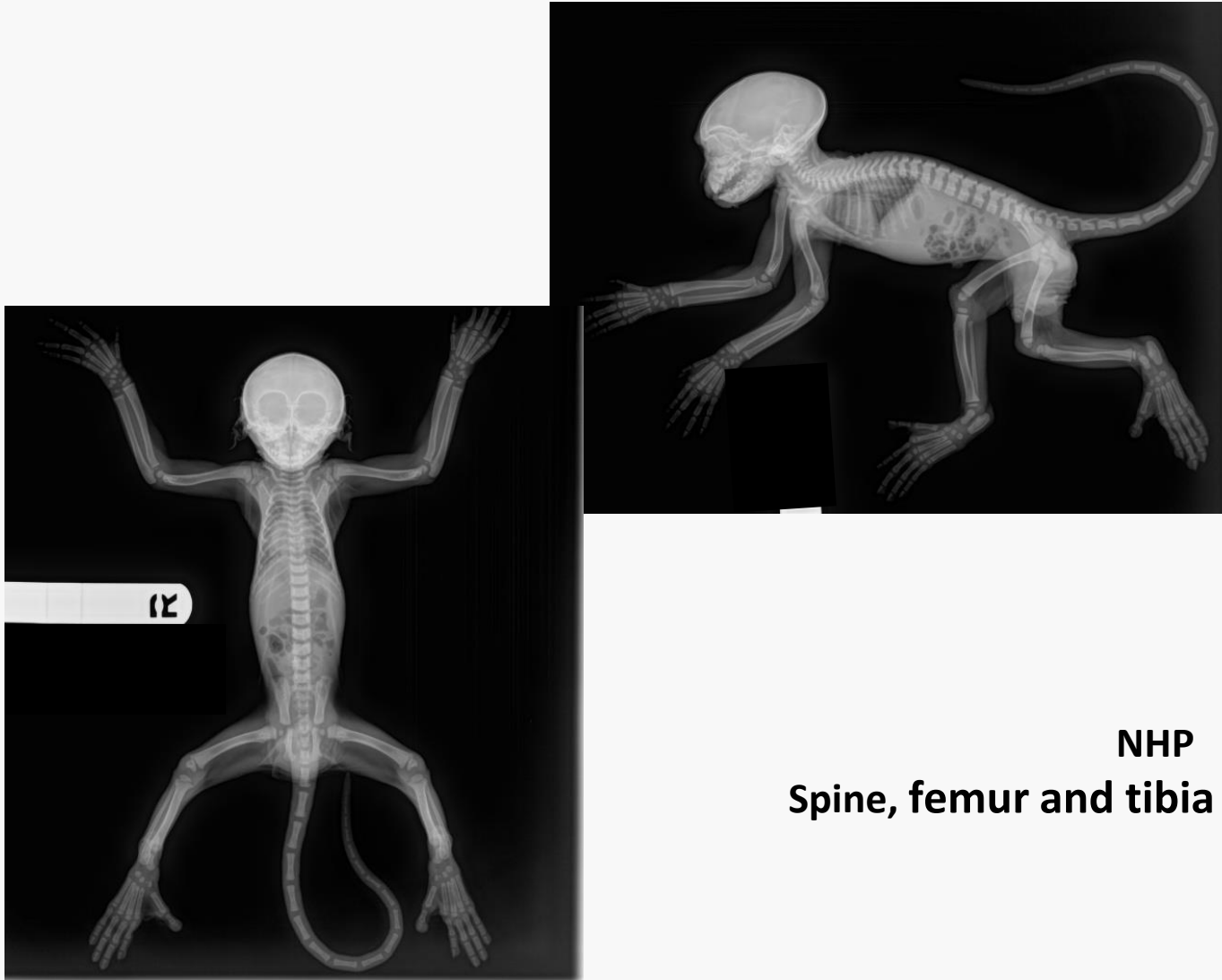


Femur and tibia



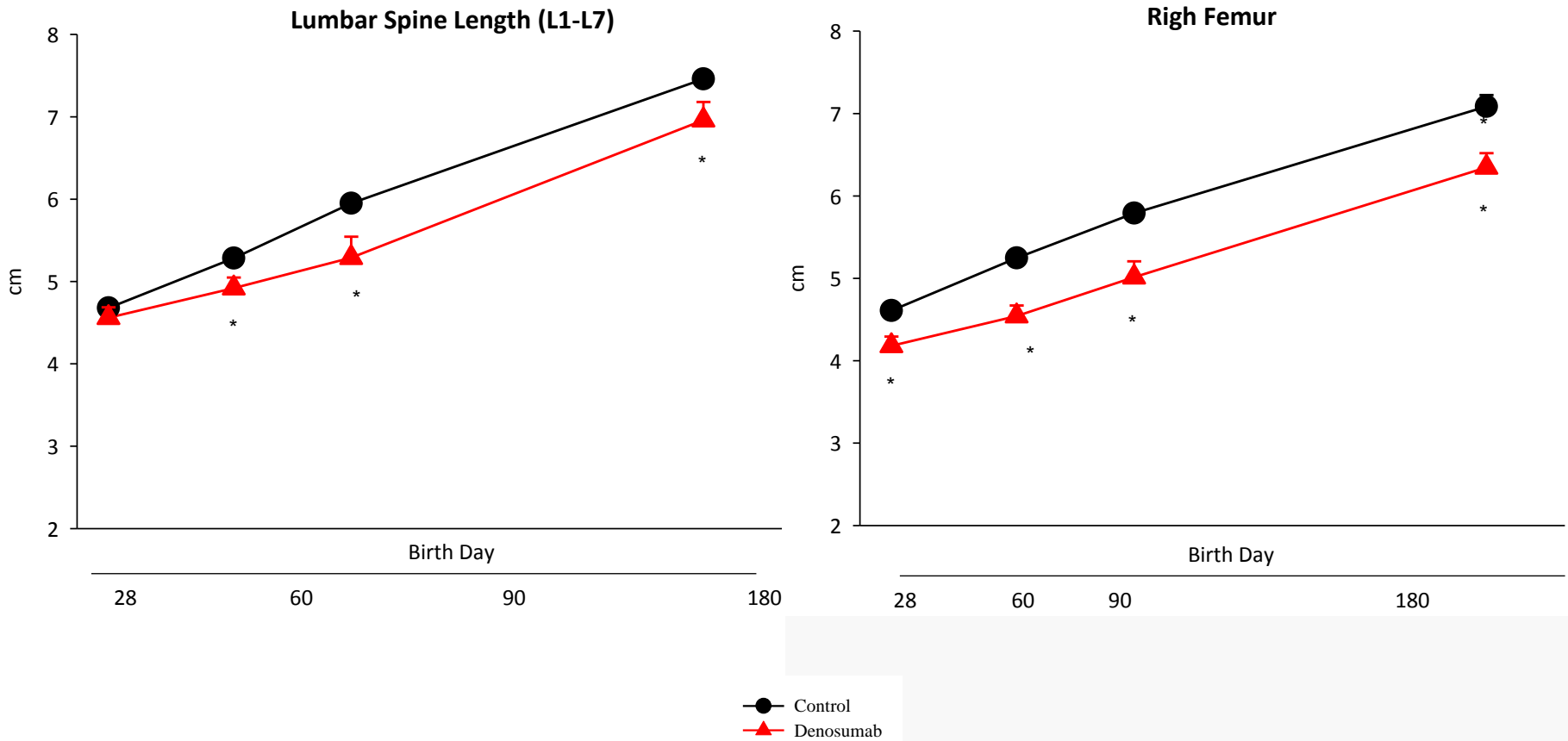
Whole body and Lumbar spine

RADIOLOGY – SKELETAL GROWTH



NHP
Spine, femur and tibia measurements

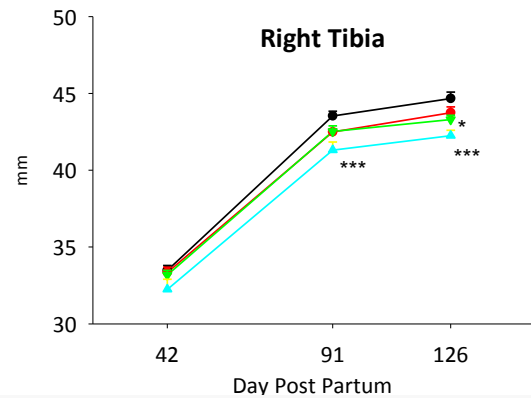
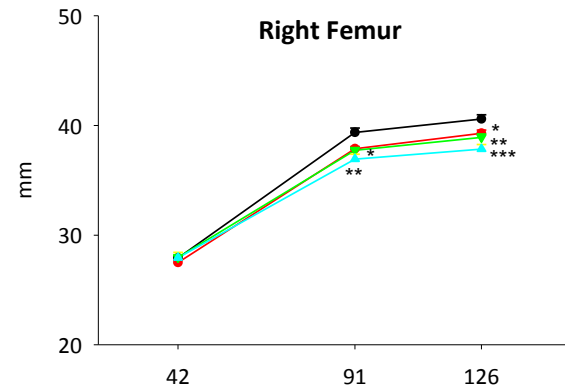
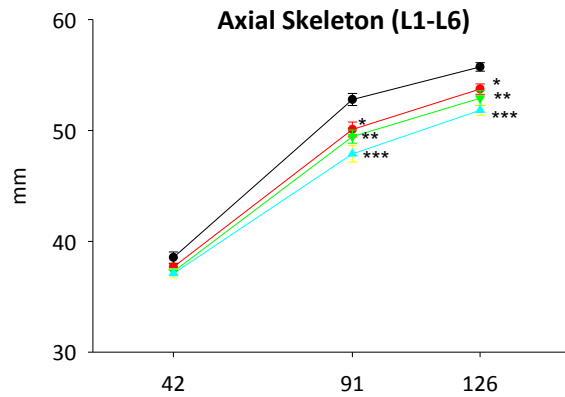
RADIOLOGY – SKELETAL GROWTH



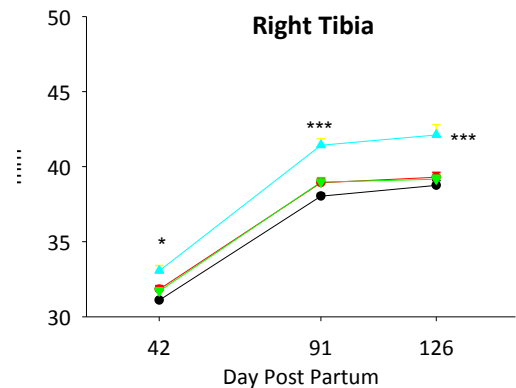
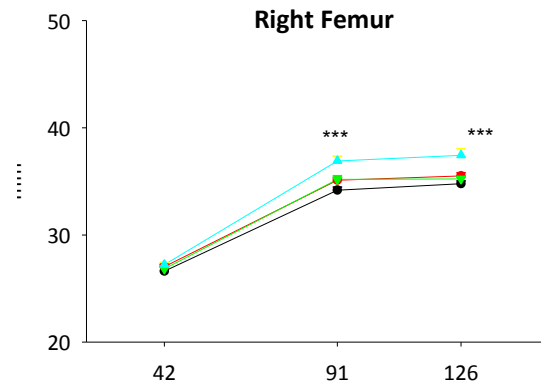
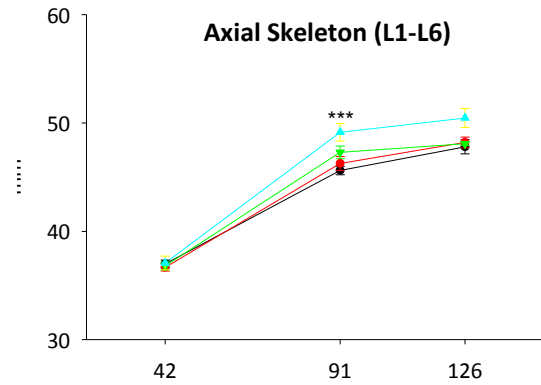
Modified from Rogely W. Boyce, Aurore Varela, Luc Chouinard, Jeanine L. Bussiere, Gary J. Chellman, Michael S. Ominsky, Ian T. Pyrah. Infant cynomolgus monkeys exposed to denosumab in utero exhibit an osteoclast-poor osteopetrotic like skeletal phenotype at birth, Bone (2014).

RADIOLOGY – SKELETAL GROWTH

Males



Females



- Control
- Letrozole 0.003 mg/kg/day
- ▼ Letrozole 0.03 mg/kg/day
- ▲ Letrozole 0.3 mg/kg/day

Significantly different from control value:

* - $P <= 0.05$ ** - $P <= 0.01$ *** - $P <= 0.001$

Modified from L Pouliot, M Schneider, M DeCristofaro, R Samadfam, SY. Smith, and DA Beckman. Assessment of a nonsteroidal aromatase inhibitor, letrozole, in juvenile rats. Birth Defects Research (Part B) 98:374–390 (2013)

In vivo Assessments Densitometry

ADVANCED IN VIVO MEASUREMENTS - DXA



Dual energy X-ray absorptiometry:
The only bone densitometry technique validated for use clinically and approved by the FDA
“Gold” standard

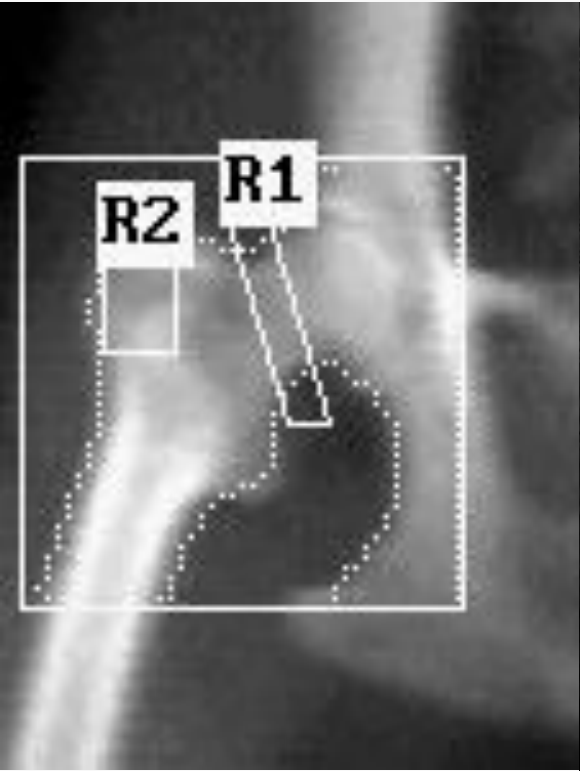
BMC, BMD, Area



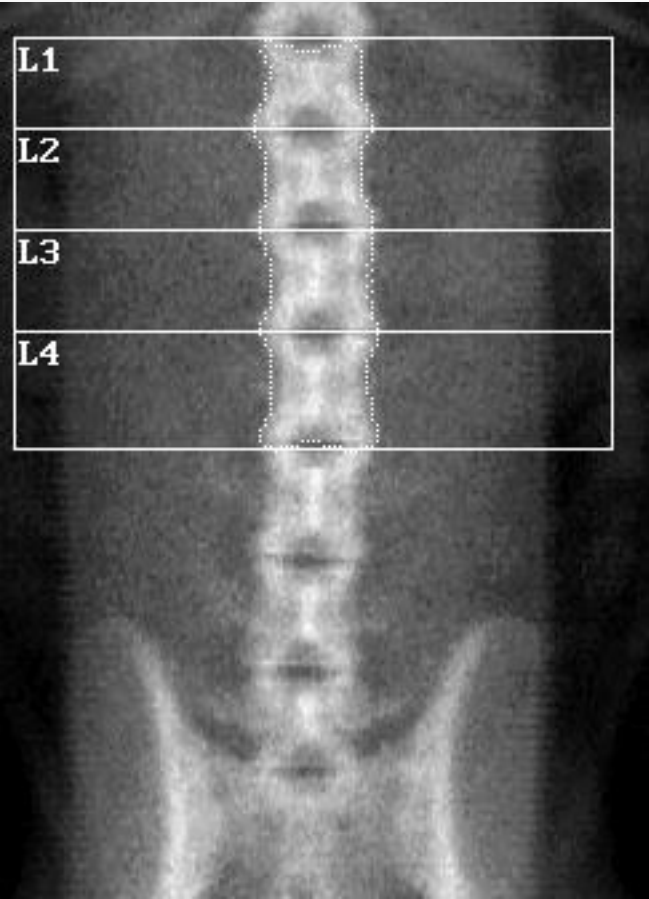
Two dimensional areal bone density
Scan large areas of bone
Anesthesia

ADVANCED IN VIVO MEASUREMENTS - DXA

Hip (proximal femur)



Lumbar Spine



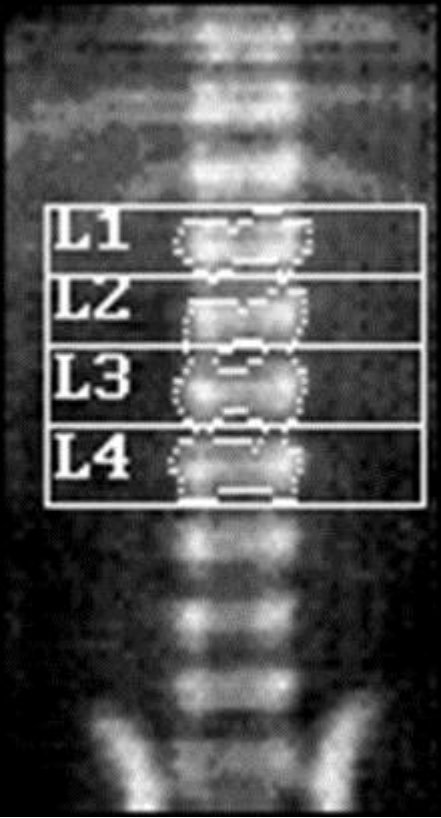
Whole Body



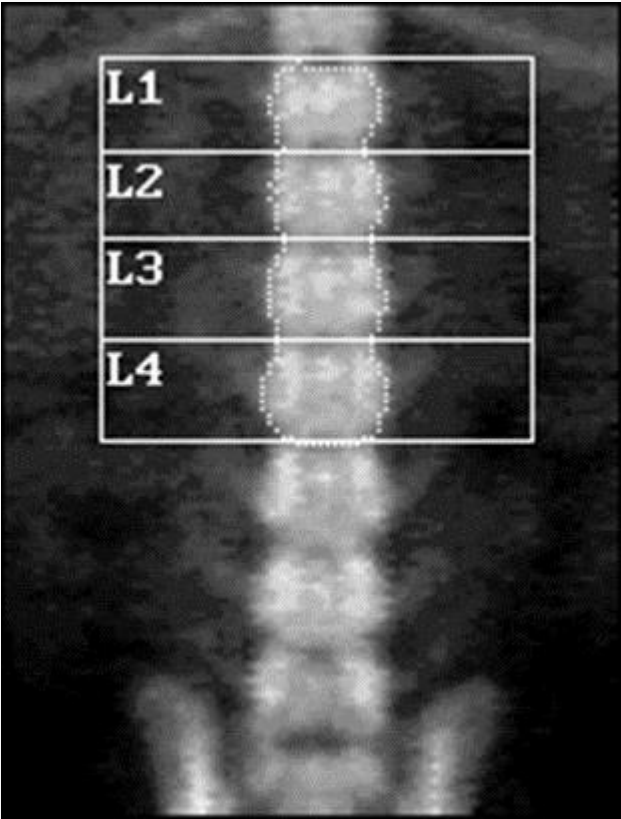
NHP

ADVANCED IN VIVO MEASUREMENTS - DXA

Lumbar Spine L1-L4 Beagle dog pups



Day 19 pp



Day 63 pp

Whole body

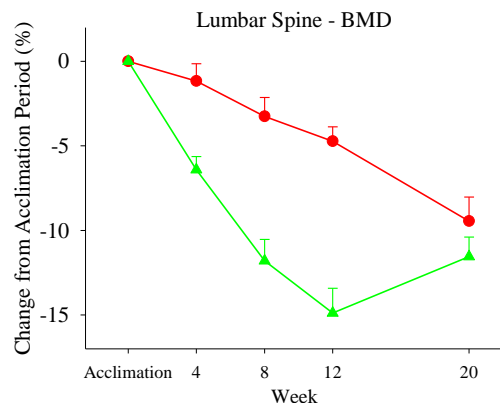
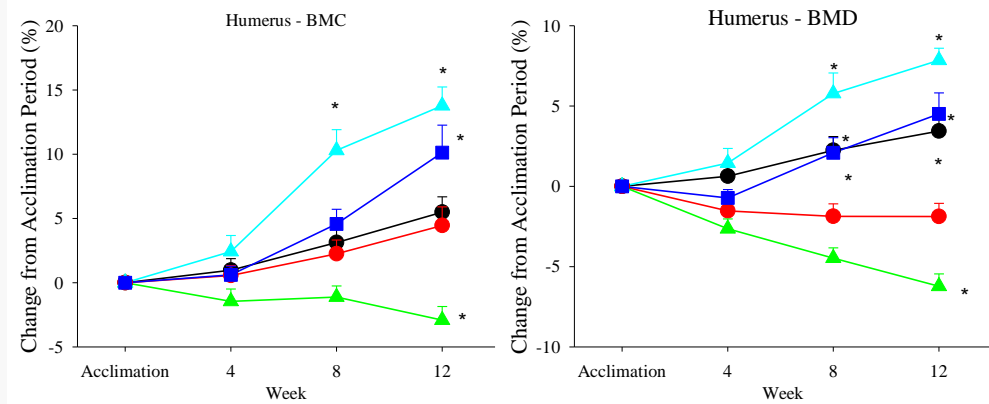
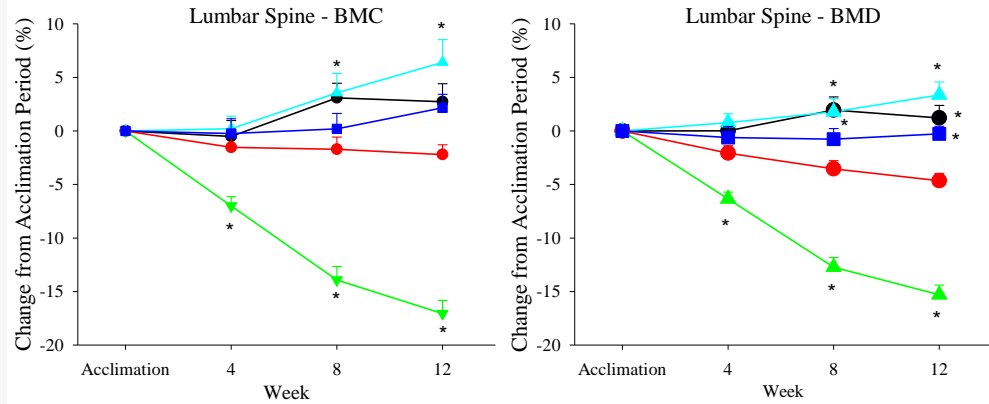


Day 1 pp



Day 19 pp

ADVANCED IN VIVO MEASUREMENTS - DXA



Changes at 12 weeks in BMC and BMD for lumbar spine and humerus

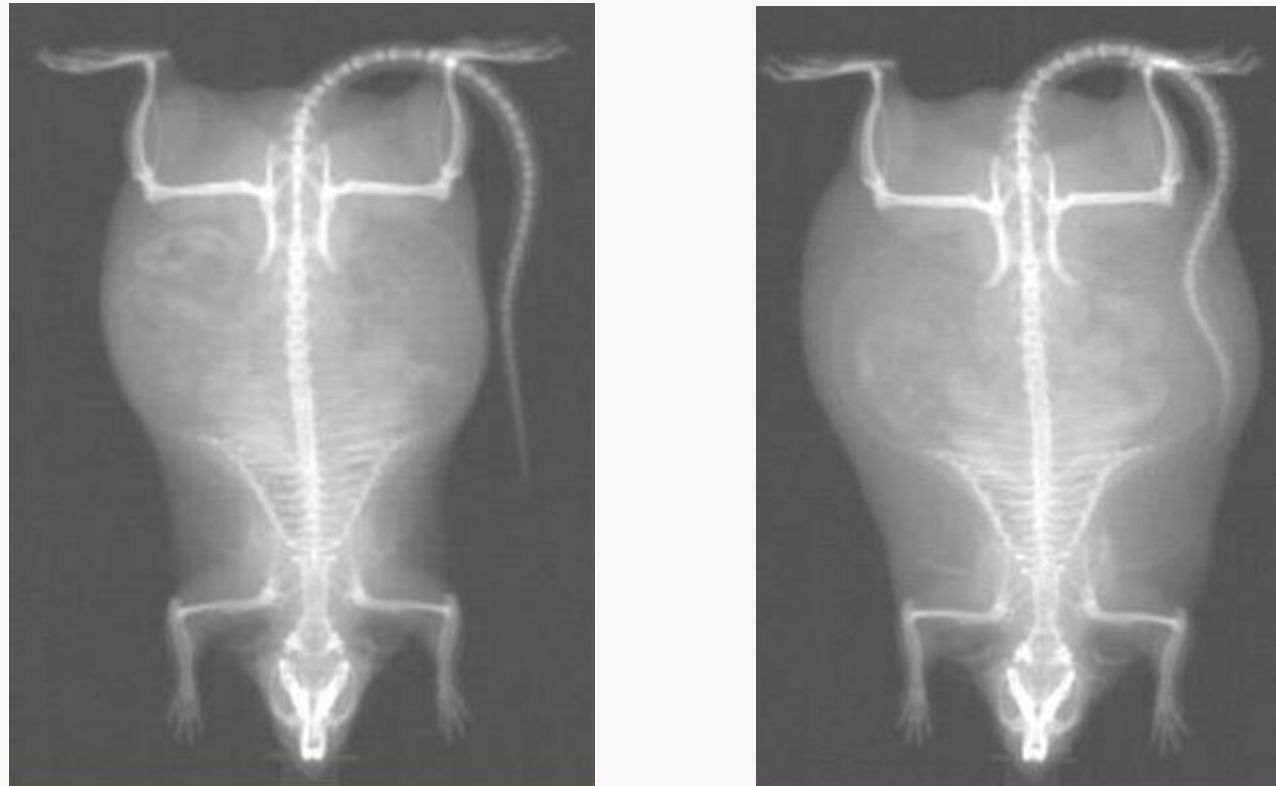
OVX = ovariectomized
RSG = rosiglitazone
ALN = alendronate

- Sham Vehicle
- OVX Vehicle
- ▲ OVX RSG 10 mg/kg/day
- ▲ OVX Alendronate
- OVX RSG 10 mg/kg/day+Alendronate

Significantly different from group 2 value: * $p \leq 0.05$

Modified from Kumar et al, Journal of Bone and Mineral Research, 28-7, 2013.

ADVANCED IN VIVO MEASUREMENTS - DXA

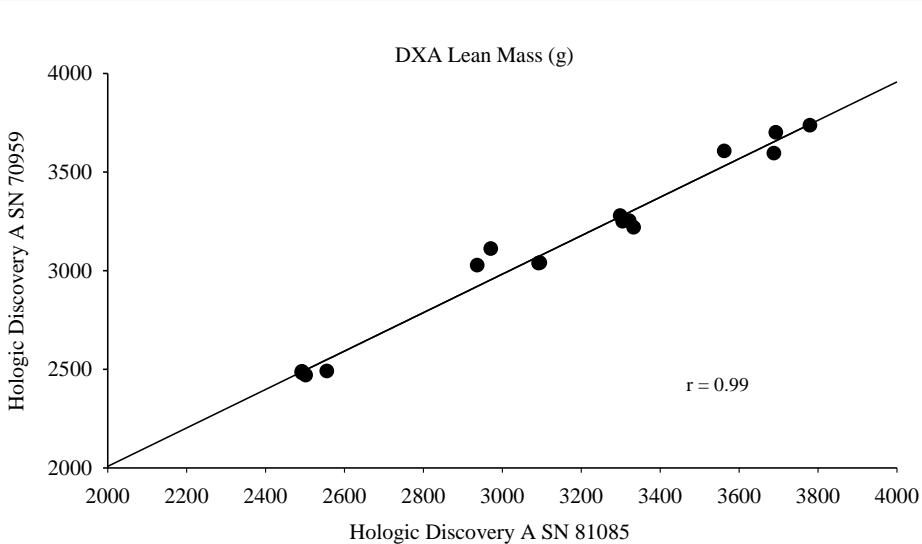


Fat...and fatter rats

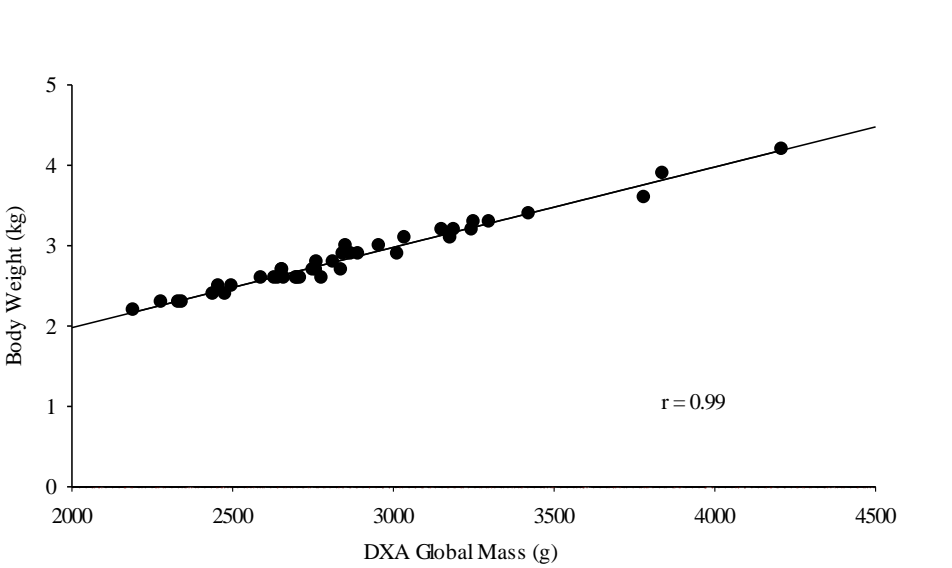
Body Composition for Fat and Lean Mass Analysis

ADVANCED IN VIVO MEASUREMENTS - DXA

Correlation of of Lean mass between two DXA machines



Correlation of Body weight vs. DXA Global Mass



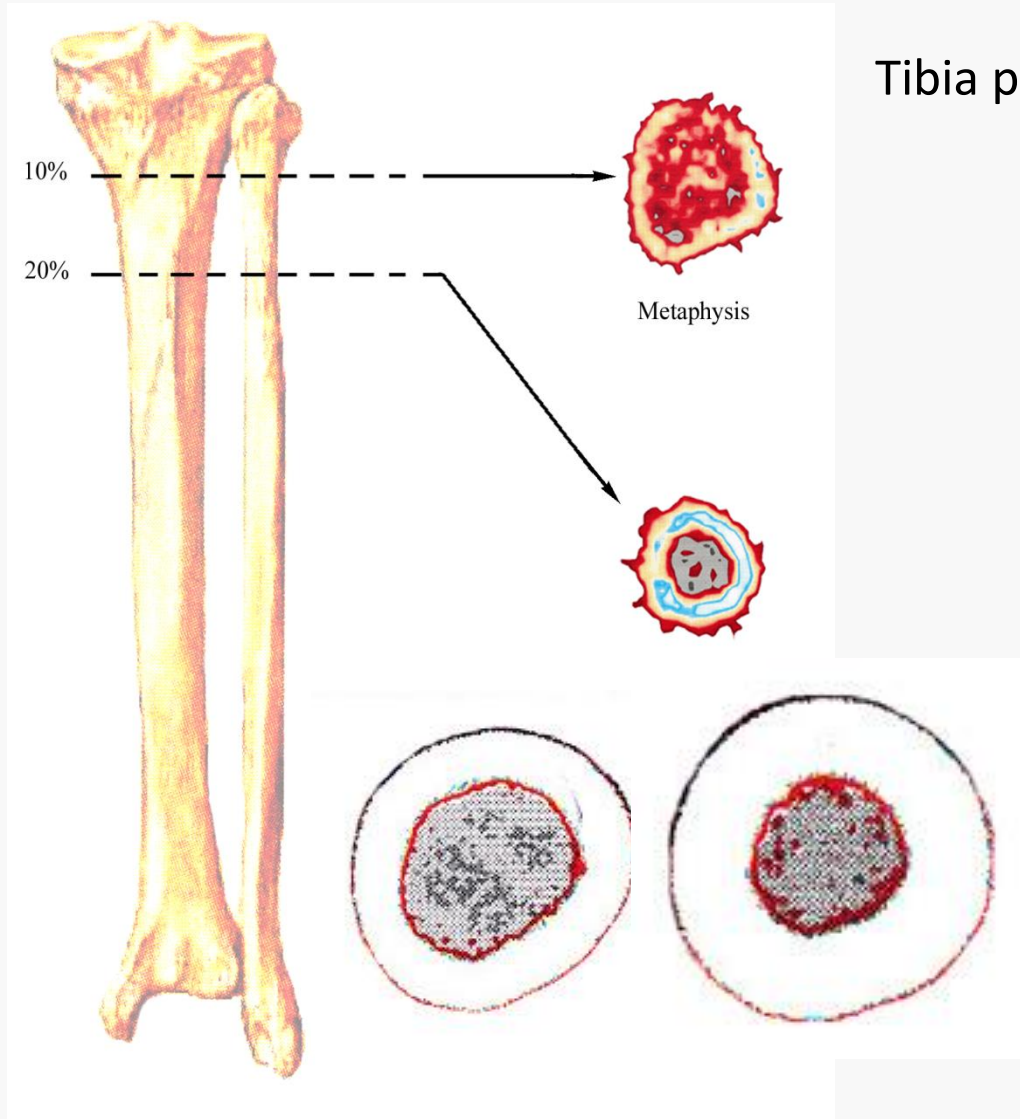
Body Composition : DXA Precision for Fat and Lean Mass Analysis.

ADVANCED IN VIVO MEASUREMENTS - pQCT

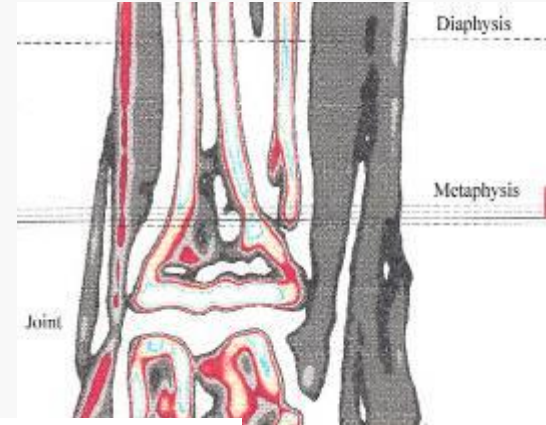


Peripheral Quantitative Computed Tomography
3D Evaluation

ADVANCED IN VIVO MEASUREMENTS - pQCT

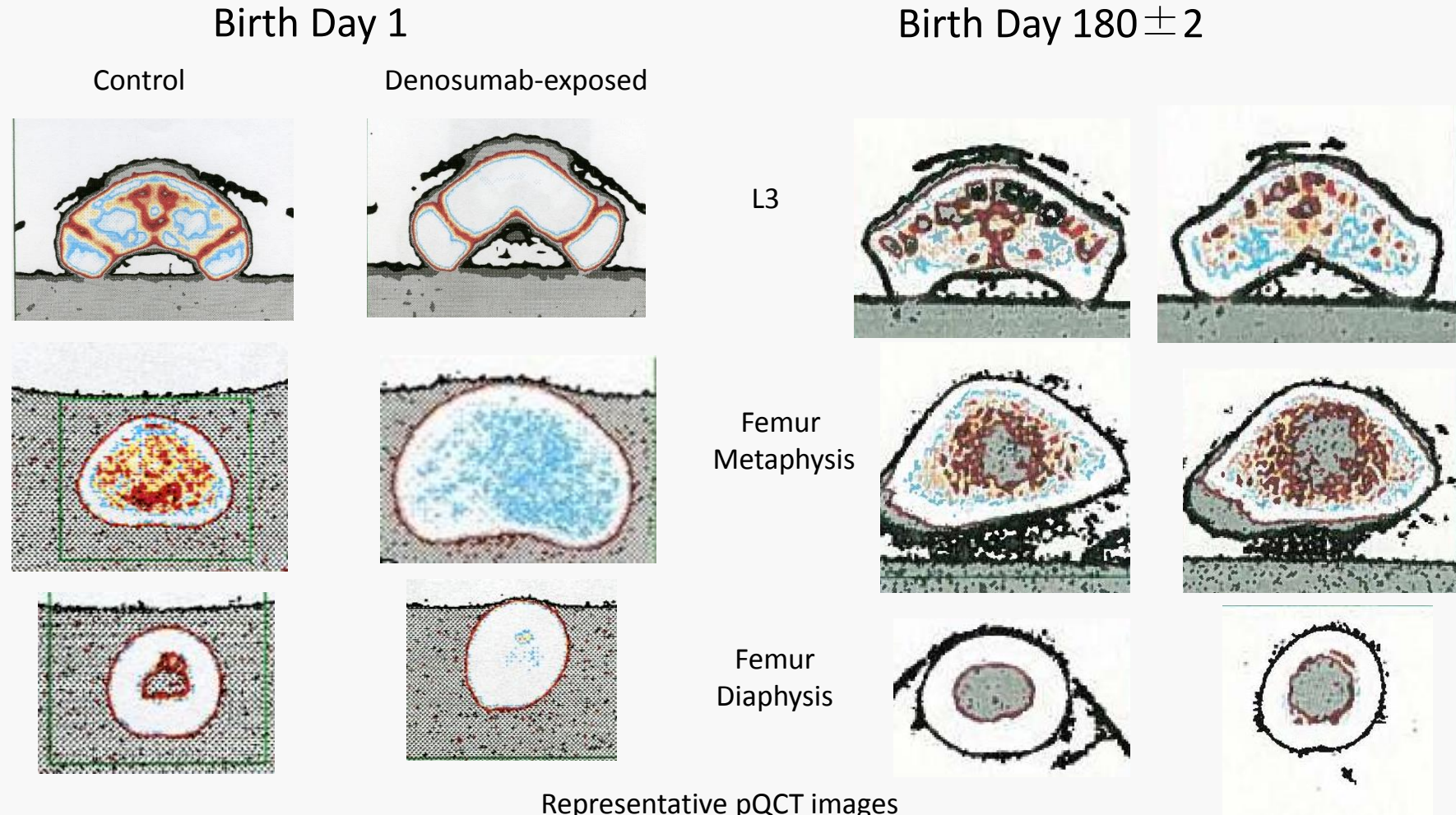


Tibia proximal



Single or multiple CT slices
Metaphysis and diaphysis regions
Separation of cortical and trabecular compartment

ADVANCED IN VIVO MEASUREMENTS - pQCT



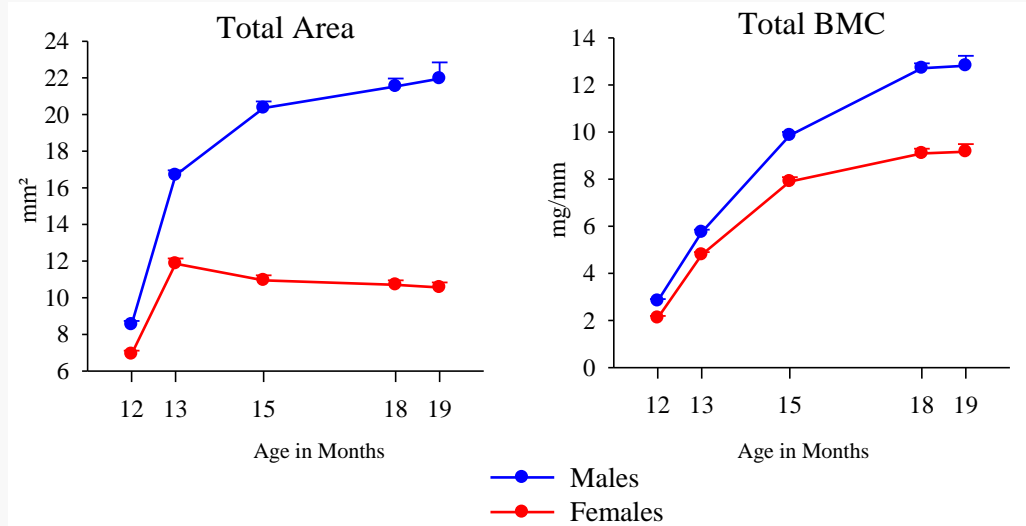
Representative pQCT images

Modified from Rogely W. Boyce, Aurore Varela, Luc Chouinard, Jeanine L. Bussiere, Gary J. Chellman, Michael S. Ominsky, Ian T. Pyrah. Infant cynomolgus monkeys exposed to denosumab in utero exhibit an osteoclast-poor osteopetrotic like skeletal phenotype at birth, Bone (2014).

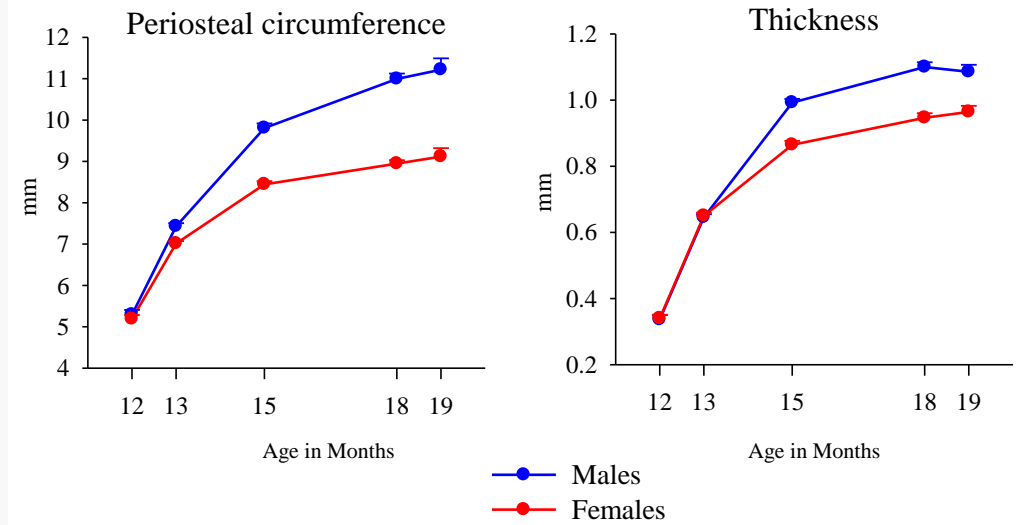
ADVANCED IN VIVO MEASUREMENTS - pQCT

pQCT IN JUVENILE NHP

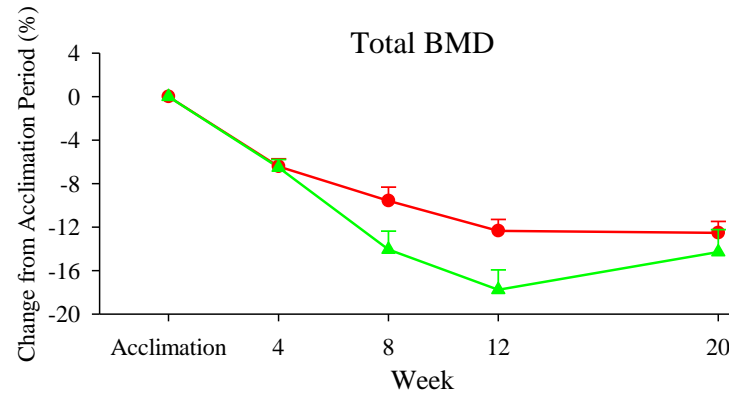
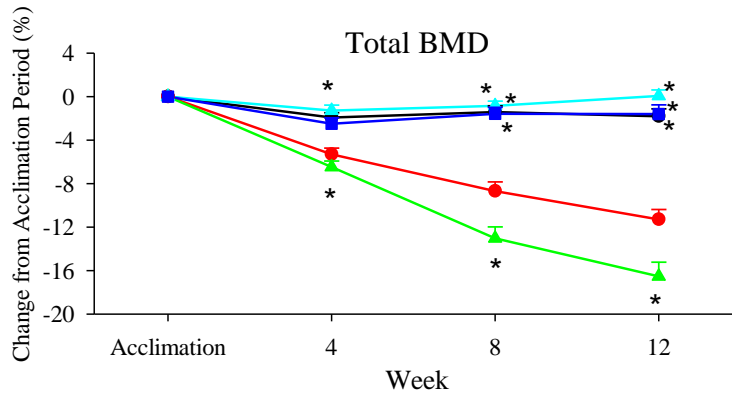
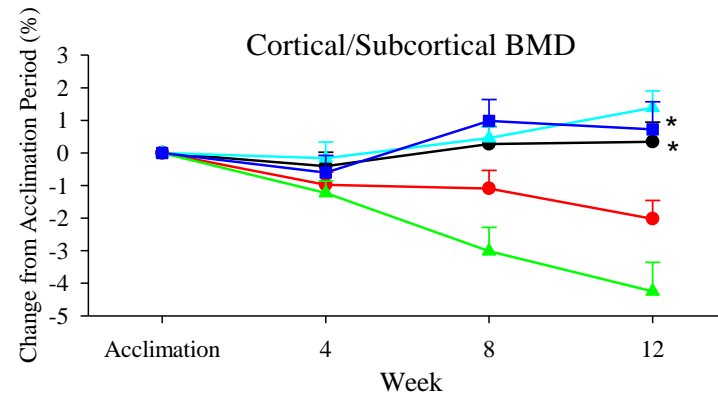
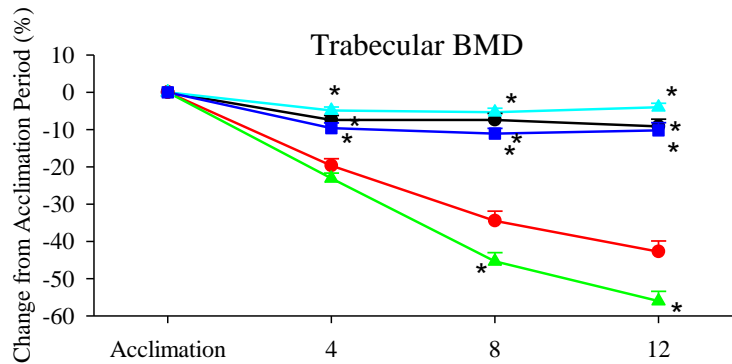
Metaphysis



Diaphysis



ADVANCED IN VIVO MEASUREMENTS - pQCT



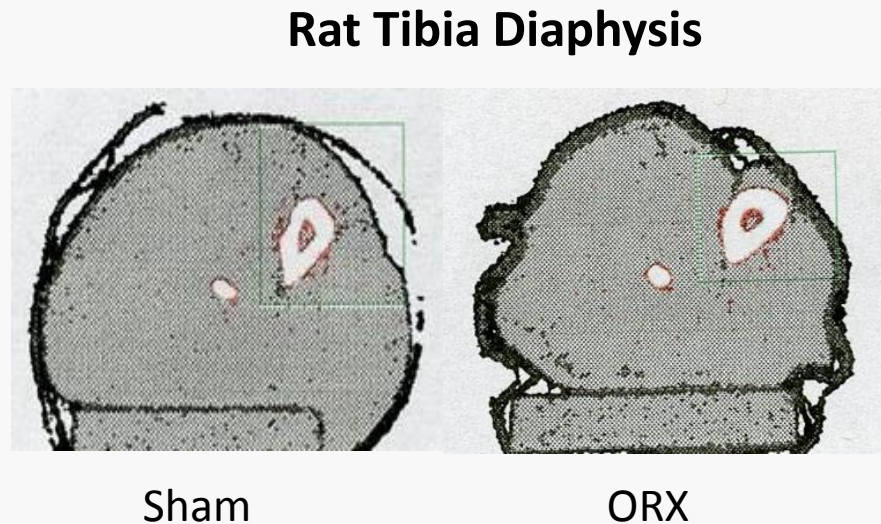
- Sham Vehicle
- OVX Vehicle
- ▲ OVX RSG 10 mg/kg/day
- ▲ OVX Alendronate 0.03 mg
- OVX RSG 10 mg/kg/day+Alendronate

Significantly different from group 2 value: *-p<0.05

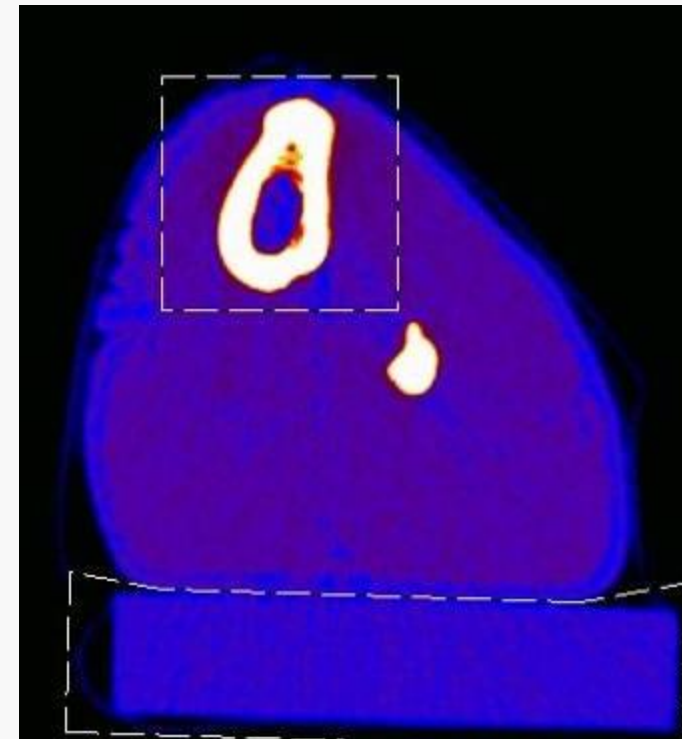
Changes in trabecular, cortical/subcortical and total bone mineral density (BMD) at tibial metaphysis over time as measured by pQCT. Changes are mean \pm SEM. OVX = ovariectomized RSG = rosiglitazone, ALN = alendronate

Advanced In Vivo Measurements - pQCT

Lean mass - Sarcopenia

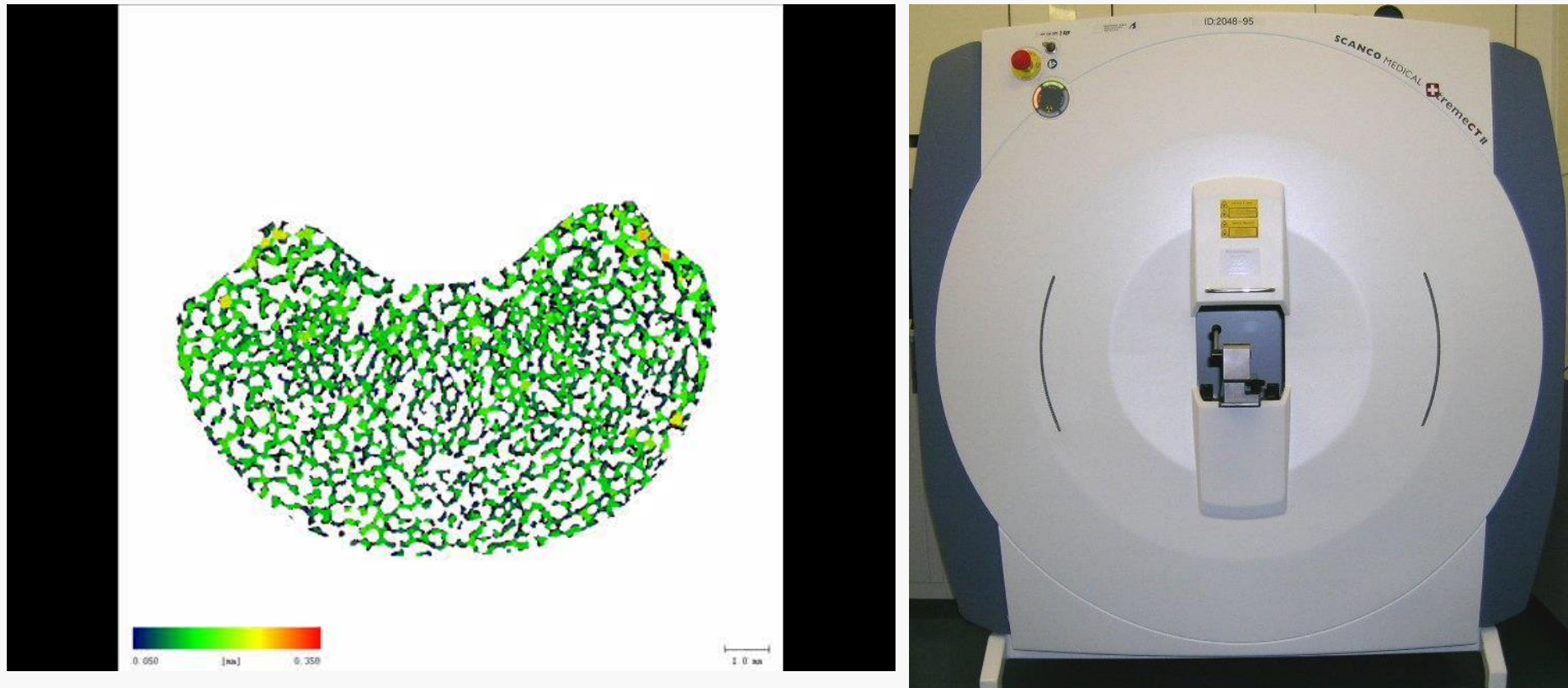


NHP Tibia Diaphysis



Both DXA and pQCT scans can be analyzed for lean mass and fat mass

Advanced In Vivo Measurements – In vivo XtremeCT



Micro-Computed Tomography: Bone Micro-Architecture, Densitometry and Geometry

Advanced In Vivo Measurements

- **Chronic Tox Studies**

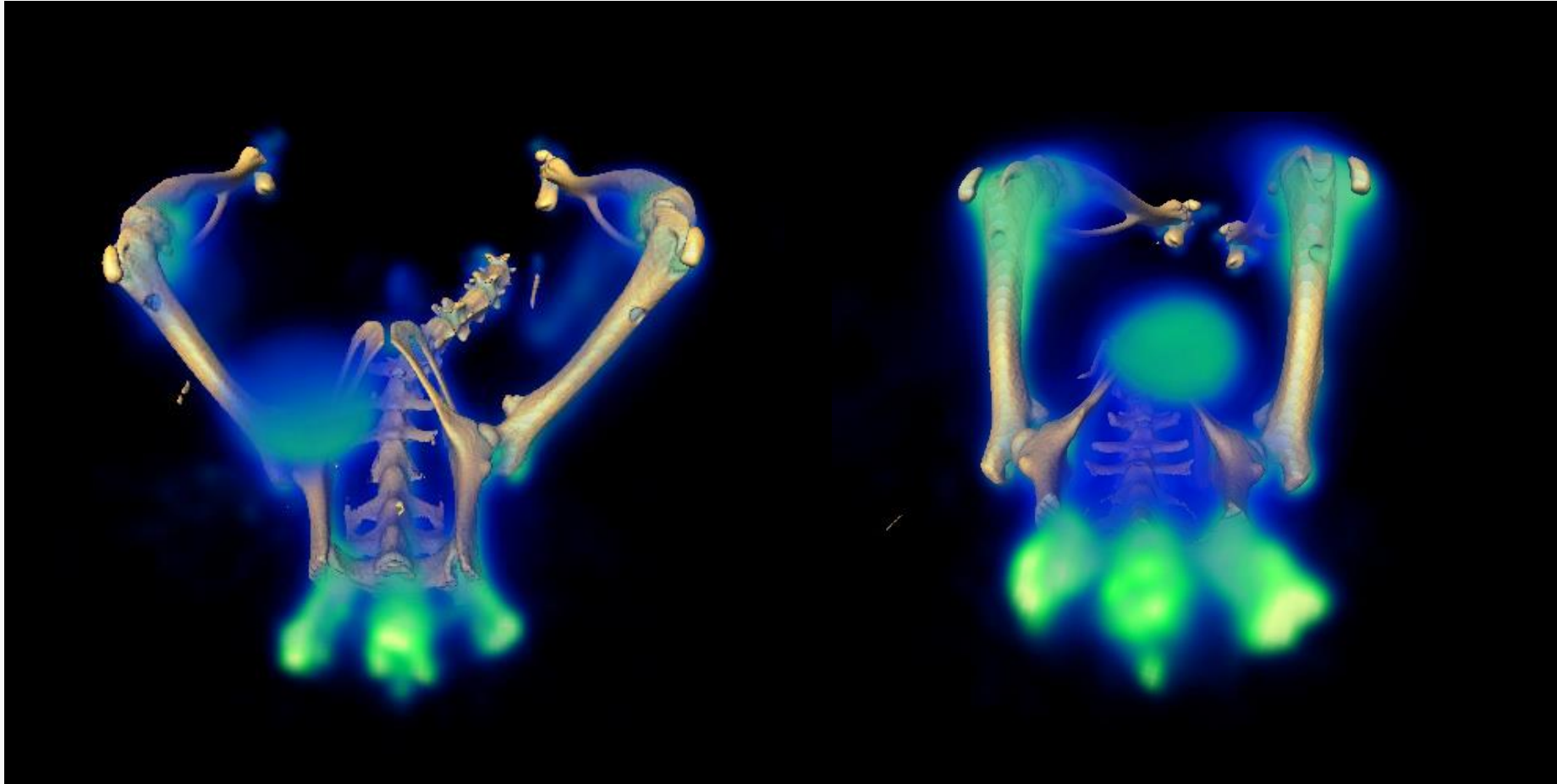
When are DXA and/or pQCT employed in toxicology studies?

- Monkeys: In vivo measurements: pretreatment, end of treatment and recovery, usually 28 days or longer. Baseline scans required due to inter-animal variability and the small group sizes
- For chronic tox studies intervals of one to three months are recommended depending on the study duration and test system

- **Juvenile Tox Studies**

- Limitations of Age
- Anesthesia: Duration may limit scanning time in young animal, Specific anesthesia cares.
- Scanning reproducibility in growing skeleton: Landmark definition
- Individual Longitudinal Evaluation – Large animals
- Sample volume for markers/hormones vs. clinical pathology

Advanced In Vivo Measurements - PET



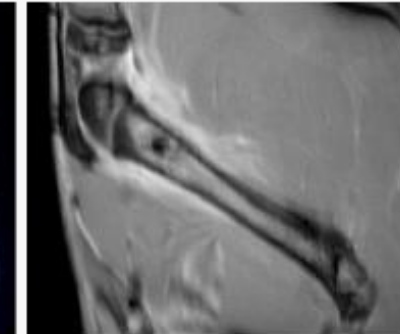
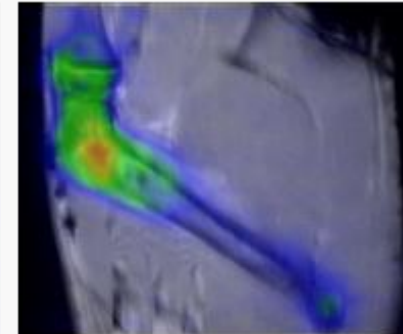
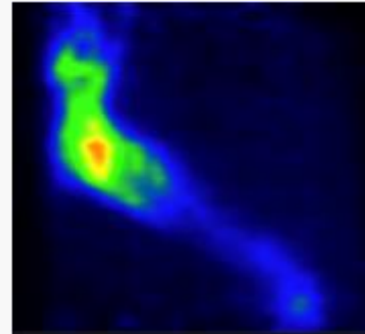
Follow-up of bone metabolic activity using Na^{18}F PET
coregistered with CT in mice

Advanced In Vivo Measurements - PET/MRI

Imaging

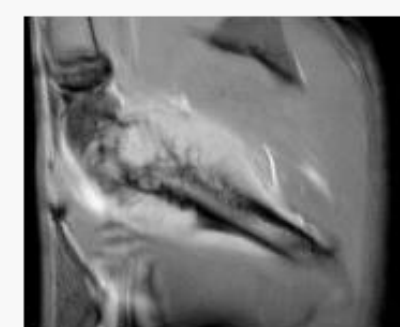
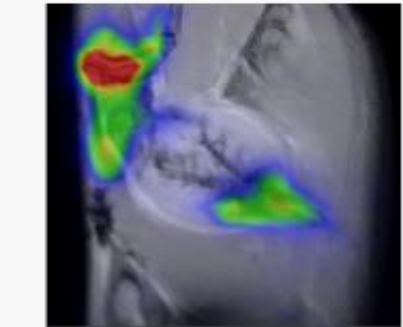
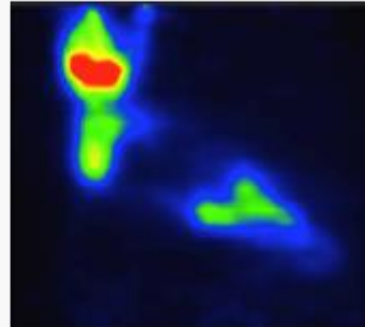
Early stage (D15)

- High Na¹⁸F in tumor area
- Bone metabolism due to tumor blastic activity

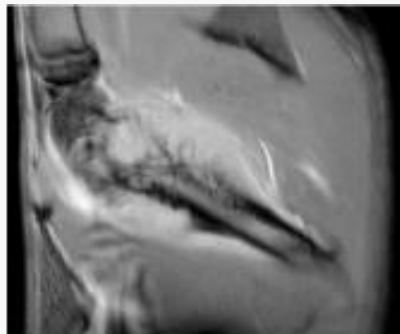
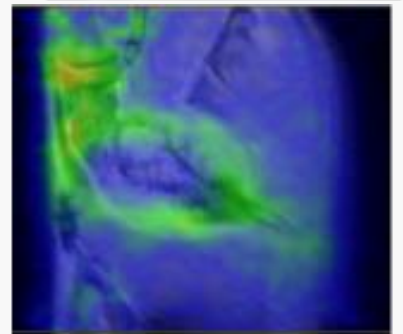
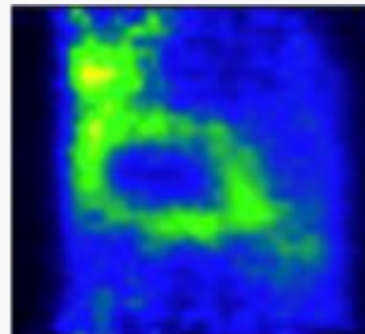


Later stage (D18)

- Decreased Na¹⁸F uptake in femoral tumor area
- Increased uptake around tumor, indicates mixed (lytic and sclerotic) tumor activity



- ¹¹C-methionine uptake indicative of high protein synthesis in outer tumor layer



Ex vivo Assessments Histomorphometry

Advanced Ex Vivo Measurements - Histomorphometry



Static or structural parameters

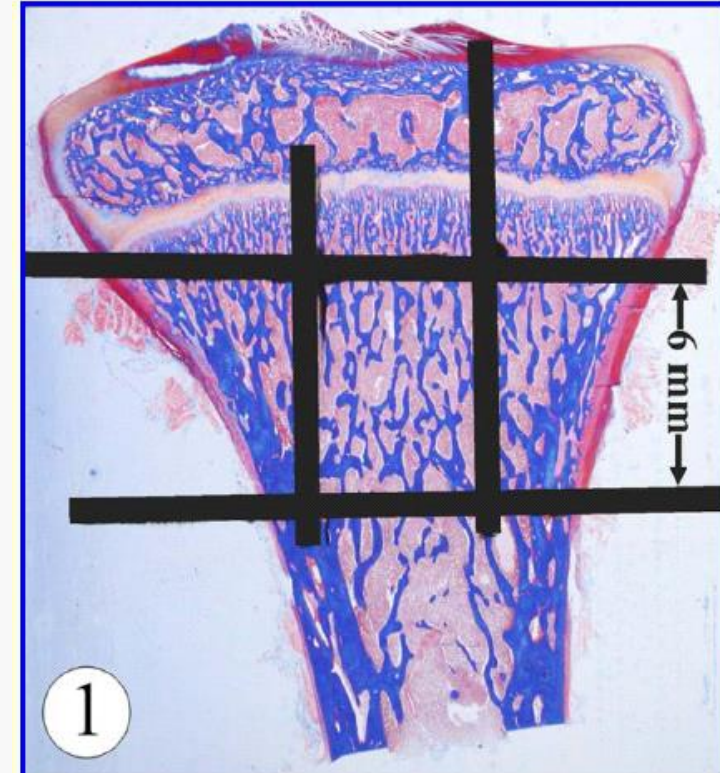
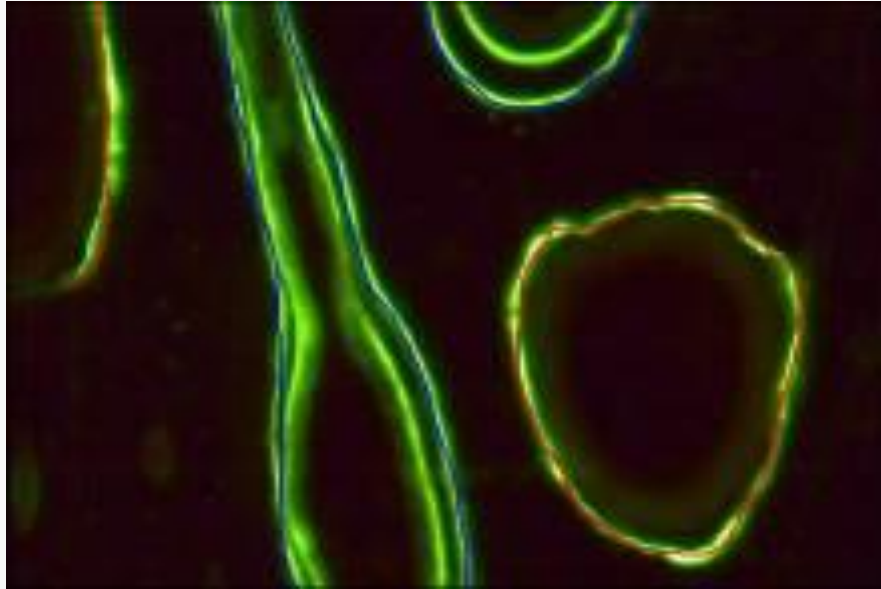


Figure 1. Region of interest measured from the central proximal tibial metaphysis is confined within a 6 x 5 mm rectangle. Goldner's trichrome.

Advanced Ex Vivo Measurements - Histomorphometry



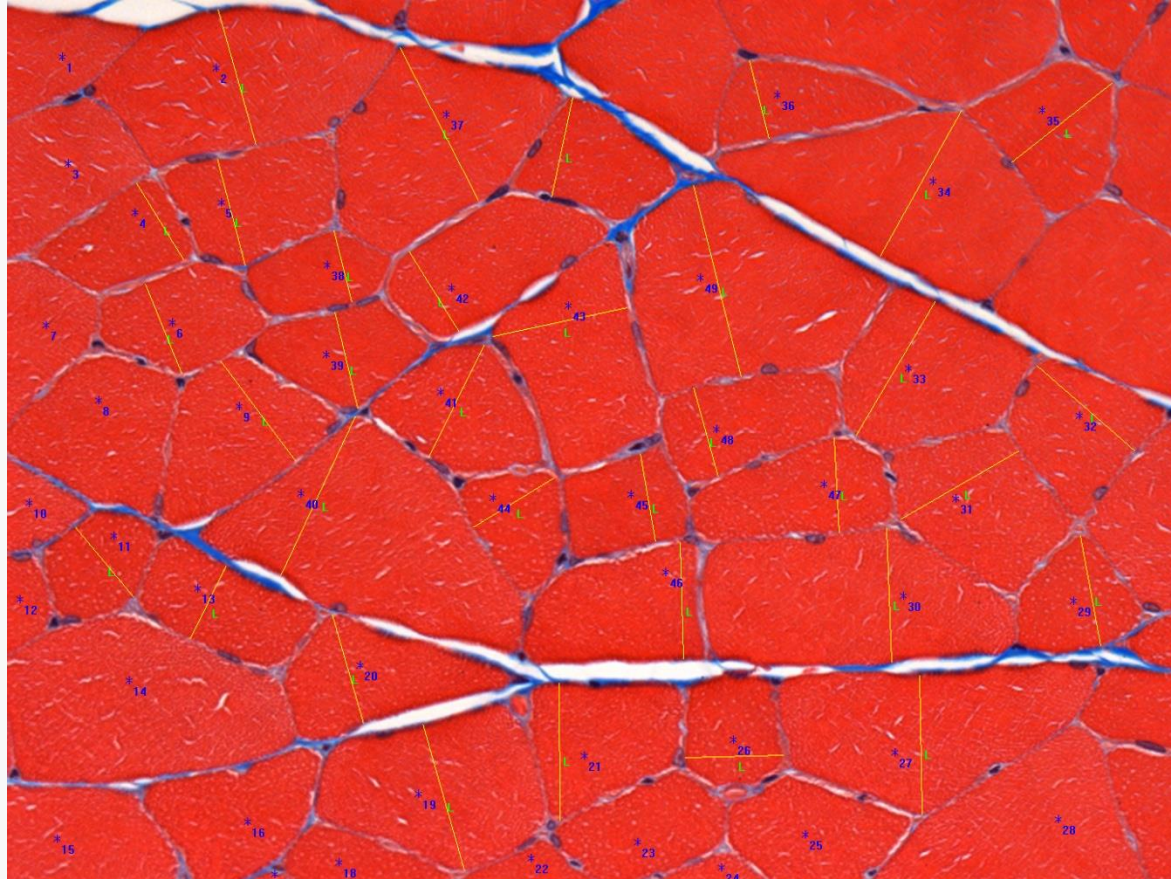
Fluorochrome labeling:

Inject with calcein green, 2
occasions prior to bone harvesting

Dynamic parameters:

Bone formation rates (BFR)
Mineral apposition rates (MAR)

Advanced Ex Vivo Measurements - Histomorphometry



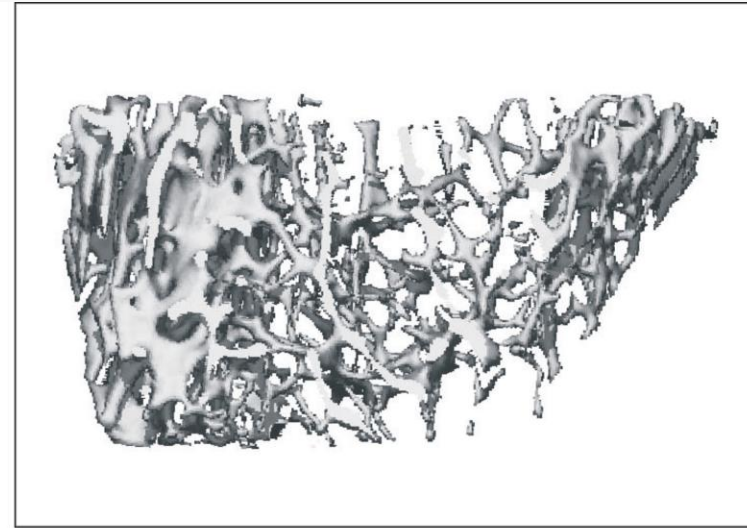
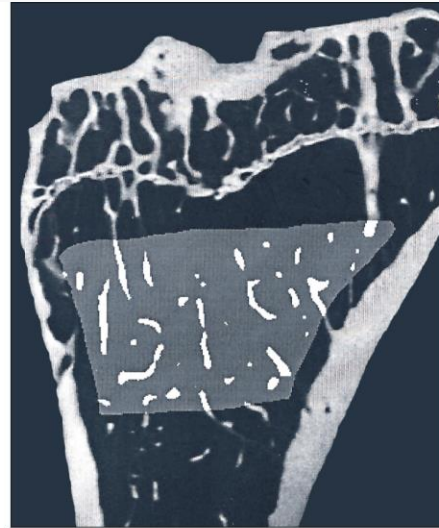
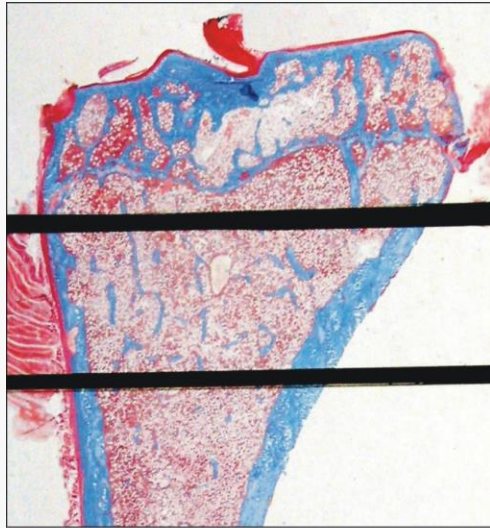
Myofiber surface
Number of myofiber
Myofiber diameter
Mean myofiber diameter
Myofiber number density

EDL muscle (Extensor digitorum longus), Masson Trichrome stain, 'Feret's diameter'

Ex vivo Assessments

Micro-CT

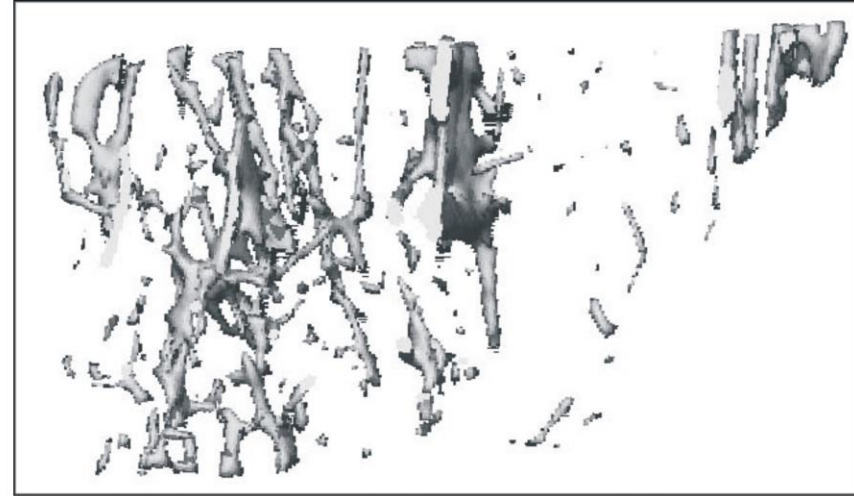
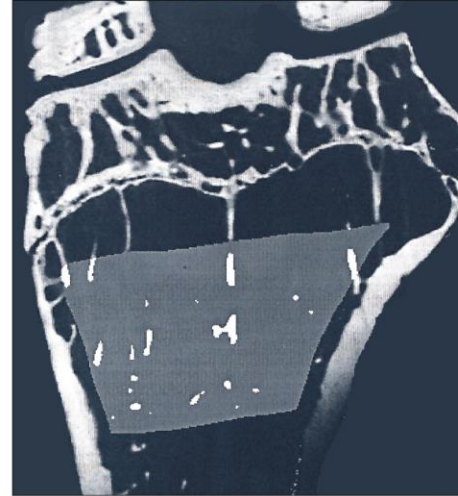
Advanced Ex Vivo Measurements - Micro-CT



ANIMAL	Bone area B.Ar mm ²	Bone volume BV mm ³	Total area T.Ar mm ²	Total volume TV mm ³	Relative Bone area BV/TV %	Relative Bone volume BV/TV %	Trabecular Thickness Tb.Th μm	Trabecular Separation Tb.Sp μm	Trabecular Number Tb.N 1/mm
INTACT CONTROL*									
Histomorphometry	0.8		9.7		8.4		52	561	1.6
2-D Morphometry	1.0274		11.6840		8.7933		59.1	612.6	1.4888
3-D Morphometry		3.0370		29.7101		10.22	90.1	527.3	1.8610

Modified from Gunson, D., Gropp, K.E., Varela, A., 2013. Bone and Joints. In: Haschek, W.M., Rousseaux, C.G., Wallig, M.A. (Eds.), Haschek and Rousseaux's Handbook of Toxicologic Pathology. Elsevier Inc., Academic Press, pp. 2761–2858.

Advanced Ex Vivo Measurements - Micro-CT



ANIMAL	Bone area B.Ar mm ²	Bone volume BV mm ³	Total area T.Ar mm ²	Total volume TV mm ³	Relative Bone area BV/TV %	Relative Bone volume BV/TV %	Trabecular Thickness Tb.Th μm	Trabecular Separation Tb.Sp μm	Trabecular Number Tb.N 1/mm
ORCHIDECTOMIZED*									
Histomorphometry	0.5		10.5		5.0		51	980	1.0
2-D Morphometry	0.4243		12.6181		3.3625		56.7	1630.5	0.5927
3-D Morphometry		0.6867		22.3129		3.08	87.7	806.7	1.2665

Modified from Gunson, D., Gropp, K.E., Varela, A., 2013. Bone and Joints. In: Haschek, W.M., Rousseaux, C.G., Wallig, M.A. (Eds.), Haschek and Rousseaux's Handbook of Toxicologic Pathology. Elsevier Inc., Academic Press, pp. 2761–2858.

Advanced Ex Vivo Measurements - Micro-CT

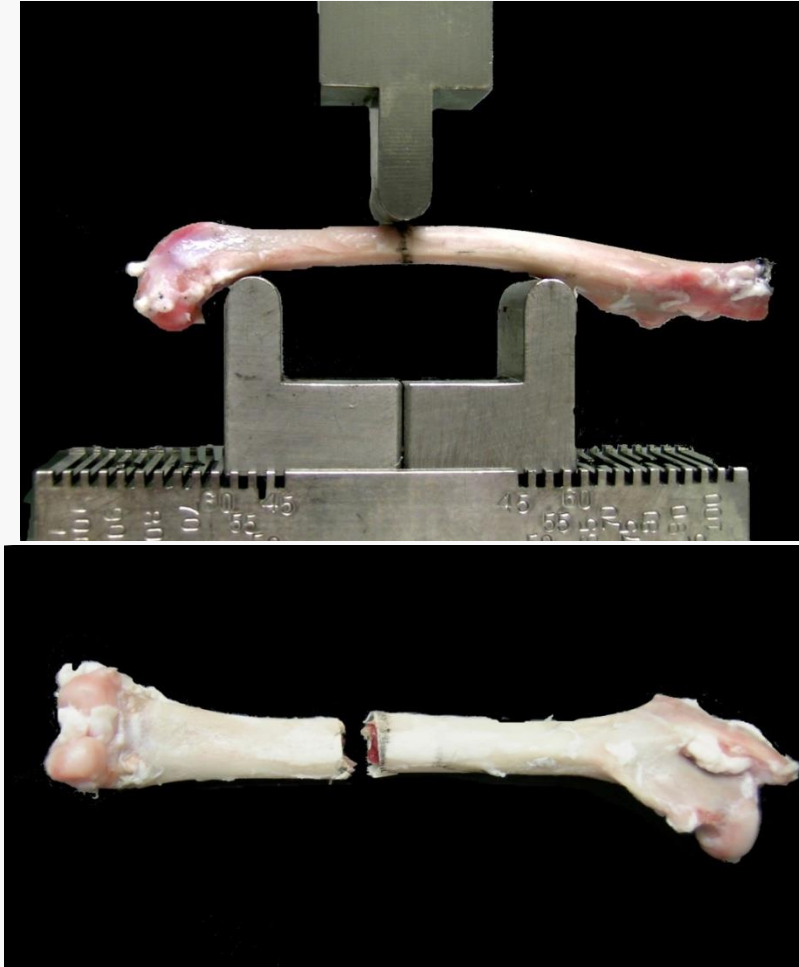


- Representative Micro-CT scans from the middle of the T10 vertebral body from sham or OVX rhesus monkeys treated daily with vehicle or PTH(1–84) (5, 10, or 25 µg/kg) for 16 months.
- Trabecular bone content is lower in the OVX control monkey and increased in PTH(1–84)-treated animals.
- Trabecular number and connectivity density increased, particularly in monkeys given the 25 µg/kg dose of PTH(1–84). BV/TV increases were similar to histomorphometry results at the lumbar spine validating extrapolation of results from lumbar to thoracic spine.

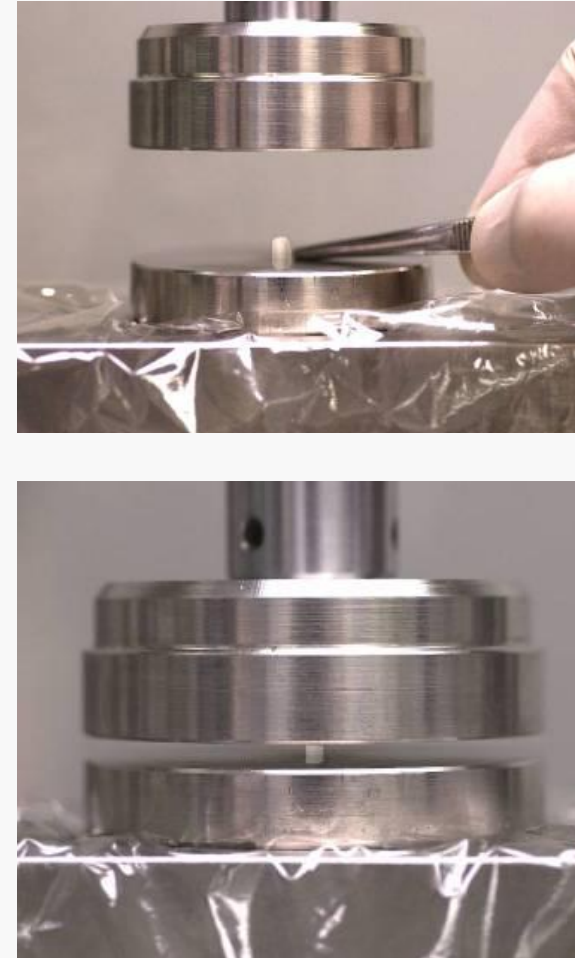
Ex vivo Assessments Biomechanical Testing

Advanced Ex Vivo Measurements - Biomechanical Testing

3-Point Bending



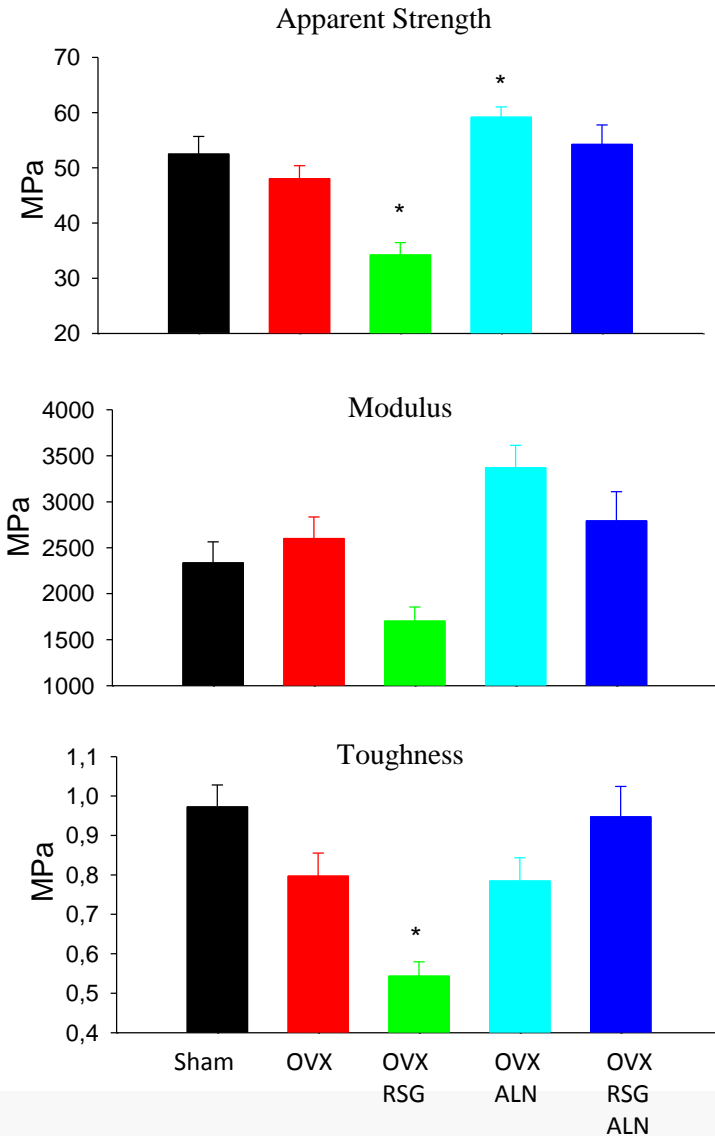
Vertebral Compression



Advanced Ex Vivo Measurements - Biomechanical Testing

- Bones are scanned (DXA, pQCT) to obtain information on bone geometry to normalize strength parameters for bone size
- Bone density and biomechanics parameters are used to assess the relationship between bone mass and strength
- Increases in bone mass should correlate with increases in bone strength
- Correlation analyses to test this relationship

Advanced Ex Vivo Measurements - Biomechanical Testing



Bone biomechanical values for the lumbar spine compression

Values are mean \pm SEM

Sham

OVX = ovariectomized

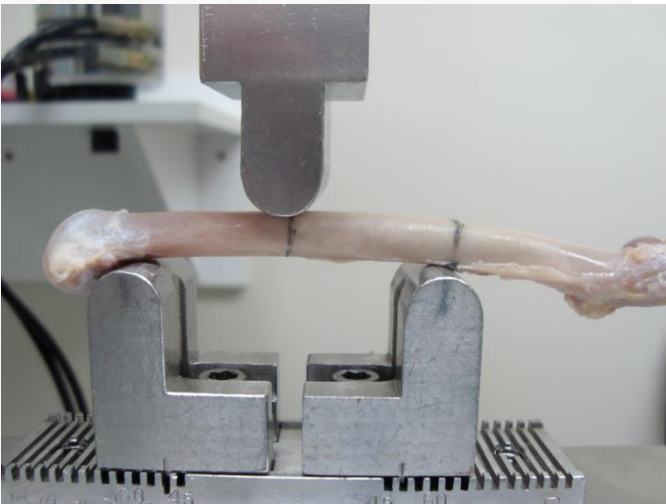
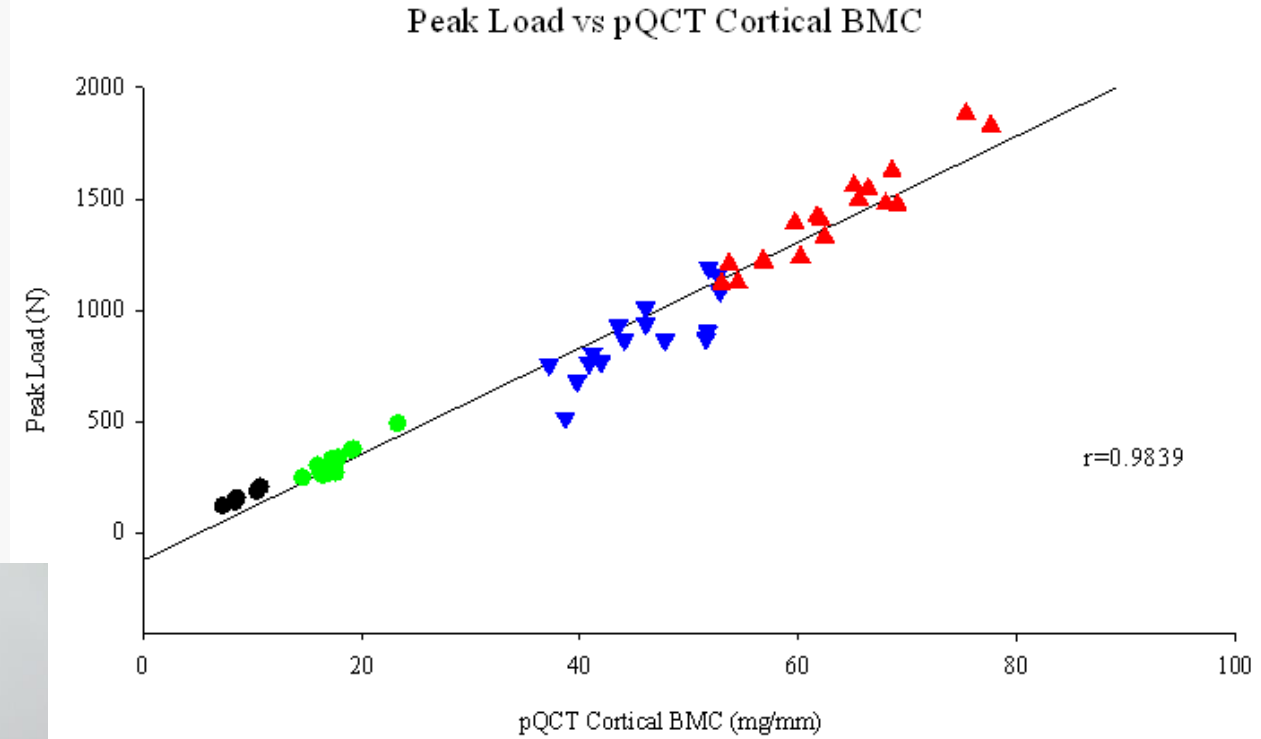
RSG = rosiglitazone

ALN = alendronate

Modified from Kumar et al, Journal of Bone and Mineral Research, 28-7, 2013.

Advanced Ex Vivo Measurements - Biomechanical Testing

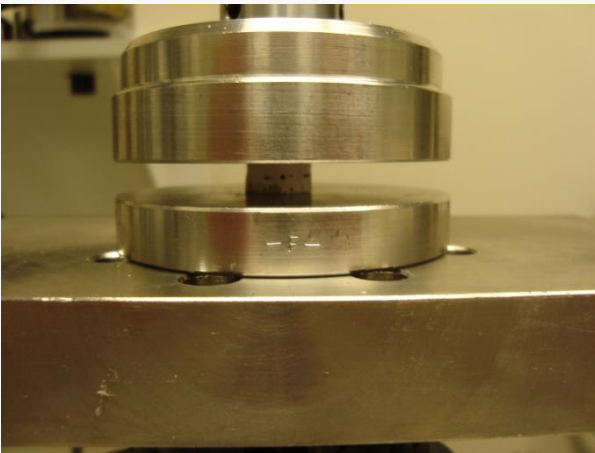
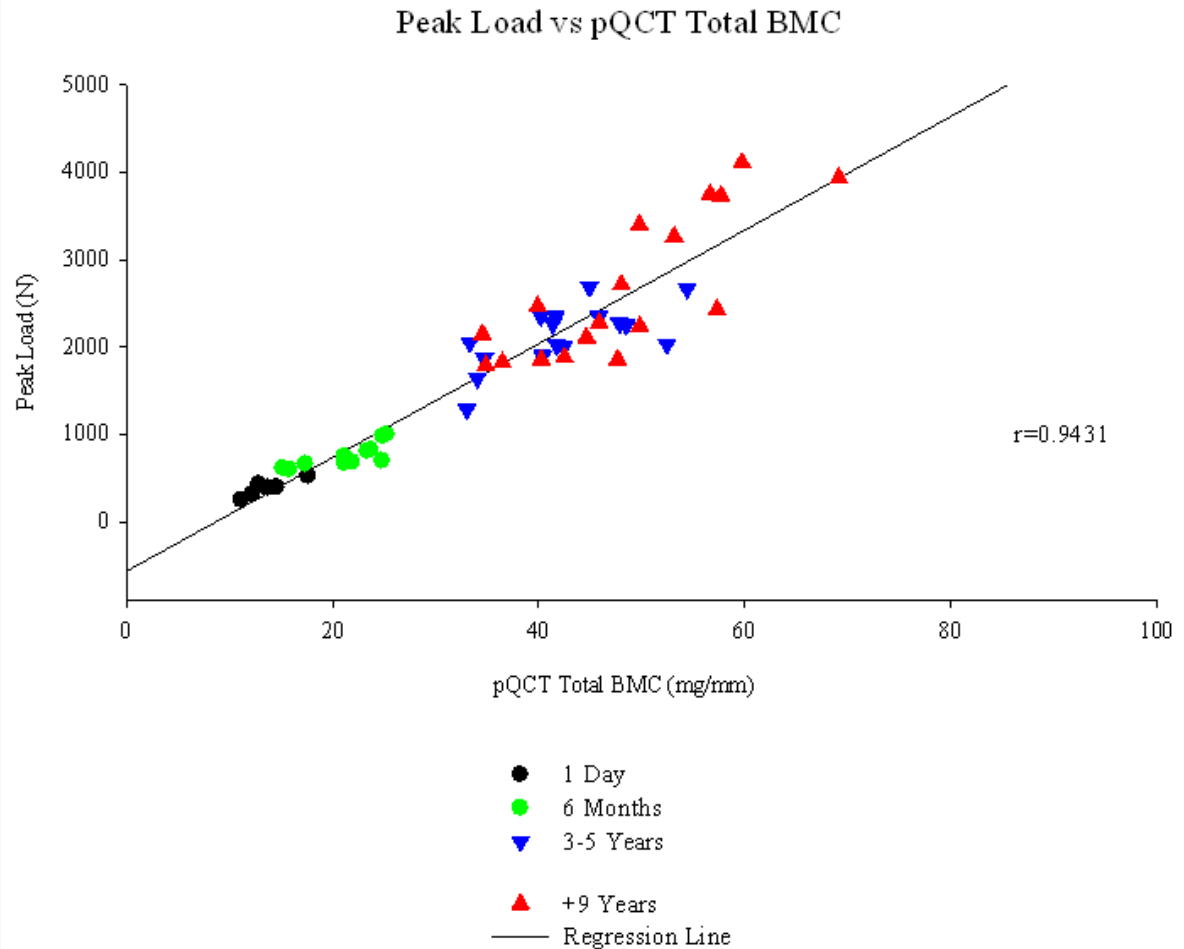
Regression Plot of Biomechanics vs. Bone Mass Cynomolgus Monkeys
Femur 3-Point Bending



- 1 Day
- 6 Months
- ▼ 3-5 Years
- ▲ +9 Years
- Regression Line

Advanced Ex Vivo Measurements - Biomechanical Testing

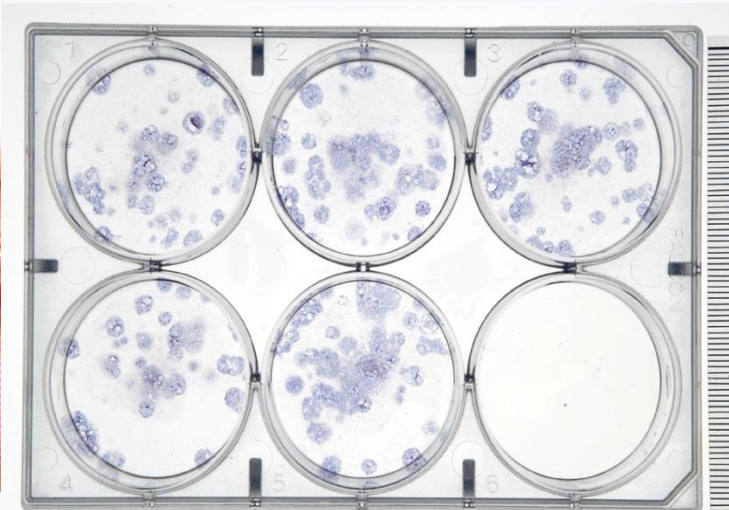
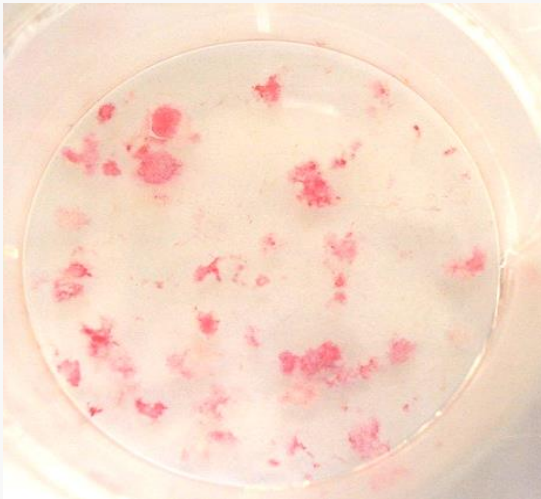
Regression Plot of Biomechanics vs. Bone Mass Cynomolgus Monkeys
Vertebral Compression



Ex vivo Assessments In Vitro Cell Assay

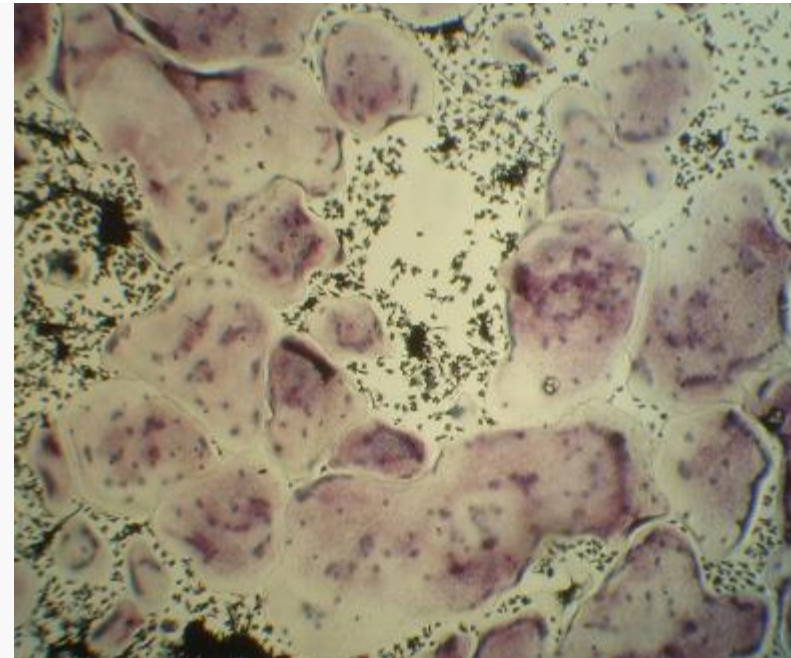
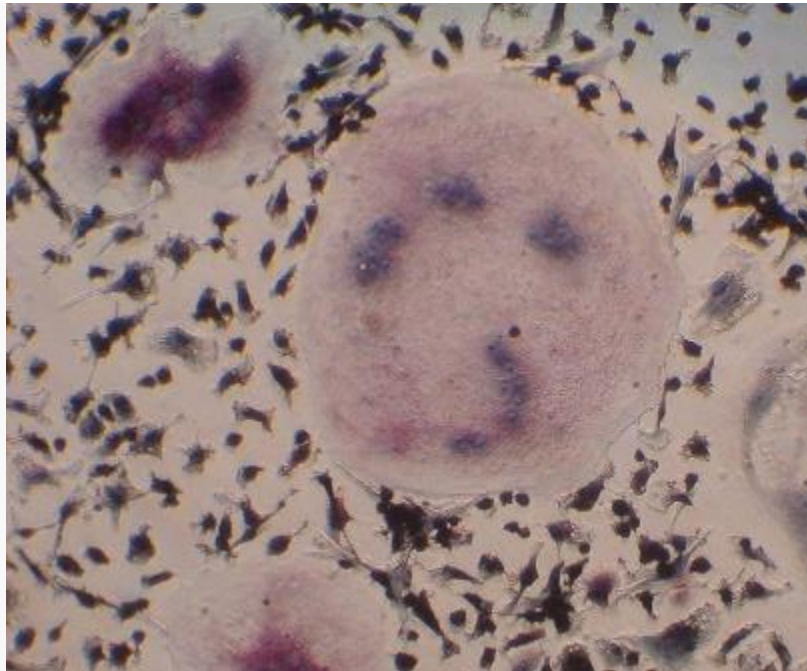
Toxicity Testing – In Vitro Cell Assay

- **ALP staining.**
- **Vonkossa staining**, Von kassa staining is used to detect the mineralization in culture.
- **Methylin blue staining.** This staining is used to detect all colonies including non-osteoblastic colonies.
- **Gene analysis.**
- **The collected media and/or cells can be subjected to immunochemistry analysis to detected expression of cytokins (such as RANKL) or PGs.**



Toxicity Testing – In Vitro Cell Assay

- **TRAP staining:** TRAP staining is used to count the number of osteoclasts in culture. Using image analysis the average size of osteoclasts can also be calculated.
- **Pit assay:** In the pit assay, the cells are removed from the osteologic slides or dentine and then stained with Von Kossa. The number of resorption pits and their average size are measured using an image analysis system.
- **Gene analysis:** At the end of the culture period, cells are washed with icy cold PBS and used for gene analysis using real time RT-PCR.
- **Immunocytochemistry:** The collected media and/or cells can be subjected to immunocytochemistry analysis to measure the concentration of cytokines (such as RANKL), small molecules such as PGs, or markers such as C-telopeptide.



Ex vivo Assessments Protocol Considerations

Toxicity Testing – Protocol Considerations

- Tier Approach:
 - Biochemical markers of bone turnover
 - In vivo Radiography, DXA - pQCT
 - Recovery
- Protocol Provisions: Recommended Retention
 - A long bone (tibia) and lumbar vertebrae in formalin then alcohol for possible histomorphometry
 - A long bone (femur) and lumbar vertebrae retained frozen at -20°C for possible biomechanical strength testing
- Additional evaluations: Ex vivo Histomorphometry - Biomechanical strength testing, Bone Quality.
- Integrated Study/Report

Conclusion

- Techniques and study designs are available to evaluate concerns for skeletal safety and development
- Simple, non-invasive techniques can be used to measure in vivo skeletal growth and skeleton in rodents and non-rodents using standard group sizes typical in toxicology studies
- Ex vivo bone measurements can be readily added to toxicology studies
- Tier approach

Questions

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